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Predicting the effects of climate change on future freshwater fish diversity at global scale

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Abstract

The aim of the present study was to predict future changes in biodiversity attributes (richness, rarity, heterogeneity, evenness, functional diversity and taxonomic diversity) of freshwater fish species in river basins around the world, under different climate scenarios. To do this, we use a new methodological approach implemented within the ModestR software (NOO3D) which allows estimating simple species distribution predictions for future climatic scenarios. Data from 16,825 freshwater fish species were used, representing a total of 1,464,232 occurrence records. WorldClim 1.4 variables representing average climate variables for the 1960–1990 period, together with elevation measurements, were used as predictors in these distribution models, as well as in the selection of the most important variables that account for species distribution changes in two scenarios (Representative Concentration Pathways 4.5 and 6.0). The predictions produced suggest the extinction of almost half of current freshwater fish species in the coming decades, with a pronounced decline in tropical regions and a greater extinction likelihood for species with smaller body size and/or limited geographical ranges.

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Keywords
Distribution models, evenness, heterogeneity, Niche of Occurrence, species richness, rarity, taxonomic diversity

Introduction

Predicting the consequences of climate change on organisms' geographical distribution and, consequently, on their biodiversity, is a complex and monumental task, but one which is necessary (Warren et al. 2018). A simplistic, but common, approach to achieve this aim has been the creation of so-called Species Distribution Models (SDMs). SDMs relate a dataset of species occurrences with the environmental characteristics of the areas in which these occurrences are observed, so as to derive predictions for various climatic scenarios. SDMs, however, can also be used to derive projections for other types of global change such as land-use or human density, so they are not restricted to climate change. SDMs may thus be considered forecasting models, in which prediction reliability is highly dependent on the quality of the data employed and the strength of the relationship between global change and species distributions. Unfortunately, when the outputs of these correlational models are evaluated using occurrence data from a different period, whether it be for hindcasting or forecasting, the results obtained are often unsatisfactory (Kharouba et al. 2009; Rubidge et al. 2011; Rapacciuolo et al. 2012; Eskildsen et al. 2013; Ko et al. 2013; Watling et al. 2013). The inability to correctly forecast the future distribution of species under other climatic scenarios is likely not only due to our lack of species-specific knowledge of the effects of the explanatory variables, but also due to certain characteristics of the modelling procedures used. SDMs require the inclusion of non-climatic variable predictors, which also influence species distributions; and it should also be considered that the general lack of adequate survey effort precludes the use of reliable absence data. These two major shortcomings hinder the estimation of the 'true' causal influence of climatic variables (Lobo 2016).

Freshwater fish appear to be a group which is especially vulnerable to climatic changes (Markovic et al. 2017) and at high risk of extinction (Collen et al. 2014) under predicted changes in hydrological regimes and warming climate. Freshwater fish have already experienced important shifts and changes in their distributions in response to recent climatic changes (Comte and Grenouillet 2013), which have dramatically affected riverine fish assemblage composition (Conti et al. 2015). SDMs have often been used to estimate the effect of climate change on freshwater fish species. However, the majority of studies have mostly been focused on species that populate cold-water habitats in temperate North American and European regions (Hauer et al. 1997; Comte et al. 2013; Filipe et al. 2013; Edwards et al. 2016; Ruiz-Navarro et al. 2016; Radinger et al. 2017). Consequently, there is an extreme geographical bias in these studies and very limited information is available on the potential responses of freshwater fish species inhabiting the southern hemisphere or the tropics (see Pelayo-Villamil et al. 2018). There have been papers pub-

lished on research at country-wide or continental scales (Booth et al. 2011; Markovic et al. 2012; Comte and Olden 2017; Markovic et al. 2017; Jarić et al. 2019). However, to date, no study has attempted to estimate the potential effects of future climate changes on freshwater fish at a global scale. In this approach, we consider a fundamentally holistic approach to studying many species at a global scale, in order to better understand the possible effects of climate change, rather than relying on the single species studies that have dominated the field of freshwater species research to date (Gallo et al. 2017).

The present study is not characterised by the use of complex algorithms with unreliable absence data; instead, we make use of the unique empirical data frequently available (occurrence observations) to infer the environmental conditions under which particular species seem to be able to maintain sustainable populations. This information was subsequently used to transfer spatial data on these suitable environmental conditions to potential future climatic scenarios. The purpose of this study, therefore, is to map the future location of the climatic conditions under which freshwater fish species are currently observed, assuming the general incapacity of freshwater fish species to colonise new river basins (Drakou et al. 2009) and obviating the role played by micro-evolutionary processes and phenotypic plasticity. Thus, the main aim of this study was to provide a general assessment of the most likely changes in freshwater fish species richness, diversity and body size in world watersheds, as well as to estimate the most plausible modifications in the distributional area of these species.

Material and methods

Occurrence records of freshwater fish

The dataset of geographical records for freshwater fish, developed by Pelayo-Villamil et al. (2015), was updated to reflect the taxonomic changes and new species described up to the end of October 2018. Appendix 1 in Suppl. material 1 describes all sources obtained from the Global Biodiversity Information Facility (GBIF; see https://www.gbif.org/), which were used in this study, in detail. Other sources, such as web pages, museum collections and published manuscripts are described by Pelayo-Villamil et al. (2015). Records were downloaded and filtered using the data cleaning capabilities available in the ModestR software (Pelayo-Villamil et al. 2012; García-Roselló et al. 2013, 2014, 2015). GBIF records were filtered as follows: i) records with the same latitude and longitude were excluded, ii) records with 0° latitude or longitude were also excluded and iii) habitat data were cleaned, in order to eliminate occurrences in habitats other than those corresponding to terrestrial freshwater ecosystems (see García-Roselló et al. 2014 for details). At the end of October 2018, 16,825 species of freshwater fish were recognised as valid by taxonomists and are available at IPez (http://www.ipez.es, Guisande et al. 2010). Of these, 16,662 species (99.6% of the total) had associated geographical information, for a total of 1,464,232 occurrence records (unduplicated). See Suppl. material 2: Table S1 for a detailed description of the

species included in the analysis, as well as future species predictions in 2050 and 2070 under both scenarios and the maximum body length of each species, all of which were obtained from https://www.fishbase.org/ or from the original manuscripts, when not available at FishBase.

Biological species traits to measure functional diversity

We used the classification described by Buisson et al. (2013), slightly modified, for functional description of the fish species. We used six traits divided into three biological functions: food acquisition, life habitat and locomotion. Food acquisition traits include the feeding habitat (pelagic, benthopelagic and benthic) and the trophic guild (primary consumer, secondary consumer, top-predator, omnivorous and detritivorous). Life habitat traits comprise habitat type (pelagic, benthopelagic and demersal) and migration type (potamodromous, anadromous, catadromous, amphidromous, oceanodromous and no migration). Finally, locomotion traits include body length (in cm: small < 15, medium 15–50, large 50–150 and extra-large > 150) and rheophily (rheophilic, limnophilic and eurytopic). The biological traits assigned to each species are available in Appendix 2 of Suppl. material 3. This information was obtained from https://www.fishbase.org/ or from the original manuscripts, when not available at FishBase (Froese and Pauly 2019).

It was not possible to include reproduction traits, such as life span, parental care or reproduction habitat, because of the difficulties inherent in the assignment of these functional traits to over 16,000 species.

Predicting future species distributions

The future distribution of species was estimated by a modelling procedure (NOO3D) available in the ModestR software (Pérez-Costas et al. 2019), which is based on a simple method called Niche of Occurrence (NOO; García-Roselló et al. 2013, 2014, 2019). NOO3D can be used both to estimate the 3D distribution of species using 3D occurrence samples and 3D environmental datasets (e.g. at different depths) and to extrapolate the probable distribution of a species at different time slices taking into account the occurrences available in one or more time periods. A full description of this procedure is provided in Suppl. material 4 (Appendix 3) and there is a step-by-step tutorial freely available at http://www.ipez.es/modestr/Manual_Tutorial.html (see also García-Roselló et al. 2019 and Pérez-Costas et al. 2019).

In essence, both NOO and NOO3D aim to overcome the drawbacks associated with the general lack of reliable absence information (Lobo et al. 2018), as well as the frequent use of arbitrary geographical extents (Acevedo et al. 2017). The procedure firstly delimits the Extent of Occurrence or the accessible area for each species using a convex hull, an α-shape or a Kernel density distribution. In this study, three separate delimitation methods were used to determine the accessible area, which included different α values, in the case of α-shapes and several smoothing values, in the case of Kernel

densities. Subsequently, the 19 bioclimatic variables of the WorldClim 1.4 database representing average climate data for the 1960–1990 period (www.worldclim.org; Hijmans et al. 2005) were used in ModestR to estimate the environmental values of the occurrence records. Future bioclimatic data of the RCP 4.5 and RCP 6.0 Representative Concentration Pathways (RCPs) coming from the same source were also used. We did not include other important variables in our analysis, such as catchment area, connectivity with other catchments, intensity of human activities etc., due to the worldwide scale of this study and the well-known relevance of climate variables in explaining large-scale species distributions (Pearson and Dawson 2003). From the available bioclimatic variables, only those that best explained the distribution of freshwater fish species were used (Manjarrés-Hernández et al. 2018); those with a Variance Inflation Factor (VIF) of less than 20 were selected. The VIF quantified the multicollinearity of predictors (Guisande et al. 2011) and, in this case, the VIF value was selected by deleting those variables which displayed the most severe multicollinearity because we are only interested in estimating the explanatory capacity of each predictor. Consequently, the following 10 bioclimatic variables were finally selected: annual mean temperature (BIO1), mean diurnal range (BIO2), isothermality (BIO3), temperature seasonality (BIO4), mean temperature of the wettest quarter (BIO8), annual precipitation (BIO12), precipitation of the driest month (BIO14), precipitation seasonality (BIO15), precipitation during the warmest quarter (BIO18) and precipitation during the coldest quarter (BIO19). Elevation was also included as a predictor because it seems to be a temporary, invariable, but important factor affecting the distribution of freshwater fish (Manjarrés-Hernández et al. 2018). Appendix 3 in Suppl. material 4 details the procedure followed to include the WorldClim 1.4 database as a 3D set of variables in the ModestR software. All these variables were handled at a resolution of 5′ × 5′.

Subsequently, the relevance of these environmental variables to explain the distribution of each one of the species was estimated by using the Instability Index described by Guisande et al. (2017a) and Guisande (2018b) that does not require normalised data. Dividing each predictor into a number of intervals or bins determined by the user, the number of records in each bin was calculated considering separately the cells where the species occurs and those of the selected studied area. A peak of instability is observed when there are important differences in the predictor comparing the bins of presence with the corresponding ones of the study area. This index outperforms other methods proposed to identify the most appropriate environmental factors (Guisande et al. 2017a; Fan et al. 2018). The explanatory variables with the highest percentage contributions to the Instability Index would be those that most affect the distribution of the species in the accessible area. In order to include only those variables with a higher contribution, it is possible to select an accumulated percentage of contribution (the default option is 80%) so that, if 100% is selected, all variables will be included. It is important to highlight the fact that the software identifies the most important variables for each species and that these variables may vary between species.

Once the most important environmental variables which affect the distribution of each species in their accessible area were identified, all cells with environmental

conditions similar to those existing in the occurrence localities were delimited, thus predicting the changes in species diversity in the years 2050 and 2070, under the RCP 4.5 and RCP 6.0 climate scenarios. The most appropriate model outputs to be applied in NOO3D were selected by comparing estimated river basin species richness with the results of accumulation curves derived from occurrence records (Lobo et al. 2018). This entire process was automatically implemented in ModestR (see Suppl. Material 4).

Pelayo-Villamil et al. (2018) demonstrated that over 71% of countries worldwide have inventories of freshwater fish species that may be categorised as of poor quality. Furthermore, even those countries with relatively accurate reliable national inventories possess high degrees of variability in the completeness of their provincial or regional inventories. Therefore, species richness in river basins is doubtlessly underestimated when the raw geographical occurrence data are used. Species richness of freshwater fish in each river basin, then, was approximated with the *RWizard* (Guisande et al. 2014) application, *KnowBR* (Lobo et al. 2018; www.ipez.es/RWizard), also available as an R package on CRAN (Guisande and Lobo 2018). This application enables the use of information, which originates from exhaustive database records, to build species accumulation curves that describe the relationship between the accumulated number of species and a surrogate survey effort for different spatial units (such as cells, countries or river basins, as is the case here) simultaneously. These accumulation curves are adjusted to different asymptotic or quasi-asymptotic functions, in order to estimate the predicted number of species when the survey effort tends toward the infinite (Clench 1979; Soberón and Llorente 1993; Hortal and Lobo 2005). The default exact estimator was utilised to derive the accumulation curve and the Clench function applied for curve adjustment in order to estimate the potential number of species when the number of records tends toward the infinite. Occurrence records in level-two river basins (González-Vilas et al. 2016), which are available in ModestR (García-Roselló et al. 2013), were used as target spatial units. The species richness estimations obtained from *KnowBR* were considered to be the most probable species richness values present in the river basins and were subsequently compared to the species richness figures obtained after overlaying the outputs of the individual species models created by the aforementioned modelling procedure.

The final consequence of all this process is a geographic representation of each species' distribution area both for present and future scenarios, taking into account the climatic conditions of the observed occurrences. In this manner, the most probable future distribution of each species was estimated according to the information derived from current occurrences and in accordance with the values of the environmental variables in potential future scenarios (see García-Roselló et al. 2019). For those species with few records, for which it was not possible to estimate the probable distribution following the formerly mentioned procedure, the ModestR software was used to verify whether the current environmental conditions where the species is present will be available under future scenarios. In the case that the current environmental conditions in which the species are present were unavailable under future scenarios, the species was considered to be locally extirpated. We must emphasise that the performance of NOO3D relies on two fundamental assumptions: i) that current geographical occurrences can

infer suitable environmental conditions for each species, at least partially and ii) that these environmental conditions are the main drivers of species distribution or act as surrogates for the true causal variables. Presence is very frequently the only available empirical evidence that provides an image of the environmental conditions in which each species is able to maintain sustainable populations. Hence, transfer to other temporal scenarios in the climatic or environmental ranges obtained from observed occurrences must be considered provisional map representations, which are highly dependent on their capacity to colonise distant, but suitable, localities. Therefore, our study strives to describe the future location of areas which are accessible to the considered species and which will have climatic conditions similar to those in which the species is observed in the present day (Lobo 2016), while excluding the role played by microevolutionary adaptations.

River basins as spatial units for the estimation of diversity

The geospatial data for river basins imported into ModestR was obtained from the WaterBase project website (http://www.waterbase.org). WaterBase global river basin data were taken from the drainage basin dataset distributed with HYDRO1k, a hydrological database developed by the EROS Data Center of the U.S. Geological Survey (USGS). This database provides a collection of global geo-referenced layers at a 1 km resolution derived from GTOPO30, a 30 arc-second digital elevation model (DEM) of the world. The drainage basins dataset from HYDRO1k was projected on to latitude/longitude geographical coordinates. Vertices were smoothed by applying a 500 m threshold in order to generate the ESRI Shape files available via the WaterBase website.

The river basins dataset was originally obtained by combining flow accumulation and flow direction layers, which were, in turn, derived from the hydrologically-corrected DEM, based on the GTOPO30 dataset. The basins were organised according to the procedure first proposed by Pfafstetter (1989) and later adapted for use in the HYDRO1k dataset (Verdin and Greenlee 1998). River basins were divided into six levels and each sub-basin was identified using a unique Pfafstetter code, (i.e. a six-digit code with information about the interconnections of the basins).

We used level-two of the river basins data set (González-Vilas et al. 2016) as the spatial unit for the estimation of the diversity, because this level is the geographical extent that best illustrates the effect of environmental parameters on the distribution of freshwater fish species (Manjarrés-Hernández et al. 2018).

Diversity indices and statistical analysis

For each river basin, both in present and in future projections (2050 and 2070), according to the results obtained for the RCP 4.5 and RCP 6.0 scenarios, several diversity indices were estimated. The DER function from the R package *EcoIndR* (Guisande et al. 2017b; Guisande 2018a) is used for this purpose, which allows the calculation of 31 different indices for each river basin belonging to five categories: rarity (two indices),

heterogeneity (14 indices), evenness (seven indices), taxonomic diversity (two indices) and functional diversity (six indices; see table 1 in Guisande et al. 2017b). Since there is no index universally applicable to all ecological assemblages (Magurran 2004), the DER algorithm was also employed for the selection of the diversity indices that best showed the differences between river basins. Therefore, from all of the estimated indices, only one of each of category was chosen.

All statistical analyses were run with the *RWizard* application *StatR* (Guisande et al. 2014). Stepwise multiple regressions were performed with the stats R package (R Development Core Team 2018). A backward-forward approach, based on Akaike Information Criterion (AIC), was used to select the most parsimonious model. The relative contribution of each variable in the regressions was estimated with the LMG method (the r^2 contribution averaged over orderings amongst regressors) with the R *relaimpo* package (Grömping 2006, 2018). The *dwtest* function from the *lmtest* package was utilised (Hothorn et al. 2018) to estimate autocorrelation with the Durbin-Watson statistic (Durbin and Watson 1951).

Results

As expected, the regression slope between river basin species richness, estimated with accumulation curves and observed species richness, obtained from records (Fig. 1, red line) is significantly lower than one (ANCOVA, $P < 0.001$), with a value of 0.81. This means that river basin species richness is underestimated when using the available raw data and that the under-prediction is greater when basin species richness is high. This is likely due to the poor quality of existing inventories in many countries, especially in tropical areas which host a higher number of species (Pelayo-Villamil et al. 2018).

Amongst all of the procedures used to select the accessible area in the modelling procedures (convex hull, alpha shape with different α values, Kernel density with different smoothing values etc.), the model displaying the best fit when compared with the species richness estimated from accumulation curves was generated with a Kernel density estimator using a smoothing value of two. The intercept of this relationship was not significantly different from zero (ANCOVA, $P = 0.292$) and the slope was not significantly different from one (ANCOVA, $P = 0.512$) with a value of 0.99 (Fig. 1, blue line). As a result, the species richness data predicted with the kernel density estimator were selected to be imported into the temporal transfer modelling procedure, because the species richness was quite similar to that obtained from the accumulation curves.

The decline in species richness was very similar between scenarios RCP 4.5 and RCP 6.0. These models predicted the complete disappearance of the distributional areas of half of all freshwater fish species (from 45.3% to 46.7%, independent of the year or the climatic scenario). As an example of the predicted decline, Fig. 2 shows the predicted change in species richness in the year 2070, under the RCP 4.5 scenario. This decline was more pronounced in tropical river basins and was particularly high in Vietnam and south-eastern China (Fig. 2).

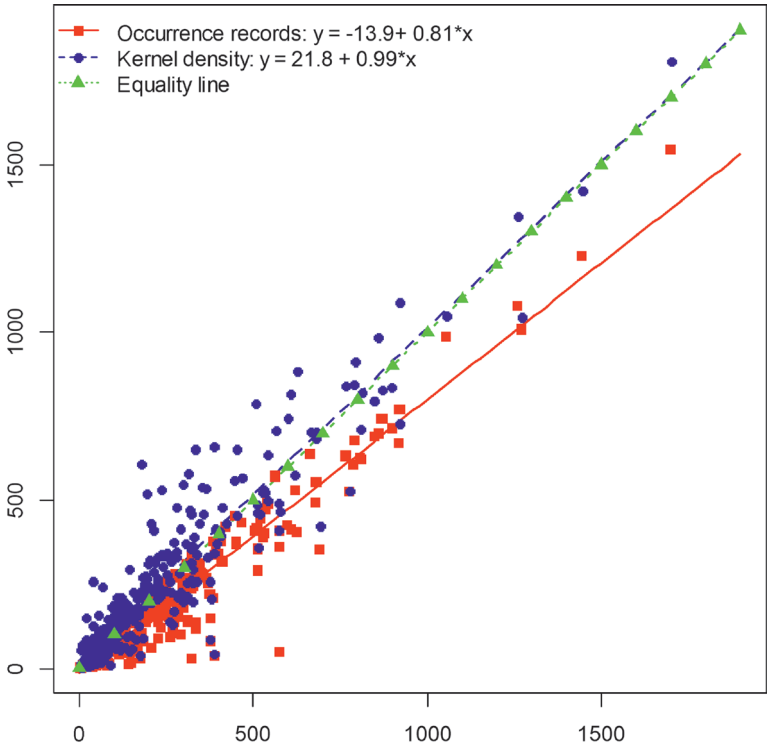

Figure 1. Relationships between the species richness in level-two river basins predicted by accumulation curves (abcissa), using the *KnowBR* package and those obtained with occurrence records (red) and after applying the proposed model approach with a Kernel density (smoothing value of 2) (blue; ordinate). Green line shows the 1:1 fit.

Fig. 3 shows the significant explanatory variables obtained from stepwise multiple regressions, considering the predicted change in species richness in the year 2070 (RCP 4.5 scenario). All the considered explanatory variables aggregated are able to explain 54% of the variance in the obtained species richness without showing a strong spatial autocorrelation (Durbin-Watson statistic 1.87, P = 0.047). The variable with the highest contribution was BIO12 (annual precipitation), so that species richness decline at higher rates in those river basins that currently have high annual precipitation values. The second variable in importance was isothermality in river basins (BIO3). Isothermality is a measure of the annual temperature range experienced on a daily basis, such that the decline was higher where current river basin temperature seasonality is high (Fig. 3).

The values of the different diversity components (richness, rarity, heterogeneity, evenness, taxonomic diversity functional diversity) were very similar between the two scenarios (Fig. 4A). The ratio of species richness in the years 2050 and 2070, as compared to the present were 0.80 and 0.78, respectively (Fig. 4). In other words, on average, in all river basins, approximately 20% of species are to be lost. Rarity was also lower in 2050

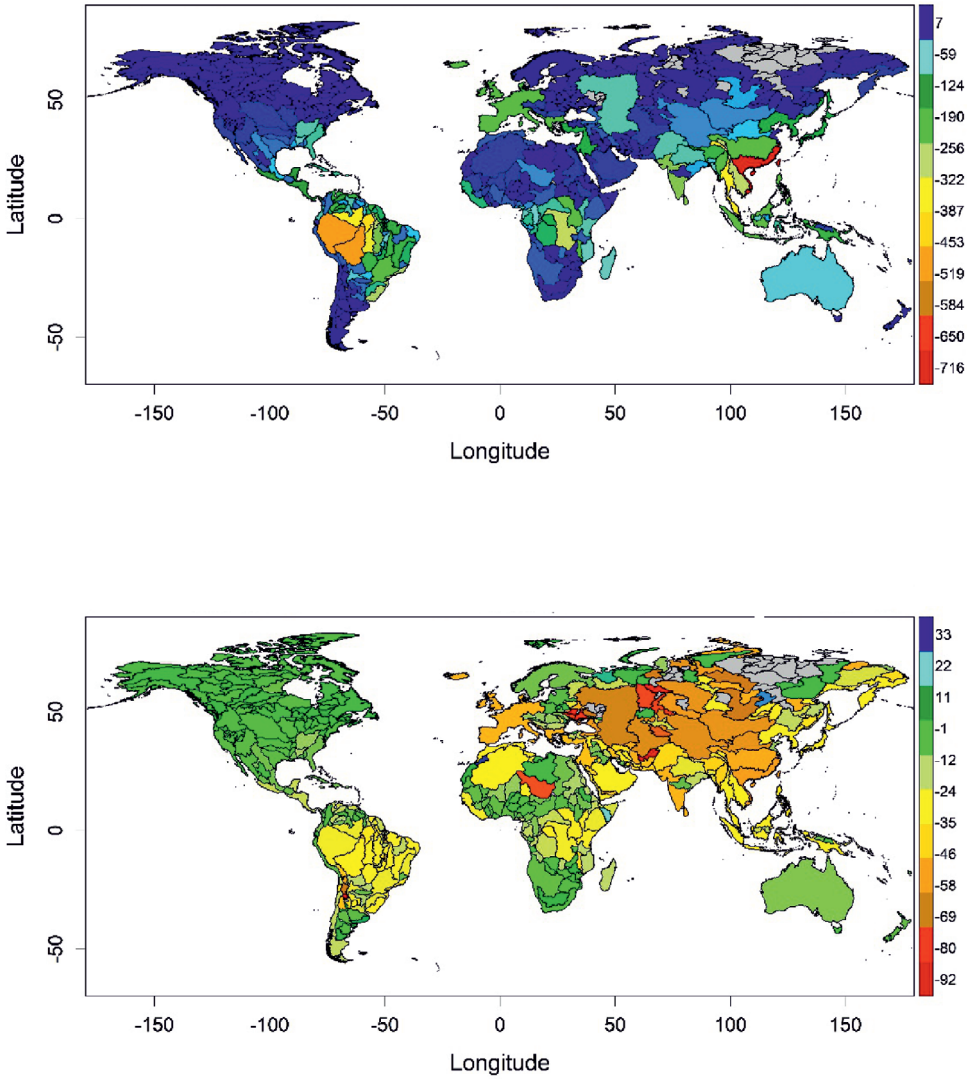

Figure 2. Predictions of the changes in species richness in river basins (in numbers in the upper panel and in percentages in the lower panel), by the year 2070 under the RCP 4.5 scenario, as compared to current species richness. The river basins with grey backgrounds had no records, no species and/or distribution model estimation was impossible. High negative values represent basins with high species extinction rates.

(mean values of 0.975 for RCP 4.5 and 0.973 for RCP 6.0) and 2070 (mean values of 0.973 for RCP 4.5 and 0.973 for RCP 6.0), as compared to the present (Fig. 4B), which means that the predicted species richness decline is partly due to the extinction of endemic or narrowly-distributed species. Heterogeneity was also lower in both years and in both scenarios, which displayed intermediate values for richness and evenness (Fig. 4C). This may be explained because heterogeneity is an index that combines the richness and

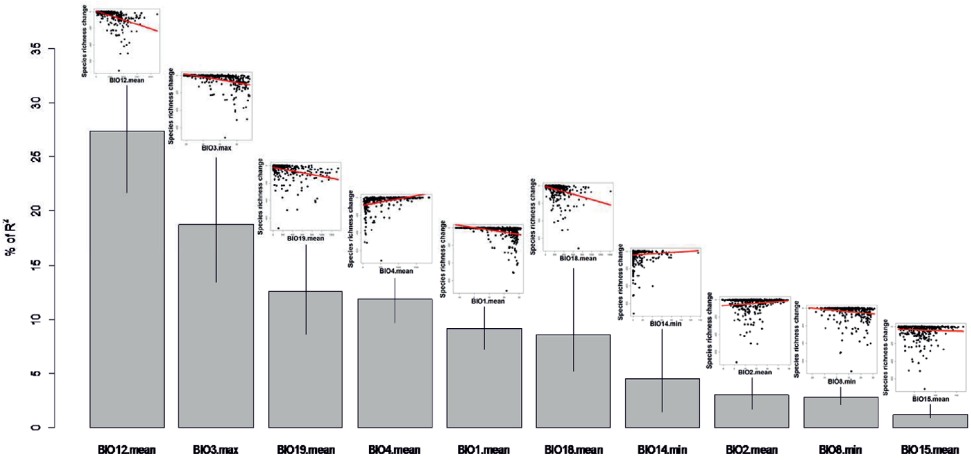

Figure 3. Relative contribution, with LMG method, of the significant climatic predictors obtained from a stepwise multiple regression, in which the dependent variable is the predicted change in species richness from the present to the year 2070 (RCP 4.5 scenario). The explanatory variables were the minimum, maximum and mean values of the climatic WorldClim variables mentioned in the Material and methods section, which were averaged for each level-two river basin. Plots above the bars show the relationships between the dependent variable and each one of the statistically-significant independent variables.

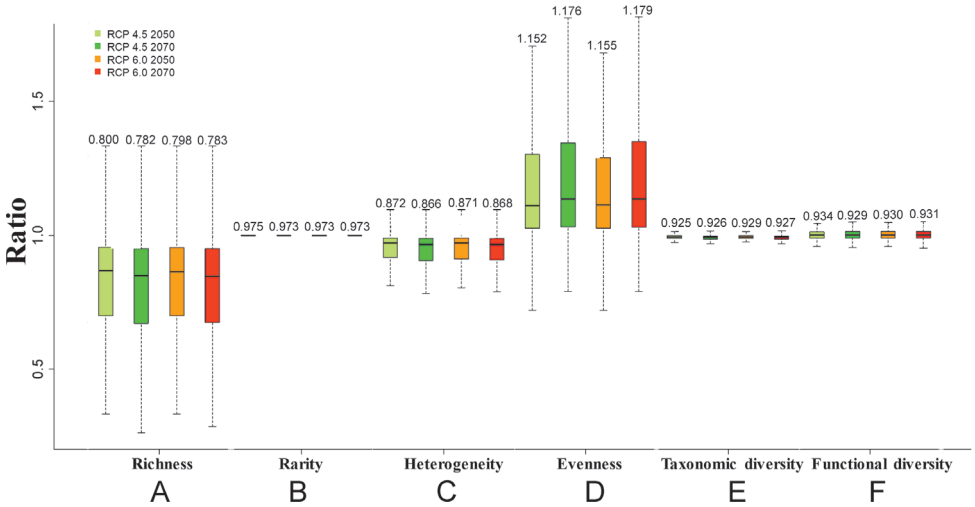

Figure 4. Boxplots of the rate of change in richness, rarity, heterogeneity (Shannon-Wiener), evenness (Simpson evenness), taxonomic diversity (taxonomic distinctness) and functional diversity (functional richness) in each river basin, as predicted for the years 2050 (RCP 4.5 scenario) and 2070 (RCP 6.0 scenario). A value less than 1 means that the Diversity Index is lower in the future scenarios than in the present and vice versa. Outliers are not shown in the boxplots. The numbers indicate median values for all river basins.

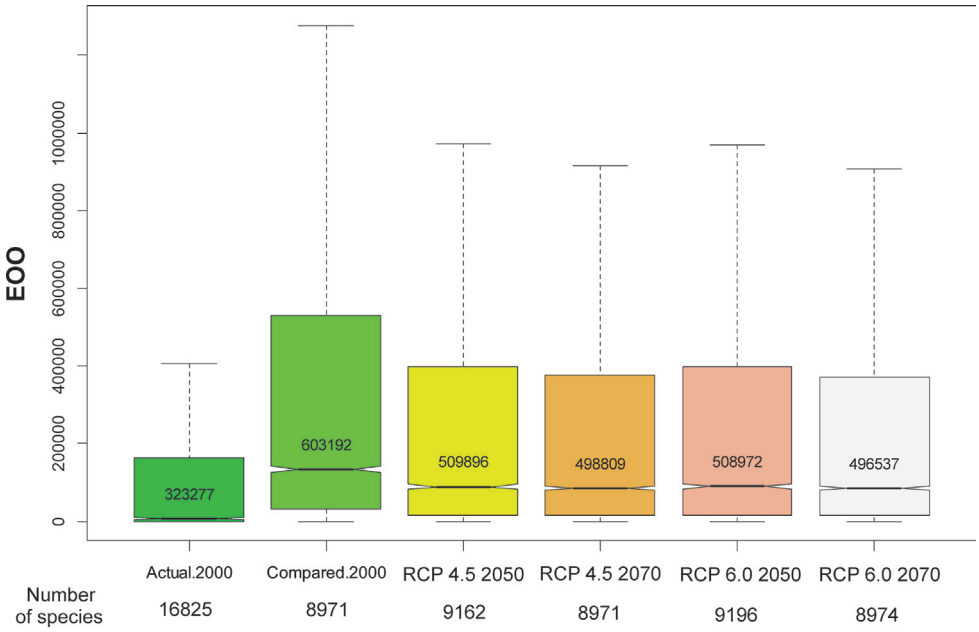

Figure 5. Boxplot showing the extent of occurrence (EOO, in km^2) of the species for each scenario and year. The numbers within each plot indicate mean EOO values for all species present in each scenario. The numbers of species predicted as present in each scenario are indicated in the x-axis. The category "Compared.2000" is the mean EOO of the species in the present, but only considering those species predicted as present in the scenario with a higher number of species projected to be extinct (RCP 4.5 2070). Notched box plots show median values (horizontal line), interquartile range values between upper and lower quartiles (top and bottom of the box), distribution of 99% of data (upper and lower dashed lines) and notch lengths representing classic 95% confidence intervals. Note that, when notches do not overlap, medians may be seen to differ significantly (Krzywinski and Altman 2014) and that the difference between "Actual.2000" and "Compared.2000" scenarios is due to the predicted disappearance of species in the future.

the proportional abundance of species (Magurran 2004). Evenness, however, increased in 2050 and 2070, as compared to the present (Fig. 4D). Finally, the predicted species richness decline also led to a reduction in river basin taxonomic diversity and functional diversity (Figs 4E, F).

Fig. 5 shows further changes in the EOO estimated by the species distribution models for 2050 and 2070 in both scenarios. In addition to the above-mentioned loss of species with small geographical ranges, our results predict a general decrease in species' distributional ranges. When the median EOO of the species, for both scenarios, is compared to the median EOO from the present, it is clear that most species lose their suitable distribution area in the future (Fig. 5). These reductions in the suitable distributional area were most pronounced in the tropical areas of South America and Africa (Fig. 6), whereas at medium latitudes (approximately between 10° and 50° both North and South), the mean EOO of the remaining species in river basins was higher (Fig. 6). Interestingly, this remarkable reduction in the distribution area would be affecting mainly those species with a smaller body size (Fig. 7).

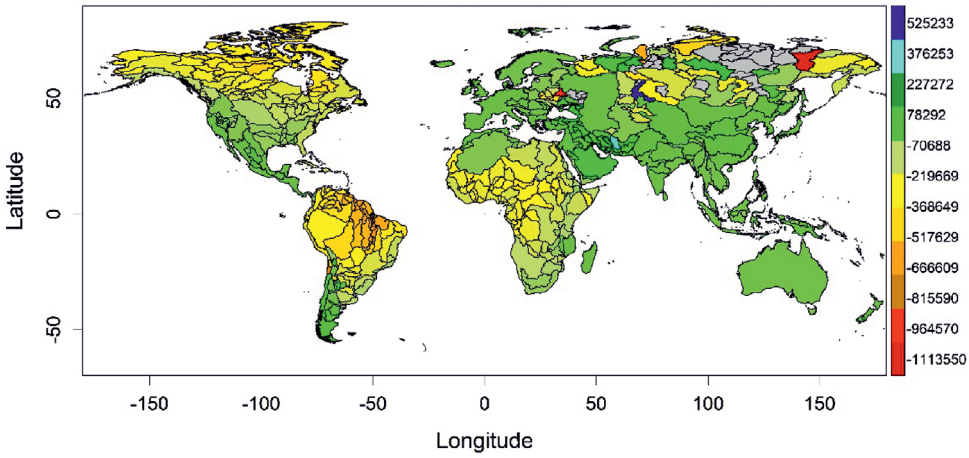

Figure 6. Predictions of the change in the Extent of Occurrence (EOO, mean value of all species present in the river basin in km^2) in river basins for the year 2070, with the RCP 4.5 scenario, as compared to the current species EOO. River basins with grey backgrounds had no records, no species and/or distribution model estimation was impossible.

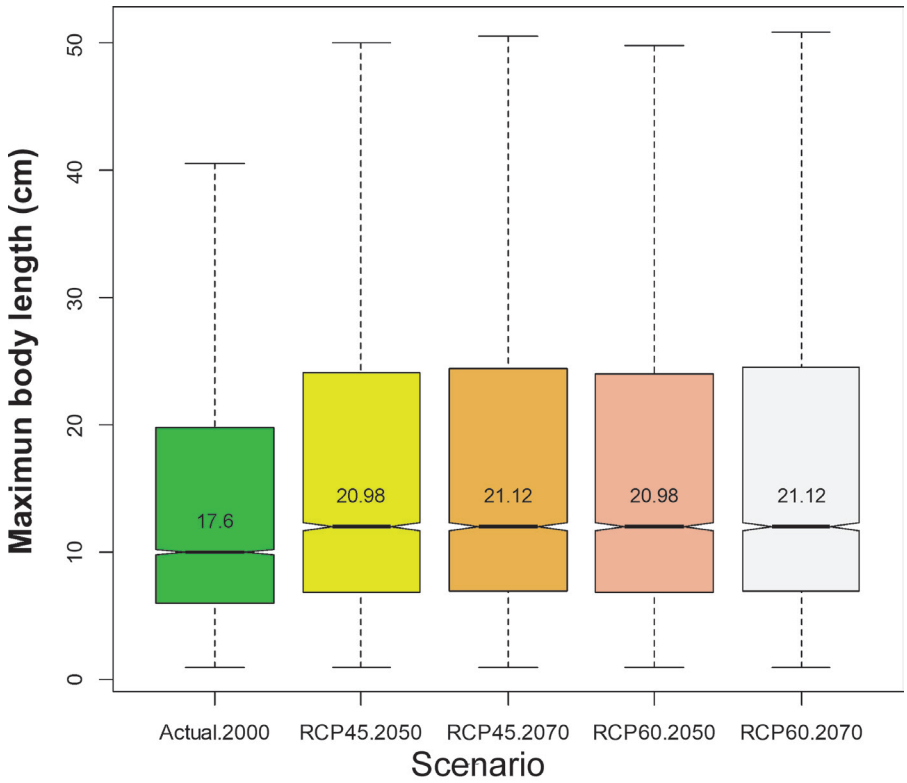

Figure 7. Boxplots with the value of the maximum body length (in cm) of species for each scenario and year. Outliers are not shown in the boxplot. The numbers indicate the mean values for all species present in each scenario.

Discussion

The comparison between the freshwater fish species richness scores, derived from accumulation curves and those generated by stacking individual SDMs, allowed the selection of the most appropriate geographical extent or accessible area (Acevedo et al. 2017), from which to derive predictions for the distribution of each species. Assuming that the congruence in the species richness estimations, provided by these two independent methods, is an indication of the accuracy of the models applied, the future geographical representations of each species were then used to estimate the potential decline and variation in the distributional area of freshwater fish. It is not possible, however, to check whether there would be changes in species composition, because the accumulation curves only provide information on species richness.

The predictions, provided by this study, are similar to those suggested by Xenopoulos et al. (2005) for freshwater fish species. However, the results obtained by Tedesco et al. (2013) contrasted greatly with the often-alarming predictions regarding climate change-driven extinction for freshwater fish (Xenopoulos et al. 2005). Furthermore, Tedesco et al. (2013) suggest that conservation action should be directed towards the current anthropogenic threats that have spurred and continue to drive, freshwater fish biodiversity loss. Although the capacity to anticipate the effects of climate change on species distributions is plagued with uncertainties (Lobo 2016), we consider that our results are consistent in suggesting the probable trajectory of freshwater fish diversity at a global scale. Future predictions are often hampered by insufficient knowledge about the effects of microclimatic variations, phenotypic plasticity, microevolution and new biological interactions, which alleviate or limit the long-term effects of the predicted climatic changes (Hoffmann and Sgro 2011; Maclean et al. 2015; Merlin et al. 2018). This would mean that the loss of diversity would be lower than shown by our correlative models. In our study, a high rate of species richness loss is predicted. We consider that this result may be partially explained by the wrong practice of extrapolating suitable areas beyond accessible river basins with current observed occurrences. Freshwater fish species are often poor dispersers (Shurin et al. 2009) and this dispersal constraint is supported by the general lack of capacity of freshwater fish species to track suitable climate conditions (Bush and Hoskins 2017; Comte and Grenouillet 2015) outside of their inhabited stream networks. This may be a consequence of river basin boundaries, which act as geographical barriers (Pelayo-Villamil et al. 2015). Thus, our previous selection of the accessible area for each species and the limitation of future predictions to these areas seem to be especially appropriate for the provision of reasonable projections. Species Distribution Models extrapolations to other time periods show, in general, that these forecasting exercises have limited capacities (Araújo et al. 2005; Maguire et al. 2016), probably as a consequence of the high number of non-climatic factors influencing species distribution and range limits (Ricklefs 2004). However, the capacity of predicting future species distribution should be higher when the ability of individuals to relocate is limited, as is the case of freshwater fish (Griffiths 2015). The vulnerability of freshwater fish communities to altered climatic patterns has been highlighted by their

isolation and fragmentation within river basins, where they act as biogeographic islands (Gozlan et al. 2010; Olden et al. 2011). In the case of freshwater fish, then, we suggest that forecasting their future spatial distribution could be facilitated by their dispersal limitations, obviating exceptions of biological invasions with human help.

The estimated predicted loss of species richness may also be attributed to the inclusion of poorly-studied tropical areas, which support elevated levels of endemism. Such endemic species may be less likely to adapt to climate change (Thieme et al. 2010). Recent findings indicate that freshwater fish species from lower latitudes and tropical warm-water habitats are at greater risk under climate change situations (Comte and Olden 2017; Payne and Smith 2017; Jarić et al. 2019). Furthermore, the results obtained suggest that the rate of extinction would be higher for species with smaller body size and/or geographical range, in agreement with previous findings (Ficke et al. 2007; Chessman 2013; Jarić et al. 2019).

These results support the species-energy theory (Hawkins et al. 2003), which predicts that cool-temperate regions experiencing warming conditions, as well as very dry regions facing increased moisture availability, are both expected to exhibit richness increases. Our models predict a higher species richness decline in those river basins with greater precipitation and also in those located in warm regions (see Figs 3, 4). The great importance of the variables related to precipitation in accounting for temporal species richness variations, in our study, differs somewhat from the results provided by other worldwide research (Buisson et al. 2008; Graham and Harrod 2009; McCullough et al. 2009), which has strongly implicated temperature warming as the major driver that will generate future changes in freshwater fish assemblages. However, our results are in agreement with those studies carried out in warmer areas (Morrongiello et al. 2011). This key difference likely reflects the primary focus of many of the accomplished studies from northern hemisphere or high-latitude freshwater environments, whereas this study was performed at the global scale.

There is an important gap, which was not considered in this study: the effect of changing flow regimes on freshwater fish diversity (Döll and Zhang 2010; Döll and Bunn 2014). The climatic variables related to precipitation (BIO12, BIO15, BIO18 and BIO19) could be considered proxies of river flow measurements (Heino et al. 2009). However, changes in seasonality or high/low flows may be not fully explained by the climatic variables applied here. One third of total fish species and one fifth of the endemic fish species in Africa occur in eco-regions that may experience a change in discharge or runoff of over 40% by the 2050s (Thieme et al. 2010). Moreover, there may also be synergies created between climatic and non-climatic anthropogenic stressors, such as deforestation, overexploitation, habitat degradation and modification, water pollution, flow modification and hydropower generation (Döll and Zhang 2010).

In addition to the effect of climate change on species richness and geographic species range size, the climate-induced changes in taxonomic diversity observed in the present study, which have rarely been addressed for freshwater fish (Buisson and Grenouillet 2009), may result in changes in biotic interactions (competition, predation etc.) and compositional changes (Comte et al. 2013). In any case, these potential changes in

biotic interactions, which may affect species distribution, do not invalidate the use of purely climate-based model estimations for the quantification of the probable impacts of climate change on species distributions (Araújo and Luoto 2007), as shown herein. In conclusion, our results suggest a deep change in the basin assemblages of freshwater fish with the extinction of almost half of current freshwater fish species in the coming decades. This alarming prediction would particularly affect tropical regions and areas with changing water flow regimes by the greater extinction likelihood of those species with smaller body size and/or limited geographical ranges.

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Supplementary material 1

Appendix 1

Authors: Ana Manjarrés-Hernández, Cástor Guisande, Emilio García-Roselló, Juergen Heine, Patricia Pelayo-Villamil, Elisa Pérez-Costas, Luis González-Vilas, Jacinto González-Dacosta, Santiago R. Duque, Carlos Granado-Lorencio, Jorge M. Lobo

Data type: data occurrences

Explanation note: Sources describing all the data downloaded from the Global Biodiversity Information Facility (GBIF; see https://www.gbif.org/), which were used in this study.

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Link: https://doi.org/10.3897/natureconservation.43.58997.suppl1

Supplementary material 2

Table S1

Authors: Ana Manjarrés-Hernández, Cástor Guisande, Emilio García-Roselló, Juergen Heine, Patricia Pelayo-Villamil, Elisa Pérez-Costas, Luis González-Vilas, Jacinto González-Dacosta, Santiago R. Duque, Carlos Granado-Lorencio, Jorge M. Lobo

Data type: species data

Explanation note: Description of the species included in the analysis, as well as future species predictions in 2050 and 2070 and under both scenarios. If a species is categorised as "NOT", the complete disappearance of their distributional area is predicted. The maximum body length of each species is also included, obtained from https://www.fishbase.org/ or from the original manuscripts, when not available at FishBase.

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Link: https://doi.org/10.3897/natureconservation.43.58997.suppl2

Supplementary material 3

Appendix 2

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Data type: species data

Explanation note: Biological traits assigned to each one of the considered species. We used 6 traits divided into three biological functions: food acquisition, life habitat and locomotion. Food acquisition traits include the feeding habitat (pelagic, benthopelagic and benthic) and the trophic guild (primary consumer, secondary consumer, top-predator, omnivorous and detritivorous). Life habitat traits comprise habitat type (pelagic, benthopelagic and demersal) and migration type (potamodromous, anadromous, catadromous, amphidromous, oceanodromous and no migration). Finally, locomotion traits include body length (in cm: small < 15, medium 15–50, large 50–150 and extra-large > 150) and rheophily (rheophilic, limnophilic and eurytopic).

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Supplementary material 4

Appendix 3

Authors: Ana Manjarrés-Hernández, Cástor Guisande, Emilio García-Roselló, Juergen Heine, Patricia Pelayo-Villamil, Elisa Pérez-Costas, Luis González-Vilas, Jacinto González-Dacosta, Santiago R. Duque, Carlos Granado-Lorencio, Jorge M. Lobo

Data type: tutorial

Explanation note: Full description of the NOO3D procedure followed to predict the future distribution of world freshwater fish.

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The illegal exploitation of the Javan Leopard (*Panthera pardus melas*) and Sunda Clouded Leopard (*Neofelis diardi*) in Indonesia

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Abstract

Indonesia is home to the Javan Leopard (*Panthera pardus melas*) and the Sunda Clouded Leopard (*Neofelis diardi*), both of which are threatened by habitat loss, human-wildlife conflict issues and the illegal wildlife trade. Leopards and clouded leopards are threatened by the illegal wildlife trade across their range, however, very little is known of the illegal trade in these two species in Indonesia, or of the efforts made to tackle this crime. Both the Javan Leopard and Sunda Clouded Leopard are protected species in Indonesia and both species are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), meaning commercial international trade is generally prohibited. To better understand the trade, and efforts to end this trade, we collected records of seizures and prosecutions relating to Javan Leopards and Sunda Clouded Leopards in Indonesia for the period 2011–2019. Despite both species being protected by law, this study reveals the prevalence of extensive poaching, illegal domestic trade and international trafficking of both species. A total of 41 seizure records were obtained from 2011 to 2019, which was estimated to amount to approximately 83 animals, which likely represents only a fraction of the total number of cases and therefore the risk may be substantially greater. Approximately half of the cases resulted in successful prosecution and of these, the highest sentence given was 2 years in jail and a fine of IDR50mil (~USD3300). The majority of the penalties handed down for these crimes were far below the maximum potential penalties and are unlikely to be effective deterrents.

Keywords

Big cats, conservation, crime, Java, poaching, wildlife trade

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Introduction

The Indonesian archipelago is made up of a diverse landscape that supports a large proportion of the Earth's fauna and flora including a high number of endemic species (Meijaard et al. 2005; von Rintelen et al. 2017). The country has extremely high biodiversity and for precisely this reason, it is also one of the most significant illegal wildlife trade hubs globally (Samedi and Iskandar 2000; Shepherd 2010; Lyons and Natusch 2012; Chng and Eaton 2016; Gomez and Shepherd 2019). Indonesia is both a source and end use destination for a wide range of species, many of which are threatened with extinction (Samedi and Iskandar 2000; Shepherd and Magnus 2004; Meijaard et al. 2012; Shepherd et al. 2015; Morgan and Chng 2017; Gomez et al. 2017; Nijman et al. 2018). Further rare, endemic and range-restricted species are frequently in high demand in the international commercial market for exotic pets (Courchamp et al. 2006; Nijman and Stoner 2014; Janssen and Shepherd 2018). Two species of high conservation concern that continuously feature in seized wildlife shipments in Indonesia are the Javan Leopard (*Panthera pardus melas*) and the Sunda Clouded Leopard (*Neofelis diardi*), though there is very little documentation or published literature pertaining to their exploitation. Both species are assessed by the IUCN Red List of Threatened Species (hereafter referred to as the Red List) as being threatened with extinction and have a restricted range, making them extremely vulnerable to exploitation.

The Javan Leopard is considered one of the most threatened subspecies of Leopard (Wibisono et al. 2018) and among the most threatened of all big cats. Endemic to the island of Java in Indonesia, deforestation and habitat conversion have significantly depleted wild populations and greatly reduced the species range which is now limited to fragmented and isolated pockets of forested areas around the island (Wibisono et al. 2018; Stein et al. 2020). According to Wilting et al. (2016), only 5% of the island is actually habitable for the species. The conservation status of this species was last assessed in 2008 as Critically Endangered by the Red List, with fewer than 250 mature breeding adults and reported populations in decline (Stein et al. 2020). The main threats to the species are habitat loss and prey base depletion (Stein et al. 2020) which is resulting in increasing human conflict as Leopards prey on livestock or pets (Partasasmita et al. 2016; Wilting et al. 2016).

The Sunda Clouded Leopard is only found on the island of Borneo (encompassing Brunei, the Malaysian states of Sabah and Sarawak, and Kalimantan, Indonesia) and the island of Sumatra, Indonesia. The species was recognised as a separate species from the Clouded Leopard *N. nebulosa*, in 2008 based on distinct differences in molecular and morphological data (Buckley-Beason et al. 2006; Kitchener et al. 2006). Wilting et al. (2011), further designated populations of Sunda Clouded Leopard on Borneo and those on Sumatra as two distinct subspecies i.e. Bornean Clouded Leopard *N. d. borneensis* and Sumatran Clouded Leopard *N. d. diardi*. On a global scale the species is assessed as Vulnerable by the Red List, with populations reported as declining across their range predominantly due to forest loss and degradation (Hearn et al. 2015), but on a subspecies level, both are classified as Endangered (Hearn et al. 2008; Sunarto

et al. 2008). Poaching is also noted as a threat in some parts of its range (Hearn et al. 2016), and it is suspected that poaching for commercial trade in Indonesia may be a key threat.

The "Act of the Republic of Indonesia No.5 of 1990 concerning conservation of living resources and their ecosystems", widely known as the "Conservation Act (No.5) 1990", is the principal legislation pertaining to the regulation of wildlife trade in Indonesia. Under this Act, species are categorised as either "Protected" or "Unprotected". Protected species are listed under "Government Regulation No.7, 1999, Concerning the preservation of flora and fauna". Protected species are not allowed to be caught, injured, killed, kept, possessed, cared for, transported, or traded whether alive or dead. Exceptions in this regard are permitted by the Government for the purposes of research, science and/or safeguarding a species. Violation of this Act can result in imprisonment for a maximum of five years and a fine of up to IDR100 million (~USD6700). Both the Javan Leopard and Sunda Clouded Leopard are classified as Protected species in Indonesia. Under "Government Regulation No. 8, 1999 concerning the utilization of wild plants and animals", the trade of a Protected species is permitted if the specimens are captive-bred. Captive-bred animals are subject to regulations under the "Decree of the Ministry of Forestry, No.P.19/Ministry of Forestry-II/2005 concerning captive management of wild plant and animal species" and Article 10 in "Government Regulation No. 8, 1999", defines that only second and subsequent generations of captive-bred Protected animals may be traded, and that all breeders must be registered with the Ministry of Environment and Forestry's Department of Forest Protection and Nature Conservation (KKH) (for exporters) and Nature Conservation Agency (BKSDA) (for breeders supplying to exporters, but not exporting themselves). At an international level, both species are also listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which means any commercial international trade in wild animals is effectively prohibited.

In this study, we examined seizure data involving these two species in Indonesia to gain an understanding of the extent of the trade and of the efforts being made to tackle this crime. We provide documented evidence of the trade which can be used to better assess the conservation status of remaining populations and identify appropriate measures to mitigate illegal exploitation of both species.

Methods

We collected records of seizures and prosecutions relating to Javan Leopards and Sunda Clouded Leopards in Indonesia for the period 2011–2019. Data were extracted from various sources including from media reports, published literature and the government website, Sistem Informasi Penelusuran Pekara (SIPP) (an open access information database of the courts for each district). Online searches for related seizures were conducted in both English (search terms: Javan Leopard, Sunda Clouded Leopard, leopard followed by seizures, hunting, killing, trade) and Indonesian (search

terms: macan, macan tutul, macan dahan, neofelis, macan kumbang and panthera). We only included records where Indonesia is reported as the location of the seizure in the analysis or reported as the origin of shipment seized elsewhere. All reported seizures and prosecutions were carefully checked to avoid duplication.

From each record, we extracted information on date of seizure, species seized (assumed to be accurately reported), commodity (live animals, paws, skull, skin, etc), quantities of each commodity, purpose of hunting/trade (i.e. for consumption, pets, trophies, etc), location of seizures and trafficking routes, suspects arrested and prosecution outcomes. Using the seizure data, we mapped important trade hubs and centres where trade exists. We have estimated a minimum number of Javan Leopards and Sunda Clouded Leopards recorded in trade from commodities seized, by either counting whole or near-whole specimens seized (e.g. live animals, skins), or by tallying quantities of body parts seized (e.g. claws, teeth, paws, skull) that form one whole individual per seizure record. In terms of tallying body parts, a leopard is naturally assumed to have four paws, 18 claws and four canines.

Due to inherent biases in the way seizure data are reported (given varying levels of law enforcement, reporting and recording practices, language biases, etc.), this dataset is interpreted with caution. Reported seizures are likely to represent only a fraction of the illegal trade and so underestimate its full extent (Burgess et al. 2014; Nijman 2015). As such, the dataset presented here is not to be assumed as representing absolute trafficking trends or volumes.

Results

A total of 41 seizure records were obtained involving Javan Leopards and Sunda Clouded Leopards in Indonesia from 2011 to 2019, which was estimated to amount to approximately 83 animals (i.e. 51 Javan Leopards; 32 Sunda Clouded Leopards). There were no seizure data found for 2013. At least 3 of these incidents were considered retaliatory killings of Javan Leopards that had entered villages or preyed on livestock and pets. The majority of obtained seizure records involved the Javan Leopard with 24 incidents involving an estimated 51 animals while the Sunda Clouded Leopard was reported in 18 incidents involving an estimated 32 animals. The greatest number of seizures were recorded in 2019 (10 seizures) followed by 2015 (9 seizures) and 2018 (8 seizures) (Fig. 1).

The most frequent commodity seized throughout the study period were skins (seized in 18 incidents which encompassed whole specimens, pieces or items for which skin had been used in accessories e.g. bag, hat and Reog art (used in traditional Indonesian dance) (Table 1, Fig. 1). This was followed by the seizure of live animals (9 incidents) and taxidermy products (8 incidents). The most abundant commodity seized were canines amounting to 30 pieces in 6 incidents that occurred in 2018 and 2019. Overall, body parts consisting of canines, claws, paws, skin and skulls along with taxidermy products made up the greatest percentage of commodities seized (Fig. 1).

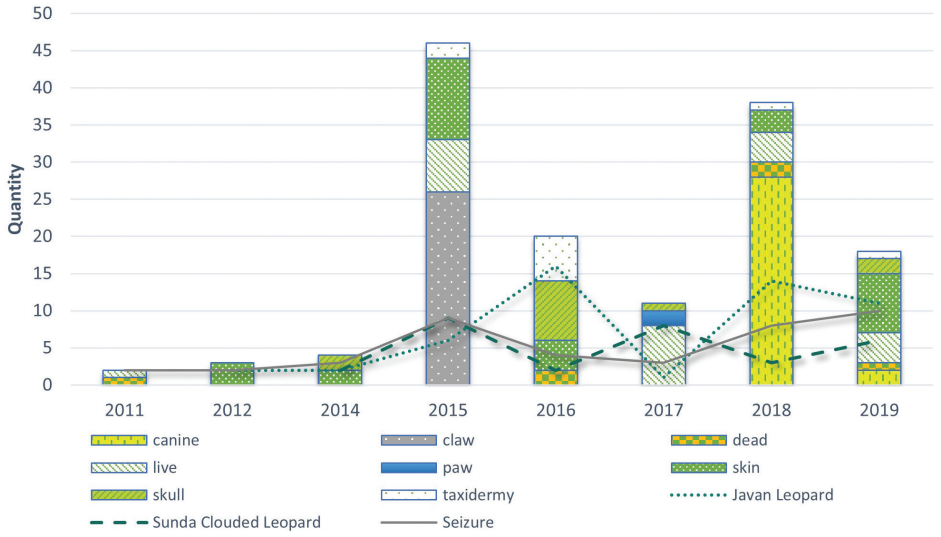

Figure 1. The number of seizures (grey line) obtained for Indonesia that involved either the Javan Leopard or the Sunda Clouded Leopard in Indonesia from 2011 to 2019 (no data for 2013) based on data extracted from media sources, published literature, CITES Trade Database, Indonesian government website (SIPP), etc., including various commodities seized (e.g. live, skin, canines, claws, taxidermy, etc) (bar graph) with an estimated number of both leopard species involved based on commodities seized per record (dotted lines).

Table 1. The various types of commodities from Javan Leopard and Sunda Clouded Leopard seized in Indonesia from 2011 to 2019 including the frequency of each commodity seized and quantities.

Commodity	Seizure (#)		Quantity	
	Javan Leopard	Sunda Clouded Leopard	Javan Leopard	Sunda Clouded Leopard
canine	3	3	20	10
claw	1	1	22	4
dead	3	2	4	2
live	3	6	6	17
paw	1		2	
skins				
– skin (pieces)	5	5	12	6
– skin (head)	1		2	
– skin (whole)	3	1	7	1
– other (bag, hat, Reog art)	2	1	2	1
skull	5		13	
taxidermied	5	3	7	3

All seizure records obtained, with the exception of 2, occurred in Indonesia (39 incidents) i.e. the islands of Java (18 incidents), Sumatra (18 incidents) and Kalimantan (3 incidents) (Fig. 2). The province of West Java and Jakarta, located on the island of Java, appear to be trade hotspots with 7 and 6 incidents obtained for each location respectively. That said, it should also be noted the lack of, or fewer, seizures in other locations could reflect poorer enforcement effort.

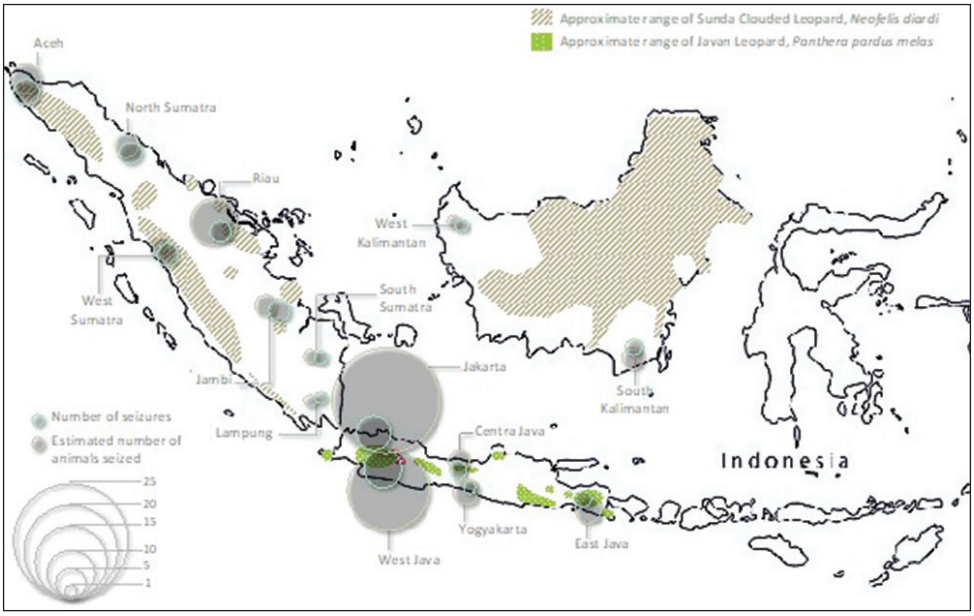

Figure 2. Location of seizure incidents involving Javan Leopard and Sunda Clouded Leopard that occurred in Indonesia from 2011 and 2019. Locations are grouped by provinces in Indonesia. A total of 41 seizure incidents were obtained of which 39 are mapped out above. The remaining two incidents occurred in Russia and UK respectively with origins reported as Indonesia. Approximate range for both species is extracted from the IUCN Red List of Threatened Species (Hearn et al. 2015; Stein et al. 2020).

The international trafficking of both species from Indonesia occurred in 3 incidents. Two incidents involved the Javan Leopard: one occurred in Russia and one in the UK but with origins of seized shipments reported as Indonesia. The seizure in the UK occurred in 2014 and involved 2 leopard skulls among various wildlife items seized while the incident in Russia occurred in 2015 involving various live animals from Indonesia including a Javan Leopard destined for the pet trade. The third incident occurred in Jakarta in 2015 involving a live Sunda Clouded Leopard ordered by a buyer in Kuwait.

At least 31 of the seizure incidents included multiple species seized along with Javan Leopard and Sunda Clouded Leopard, including live animals, body parts or taxidermy/ preserved animal parts. Tigers (*Panthera tigris*) (in 17 seizures) and Sun Bears (*Helarctos malayanus*) (in 13 seizures) were the most frequent species seized together with both species.

Of the 41 seizure records obtained, we could only find successful prosecution records for 20 cases (48.8%; 10 cases involving Sunda Clouded Leopard, 9 cases involving Javan Leopard, and 2 cases involving both species) involving 29 suspects (Table 2). The highest sentence given was 2 years in jail and IDR50mil (~USD3550) fine to a trader caught in 2018 for possession of 4 canines belonging to a Sunda Clouded Leopard and a bag made from the skin of a Sunda Clouded Leopard. Remaining convictions ranged from 1–1.6 years with fines (7 cases, 15 suspects) to <1 year with fines (11 cases, 12 suspects).

Table 2. The number of arrests and convictions (20 cases) involving Javan Leopard and Sunda Clouded Leopard in Indonesia, 2011–2019 based on the Indonesian government website (SIPP).

Date	Seizure location	Species	Commodity	Quantity	Other species seized	No. of suspects arrested	Prosecution
11-Dec 2011	West Sumatra	SCL	dead	1	None	1	9 months in jail + IDR5 million (~USD355) fine or additional 1 month in jail.
19-Dec 2012	Riau	JL	skin (pieces)	2	Tiger skins, Sun Bear head, deer skull, deer skin, goat head, monkey heads and Leopard Cat (*Prionailurus bengalensis*) heads	1	3 months in jail + IDR1 million (~USD70) fine or additional 1 month in jail.
3-Jan 2014	Aceh	SCL	skin (pieces); skull	1	body parts of Tiger, Golden Cat, Leopard Cat, Sumatran Serow (*Capricornis sumatraensis*), hornbill, Sun Bear	2	1 year in jail + IDR10 million (~USD700) fine or additional 4 months in jail
20-May 2014	West Kalimantan	SCL	skin (pieces)	1	body parts – species not specified	1	2 months + 10 days in jail + IDR500 thousand (~USD35) or additional 1 month in jail
6-Nov 2015	Jakarta	SCL	live	1	live gibbon, birds of paradise, Sun Bear	5	1.6 years in jail + IDR10 million (~USD700) fine or additional 3 months in jail – 4 SUSPECTS 1.3 years in jail + IDR10 million (~USD700) fine or additional 3 months in jail – 1 SUSPECT
14-Oct 2015	Lampung	SCL	skin (pieces)	1	Sun Bear, Leopard Cat	3	1.6 years in jail + IDR50 million (~USD3550) fine or additional 1 months in jail – 1 SUSPECT 1 year in jail and IDR50 million (~USD3550) fine or additional 3 months in jail – 2 SUSPECTS
16-Sep 2015	West Sumatra	SCL	claw	4	body parts – species not specified	1	6 months in jail + IDR100 million (~USD7100) fine or additional 1 month in jail.
30-Jul 2015	West Java	JL	claw; skin (pieces); leopard skin hat	22; 5; 1	body parts of marine turtles, deer, Tiger, mouse deer, Sunda Pangolin (*Manis javanica*), Asian Elephant (*Elephas maximus*), Sun Bear, birds of paradise	1	3 months in jail + IDR1 million (~USD70) fine or additional 1 month in jail.
15-Jan 2016	Jakarta	JL SCL	skin (whole); skin (pieces), taxidermy; skulls taxidermy	1; 3; 3; 8 1	taxidermy parts of Sumatran Serow, deer, Sun Bear, python, Tiger, Golden Cat (*Catopuma temminckii*), birds of paradise, parrots, eagle and Bali Starling (*Leucopsar rothschildi*)	1	1 year in jail + IDR50 million (~USD3550) fine or additional 1 month in jail.
2-Aug 2016	Jambi	SCL	taxidermy	1	taxidermy parts of Sunda Pangolin, Leopard Cat, Golden Cat, Chital (*Axis axis*), Sambar Deer (*Rusa unicolor*), Tiger	2	8 months in jail + IDR1 million (~USD70) fine or additional 1 month in jail – 1 SUSPECT No SIPP record for SUSPECT 2
3-Oct 2017	South Kalimantan	JL	head; paw	1; 2	None	1	2 years in jail + IDR50 million(~USD3550) fine or additional 3 months in jail*
22-Feb 2017	Riau	SCL	live	7	Black Eagle (*Ictinaetus malaiensis*), langurs, otters	2	1.2 years in jail + IDR25 million (~USD1700) fine or additional 1 month in jail – 1 SUSPECT 8 months in jail + IDR50 million (~USD3550) fine or additional 2 months in jail – 1 SUSPECT

Date	Seizure location	Species	Commodity	Quantity	Other species seized	No. of suspects arrested	Prosecution
29-Jan 2018	North Sumatra	SCL SCL	canine leopard skin bag	4 1	body parts of Tigers, Sun Bears	1	2 years in jail + IDR50 million (~USD3550) fine or additional 2 months in jail.
11-May 2018	South Kalimantan	SCL	canine; skin (pieces)	5; 2	Sun Bear, deer	1	1 year in jail + IDR5 million (~USD355) fine or additional 2 months in jail.
11-Apr 2018	Jakarta	JL	canine	18	body parts of Sun Bear, Tiger, Lion	1	9 months in jail + IDR50 million (~USD3550) fine or additional 1 month in jail
21-Nov 2018	West Java	JL	canine	1	body parts of Tiger, elephant accessories	1	8 months in jail + IDR10 million (~USD700) fine or additional 1 month in jail.
23-Apr 2018	Jambi	JL	taxidermy	1	taxidermy parts of Tiger, Sun Bear, Common Muntjac (*Muntiacus muntjac*), Binturong (*Arctictis binturong*), deer	1	1 month in jail + IDR5 million (~USD355) fine or additional 1 month in jail.
5-Aug 2019	Yogyakarta	JL SCL	skin (whole); skull canine	2; 1 1	Reog art made from Tiger skins, peafowl feathers, deer antlers	1	1.6 years in jail + IDR2 million (~USD140) fine or additional 2 months in jail.
29-Nov 2019	Central Java	JL	skin (pieces); skull	1; 1	live peafowl	1	9 months in jail + IDR500 thousand (~USD35) or additional 1 month in jail.
30-Oct 2019	Central Java	JL	canine	1	body parts of babirusa (*Babyrousa* sp.), deer, hornbill, Tiger, Sun Bear	1	7 months in jail + IDR10 million (~USD700) fine or additional 1 month in jail.

Note: JL: Javan Leopard, SCL: Sunda Clouded Leopard. *Sentence suggested by the court: the actual sentence given was unknown. Conversion to USD is based on exchange rate of USD1=IDR14,059 (https://www1.oanda.com/currency/converter/; 10 June 2020).

Discussion

Based on the analyzed seizure data from 2011 to 2019, there is a substantial illegal trade in the Javan Leopard and the Sunda Clouded Leopard in Indonesia. The trade in both species predominantly supplies a demand for wildlife trophies and ornaments as illustrated by the fact that mostly body parts, particularly skins, as well taxidermy products were seized. This corresponds to findings of Partasasmita et al. (2016) which revealed targeted hunting of the Javan Leopard by a village community in Sukabumi, West Java, due to an increased demand for animal skins and other valuable body parts, as well as other studies in Asia that reveal the availability of leopard skins of various species in wildlife markets (D'Cruze and Macdonald 2015; Nijman and Shepherd 2015; Ghimirey and Acharya 2020), indicating the demand for and their value as trophies/ornaments. In a study on the global trade in clouded leopards, Indonesia was frequently cited as a source country of concern particularly in reference to the trade in skins, meat and bones (D'Cruze and Macdonald 2015). Trade in body parts of both species could also be feeding a demand for traditional medicine. For example, leopard skin is used in Indonesia to treat skin disease (Partasasmita et al. 2016). In fact, much like the Tiger, almost every part of the leopard is used in traditional medicine – meat and bones are used to enhance male strength and virility, ash from burnt hair for foot and mouth disease, liver and bile as an antibiotic, brain for lung and heart disease, heart for asthma, while teeth, claws and tongue are prized as amulets/charms (Partasasmita et al. 2016). This raises further concerns as leopards are increasingly being used as substitutes for Tigers, which are locally extinct in many parts of their range and fast depleting in others (Raza et al. 2012). The data also revealed live individuals of both species exploited for the exotic pet trade (9 incidents and 23 animals, i.e., 6 Javan Leopards and 17 Sunda Clouded Leopards). Three of these incidents reported cubs seized, two of which occurred as recently as 2019 in Sumatra: one incident took place in Riau where two suspects were arrested for trying to smuggle one Javan Leopard cub, four Lion (*Panthera leo*) cubs and close to 60 Indian Star Tortoises (*Geochelone elegans*) and the other in Aceh, where a villager was caught trying to sell two Sunda Clouded Leopard cubs.

Aside from the clear intention of illegal exploitation of both species for trade, there were at least three cases involving the retaliatory killing of Javan Leopards that reportedly wandered into villages or preyed on livestock. All three incidents occurred in West Java involving at least seven animals. In two of the incidents, the leopards were either shot or poisoned, but the bodies were not found by the authorities (only pictures posted of the dead leopards). In the third incident, a leopard was caught by locals in the Ciamis-Garut area citing human-leopard conflict. When authorities came to retrieve the animal, the locals asked for money before they would hand over the animal. This had reportedly happened a few times in this area, and authorities were suspicious as this was generally coordinated by one individual person. These three incidents raise doubts as to the genuine nature of these conflicts and suggest that to some extent, loopholes in the law are being exploited (i.e. authorities overlook the killing of leopards

if they are deemed to be a threat to human life or livestock) to harvest these protected species for trade. Human-leopard conflict in Indonesia is reportedly becoming more frequent due to increasing fragmentation and loss of suitable habitat as well as depleting prey base (Partasasmita et al. 2016; Gunawan et al. 2017; Wibisono et al. 2018). This presents a significant conservation threat to the Javan Leopard and potentially the Sunda Clouded Leopard if not resolved urgently and further complicates efforts to eradicate illegal trade in these species.

Our study also reveals the international trafficking of both species from Indonesia to Kuwait, Russia and the UK, in violation of national legislation and CITES regulations. This was derived from three seizure incidents, two of which involved live animals for the pet trade, and one involving skulls. Live individuals of both species were despatched to Kuwait and Russia. In the first incident, the seizure took place in Jakarta in 2015 and involved a Sunda Clouded Leopard that was being smuggled to Kuwait. This was part of a larger investigation into the international trafficking of wildlife (including orangutan, Sun Bear, birds of paradise) sourced from Sumatra and Indonesian Papua to Middle Eastern countries. The second incident occurred in Russia in 2015, where a woman was caught at the Domodedovo Airport with plastic boxes containing animals sourced from wildlife markets in Indonesia including a live Javan Leopard. The incident of leopard skulls occurred in the UK in 2014, where an illegal wildlife trader was caught for selling two leopard skulls and 134 primate parts on e-Bay which had reportedly been sourced from a wildlife trader based in East Java. Given the close proximity to the Malaysian States of Sabah and Sarawak, cross border trade in these species between Indonesia and Malaysia should be considered a possibility as well.

The Javan Leopard and Sunda Clouded Leopard are strictly protected under Indonesia's national laws and by international regulations, yet this study reveals the prevalence of poaching, illegal domestic trade and international trafficking of both species. More than half of seizure incidents obtained were a result of intel and further investigation by enforcement authorities. Most of the seizure data (with the exception of 7 incidents) involved numerous wildlife species seized and not just leopards. This included high-profile species such as Tigers (41% of seizure incidents obtained for this study) and orangutans (in 2 incidents) as well as various other wildlife encompassing birds, deers, gibbons, pangolin, serow, snakes, slow loris, bears and turtles. This indicates enforcement efforts are not specifically targeted at Sunda Clouded Leopards or Javan Leopards but rather focused on dismantling Tiger poaching rings or general wildlife smuggling syndicates. This in itself suggests that true trade or poaching levels are vastly underestimated. The ongoing poaching and commercial trade is a conservation concern as both the Javan Leopard and the Sunda Clouded Leopard are highly threatened species and even low levels of removal could have major repercussions on remaining populations. For instance, recent studies on the distribution and population size of the Sunda Clouded Leopard in Borneo failed to detect the species in some areas and suggest that the species occurs in extremely low densities (Hearn et al. 2019). Approximately half of the cases resulted in successful prosecution and of these, the

highest sentence given was 2 years in jail and a fine of IDR50mil (~USD3300) to a wildlife trader caught in 2018 for attempting to sell 4 canines belonging to a Sunda Clouded Leopard, a bag made from the skin of a Javan Leopard and parts from Tigers and Sun Bears. All four species are strictly protected for which the maximum penalty afforded by Indonesia's wildlife laws is 5 years in prison and a fine of IDR100mil (~USD6700). Remaining convictions ranged from <1–1.6 years with fines ranging from IDR500,000 to IDR1mil (~USD35-70). The penalties handed down for these crimes are hardly deterrents and it is not known why maximum penalties as provided for under Indonesian law are not utilised further considering that multiple highly vulnerable and protected species are involved in these incidents and that such low penalties undermine enforcement efforts.

Conclusion

Poaching for commercial trade is pushing many wild cat species to the brink of extinction across their range in Asia (D'Cruze and Macdonald 2015; Nijman and Shepherd 2015; Nijman et al. 2019). The hunting of wildlife, particularly by indigenous communities for ceremonial clothing, food, medicine, protective charms and hunting trophies, has traditionally always been part of Indonesian culture (Meijaard 1999). However, hunting motives have since evolved, e.g., from one of subsistence to being commercially driven due to high commodity value (Meijaard 1999; Bennet and Robinson 2000; Harrison et al. 2016; Voigt et al. 2018). This, combined with modern hunting methods (including indiscriminate snaring), increasing accessibility of forests and increasing human-wildlife conflict, has resulted in significant detrimental impacts on wildlife populations across Asia (Gray et al. 2018; Symes et al. 2018), including Indonesia (Gunawan et al. 2017; Wibisono et al. 2018). Indonesia also has a notorious reputation for illegal and/or unsustainable trade in wild and exotic animals as pets, both as a source and demand country, involving a tremendous diversity and abundance of species (Nijman et al. 2009; Shepherd 2010; Harris et al. 2017; Morgan and Chng 2017; Gomez and Bouhuys 2018), including wild cats (Nijman et al. 2019). Over-harvesting of wildlife for the pet trade has been persistently raised as a key threat to Indonesia's biodiversity, particularly when it involves rare, endemic and restricted range species (Nijman and Stoner 2014; Janssen and Shepherd 2018), such as the Javan Leopard and Sunda Clouded Leopard. Hence, the illegal exploitation of the Javan Leopard and the Sunda Clouded Leopard for trade in Indonesia should be considered a significant threat to both species because of their threatened status in the wild (i.e. both species populations are small, in decline and vulnerable to extinction) and considering the dataset underrepresents true trade volumes.

We recommend further research into the drivers behind the poaching and trade in Javan Leopards and Sunda Clouded Leopards, including a focus on the links between human-wildlife conflict and trade. We encourage the Government of Indonesia to ramp up efforts to prioritise this issue and to investigate and dismantle

criminal networks involved in the commercial trade in both species and their parts and derivatives. We strongly suggest penalising offenders involved in the poaching and commercial trade in these species in Indonesia to the full extent of the law to create a deterrent. Cases resulting in prosecutions should be highlighted in the media to assist in raising awareness of the conservation plight these two species face as well as to educate the public regarding the legislation and penalties in place to prevent poaching and illegal trade. Finally, further monitoring of the poaching and trade in the Javan Leopard and Sunda Clouded Leopard, and of the efforts made to eradicate these crimes should continue to measure progress and to aid in informing future conservation and enforcement efforts.

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RESEARCH ARTICLE

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Community perceptions towards nature conservation in the Eastern Cape Province, South Africa

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Abstract

Relationships between protected area managers and adjacent communities, as well as communities' attitudes, views and perceptions of these areas, are critical for the success of conservation efforts. It is important for protected area managers and administrators to understand how local communities view these areas and their management, so that they can build sustainable working rel ationships. This paper is based on a survey of 375 semi-structured questionnaires administered to household heads, living at distances ranging from the edge of the reserves to 50 km away from the reserve boundary across the Great Fish River, Mkambati, Hluleka, and Tsolwana nature reserves in the Eastern Cape Province, South Africa. The paper provides a longitudinal assessment of households' knowledge about the role of reserves and the reserves' impacts on livelihood assets. In addition to households' knowledge about the role of reserves, the paper also provides an assessment of people's attitudes towards their location and management, as well as views on the best way to manage the reserves. For 79% of community members, reserves were important as they were seen to conserve biodiversity and valuable ecological systems necessary for sustaining life. Most (75%) respondents indicated that closely located reserves gave them opportunities to learn about nature conservation and to subsidize their incomes through tourism ventures. However, 58% had a problem with reserves' staff, due to restrictions on resource use, which negatively impacted their livelihoods. Over half (51%) of the households argued that sustainable conservation can only be achieved through an integrated approach where conservation and local communities' needs are given equal weight. We concluded that reserve managers should look at communities as active partners in the management of protected areas if sustainable conservation objectives are to be realised.

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Keywords
Conservation knowledge, local communities, nature conservation, protected areas

Introduction

For decades protected areas have been seen as the cornerstone for conserving biological resources and systems (Mutanga et al. 2015). From 1872 through to the 1960s, nature was conserved as an unspoiled wilderness, where people were removed from areas designated for conservation, and economic development controlled to reduce conflict with natural systems (MacKenzie et al. 2017). Areas designated for establishment of protected areas are usually surrounded by large numbers of people, who depend on them for their livelihoods (Andrade and Rhodes 2012). Therefore, separating protected areas from their adjacent communities negatively affects these communities (Soliku and Schraml 2018).

The negative socio-economic impacts of protected areas on adjacent communities are well-documented. Wild animals that escape from the reserves attacked, injured and killed people as well as their livestock (Matema and Andersson 2015). In addition to injury and death of people and their livestock, wild animals also cause crop damage and increase labor costs of crop defence (Subakanya et al. 2018). When rangers find animals grazing inside protected areas 'illegally' they are impounded and their owners fined for breaking the regulations that govern protected areas management (Mackenzie 2012). Restrictions on the use of resources in protected areas force the community to resort to 'illegal' harvesting of these resources and, when found, they are arrested, harassed and heavily fined (Mackenzie 2012). Resource use restrictions lead to food and financial insecurity for communities residing around these areas, so exacerbating their poverty levels (Widianingisih et al. 2016). Food insecurity exacerbates hunger and malnutrition in communities around protected areas (Subakanya et al. 2018). Because of poor infrastructure around protected areas, these areas are devoid of services, hence people are forced to walk long distances to access these services as well as resources they need for their livelihoods (Bond and Mkutu 2018).

Tensions arise whenever local communities are negatively affected by protected areas as a result of restricted access to livelihood resources (Soliku and Schraml 2018). On the other hand, if local communities' needs and aspirations are considered and addressed, the relationship between local communities and protected areas management is likely to improve (Nguyen 2019). Mutually supportive relationships between local communities and protected areas are, therefore, critical to successful protected area management (Yatesa et al. 2019). Understanding and incorporating the views of local people in the process of decision-making and provision of alternative livelihood solutions are important steps towards successful conservation (Mir et al. 2015).

The Eastern Cape Province is the poorest province in South Africa (Statistics South Africa 2017). For this reason, natural resources, specifically non-timber forest prod-

ucts (NTFPs) play a crucial role in sustaining livelihoods (Shackleton and Shackleton 2004). If resources are not equitably shared between communities and reserve management, then poverty and the increased demand for NTFPs is likely to make nature reserves viable areas for exploitation by adjacent communities. The objective of this study was to provide a longitudinal assessment of households' views on the location and management of nature reserves as well as knowledge about the importance of reserves and how they are managed. The study therefore covered (1) knowledge on the role of reserves and how the reserves were managed, (2) attitudes towards the location and management of the reserves, and (3) views on the best way to manage the reserves.

Methods

Study area

The study was conducted in rural communities around four nature reserves located in the Eastern Cape Province of South Africa. These reserves were Hluleka Nature Reserve, Mkambati Nature Reserve, Tsolwana Nature Reserve and Great Fish River Nature Reserve (Fig. 1).

The study sites are located in a remote rural area. The residents around the sites are predominantly black and poor Statistics South Africa (2017). The report further stated that the majority of persons in the study area were without a formal education. Unemployment in the study area stood at 94% and the majority of those in work are employed in informal sectors (Statistics South Africa 2017). The report noted that

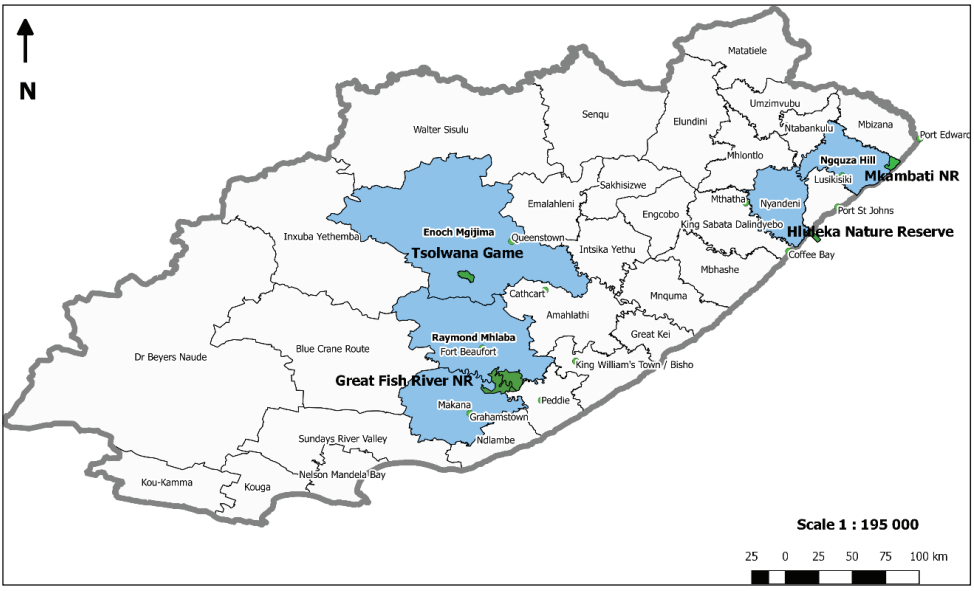

Figure 1. Location of the study sites in the Eastern Cape Province.

79% of people residing around the reserves lived in informal traditional dwellings. According to Statistics South Africa (2017), 42% of residents around the study area do not have toilets while 74% do not have access to clean water.

Data collection

The study sites were visited during 2015–2017 for the collection of data. Data on views and perceptions of communities on the role of reserves, how the reserves were managed, as well as perceptions on the location of the reserves, were collected. The investigation started with focus group discussions with communities adjacent to the reserves. For this purpose, eight focus groups were established, consisting of between four and eight household heads. Participants were selected by households on the basis that they were knowledgeable about the reserves and could represent the interests of the communities. Meetings were held at the homes of participants and facilitated in isiXhosa (by trained field assistants). Four focus group sessions were conducted per site – one for each distance category (0–10 km, 11–20 km, 21–30 km, and 31–50 km). The aim was to obtain a general picture of communities' knowledge on the roles of reserves and how the reserves were managed, attitudes towards the location and management of the reserves, as well as views on the best way to manage the reserves.

Focus group discussions were supplemented with 375 self-guided (where respondents filled the questionnaires by themselves) semi-structured questionnaires, administered to household heads adjacent to the four reserves. These household heads were selected via stratified random sampling (0–10 km (86 questionnaires), 11–20 km (81 questionnaires), 21–30 km (42 questionnaires) and 31 km and further to a maximum of 50 km (166 questionnaires)). The age of the informants ranged from 20 to 92 years. The questionnaire included questions across three broad categories: (1) knowledge about the role of reserves and how the reserves were managed, (2) attitudes towards the location and management of the reserves, and (3) views on the best way to manage the reserves.

Data on the specific distances were classified into suitable categories based on households' views and perceptions and summaries extracted. Descriptive statistics of communities' knowledge on the roles of reserves and how the reserves were managed, attitudes towards the location and management of the reserves, as well as views on the best way to manage the reserves, were computed. Cross-tabulation was performed to determine whether there were significant variations between distance and communities' knowledge about the roles of reserves, how they were managed, attitudes towards the location, management of the reserves, as well as views regarding the best way to manage them.

Ethical clearance

Ethical clearance was obtained from Rhodes University before fieldwork commenced. Before administering the questionnaires to the respondents, permission was sought from the chief and respective headmen in the study area. Respondents' consent was

also sought before the commencement of the survey. The purpose of the research was clearly explained to the respondents before the questions were administered to them. All respondents' concerns and fears about anonymity were attended to by maintaining confidentiality.

Results

Communities' knowledge of the role of the reserves and how they are managed

On average, 61% of the household heads knew the role of the reserves. The proportion of household heads who knew the role of reserves significantly decreased with distance from the reserves' boundaries (P = 0.01) (Table 1). Generally, 60% of the respondents knew how reserves were managed. The percentage of household heads' with this knowledge, significantly increased with distance from the reserves' boundaries (P = 0.02) (Table 1).

Communities' attitudes towards the location and management of the reserves

On average, 75% of the household heads were satisfied with the location of the reserves. The satisfaction significantly increased with distance from the reserves (P = 0.001). Those who were in favor of reserves stated that it gave them an opportunity to learn more about the fauna and flora, that they could subsidize their incomes through tourism ventures, and that they acquired employment directly or indirectly through opportunities created by reserves (Table 2).

Fifty-eight percent of household heads resented the way the reserves were managed. The percentage of household heads that resented the way the reserves were managed decreased with distance. There were statistically highly significant (P = 0.001) variations in terms of resentment at how the reserves were managed (Table 2). The most prominent reasons for respondents' dissatisfaction are that they incurred hardship due to restrictions on resource use and their movements (especially in Hluleka Nature Reserve).

Communities' views on the best way to manage the reserves

Eighteen percent of the household heads held the view that if the reserves could implement projects that created jobs for the local communities, and that if these jobs were shared equitably among the beneficiaries, then sustainable conservation could be achieved. Generally, the percentage of household heads holding the view that the reserves should provide jobs to them decreased with distance from the reserves' boundaries (Tables 3, 4).

Eighteen percent of the household heads said that involving communities in reserves' meetings and considering communities' views in all decision making will achieve sustainable conservation. Generally, the percentage of household heads who favored participating in the day-to-day management of the reserves, significantly increased

Table 1. Communities' knowledge (percentage) of the role of the reserves and their management.

Distance from reserve (km)	Knowledge on role of reserves	Knowledge on how reserves were managed
0–10	82	54
11–20	67	57
21–30	54	63
31–50	41	67

Table 2. Communities' attitudes towards the location and management of the reserves.

Distance from reserve	Percentage	
	Attitudes towards the location (satisfaction)	Attitudes towards the management approach (resentment)
0–10 km	43	96
11–20 km	76	74
21–30 km	84	49
31–50 km	93	12

Table 3. Communities' views (percentage*) on the best way to manage the reserves.

Ideas proposed for best management practices	Distance from reserve (km)			
	0–10	11–20	21–30	31–50
Implement projects that create jobs for local communities	23	14	16	17
Involving communities in reserves' meetings and considering communities' views in all decisions	16	11	25	19
Empowering local communities with knowledge on the importance of protected areas	6	2	10	4
Strengthening the security of wild animals	12	16	6	3
Aligning conservation with local communities' needs	18	13	6	0
Allowing communities uncontrolled access to natural resources in the reserves	5	5	0	1
Allowing communities controlled access to natural resources in the reserves	3	2	0	1
Strengthening law enforcement of trespassers	7	10	20	16
Willingness to participate in reserve activities	90	78	66	47

*Percentages do not add up to 100 due to the multiple choice options available to respondents.

Table 4. Variation of communities' knowledge, views and perceptions with changes in distance from the reserve boundaries.

Variable	Variation with distance	χ^2	P-Value
Knowledge about the role of reserves	Decreased	9.656	0.01
Knowledge about how the reserves are managed	Increased	3.343	0.02
Satisfaction of location of the reserves	Increased	54.98	0.00
Resentment of reserves' management styles	Decreased	42.068	0.00
Inclusion of local communities in reserves management	Increased	65.088	0.00
Uncontrolled access to natural resources in the reserves	Decreased	42.719	0.67
Reserves should provide jobs to the locals	Decreased	38.825	0.046
Strengthening law enforcement to keep off trespassers	Increased	120.749	0.84
Strengthening security to ensure safety of the locals and their livestock	Decreased	84.114	0.86
Empowering locals with conservation knowledge	Decreased	17.243	0.81
Meet communities' needs (roads, water and electricity)	Decreased	55.359	0.02
Controlled access to natural resources in the reserves	Decreased	40.98	0.00
Willingness to participate in reserves' activities	Decreased	44.576	0.00

with distance from the reserves' boundaries ($P = 0.001$) (Tables 3, 4). Household heads argued that involving locals in the day-to-day running of the reserves will inform them about the decisions managers make. Household heads also stated that reserve managers

should stop harassing community members and impounding their livestock, should they happen to accidentally enter the reserves.

Ten percent of household heads who held the opinion that empowering local communities with information on the importance of protected areas, will make them embrace the reserves, were located 21–30 km from reserves. Thereafter the percentage of household heads who agreed with this statement declined with distance from the reserves' boundaries (Tables 3, 4).

Nine percent of household heads stated that preventing wild animals from escaping and injuring or killing people and domestic animals will achieve sustainable conservation. Generally, the percentage of household heads who noted that enhancing security around the reserves would ensure the safety of locals and their livestock, decreased with distance from the reserves' boundaries (Tables 3, 4).

Nine percent of household heads indicated that aligning conservation with local communities' needs (improving physical infrastructure (roads, electricity and water) will achieve sustainable conservation. The percentage of household heads with this opinion decreased with distance from the reserves' boundaries (Tables 3, 4).

Three percent of household heads noted that allowing uncontrolled access to natural resources in the reserves was the only way to achieve sustainable conservation. Household heads who were in favour of uncontrolled access decreased with distance from the reserves' boundaries (Tables 3, 4).

A minority of household heads (3%) said that allowing controlled access to natural resources in the reserves will achieve sustainable conservation. The number of household heads holding this view decreased with distance from the reserves' boundaries (Tables 3, 4).

Thirteen percent of household heads favored strengthening law enforcement to ward off trespassers as a way of attaining sustainable conservation. Communities indicated that this will help curb unsustainable resource use by the locals, thereby sustaining them. Furthermore, 70% of household heads were willing to participate in reserve activities. The percentage of willingness to participate decreased with distance from the reserves (Tables 3, 4).

Discussion

Communities' knowledge of the role of the reserves and their management

Communities' knowledge about the role of the reserves significantly decreased with distance from the reserves' boundaries. Seventy-nine percent of household heads indicated that the reserves conserved biodiversity for future generations. They noted that when biodiversity is conserved, protected areas can supply essential goods and services important for sustaining both humans and ecosystem functioning. According to Gandiwa et al. (2014), because of the longstanding relationships between communities and protected areas, local communities have developed knowledge about the environments in which they live. Gandiwa et al. (2014) further noted that this knowledge and perspective are based on locally developed practices of resource use. It is therefore

not surprising that the knowledge about the role of reserves decreased with distance from the reserves' boundaries. Communities closer to the reserves have longstanding interactions with the reserves and, therefore, have more interactions with the reserves compared to those far from these areas.

Our study established that local communities knew and appreciated the importance of nature and natural resources. This is an indication that if local communities are given an opportunity to participate in the running of reserves, they are likely to be effective co-custodians. According to Soliku and Schraml (2018), social ecological systems are important for sustainable conservation. Gandiwa et al. (2014) assert that information about local people's knowledge and perceptions about conservation is important to the success of wildlife conservation because understanding and acknowledging residents' knowledge and perceptions about wildlife conservation can build constructive relationships between residents and protected area management. In our study, local communities knew and appreciated the importance of nature and natural resources. This is an indication that if local communities are given an opportunity to participate in the running of reserves, they are likely to be effective co-custodians. Seoraji-Pillai and Pillay (2017) found that communities and protected area managers had developed an understanding that led them to co-exist. Soliku and Schraml (2018) argue that locals have long been managing natural resources either consciously or unconsciously through local rules, taboos, and belief systems. For local communities to be effective custodians of natural resources, however, will require building trust between communities and reserve management. Mir et al. (2015) argued that putting humans at the centre of ecosystem management requires the building of ecological knowledge and relationships because ecosystems are complex adaptive systems characterized by historical dependency.

Knowledge about how the reserves are managed significantly increased with distance from the reserves' boundaries. This variation can be associated with the negative attitudes that locals had towards the way the reserves were managed, where communities closer to the reserves resented the way the reserves were managed. Bennett and Dearden (2014) noted that most people residing adjacent to protected areas resent these areas, ending up not interested in conservation efforts simply because they see no benefits flowing to them, resulting in a case of "us"- and -"them". Soliku and Schraml (2018) further noted that when local communities lack interest in the affairs of protected areas, they are likely not to know what is happening there. For effective conservation, therefore, it is important that conservationists find ways to entice local communities to be interested in the protected area management, and thus avoid making mistakes that can arise from central policies which ignore local realities (Pekor et al. 2019).

Local communities' attitudes towards the location and management of the reserves

Satisfaction with the location of reserves significantly increased with distance from the reserves' boundaries. Most people closer to the reserves were not satisfied with their

location because frequent disease outbreaks affected them and their livestock, as well as escaping wild animals that attacked and injured or killed them and their livestock. Most local communities also noted that restriction of access to natural resources negatively impacted on their livelihoods. Our findings differ from those of Mir et al. (2015) who found that despite 75% of surrounding communities suffering crop damage and 23% suffering livestock predation from wild animals, a majority (84%) of the respondents were still in favor of wildlife conservation. Our study is, however, in line with Soliku and Schraml (2018) who indicated that restricting access to natural resources which are crucial for people's livelihoods, results in negative attitudes toward protected areas. Therefore, protected areas and local communities can only co-exist if their needs and views of those living around them are incorporated in the decision-making process.

The number of people who resented the way the reserves were managed decreased with distance from the reserves' boundaries. According to Mutekwa and Gambiza (2017), when locals view protected areas as places and resources stolen from them, they are likely to resent them. People were restricted access to resources such as building materials, medicinal plants, mussels and fish that they needed for their livelihoods. They developed negative attitudes towards the reserves. Those far from the reserves depended less on the reserves' resources for their livelihoods, hence had no interest in how they were managed. Soliku and Schraml (2018) argued that restricting communities' access to natural resources that are crucial for their livelihoods can result in retaliation and hostile attitudes towards protected area objectives. This was the case in our study with communities perceiving the government as having 'stolen' their resources, leading to conflicts between reserve managers and local communities, especially in Mkambati and Hluleka.

Most local communities closer to the reserves disapproved of the managers that were in charge of the reserves. Most locals felt the managers were imposed on them from East London (the headquarters of Eastern Cape Parks and Tourism Authority). This created mistrust between local communities and reserve management. Soliku and Schraml (2018) noted that making decisions that affect people without consulting them can result in retaliation and hostile attitudes. This was the case in our study as people felt that they were not consulted or informed about the appointment of the managers. Local communities argued that reserve managers served the interests of the Eastern Cape Parks and Tourism Authority at the expense of their livelihoods.

Local communities' views on the best way to manage the reserves

The number of household heads who agreed with the statement that inclusion of local communities in day-to-day running to the reserves would enable sustainable conservation, decreased with distance from the reserves' boundaries. Those closer to the reserves felt that the reserves were too important to their livelihoods and that it was unethical to exclude them in decision making. Andrade and Rhodes (2012) stated that if communities do not benefit from protected areas, they do not have any interest in their management. Incorporating locals in conservation efforts can indeed promote effective

conservation. According to De Pourcq et al. (2017), understanding and incorporating the views of local people in decision-making and providing alternative livelihood solutions are important steps towards successful conservation. According to Mir et al. (2015), an inclusive approach to conservation improves trust and reduces transactions costs for managing protected areas. Stakeholder participation also reduces the likelihood that those in the periphery of the decision-making context are marginalized (Mutanga et al. 2017).

The percentage of household heads who indicated that law enforcement should be strengthened to keep trespassers away from the reserves increased with distance from the reserves' boundaries. This was expected because households closer to the reserves suffered hardships due to resource restrictions and constrained movement compared to those far from the reserves. Therefore, strict protection was inversely proportional to suffering and hardships among communities living around the reserves. According to Dewu and Roskat (2017), when communities living adjacent to protected areas see the benefits of protected areas, they are likely to support conservation efforts but where they face hardships and suffering they will not support any efforts that will bring more hardships and suffering to them. This was the case in this study.

The percentage of household heads who held the view that the reserves should provide jobs to them decreased with distance from the reserves' boundaries. This was expected because the unemployment rate decreased with distance from the reserves' boundaries. Galvin et al. (2018) found that when conservation projects in Zambia created jobs and enhanced household incomes of many local communities, their attitudes towards protected areas improved, reducing poaching, and ultimately enhanced conservation.

The percentage of household heads who indicated that empowering locals with conservation knowledge will help achieve sustainable conservation decreased with distance from the reserves' boundaries. According to Chevallier and Milburn (2015), when people have a clear understanding of environmental concerns, they will follow sustainable development practices. The higher percentage of people closer to the reserves who supported this view is associated with the interests they have, as well as their knowledge of the importance of protected areas to their livelihoods and ecosystem functioning.

Sustainable use of natural resources in protected areas can promote ecological integrity and at the same time improve the livelihoods of those living around protected areas. Access to resources is likely to be advocated by those who benefit more from them. It is, therefore, not surprising that the percentage of household heads supporting the view that controlled access will promote sustainable conservation decreased in line with distance from the reserves' boundaries. Soliku and Schraml (2018) attest that it is those who have more to gain from natural resources that will want access to them.

Since there were more people closer to the reserves who resented the way the reserves were managed than those farther afield, it is not surprising that the percentage of those who were willing to participate in reserve activities was higher closer to the reserves compared to far from them. The findings of our study therefore are in line with those of Amin et al. (2015) who argued that people residing adjacent to protected areas will participate in wildlife conservation activities when opportunities arise to try influ-

ence decisions. The findings of our study also concur with Dewu and Roskat (2017), who found that local communities are more likely to comply and to commit themselves to long-term conservation strategies when their knowledge and opinions are incorporated into protected area decision-making processes. The high number of people who were willing to participate in our study, can be attributed to the belief by locals that the reserves are important to their livelihoods and sustainable ecosystem functioning.

Conclusions

We found that communities around the reserves knew the importance of reserves and were willing to participate in any reserves' activities, if the opportunity is extended to them. On this basis, it can safely be said that if communities around the reserves are involved in the activities of the reserves, and their views incorporated in decision making, sustainable conservation can be achieved in the province.

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How do red deer react to increased visitor numbers? A case study on human-deer encounter probability and its effect on cortisol stress responses

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Abstract

The numbers of visitors to greenspaces in the United Kingdom has increased over the last few years as the health benefits of spending time in greenspaces have become better known. This has led to problems for conservation ecologists due to increased numbers of reported human-wildlife encounters. Deer are often found in public spaces and are of particular concern. Previous research suggests elevated levels of stress hormones (e.g., cortisol) in deer is a result of increased human activity. This has been linked to several negative effects on the deer's health. From a practitioner's point of view, it is therefore important to implement effective management strategies that are based on scientific evidence to help ensure the welfare of managed deer populations. In an effort to identify the impact of visitor numbers on faecal cortisol concentrations, samples from 2 red deer (*Cervus elaphus*) herds in Lyme Park (Cheshire), United Kingdom, were collected and analysed. A predictive spatial model was developed based on logistic regression to identify areas within the park of low and high human-deer encounter probability. The faecal cortisol levels were found to be significantly higher on days with a high number of visitors. In addition, landscape features such as buildings and roads increased the probability of human-deer encounters, whereas woodland and scrub decreased the probability. However, human-deer encounter probability changed with distance to the features. By providing local park managers with this scientific data, these findings can directly inform current management efforts to reduce deer stress levels in Lyme Park. In addition, the spatial modelling method has the capacity to be implemented in other parks across the country with minimal cost and effort.

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Keywords
Assay, *Cervus elaphus*, cortisol, modelling, red deer, visitors

Introduction

A Natural England report found that over a seven-year period there has been a 4% increase in the number of adults visiting a greenspace at least once a week; up to 28 million people or 58% of England's adult population (Natural England 2011; Natural England 2017). Recreational use of green spaces has been promoted due to the physical and mental health benefits it offers such as lowering rates of depression, anxiety, and high blood pressure (Sandifer et al. 2015; Shanahan et al. 2016; Cox et al. 2017), as well as reducing brain activity in areas linked to increased mental illness risk (Bratman et al. 2015). The importance of these health-related services has been identified in UK policy, which highlights them as vital components of nature conservation (DEFRA 2011). This has resulted in the proportion of health motivated visits to nature rising from approximately a ⅓ (0.97 billion) of all visits in 2009–2010, to approximately ½ (1.51 billion) of all visits in 2015–2016 in England (Natural England 2011; Natural England 2017).

This increase in visitors has led to problems for conservation ecologists, as intensity of visitors to a habitat has been found to negatively impact various different organisms, most notably by directing their habitat selection to areas which are less desirable. The richness and distribution of plants were found to be reduced in highly visited areas (Jägerbrand and Alatalo 2015; Malavasi et al. 2016); invertebrates, such as *Bathyporeia pelagica*, have found to be absent from their preferred and expected habitat solely due to trampling resulting from human activity (Reyes-Martínez et al. 2015). Both birds (Thiel et al. 2008; Coppes et al. 2017b, D'Acunto et al. 2018). Mammals were also found to avoid areas with high human activities which may prevent them from utilising their optimal habitat (Haigh et al. 2017; Reilly et al. 2017.) Deer are a common parkland species in the UK, found in over 90 public accessible parks across Britain (British Deer Society 2018). Previous studies have investigated the differing impacts of human activity on deer habitat usage (George and Crooks 2006; Sibbald et al. 2011; Bonnot et al. 2013; Scholten et al. 2018), diet composition (Jayakody et al. 2011) and behavioural responses (Reimoser 2012). Each of these impacts were found to be linked to human-induced stress.

Deer stress is an important management consideration as it is a contributing factor for the general welfare of a herd. When stressed, the ability of the deer to react quickly to changes in their environment is hampered and how the deer react to these changes may be altered (Broom 1991). These altered reactions as a response to stress could be behavioural: for example, a stressed deer being more likely to respond aggressively to additional stressors, such as a visitor or dog who gets too close – an issue for parkland sites where the safety of visitors is under constant scrutiny (Hubbard and Nielson 2009). The reaction could also be physiological, with higher stress levels being

linked to a decrease in immune system function, reproduction and metabolic processes (Cherry et al. 2016; Sapolsky et al. 2000).

Site managers are faced with the challenges of mitigating negative impacts to their deer populations; this includes the growing issue of rising human utilisation of these greenspaces. This study was carried out in response to this challenge and it was hoped that the work we carried out could help to bridge the gap between understanding the impact of human activity on deer stress, and the predicted occurrence of stress events across a landscape. The results of this study were readily applicable to the herds of red deer (*Cervus elaphus*) at a specific site, Lyme Park in Cheshire, UK. The methods and findings of this study can then be used to inform management in a broader context and can be utilised at other similar sites. Thus, the main aims of this study were to (a) explore whether an increase in stress hormones can be attributed to increased visitor numbers, and to (b) inform the current management practices through the development of a spatial model that can predict the chance of a human-deer encounter occurring. Throughout this paper the term human-deer encounter probability will be used to describe the likelihood of human activity occurring within close proximity to the deer.

Study area

The study was conducted at Lyme Park, Disley, Cheshire, United Kingdom (53.338041, -2.0547761). The site covers approximately 590 hectares; the native, wild red deer were enclosed in the park during the 14th century. The site is a large natural area just outside of Greater Manchester and is popular with visitors from this built-up urban area, and from further afield. Lyme Park is open to visitors around the year. The site vegetation is predominantly grassland, but the park also contains a range of habitats typical of Northern England (Fig. 1). Grazing by sheep (*Ovis aries*), highland cattle (*Bos taurus*) and red deer occurred throughout the park with the objective of promoting plant diversity. Prior to 2002 deer were able to graze in the whole park until they were fenced out of a central zone (Fig. 2) due to concerns that increasing visitor numbers would generate greater disturbance for the deer. When the deer were excluded from the central zone they were separated into two herds: one herd was located closer to the main visitor attractions of the park (the "Park Herd") and the other was in the less visited moorland area (the "Moor Herd"). During study period the Park Herd consisted of approximately 160 animals, and the Moor Herd of approximately 90 animals (Fig. 2).

Over the last 5 years the number of visitors to Lyme Park has risen by approximately 150,000 individuals based on the number of vehicles entering the site. The number of total visitors in 2018 is likely closer to 750,000 when accounting for visitors taking public transport to the site, or walking into the park through alternate entrances (National Trust 2018a, b). The site is well developed for tourism and is visited all year round; a large number of these visitors are brought to the park to see its historical features. These features include the 16th century mansion house and associated gardens, a hunting lodge named "the cage" and the deer park (and by extension the deer) itself. Aside from tourists, the site is frequently visited by local walkers, families and hikers.

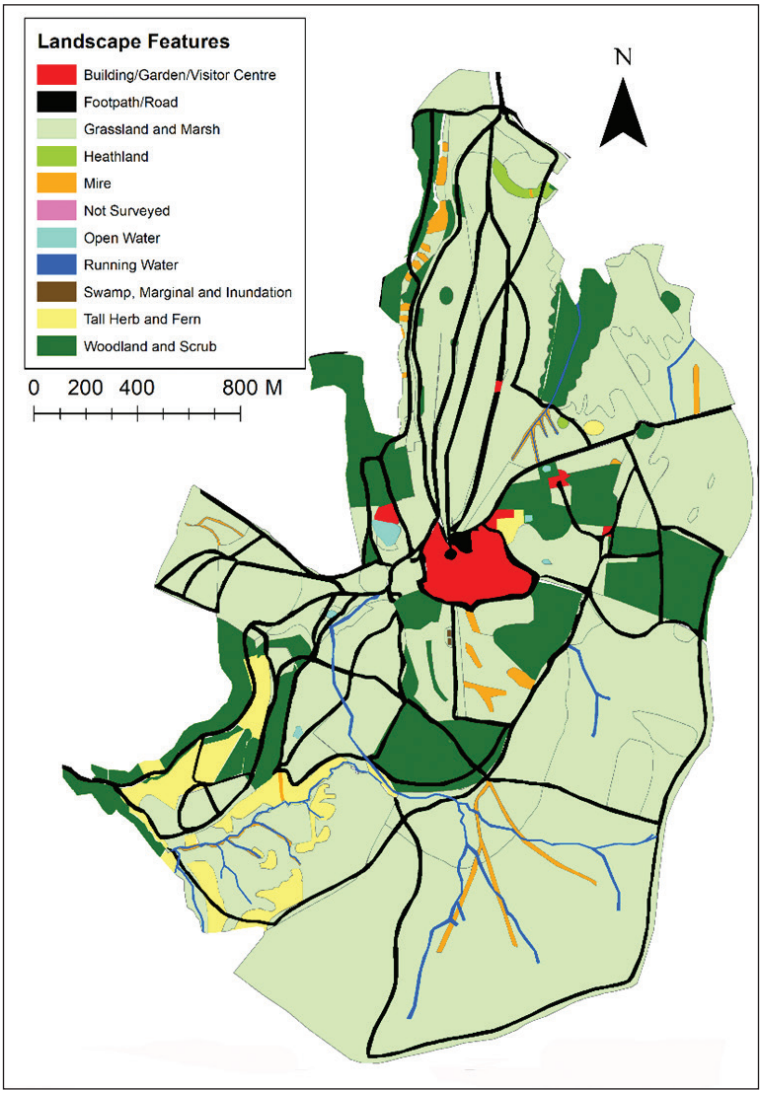

Figure 1. Map of landscape features. Each landscape feature is shown as a different colour. Data collected from Lyme Park, Disley, UK, in 2018.

Methods

Identifying areas of shared habitat use and landscape features

The experienced park ranger team, made up of four rangers, work in the park every day of the week for nine hours per day. During the study the rangers had 20 years of experience visually tracking the movements of the deer daily throughout the year to carry out management tasks such as feeding and culling the herds. Attention is paid to which areas of the park the deer use to ensure that the management of the habitat

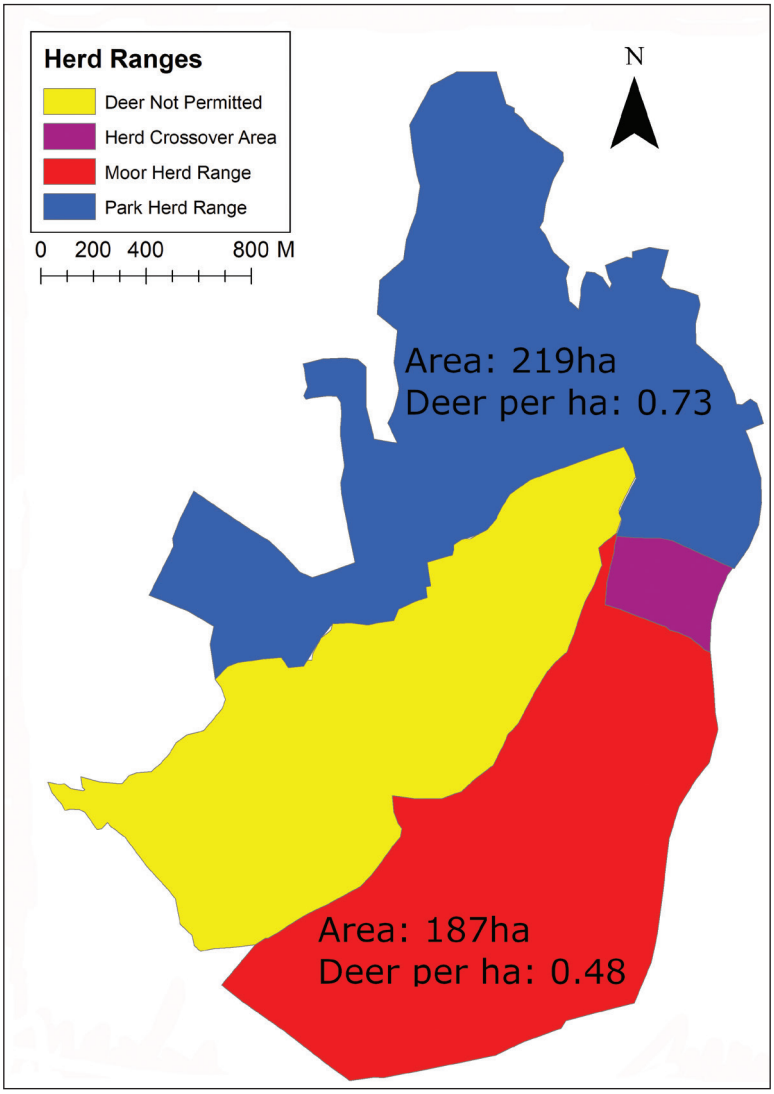

Figure 2. Map showing the area open to each of the herds. The herd crossover area (area accessible to both herds) is included in the total area of both herds. Area where deer are excluded also shown. Data collected from Lyme Park, Disley, UK, in 2018.

is well informed. We used these historic space-use observations to compile and create two qualitative maps; one which displayed the areas of the park which were heavily utilised by the two herds of deer and one which displayed the areas of the park most heavily utilised by the human visitors. To ensure that the deer and visitor movements were accurately represented by these maps, observations were visually confirmed by the authors over approximately 100 hours between June and August in 2018. This was done by counting the number of visitors and deer in each area of the park for a mini-

mum of three hours per day, four days a week over the seven-week study period. This method was chosen because observational data is usually the only data available to park managers, due to the high costs and specialised knowledge required to install GPS tags. Although GPS tracking data would have been desirable, we were not given permission to tag the deer during the period the study took place. Similarly, our activity data for visitors relied on observational accounts of the park managers and on visual confirmation of these accounts by the authors of this study.

The two sets of activity data, human and deer, were used to create two maps; these maps split the park into areas of high activity (where the visitors/deer were most often found) and areas of low activity (areas where visitors/deer rarely visited). The two separate activity maps were overlaid to identify the areas of the park which are shared and utilised by both the deer herds and human visitors; these areas were categorised as areas of "High likelihood of encounter". Conversely, the areas where deer and human usage did not overlap were categorised as areas of "Low likelihood of encounter" (Fig. 3).

Geographical landscape feature information was obtained from the National Trust as a shapefile (National Trust 2018c). The footpaths and roads were digitised and added to the maps manually using ArcGIS (2011) and the highest resolution aerials images available. There was a total of nine landscape features, which could affect the likelihood of human-deer encounters, identified (Table 1). The area covered by each landscape feature within each of the herds' range is presented in Table 2.

Generating distance matrix data

To create a dataset from which encounter probability could be modelled, information provided by two maps were used to generate a distance matrix. Two hundred random points were selected from both the low and high encounter zones (Fig. 3) using the ArcGIS (Environmental Systems Research Institute, Inc., Redlands, CA, USA) 'Create Random Points' tool from the 'Sampling Toolset'. This resulted in 5,494,400 probability calculations, which was also the maximum number of calculations that we were able to run on our computer systems. No points were selected from the deer exclusion zone in the park (Fig. 3).

The distance matrix required the landscape feature shapefile to be converted into a raster file; this conversion was carried out using ArcGIS and resulted in the map being divided into 20×20 m grid cells. Each cell was assigned a landscape cover feature (Table 1), based on the highest proportion of each feature in a cell. Cells containing footpaths or roads were prioritised during this assignment, as they are important access routes for visitors. This was necessary due to the long, narrow nature of footpaths and roads, resulting in a low relative area within each of the grid cells. In the case of running water, we did not prioritise this feature during our assignment because it was mainly located in areas that visitors did not utilise and because the few small streams that were in the study area, were mostly seasonal.

The distance matrix calculated the distance from each selected low and high-risk points (Fig. 3) to each separate landscape feature in the raster file using the 'Point Distance' tool in ArcGIS.

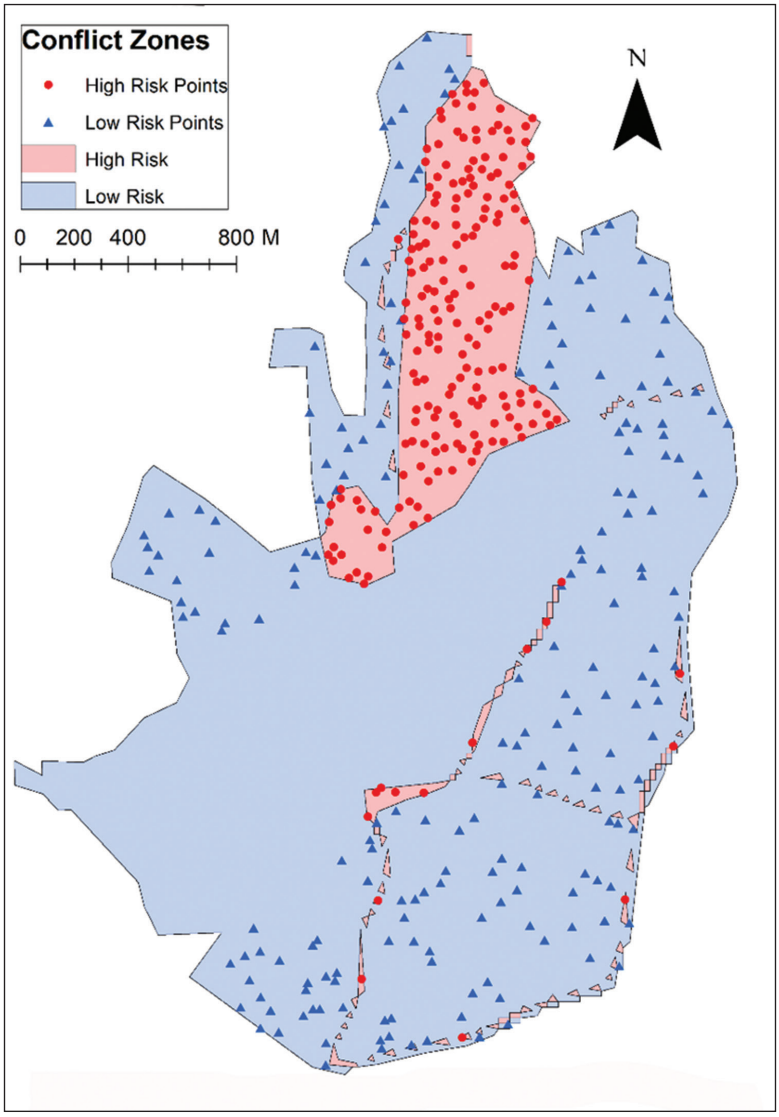

Figure 3. Map showing zones where human-deer (*Cervus elaphus*) encounters have occurred. Locations of randomly sampled points are shown as red dots (high risk points) and blue triangles (low risk points). Data collected from Lyme Park, Disley, UK, in 2018.

Mapping encou l nter probability

To determine how landscape features influenced the probability of human-deer encounters, a binary logistic regression model was built using the data provided by the distance matrix as described by Miller et al. (2016). Low and high-encounter points were used as the binary responses (Fig. 3). The feature predictor variables used in the model are shown in Table 1. The 'Not surveyed' and 'Swamp, Marginal and Inundation' features covered only a small area in the two herd ranges (>0.01%) and thus did not

provide enough sample points to include them in any further analysis. Starting with a global model, ranked models were generated using all combinations of habitat variables (Table 2); this was carried out using the "gmulti" package in R (R Core team, 2013. R Version 3.5.1, www.r-project.org, Accessed 12 Dec 2019)

Akaike's Information Criterion (AIC) was used to select the best fit model. As the highest scoring model did not reach an Akaike weight > 0.90, the top 8 models (Table 3) were averaged to produce the final model. The sum of the AIC scores also allowed a comparison between the contribution of each habitat variable to the best fit model using relative importance. Relative importance ranged from 0 to 1, with 1 indicating that the variable made a strong contribution to the model.

Table 1. Predictor variables used in the study. Showing evidence of importance in relation to human-ungulate encounters.

Category	Predictor variable – landscape feature (unit)	Evidence of effect on encounter probability
Human Presence	Distance to buildings/gardens/visitor centres (m) Distance to recreational routes (roads/paths) (m)	Increased human activity stresses deer. [a,b,c,d]
Land Use	Distance to woodland/scrub (m) Distance to grassland/marsh (m) Distance to heathland (m) Distance to mire (m) Distance to tall herb/fern (m)	Provide refuge space[c,e] and effects vigilance levels of deer. [f] Human disturbance can affect foraging.[c] alongside other deer habitat uses.[f]
	Distance to running water (m) Distance to open water (m)	Drinking requires the entry to high risk areas, prioritised over vigilance. [g]

[a] D'Amico et al. 2016; [b] Sibbald et al. 2011; [c] Coppes et al. 2017a; [d] Scholten et al. 2018; [e] Torres 2012; [f] Ciuti et al. 2012; [g] Crosmary et al. 2012

Table 2. Landscape features in Lyme Park and their relative percentage covered and area, separated for the Park and Moore deer herd.

Landscape feature	Area (hectares)		Area (%)	
	Moor	Park	Moor	Park
Building/Garden/Visitor centre	0,52	1,47	0,28	0,67
Footpath/Road	8,28	23,81	4,43	10,87
Grassland/Marsh	146,38	146,42	78,28	66,86
Heathland	0	1,27	0	0,58
Mire	2,23	2,93	1,19	1,34
Open water	0	0,83	0	0,38
Running water	2,56	0,7	1,37	0,32
Tall herb/Fern	11,93	0,35	6,38	0,16
Woodland/Scrub	15,09	41,22	8,07	18,82

Table 3. Explanatory variables which were included in each of the top 8 models produced. All explanatory variables were included in the final average model.

Explanatory variable	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7	Model 8	Average
Build./Gard./Visitors centre	✓	✓	✓	✓	✓	✓	✓	✓	✓
Footpath/Road	✓	✓			✓	✓			✓
Grassland/Marsh	✓	✓	✓	✓					✓
Heathland	✓	✓	✓	✓	✓	✓	✓	✓	✓
Mire	✓		✓		✓		✓		✓
Open water	✓	✓	✓	✓	✓	✓	✓	✓	✓
Running water	✓	✓	✓	✓	✓	✓	✓	✓	✓
Tall herb/Fern	✓	✓	✓	✓	✓	✓	✓	✓	✓
Woodland/Scrub	✓	✓	✓	✓	✓	✓	✓	✓	✓

The relationship of each predictor variable to the encounter probability was examined by holding all variables constant at their mean. To measure the performance of this binary classifier, a receiver operating characteristic (ROC) curve was generated by plotting the true positive rate (TPR) against the false positive rate (FPR) at various threshold settings. The area under the curve (AUC) was then calculated to assess the model performance. The probability data of deer-human encounters was then mapped for each 20×20 m grid using ArcGIS. Encounter risk ranged from 0 to 1 and was divided into five categories (0–0.19, 0.2–0.39, 0.4–0.59, 0.6–0.79, 0.8–1) for mapping purposes.

Determining high and low visitor days

The relative proportion of visitors on each day of the week was retrieved from Google Visitor Data which uses aggregated and anonymised data from users who have opted in to Google Location History. This data was used to calculate the percentage of visitors a day visiting the park: Wednesdays and Thursdays were identified to be days of low visitor numbers (5–8% of total weekly visitors) and Saturdays and Sundays of high visitor numbers (21–27% of total weekly visitors) (Table 4).

Sample collection

Fresh faecal samples were collected in Lyme Park between June and August 2018. Samples were determined to be fresh primarily based on direct observation of defecation events; in cases where the event was not witnessed, pellets in the area immediately vacated by the deer were assessed for freshness based on three metrics; level of moistness, pellet being intact, and resting atop ground flora (Goode et al. 2014). Only samples which matched all three metrics were collected. To compare cortisol levels between the Park and Moor herd, we collected ten faecal samples from each herd, every day over a seven-day period: 25 Jun 2018–1 July 2018 (n = 140). To compare deer cortisol levels between days that experienced low and high number of visitors (Table 4) 20 additional samples were collected four times a week on the days following the expected highest and lowest visitor numbers (Thursday, Friday, Sunday and Monday). This comparison was only done between individual days. This second set of samples was only collected from the Park herd over the period 5 July 2018–6 August 2018 (n = 400). These samples were collected between 5am and 7am, 18 hours after peak visitor hours; a

Table 4. Weekly number of visitors (%) in the summer who visit Lyme Park. Values are based on relative numbers of visitors extracted from Google analytics (see methodology for further detail).

Day	Weekly number of visitors (%)
Monday	16
Tuesday	10
Wednesday	5
Thursday	8
Friday	13
Saturday	21
Sunday	27

timeframe that has been shown to fall within the gut passage time of cortisol in deer (Millspaugh et al. 2002).

In order to reduce the effect of different metabolic rates between individual animals, samples were collected over a short time frame each day and homogenised when processed in the laboratory (Goymann 2012). The time of the year and the duration of the collection of the samples (June-August), fell before the rut and before the females came into esterus. This limited the impact the sex of the deer, and the reproductive state of females, would have over the short six-week period (Gordon 1997; Huber et al. 2003). To further limit these possible impacts, we avoided the collection of samples from male deer who tended to isolate themselves from the main group and from female deer with foals. The fresh samples were stored following standard protocol at -20 °C, to prevent further metabolisation, until the extraction and assay analysis were conducted (Touma and Palme 2005; Konjević et al. 2011; Pavitt et al. 2016).

Hormone extraction and assay analysis

We used the DetectX Steroid Immunoassay Kit from Arbor Assays for the cortisol analysis (catalogue #K003-H5). This kit was validated for dried faecal extracts by the manufacturer and had been used to measure cortisol in a number of previous studies (Brand et al. 2016; Chen et al. 2017; Endo et al. 2018). The hormone extraction and assay analysis followed the manufacturer's guidelines (Arbor Assays 2009) with 2 alterations: all samples were processed wet and methanol was used in place of ethanol during the extraction procedure. These changes were based on the recommendations of a steroid extraction review (Palme 2012).

All samples were defrosted and all samples from a single day were homogenised. Two 0.5g amounts of wet homogenised sample were weighed out (treated as replicates for each weekday). We added 5 mL of 90% (v/v) methanol and incubated the samples at room temperature on an orbital shaker overnight. To remove any insoluble material the samples were centrifuged at 500 g for 20 minutes; solvents were removed from the supernatant in a SpeedVac at 60 °C until dry. All the dried homogenised pellets from each day were resuspended in a total volume of 500 µL 90% (v/v) methanol.

Resuspended samples were diluted in assay buffer at a 1:20 ratio. 50 µL of samples and standards were pipetted into the relevant wells; 75 µL of assay buffer were pipetted into each of the non-specific binding (NSB) wells. 50 µL of assay buffer was pipetted into the maximum binding (B0) wells. 25 µL of cortisol conjugate was added to each well, followed by 25 µL of cortisol antibody to each well (except the NSB wells). The plate was covered with a plate sealer and rotated on an orbital shaker at room temperature for 1 hour. The wells were aspirated before being washed four times with 300 µL wash buffer. 100 µL of the TMB Substrate was added to each well and the plates were incubated at room temperature for 30 minutes. 50 µL of the stop solution was added to each well. The optical density generated from each well was read using an ASYS UVM340 plate reader at 450 nm. Cortisol concentrations were determined from these readings using the manufacturers' online data analysis tool (MyAssays Ltd., https://www.myassays.com/arbor-assays-detectx-cortisol (extended-range) assay, Accessed 12 Dec 2019).

Statistical analysis of the cortisol levels

To test for data normality a Shapiro-Wilk test was used. To test homogeneity a Bartlett test was used. A t-test was used to test for difference in cortisol levels between the two herds. A Kruskal Wallis test was used to test for differences between visitor days. Significance threshold was set at p = 0.05. All statistical analysis was carried out in R (2013).

Results

Model validation and feature probability

The power and accuracy of the best fit model (Table 5) to predict the probability of an encounter occurring within a grid square, based on landscape features, was validated using a ROC curve (Fig. 4). The high AUC (AUC = 0.92) suggests that the model had

Table 5. Results from best fit model. All explanatory variables were included in this model.

Feature	Estimate	Standard Error	t Value
(Intercept)	7.49×10-1	2.4×10-2	31.194**
Building/Garden/Visitors centre	1.44×10-4	8.35×10-6	-17.292**
Footpath/Road	-2.91×10-5	5.24×10-6	-5.546**
Grassland/Marsh	2.98×10-5	4.87×10-6	6.118**
Heathland	2.21×10-4	4.09×10-6	-53.91**
Mire	-1.06×10-5	4.93×10-6	-2.153*
Open water	-1.52×10-4	7.62×10-6	-19.992**
Running water	1.59×10-4	5.12×10-6	31.044**
Tall Herb/Fern	1.28×10-4	5.67×10-6	22.656**
Woodland/Scrub	-6.97×10-5	5.88×10-6	-11.839**

*Significant at p<0.05 ** Significant at p<0.05

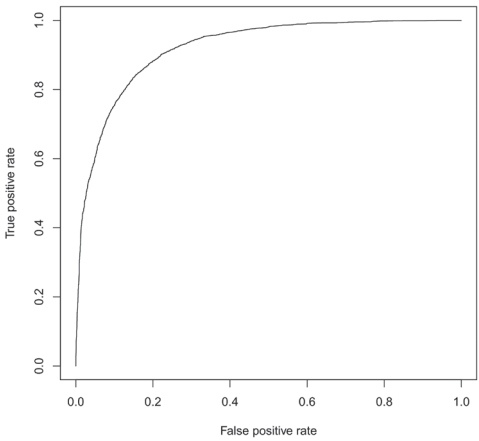

Figure 4. Receiver operating characteristic (ROC) curve showing the predictability of the human-deer (*Cervus elaphus*) encounter model. The true positive rate (sensitivity) is plotted as a function of the false positive rate (specificity). The area under the ROC curve was 0.92. Data collected from Lyme Park, Disley, UK, in 2018.

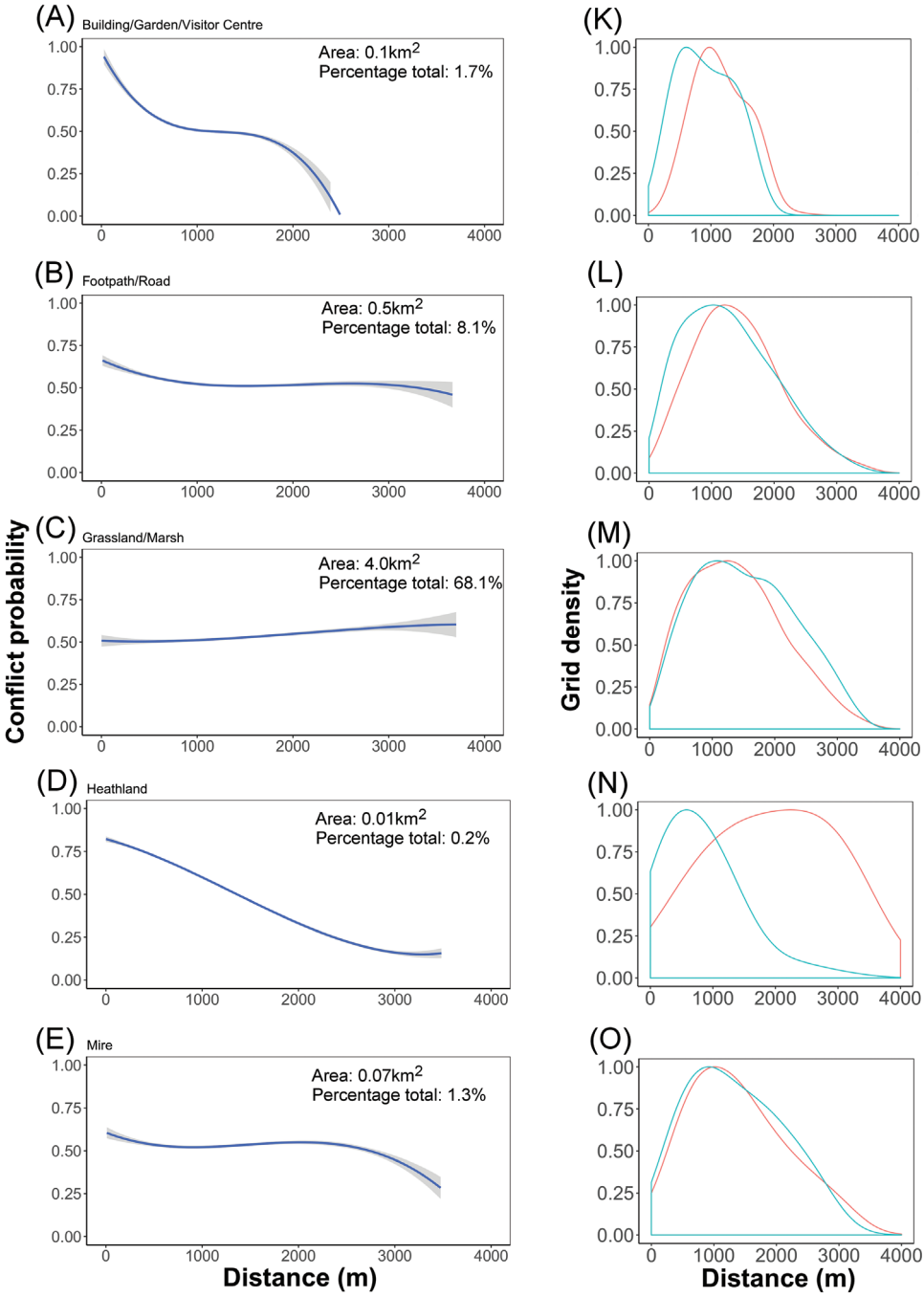

Figure 51. A–E human-deer (*Cervus elaphus*) encounter probability modelled with distance to each feature. The 95% confidence intervals are shown in grey **K–O** Density distribution of randomly sampled high-encounter points (blue) and low-encounter points (red) modelled with distance to each feature. Data collected from Lyme Park, Disley, UK, in 2018.

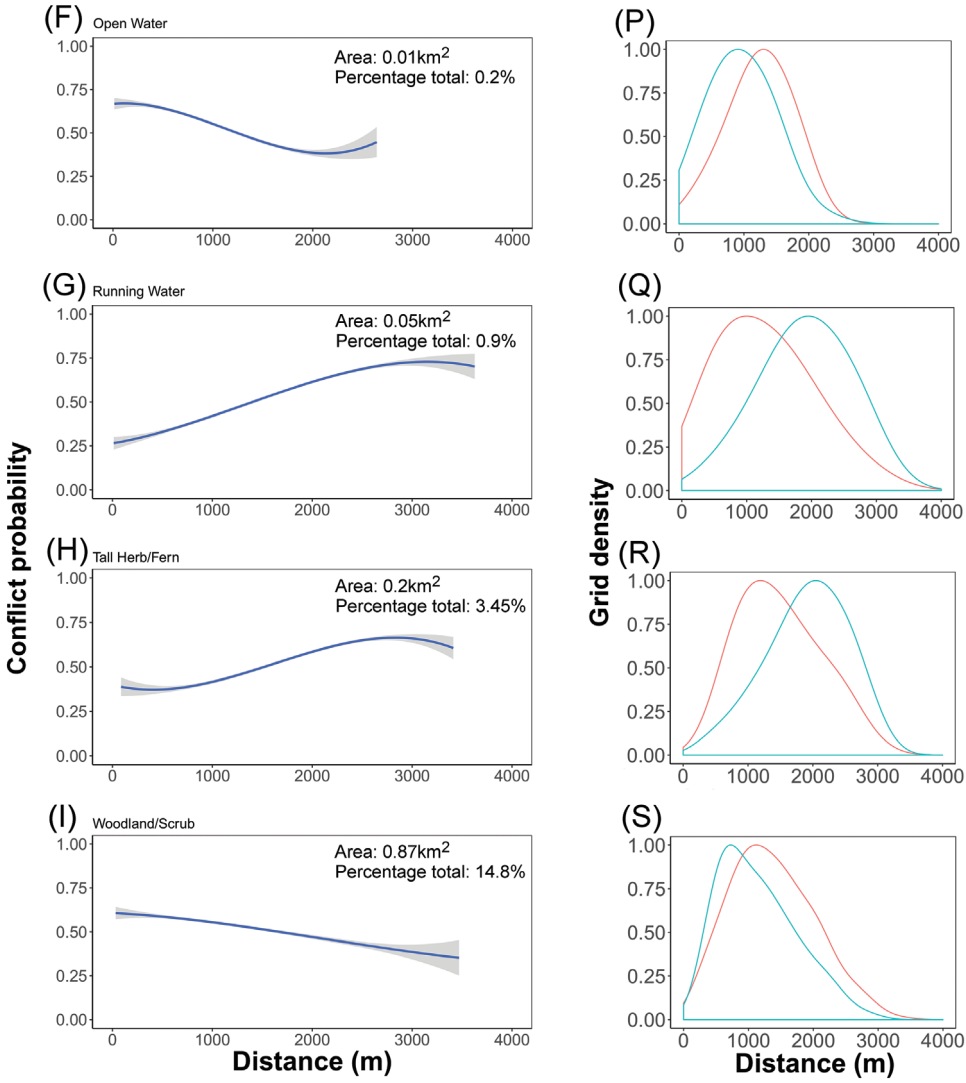

Figure 511. Continued. **F–I** human-deer (*Cervus elaphus*) probability modelled with distance to each feature. The 95% confidence intervals are shown in grey **P–S** Density distribution of randomly sampled high-encounter points (blue) and low-encounter points (red) modelled with distance to each feature. Data collected from Lyme Park, Disley, UK, in 2018.

a good predictability (Robertson et al. 1983; Swets 1988). For each modelled landscape feature, the encounter risk probability was plotted in relation to geographical distance. Running water, grassland/marsh, and tall herb/fern exhibited a positive correlation between encounter probability and increased distance. Building/garden/visitor centre, footpath/road, heathland, mire, open water and woodland/scrub exhibited a negative correlation between encounter probability and increased distance. Encounter probability was highest close to Building/Garden/Visitor Centre landscape features (Fig. 5).

Encounter heatmap

The encounter heatmap takes the results of the model and applies them to create a visualisation of the spatial distribution of encounter probability (Fig. 6). The map shows high-encounter probabilities were predicted in the central areas of the park, generally aligning with areas where high visitor activity has been observed, most notably around the main visitor attraction of the house and lake. Low probability of encounter was predicted in areas of predominately Grassland/Marsh which were not close to other landscape features. The south of the park had the greatest proportion of low-encounter area. Five percent (0.29 km^2) of Lyme Park fell into the highest risk category (0.8–1), with a mean encounter probability across the park of 0.55 (Fig. 7).

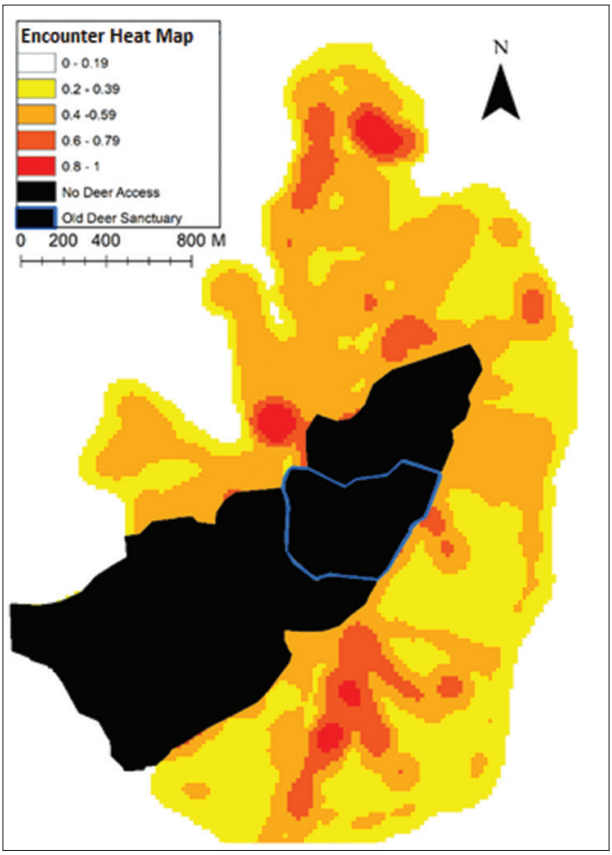

Figure 6. Modelled probability of human-deer (*Cervus elaphus*) encounters based on landscape features. Spatial grain= 20 m. The darker the colour, the greater the probability of encounters occurring. The hatched area is currently not accessible by deer but was included for the mapping of the spatial model. Area outlined in blue is an old deer refuge area that may be reopened in future. Data collected from Lyme Park, Disley, UK, in 2018.

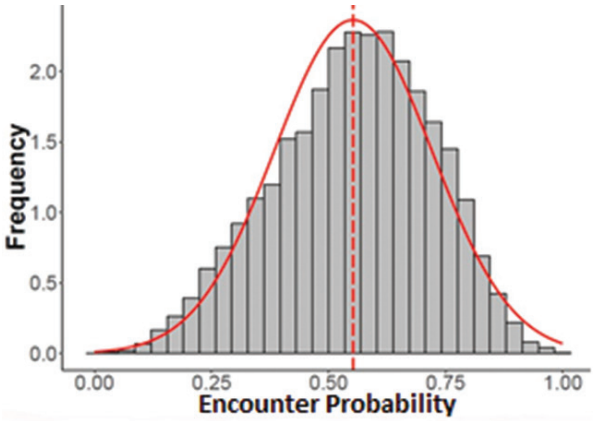

Figure 7. Frequency histogram showing encounter probability for all spatial grains (n=66,902). The y-axis is a proportional scale (2=3000). Data collected from Lyme Park, Disley, UK, in 2018.

Faecal cortisol

The assay results showed that cortisol levels differed significantly between the herds (t = 2.27, df = 26, P = 0.03), with the Moor Herd (M = 8329.14, SD = 4142.2) being significantly higher than the Park Herd (M = 5135.29, SD = 3266.05). The cortisol levels were only found to be significantly higher on Sundays compared to Wednesdays; comparisons between the other days found no significant differences (Fig. 8).

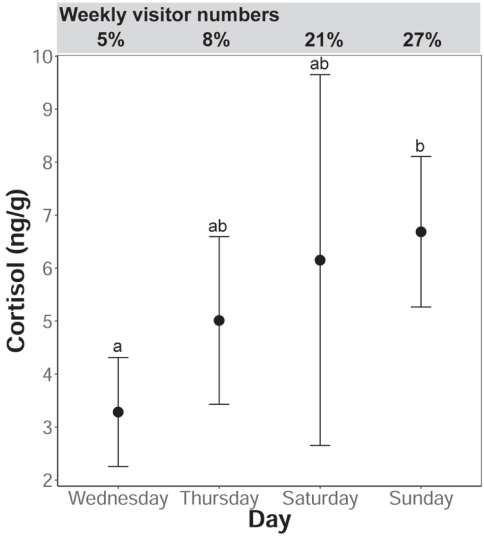

Figure 8. Mean faecal cortisol concentrations and 95% confidence interval from samples collected at different days of the week. Different letters indicate significant differences at p=0.05. The grey box above the figure shows the weekly percentage (of the total) visitor numbers during the period when the samples were collected. Data collected from Lyme Park, Disley, UK, in 2018.

Discussion

The study found a link between the numbers of visitors in the park and the amount of cortisol found in the faecal matter of the deer. This was a notable finding for the rangers at the site who could use these results to strengthen the argument that visitors are indeed having an impact on the wellbeing of the herd. The impact of human activity in green spaces is an issue that is likely to increase in the future, as the pressure on British green spaces will grow with an increase in population. Our encounter probability map can become a useful tool for rangers to inform their management practices on the ground. Although the map presented here used Lyme Park as a study system, the methodology used can be applied by other, different sites, or species of conservation/malmanagement concern, in order to better understand encounter probability between humans and wildlife.

Previous studies used several different methods to measure deer stress levels, including blood sampling (Jones and Price 1992; Huber et al. 2017), hair sampling (Caslini et al. 2016) and clinical assessments (Montane et al. 2002). A non-invasive technique, which did not require the capture of the deer, was prioritised for this study. The measurement of cortisol levels in faecal samples provided an insight into deer stress levels whilst avoiding additional stress from the collection of this data. This method had been used successfully by several other studies (Millspaugh et al. 2002; Jayakody et al. 2011; Landry et al. 2016; Zbyryt et al. 2018).

The mean cortisol levels of the herd were related to the percentage of weekly visitors to the park on each of the days. Post Hoc, pairwise comparisons using the Tukey and Kramer (Nemenyi) test with Tukey-Dist approximation for independent samples indicated that the cortisol levels were only found to be significantly higher on Sundays compared to Wednesdays; comparisons between the other days found no significant differences (Fig. 8). The large error bars displayed for the combined Saturday reading were due to this day containing the largest variance in result values. This could potentially be explained by either abnormal stress events affecting the deer on individual Saturdays, or by fluctuations in visitor numbers due to external factors. Lyme Park is free of large predators, leaving humans (and their dogs) as the primary predator-related stress factor for the deer. Zbyryt et al. (2018) also showed in their work that proximity to humans increased the frequency of stressful events and, as a result, the intensity of the animals' hormonal response also increased.

In addition, faecal cortisol levels were also compared between the two herds present in the park. The result was unanticipated as we found the moor herd, which is exposed to fewer visitors, was found to have significantly higher cortisol levels compared to the park herd and was located in the area with the lower probability of encounter.

Habituation is a possible explanatory factor for why cortisol levels in the park herd were lower. Repeated exposure to the stressor is a requirement for habituation to occur (Romero 2004). As the Park Herd is exposed to many visitors moving within their range every day, they are likely to have acclimated to visitor presence to a greater degree compared to the Moor herd. This acclimatisation may result in the park herd avoiding

areas of high disturbance, which they can recognise through repeated exposure. This type of avoidance behaviour has been highlighted as a result of habituation occurring (Bonnot et al. 2013).

In the case of Lyme Park these high disturbance areas may be the areas surrounding the gates and the car parks which are extremely busy. The possibility that the park herd has habituated to the visitors may have mitigated the stress response but it has not been eliminated completely, hence this is why the busier visitor days still had an impact on them. During the study the deer were observed being extremely wary of humans and would flee if approached, suggesting that the herd has not been completely habituated. As the moor herd was not subjected to the same level of visitor numbers with the same regularity and predictability, they may not have developed the same avoidance strategies as the park herd, meaning less mitigation of their stress response.

A second explanatory factor may be the differences in habitat types found in each of the herd's ranges. The map generated allows for the visualisation of how each landscape feature interacts across the spatial frame of Lyme Park. This allows the role of habitat to be examined in greater depth. The main feature which was comparatively sparse in the moor herds range was woodland and scrub. Studies have indicated this type of landscape provides refuge areas where ungulates can avoid stressors, particularly predators (Torres et al. 2012). As humans are perceived by deer as potential predators, tree cover could be utilised in much the same way (Ciuti et al. 2012). This refuge-seeking response to human activity has been found to be most prevalent during summer days (Coppes et al. 2017a) during a similar time when we collected our data. The increased need to maintain constant vigilance is a behaviour intrinsically linked to disturbance and has been correlated with decreasing time spent feeding and increased stress levels (Torres et al. 2012). The effects of lacking refuge could be exacerbated by the weather such as wind and precipitation in the winter, and as shelter from the heat and sun in summer. Exposure to these types of weather conditions due to a lack of cover has been found to result in higher cortisol levels in ungulates (Landry et al. 2016). Whilst this possibility has been supported by the aforementioned papers, it is important to note that the quantification of vegetation cover was not carried out as part of this study. Doing so could offer a greater insight into the suitability of the habitats at Lyme Park to act as sufficient cover.

The importance of the woodland/scrub landscape feature as a refuge can also be looked at across the entirety of the park. Our model found that the closer a point was to woodland/scrub, the lower the expected encounter probability was. This again supports its utilisation by deer as a refuge in areas where human activity is prevalent (Ciuti et al. 2012; Torres et al. 2012; Coppes et al. 2017a). Red deer in less forested sites have also been found to exhibit a stronger reaction to disturbance than those in more densely forested sites (Jarnemo and Wikenros 2014). The current management at Lyme Park reflects this thinking: a section of woodland within the park herd range is closed to the public, providing a refuge area for the herd. A similar refuge area is not present in the moor herds' range: this offers an opportunity for management to be altered to reduce the probability of human-deer encounters.

Opening the old deer sanctuary area (Fig. 6), would likely be a good option. This area is wooded, a feature which is currently lacking in the Moor Herds' range. It would be preferable to restrict human access to this area. If this is not possible it could be considered only for peak visiting hours. This would provide additional refuge area for the deer, as well as providing a buffer between the busier central area of the park and the rest of the herds' range.

To try to reduce deer cortisol levels across the entire park the map and model we produced is useful as it outlined the relationship between encounter probability and the distance to individual landscape features (Figs 5, 6). As human presence is a required factor for human deer encounters to occur, it is not surprising that the areas in the park which attract many of the visitors increase the probability of humans and deer encounters. This is especially true in the case of Lyme Park, where the deer are considered a visitor attraction and as a result are actively sought out by some visitors. An example of an area where visitors were abundant and encounter risk was high is the main house, gardens, and particularly the lake. Open water is an essential habitat resource, which has been found to be a strong driver for habitat selection by deer (Wu et al. 2016; Coppes et al. 2017a). Our model predicted that the closer to open water a point was, the higher the encounter probability. The map also shows this relationship with the lake falling into a red, high encounter probability zone (Fig. 6). The lake is a popular destination for visitors due to its location beside the café. It is also popular with deer whenever there are no visitors in the park, e.g. early morning. Despite running water similarly being an essential habitat resource it exhibits the opposite relationship; this is likely because it falls outside of the high visitor areas. Providing alternate open water accessible to the deer but not to visitors may help reduce deer stress levels.

Similarly, low-encounter areas can also be useful as they can provide a template for low stress deer habitat which can then be emulated in other areas of the park, particularly in identifying areas that can improve alternate areas of deer refuges (Torres et al. 2012; Coppes et al. 2017a). Not all landscape features showed a clear relationship between distance and encounter probability: the most notable example is the grassland/marsh feature. This covered the largest habitat area in both the high and low risk zones (Table 2). The encounter probability remained relatively stable as risk points from either encounter zones are generally near to this feature. At the greatest distances encounter probability increased as the confidence intervals widened (Fig. 8).

Conclusion and management implications

Finding ways to reduce the probability of human-deer encounters is an important consideration for the management of this site, and, by extension, other similar sites. The site managers want to reduce the stress responses these encounters cause, as it has the potential to impair biological functions and lower survival rates of their deer populations (Sapolsky et al. 2000). The impact the visitors are having on deer stress levels is

an inherent problem due to the nature of the site. Restricting visitor access to larger areas of the park where the herds are primarily situated would also greatly reduce the risk of human-deer encounters. However, this would need to be balanced against the loss of potential visitors and the funds they bring. A more realistic aim which would keep the majority of the park open, whilst giving the deer relief from human activity would be to mitigate the risk of human-deer encounters occurring specifically close to deer refuge areas. This mitigation of deer stress could be further supported through the creation of more deer refuge areas, particularly the old deer sanctuary in moor herds' range; the creation of alternate open water sources away from high visitor areas and the emulation of low encounter habitat in other higher risk areas of the park.

The methods used in this study could be adapted and transferred to inform local conservation management elsewhere. The results of our cortisol experiment support other studies which found that human disturbance can negatively impact deer stress levels. This alone should give reason for managers at sites which contain deer to consider the impact visitor numbers may be having on their herds. As the visitor pressure increases across British greenspaces this is likely to become a more prevalent problem. Although some of these negative effects may be reduced by increased habituation of the deer to visitors, this paper has shown that even herds exposed to human activity over a period of decades still exhibit an increase in cortisol levels on days when visitor numbers are highest. The mapping and modelling systems used in this paper could readily be adapted for other sites to help modify deer management to help limit the impact of human disturbance. The model and map would allow for the identification of high encounter zones which would require mitigated or low encounter zones to be promoted. Although the management recommendations are linked to the unique landscape of Lyme Park, they are rooted in the findings of other papers, making them generally applicable. In particular, the importance of refuge areas is something managers should not overlook as it is a valuable resource highlighted in our own paper and supported by other studies.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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RESEARCH ARTICLE

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Termites' diversity in a protected park of the northern Sudanian savanna of Togo (West Africa)

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Abstract

Termites occur in many ecosystems throughout tropical and subtropical areas. Their distribution is driven by several factors, including landscape and some soil characteristics. This study aims to determine soil organic matter's role on termites' diversity in a shrubby savanna park. Termites were sampled across transects in 3 sites of Galangashi park (northern part of Togo). The soil in which termites were harvested was analyzed to check organic matter's influence on termites' species richness. A total of 28 termite species belonging to 14 genera and 6 subfamilies were identified. Feeding group II (all fungus-growing termites, grass feeders, and wood feeders) was the most important among the three identified groups. Nine species (with two potentially new species for sciences: *Amitermes* sp and *Eremotermes* sp) were recorded for the first time in the country. A strong correlation was found between species richness, total organic matter, and total organic carbon, suggesting the influence of soil richness on termite distribution. The occurrence of the unique member of the feeding group I, *Coptotermes intermedius* Silvestri, 1912, as well as the occurrence of *Fulleritermes tenebricus* Silvestri, 1914 (both wood-dwelling termites), was certainly due to the vegetation. The relatively higher species richness as well as the correlation between the species richness and the organic matter of shrub savanna, suggest a better conservation of this landscape.

Keywords

Organic carbon, shrub savanna, species richness, systematic

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Introduction

Termites are ecosystem engineers (Jones et al. 1994) and are among the most dominant macroinvertebrates in tropical soils (Bignell and Eggleton 2000). Their nesting activities promote soil aeration, improve the absorption and storage of water in the soil, and facilitate carbon flux (Lobry de Bruyn and Conacher 1990; Sileshi et al. 2010). Worldwide, there are more than 3106 species of living and fossil termites classified into 12 families and 330 genera (Krishna et al. 2013), which are particularly abundant and diversified in tropical forest and savannas within the 10° North and South latitudes from the equator (Collins 1989). Thus the majority of termite species live in tropical and subtropical ecosystems. However, they are unevenly distributed: the large equatorial forests constitute an important reservoir of termite species (Jones and Eggleton 2011). Termite species richness of these forests is estimated at more than 70 species per hectare (Eggleton 2000).

In comparison to these primary forests, agro-systems hosted fewer species because termites are very sensitive to any disturbance of their environment and are thus used as bioindicators (Viana et al. 2016). The species richness and abundance of termites are extremely affected by agricultural practices (Samb et al. 2011; Dosso et al. 2013; Kaiser et al. 2015). These and other human activities, such as mining and urbanization, are among the major causes of the loss of termite diversity (Green et al. 2005). Rainfall, as well as the type of soil, are also factors that influence the diversity of termites (Lobry de Bruyn and Conacher 1990).

Several authors (Konaté et al. 2003; Dosso et al. 2010, 2012, 2013, 2017) have conducted studies on the termite in relation to natural or processed habitats in West Africa. Moreover, it appeared that termite species richness decreases with the increase of land use (essentially for farming and agro-systems establishment). Therefore protected areas are places where more termite species could be found. In Togo, only two similar types of research have recently been carried out in the forest and teak plantation in the southern part of the country (Schyra et al. 2019) and Sudanian savannas and fallows (at different ages) in the Oti Keran park located in the northern part of the country (Schyra and Korb 2019). The park of Galangashi (our study area) is located in the north of Oti Keran park and to the best of our knowledge, no data on termites from this area are available. Because of the unprospected condition of Galangashi park (for termite species), we evaluated the diversity of termites in this protected shrubby savanna park. We also analyzed a correlation between the species richness of termites and soil total organic material.

Material and methods

Study sites

This study was carried out at Galangashie protected park located in the savanna region (Fig. 1) between 10°19'–20°28'N and 0°14'–0°27'E (northern part of Togo). It covers 7650 hectares, and it had been protected since its creation in September 1954. The

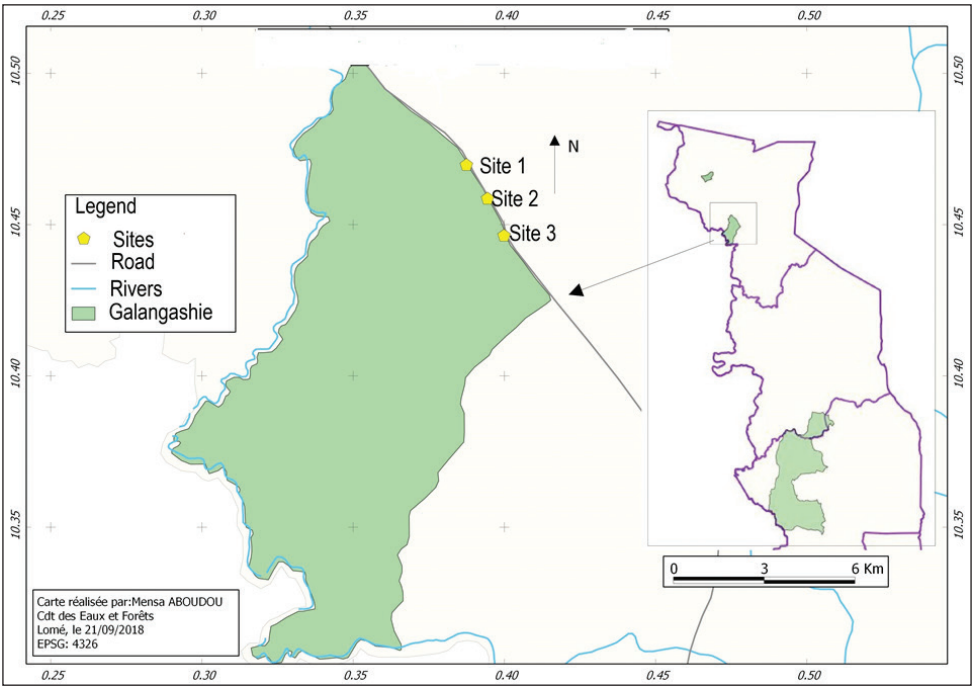

Figure 1. The location of the study area Galangashie.

study area has a Sudanian tropical climate characterized by a long dry season (November to May) and a long rainy season (June to October). The mean temperatures range from 30±3 °C (during dry season) and 29±2 °C (during the rainy season) with an annual rainfall of 986 mm. The landscape is a shrubby savanna.

Sampling of termites

Termites were sampled within the frame of belt transects. Three sets of three belt transects were laid at three sites: site 1 (10°26'42.6"–10°26'43.4"N; 000°23'52.4"–000°26'56.9"E) site 2 (10°27'30.8"–10°27'31.9"N; 000°23'39.5"–000°23'42.0"E) and site 3 (10°28'11.2"–10°28'10.8"N; 000°23'16.4"–000°23'16.6"E). The sets of three transects were at a distance of at least 2 km from each other, and two consecutive transects were separated by at least 15 m (Fig. 3). Each belt transect was 100 × 2 m divided into 20 units of 5 × 2 m as described by Jones and Eggleton (2000) for forest ecosystems. In our study, this standard protocol was adapted to the savanna ecosystems according to Dosso et al. 2010 and Hausberge et al. 2011. Each unit (5 × 2 m) was sampled for 15 minutes, according to Schyra and Korb (2019). During this period, termites were systematically searched in their microhabitats such as litter, dead wood, trees (up to 2 m above ground), grasses, uprooted grass tufts and inside mounds within each sampling unit by a well-trained collector. The search time of 15 minutes was chosen instead of 30 minutes (as proposed by Jones and

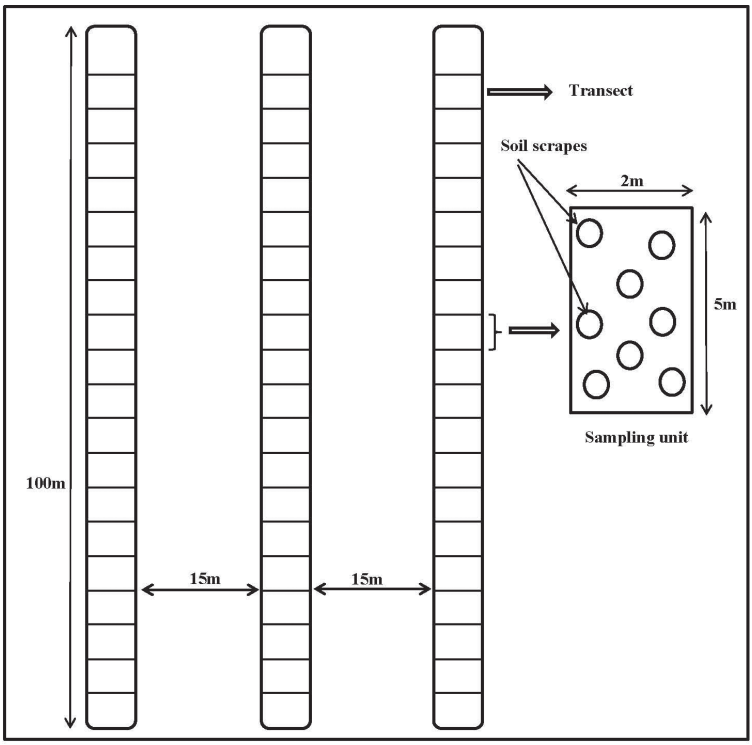

Figure 2. Scheme of transects with a sampling unit.

Eggleton 2000) because available substrates or microhabitats are lesser in savanna ecosystems than in forest ones. The length of the belt transect (100 m) was kept in our study, instead of 50 m proposed by Schyra and Korb (2019) in order to increase the sampling effort. Termites were also searched throughout eight soil scrapes of 15 × 15 × 10 cm (Fig. 2) according to Schyra and Korb (2019) within each unit. Collected termites were stored in 95% ethanol inside labeled vials and kept in the laboratory for further processing.

Termite identification

Termite specimens were sorted and grouped into morpho-species. The identification was based on morphological characters (antenna, mandible, head, labrum, pronotum, gula) and morphometric parameters (head length and width, mandible length, pronotum width, gula width, and hind tibia length). Measurements were made using a stereomicroscope (Leica EZ4) with an integrated camera connected to a computer. Las EZ software application was used for image acquisition and mensuration. The reference works of Grassé (1937), Bouillon and Mathot (1965), Silvestri (1912), Sands (1965, 1992), and Ruelle (1970) were used for the species identification.

The voucher specimens are conserved in the entomological collection of "Laboratoire d'Entomologie Appliquée" of the University of Lomé (Togo).

The obtained species list of this study was compared to previous studies (Anani Kotoklo et al. 2011; Kasseney et al. 2019; Schyra et al. 2019) carried on termites in Togo, in order to sort out the newly recorded species.

The identified species were classified within their corresponding feeding groups, according to Donovan et al. (2001).

Evaluation of soil total organic material

Soil samples were collected in transects in which termites were harvested. An amount of 500 g of soil was taken from each sampling site and kept in plastic bags. They were brought to the Laboratory of Chemistry of the University of Lomé for chemical analysis. Five grams of each soil sample (previously dried at 220 °C) were burned at 450 °C for 3 hours. After cooling, the percentage of mass loss generated by the calcinations constitutes the soil samples' total organic material content. The total organic carbon content was derived from the total organic matter. It was estimated to be 58% of the content of the total organic material.

Data analysis

For analysis smoothness, each set of three transects was considered as a replica. Data from each set of three transects were then pooled, and three replicates were obtained. We used the occurrence (presence or absence) and the relative abundance (the percentage of the occurrence of each single species in a sampling site) of specimen instead of a number of collected individuals because the occurrence of a single individual indicates the presence of a whole colony of termites around.

Alpha diversity indices, including the total observed richness (Sobs), Simpson index of diversity (1-D), and Shannon index, were calculated using Past 3.2 software (Hammer et al. 2001).

The test of Kendalls τ was run to examine the correlation between species richness, total organic material, and total organic carbon of the soils from each set of three transects. Because there were three sets of three transects, the sample size was 3: each set of three transects was combined to get one sample size. This test was carried out in SPPS 20 at the level of 0.01. The coefficient of Kendalls τ test ranges from -1 (a strong negative correlation) to +1 (a strong positive correlation) with 0 value indicating no link between the examined factors.

Results

Diversity

A total of 28 species belonging to 14 genera and 6 subfamilies were identified (Table 1). Macrotermitinae subfamily not only had a higher number of sampled genera (*Ancistrotermes* Silvestri, 1912; *Macrotermes* Holmgren, 1909, *Microtermes* Wasmann,

1902 and *Odontotermes* Holmgren, 1910) but also a higher number of species (9). Termitinae was the second most important subfamily in terms of the recorded number of genera (*Amitermes* Silvestri, 1901; *Eremotermes* Silvestri, 1911; *Microcerotermes* Silvestri, 1901) and also in term of sampled species (9). The other subfamilies (Apicotermitinae, Cubitermitinae, and Nasutitermitinae) were all represented by two genera except the Coptoterminiane subfamily, for which only *Coptotermes intermedius* Silvestri, 1912 was recorded.

Microtermes grassei Ghidini, 1955 was the most sampled species with 25.42% of occurrence (Table 1). It was followed respectively by, *Microtermes toumodiensis* Grassé 1937 (11.62%), *Trinervitermes oeconomus* Trägårdh, 1904 (11.62%), *Microcerotermes* sp (6.78%) and *Fulleritermes tenebricus* Silvestri, 1914 (5.33%). Besides these frequently sampled species, several species, including *Amitermes* sp. (0.48%), *Coptotermes intermedius* Silvestry, 1912 (0.48%), *Odontotermes* sp.1 (0.48%)and *Trinervitermes togoensis* Sjöstedt, 1899 (0.48%), were rarely found during our study. That represented precisely the double of the total occurrence (0.24%) of the less sampled species, *Amitermes spinifer* Silvestri, 1914, *Ancistrotermes crucifer* Sjöstedt, 1897, *Cubitermes* sp. and *Noditermes* sp. (Table 1).

Newly sampled species

Among the sampled specimens, 9 species to our knowledge were identified for the first time in Togo (showed in Table 1 with an asterisk). Two species, *Amitermes* sp., *Eremotermes* sp. could be new (not yet described) species for sciences.

Feeding groups

The sampled termite species (from both landscapes) belong to 3 out of 4 feeding groups: feeding group I (FG I), feeding group II (FG II), and feeding group IV (FG IV) (Table 1). FGI was represented by the unique species of *Coptotermes intermedius*, a wood-feeding termite. FG II consisted of Macrotermitinae, Termitinae, and Nasutitermitinae subfamilies, the most important feeding group with 8 genera and 21 species out of 28. Species of this group essentially feed on wood and or litter and grasses (*Trinervitermes* species). The last feeding group (FG IV) found in our study included 2 subfamilies Apicotermitinae and Cubitermitinae, with 4 genera: *Adaiphrotermes* Sands, 1972; *Aderitotermes* Sands, 1972 (both belonging to Apicotermitinae subfamily), *Cubitermes* Wasmann, 1906 and *Noditermes* Sjöstedt, 1924 (both genera of Cubitermitinae subfamily).

Diversity analysis

The Simpson index (1-D) for sampled termites was 0.89 (Suppl. material 1). This value is close to 1 (the highest possible value), indicating a great diversity. The same pattern of great diversity was also shown by Shannon index 2.63.

Table 1. List of termites' species collected, feeding group and habits, and relative abundance (RA).

Feeding group	Feeding habit	Subfamilies	Termites species	RA (%)
Group IV	S	**Apicotermitinae**	*Adaiphrotermes* sp.	2.91
			Aderitotermes sp.	2.66
Group I	W	**Coptotermitinae**	*Coptotermes intermedius* Silvestri, 1912	0.48
Group IV	S	**Cubitermitinae**	*Cubitermes* sp.*	0.24
			*Noditermes cristifrons** Wasmann, 1911	0.73
			Noditermes sp.	0.24
Group II	W/L (F)	**Macrotermitinae**	*Ancistrotermes cavithorax* Sjöstedt, 1899	0.73
			Ancistrotermes crucifer Sjöstedt, 1897	0.24
			Macrotermes bellicosus Smeathman, 1781	1.21
			Microtermes grassei Ghidini, 1955	25.42
			*Microtermes hollandei** Grassé, 1937	1.69
			Microtermes Lepidus Sjöstedt, 1924	3.15
			*Microtermes toumodiensis** Grassé, 1937	11.62
			Odontotermes erraticus Grassé, 1947	0.73
			Odontotermes sp.	0.48
Group II	W	**Termitinae**	*Amitermes evuncifer* Silvestri, 1912	3.15
			Amitermes guineensis Sands, 1992	0.97
			Amitermes sp.*	0.48
			*Amitermes spinifer** Silvestri, 1914	0.24
			*Amitermes truncatidens** Sands, 1959	1.21
Group IV	S		*Eremotermes* sp.*	2.91
Group II	W		*Microcerotermes parvulus* Sjöstedt, 1911	5.33
			*Microcerotermes solidus** Silvestri, 1912	4.12
			Microcerotermes sp.	6.78
Group II	W	**Nasutitermitinae**	*Fulleritermes tenebricus* Silvestri, 1914	5.33
			Trinervitermes occidentalis Sjöstedt, 1904	4.84
			Trinervitermes oeconomus Trägårdh, 1904	11.62
			Trinervitermes togoensis Sjöstedt, 1899	0.48

S: soil feeders, W wood feeders, W/L Wood and or litter feeders, (F): fungus-growing termites, G: Grass feeders. Newly recorded species names are followed by an asterisk (*).

Correlation between species richness and soil organic material

The total organic material of soil was positively correlated with the species richness found in this landscape (Fig. 3, Suppl. material 1). The Kendall τ test ($\tau = 1$) showed that this correlation was very significant ($p < 0.001$, $N = 3$). There was also a strong, positive correlation (Fig. 4) between species richness and its total organic carbon ($\tau = 1$, $N = 3$, $p<0.001$).

Discussion

Diversity

The distribution of termites (like many other terrestrial arthropods) is driven by several factors, including soil richness (Park et al. 1994; Bourguignon et al. 2015), vegetation (Park et al. 1994; Gillison et al. 2003), and also the degree of anthropogenic disturbance (Hausberger and Korb 2016; Netshifhefhe et al. 2019). In our study, the

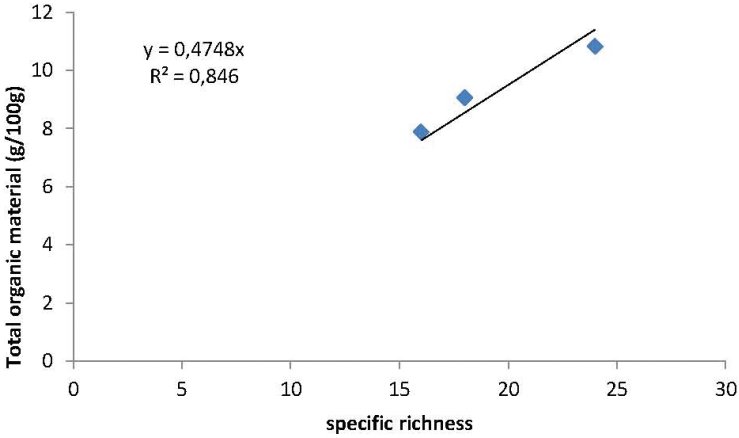

Figure 3. Correlation between species richness and total organic material (Shrub savanna).

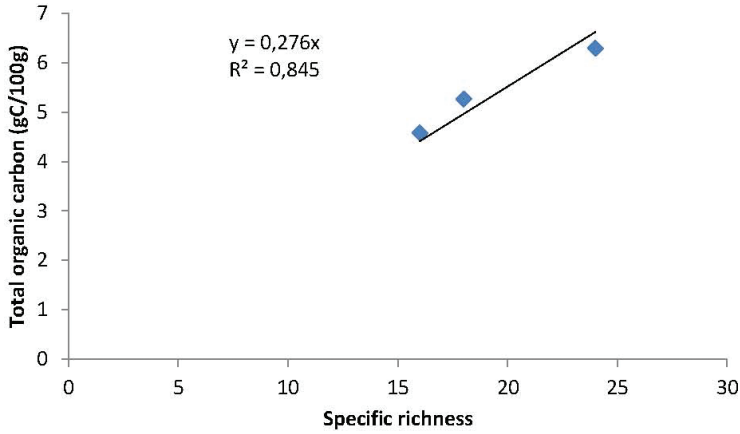

Figure 4. Correlation between species richness and total organic carbon.

main factors examined were the soil richness in organic matter and its total organic carbon. The computed diversity indices (Simpson 1-D close to 1, and Shannon over 2.5) showed that the study area was quite well diversified. Macrotermitinae subfamily, with 4 genera and 9 species, was by far the most important subfamily. Dosso et al. (2013) and Kaiser et al. (2015) carried out a similar study throughout several habitats, including semi-arid, semi-deciduous forest, food crop fields, fallow, and several agro-ecosystems. They found that fungus-growing termites were relatively more abundant in all their studied habitats.

Feeding groups

The high percentage of Macrotermitinae species occurrences is certainly due to their feeding habit and, therefore, to their feeding group. Indeed, these species could feed on

diverse sources of celluloses from dry herbs to dry wood as well as dry leaves, twigs, litter, and even animal dung (Freymann et al. 2008). All these cellulose resources can be found in several landscapes. Besides the ability to feed on large cellulose resources, Macrotermitinae species also developed a symbiosis with a basidiomycete fungus, *Termitomyces*, which contribute to the successful decomposition of lignocelluloses (Ohkuma 2003).

Coptotermes intermedius, feed essentially on wood (dry or still alive). The presence of many kinds of wood has facilitated its occurrence in the shrub savanna. Indeed, wood feeders were reported to be very sensitive to disturbance and therefore were less abundant in habitats with few and small trees (Dosso et al. 2013). Similarly, *Fulleritermes tenebricus* is also found in medium to densely shrub savanna as well as a forest ecosystem (Schyra et al. 2019).

Microtermes grassei was the most sampled species indicating its ubiquitous state and its ability to dwell on large food sources. This species was also sampled in the forest ecosystem (Schyra et al. 2019) as well as in the open grassy area of a botanical garden in the middle of a city (Kasseney et al. 2019).

Effect of soil organic matter on termite species richness

A positive correlation was found between termite species richness and soil organic matter. Indeed, this landscape, as mentioned above, was a protected area since its creation. Fallen leaves, dry twigs, and branches from sampled trees are food for many termite species that recycle these plant parts (Ohkuma 2003; Brune and Ohkuma 2010). This recycling increases the amount of soil organic matter, which is beneficial for soil-dwelling termites such as *Noditermes* species.

Newly recorded species

The occurrences of newly recorded species underlined the importance and the contribution of this study to the knowledge of termite species in the country, in keeping with previous studies (Anani Kotoklo et al. 2011; Kasseney et al. 2019; Schyra et al. 2019). Indeed 9 out of 28 termite species were recorded for the first time in the country. Moreover, besides enlarging the country's termites list, this study, with two potentially new species (*Amitermes* sp. and *Eremotermes* sp.), appeared to be a modest contribution to termites' systematic and taxonomy throughout the entire world. *Amitermes* species are commonly found in arid zones as well as in wet areas (Sands 1992). The new record of these species in our study is due to the lack of available data on termites in this area. This study was the first on termites during the post-colonial period in this area to the best of our knowledge. The species of the genus *Eremotermes* were thought to occur exclusively in arid zones. Its presence in our study area (a Sudanian savanna) support the result of Ndiaye (2014) who sampled some species of *Eremotermes* in a relatively wet savanna in Senegal. *Eremotermes* species are very cryptic (Sarr 1999; Diop et al. 2013); for this reason they are usually not seen and sampled by the collectors in the field.

Conclusion

It is the first recent study carried out on termites in the area mentioned above. The study area was quite well diversified. Twenty-eight (28) termite species were identified from the three sets of three transects. Macrotermitinae was the most important subfamily and *Microtermes grassei* was the most sampled species among them all. This study was a modest contribution to the list of termites in Togo as 9 new species (among the 28 identified) were added to the list of termites' species that occur in the country. Two of these newly recorded species (*Amitermes* sp and *Eremotermes* sp) seemed to have been hitherto undescribed to the best of our knowledge. Termites' species richness was correlated with soil organic matter.

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Supplementary material 1

Tables S1, S2

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Data type: diversity data

Explanation note: Table S1. Termites diversity index in the two habitats. Table S2. Correlation with Kendall τ test.

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RESEARCH ARTICLE

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Natural strongholds for red squirrel conservation in Scotland

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Abstract

The Eurasian Red Squirrel (*Sciurus vulgaris*) is under threat from the invasive North American eastern Grey Squirrel (*Sciurus carolinensis*) with 80% of the remaining red squirrel populations in the British Isles found in Scotland. In this study we develop a spatially explicit mathematical model of the red and grey squirrel system and use it to assess the population viability of red squirrels across Scotland. In particular, we aim to identify existing forests – natural strongholds for red squirrels – that can successfully support red squirrels under UK Forestry Standard management and protect them from potential disease-mediated competition from grey squirrels. Our model results indicate that if current levels of grey squirrel control, which restrict or reduce the distribution of grey squirrels, are continued then there will be large expanses of forests in northern Scotland that support viable red squirrel populations. Model results that represent (hypothetical) scenarios where grey squirrel control no longer occurred indicated that grey squirrel range expansion and the process of red squirrel replacement would be slow. Model results for an assumed worst-case scenario where grey squirrels have expanded to all regions in Scotland identified forest regions – denoted natural strongholds – that could currently support red squirrels under UK Forestry Standard management practice. The results will be used to inform forest management policy and support a strategic review of red squirrel management by land management agencies and other stakeholders.

Keywords

Ecological modelling, forest management, invasive species, policy

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Introduction

The Eurasian red squirrel (*Sciurus vulgaris*) is under threat in the British Isles. Recent estimates (Mathews et al. 2018) indicate that just over 80% of the remaining British populations are now thought to live exclusively in Scotland. The decline of red squirrel populations has arisen due to the expansion of the North American eastern grey squirrel (*Sciurus carolinensis*) which was introduced into the British Isles in the 19th century (Middleton 1930). The replacement of red squirrels by greys has occurred due to the disease-mediated competition that occurs between the two species, with grey squirrels acting as a reservoir for squirrelpox virus that is lethal to red squirrels, but non-lethal and endemic in grey squirrels (Tompkins et al. 2003; Rushton et al. 2006).

Current efforts to protect red squirrels in Scotland are focused on defending priority populations of red squirrels (Scottish Natural Heritage 2015) across their Scottish range. This is achieved mainly through grey squirrel control, via trapping, that aims to prevent grey squirrel expansion northwards across a chosen boundary line (a line approximately between Helensburgh and Montrose (Scottish Natural Heritage 2015), henceforth called the grey squirrel control boundary). There are also efforts to protect red squirrels in priority regions that lie south of the grey squirrel control boundary. Grey squirrels typically dominate habitat south of the grey squirrel control boundary as well as habitat in Aberdeen and surrounding areas. The grey squirrel population at Aberdeen has persisted since a separate introduction in the 1970s (Saving Scotland's Red Squirrels 2020). At present, grey squirrel control, coordinated by Saving Scotland's Red Squirrels (Scottish Natural Heritage 2015) aims to prevent grey squirrels from expanding northwards beyond the grey squirrel control boundary, to reduce the distribution of greys in Aberdeen and the surrounding area and to defend the isolated red populations in southern and central Scotland. Red squirrel conservation policy also recommends conservation action through sympathetic forest management in selected forest sites (strongholds), which are meant to provide refuge for red squirrels against the incursion and competition from grey squirrels (Scottish Forestry 2020). The native habitat of the grey squirrel is the broadleaved forests of eastern North America, meaning grey squirrels are broadleaf specialists. Consequently, they enjoy a significant competitive advantage over native Eurasian red squirrels in deciduous but not coniferous woodland. Strongholds tend to therefore be large, conifer-dominated forests (Gurnell et al. 2004; Forestry Commission Scotland 2012).

There are currently 19 sites throughout Scotland that have been designated as strongholds (Forestry Commission Scotland 2012; Scottish Forestry 2020). Forest management guidance in these strongholds aims to "use woodland management to maintain healthy self-sustaining populations of red squirrels" (Forestry Commission Scotland 2012). The guidance is based on five key principles: (i) the maintenance of a dependable tree seed food supply, (ii) a resolution of conflicts with other management objectives, (iii) planning for red squirrels at the landscape scale, (iv) long-term planning to sustain resilience and (v) the establishment of a monitoring system. Whilst the guidance acknowledges that stronghold sites should have been chosen to minimise

conflicts (e.g. sites with a low proportion of broadleaved trees that favour grey squirrels), it recognised the challenge for forest managers to integrate red squirrel conservation with other forest management objectives.

A recent study has examined red squirrel viability in designated strongholds under recommended stronghold forest management compared to UK Forestry Standard (UKFS) management for strongholds in Scotland (Slade et al. 2020). The findings showed that forest management policy specifically designed to reduce competition has little benefit in the absence of grey squirrels. Thus, the rationale for managed strongholds north of the grey squirrel control boundary could be reconsidered to account for the distribution of grey squirrels. However, when grey squirrels are present the strongholds perform as intended, with stronghold forest management benefiting red squirrels at the expense of grey squirrels. The study (Slade et al. 2020) also highlighted that some designated strongholds could not protect red squirrels from potential grey squirrel invasion regardless of forest management policy – largely due to the high connectivity of these strongholds to habitat that is favourable for grey squirrels. These designated strongholds are therefore unsuitable for red squirrel protection and reflect an unwise choice in stronghold site selection. Furthermore, the study also described the potential for natural strongholds, where red squirrel populations would persist under UKFS management (Forestry Commission 2017) despite the threat from grey expansion. Management of the designated strongholds requires resources and additional management time. Therefore, the identification of natural strongholds, both north and south of the grey squirrel control boundary, within which red squirrels can persist under UKFS management, even in the presence of grey squirrels, would free resources and management time that could be deployed elsewhere.

Mathematical models that combine accurate habitat information, such as land-cover information provided by GIS data and data on a species' ecology from field studies, with dynamic modelling to capture the population dynamics and species interaction can be utilised to inform conservation policy (Broome et al. 2014; Carter et al. 2015; Heikkinen et al. 2015). Such model frameworks have been successfully employed to inform red squirrel conservation, with models being used to examine the potential spread of squirrelpox in Scotland (White et al. 2016), to assess the importance of grey squirrel control to protect red squirrel populations on Anglesey (Jones et al. 2017), to determine how forest management practice affects red squirrel viability in the absence of grey squirrels (Jones et al. 2016) and in designated red squirrel strongholds in Scotland (Slade et al. 2020). In collaboration with Forestry and Land Scotland (FLS), the Scottish Government agency responsible for managing Scotland's publicly-owned forests and land, we extend the established spatially explicit mathematical modelling framework for the red and grey squirrel system (White et al. 2016; Jones et al. 2016, 2017; Slade et al. 2020) and use it to assess the population viability of red squirrels across Scotland. In particular we aimed to identify existing forests, natural strongholds for red squirrels, that can successfully support red squirrels against potential disease-mediated competition from greys under UK Forestry Standard management. The results will be used to inform forest management policy and support a strategic review of red squirrel management by FLS and other stakeholders.

Methods

In this study we have developed a mathematical model to assess the long-term viability of red squirrels in Scotland. The model is based on previous models of the UK squirrel system in realistic landscapes which have adapted classical deterministic approaches (Tompkins et al. 2003) to develop a spatial, stochastic model (White and Lurz 2014; Jones et al. 2016; White et al. 2016; Slade et al. 2020). The deterministic approach underpinning the model (see equations 1 and 2) allows the key population processes to be defined and understood. However, deterministic models do not include the randomness and variability that is exhibited by real systems. We develop a stochastic version of the deterministic model in which the probability of birth, death, infection, recovery and dispersal of individuals is used to determine the population dynamics. Hence, the stochastic model includes the variability seen in real systems and provides essential realism when squirrel numbers become low which gives a better representation of population extinction and the fade-out of infection. The underlying deterministic system, which assumes the existence of a shared disease, represents the dynamics of red squirrels who are susceptible (S_R) to the disease and those that are already infected (I_R) by the disease. The model also includes susceptible (S_G) and infected (I_G) grey squirrels as well as grey squirrels that have recovered (R_G) from the disease. The model we use is:

$$\begin{aligned}
\frac{dS_G}{dt} &= A_G(t) - bS_G - \beta S_G\left(I_G + I_R\right), \\
\frac{dI_G}{dt} &= \beta S_G\left(I_G + I_R\right) - bI_G - \gamma I_G, \\
\frac{dR_G}{dt} &= \gamma I_G - bR_G, \\
\frac{dS_R}{dt} &= A_R(t) - bS_R - \beta S_R\left(I_G + I_R\right), \\
\frac{dI_R}{dt} &= \beta S_R\left(I_G + I_R\right) - bI_R - \alpha I_R
\end{aligned} \tag{1}$$

where

$$A_G(t) = \begin{cases} \left(a_G - q_G\left(H_G + c_R H_R\right)\right) H_G & 0 \le t < 0.5 \\ 0 & 0.5 \le t < 1 \end{cases} \tag{2}$$

Here, $A_G(t)$ represents the periodic birth rate of grey squirrels which assumes births occur for only half of the year (between March and September each year, representing observed peak litter periods and periods with no breeding activity). The term

for $A_R(t)$, which represents the periodic birth rate of red squirrels, is equivalent to $A_G(t)$ with the subscripts for R and G interchanged. Note, $H_G = S_G + I_G + R_G$ and $H_R = S_R + I_R$ represent the total populations for grey and red squirrels respectively. The natural rate of adult mortality $b = 0.9$ (Barkalow et al. 1970) is the same for both red and grey squirrels but the rates of maximum reproduction differ, with red squirrel birth rate $a_R = 3$ and grey squirrel birth rate $a_G = 3.4$ (Tompkins et al. 2003). The competitive effect of grey squirrels on red squirrels is denoted by $c_G = 1.65$, whilst that of red squirrels on grey squirrels is denoted by $c_R = 0.61$ (Bryce et al. 2002). Squirrelpox virus is transmitted (both within and between each squirrel species) with coefficient $\beta = 1.1$ (White et al. 2016). Infected red squirrels die due to the disease at rate $\alpha = 26$ and infected greys recover at rate $\gamma = 13$ (Tompkins et al. 2003). The susceptibilities to crowding (q_R, q_G) are set to ensure the average density over one year is equal to the carrying capacity in each grid square for that year, with carrying capacities being habitat dependent (see Suppl. material 1: Section S1.1). All parameter values assume an annual timescale. To generate the stochastic model, the rates for birth, death, infection, recovery etc., in the deterministic model are converted into probabilities of events that account for changes in individual 1 km by 1 km patch level abundance (Renshaw 1993) – see Suppl. material 1: Section S1.2 for full description. The stochastic model also includes events that allows for dispersal of individuals between patches (see Suppl. material 1: Table S2 for details) as well as the possibility of control through the removal of grey squirrels (see Suppl. material 1: Section S1.4).

The stochastic model is used in conjunction with landscape information, primarily forest composition data and information on masting, which gives forest capacity dynamics. These data provides estimates for red and grey squirrel carrying capacity at the 1 km^2 level (see Suppl. material 1: Section S1 for further details).

In this paper we considered the following scenarios:

(i) The natural expansion of red and grey squirrel populations beyond the grey squirrel control boundary. This allows an examination of the threat to current red squirrel population from 'natural' grey squirrel expansion.

For this scenario the model was initialled with observed data for the presence of red and grey squirrels between 2014–2017 (using the National Biodiversity Network's (NBN) Gateway, http://data.nbn.org.uk). In regions where only one squirrel species was observed the model was initialised at the respective carrying capacity for that grid-square, based on available habitat types. In regions where both squirrel species were observed the model was initialised with red and grey squirrel densities at half their respective potential carrying capacities. Once initialised, the model was simulated 10 times, with each simulation being run for 10 years and an average taken, in order to allow for changes in density in grid-squares with both squirrels present and for squirrels to expand into nearby available habitat. The average result of the 10-year spin-up serves as the initial conditions for this scenario.

(ii) The assumption that grey squirrels initially occupy all viable habitat in Scotland. This allows the model to predict the composition and location of current natural strongholds in Scotland in which viable red populations would persist when faced with the threat from grey squirrels.

For this scenario the model was initiated by assuming red squirrel occupancy is as in scenario i (see Fig. 1a) and that grey squirrels are present at their carrying capacity in all grid squares in which the grey carrying capacity is greater than, or equal to, 5 km^{-2} (and therefore this scenario assumes that grey squirrels have dispersed to all regions in Scotland).

To generate results each scenario was simulated 10 times, with each simulation of the model being run for 150 years each to ensure that the model is predicting the long-term population dynamics. The simulation results are then averaged. Regarding occupancy, a grid square is classed as being occupied if there are 2 or more individuals of either species present. Habitat, carrying capacity and occupancy maps were generated using MATLAB R2018b. Simulations were run using Fortran90.

Results

Fig. 1a shows the initial distribution of red and grey squirrels used in the model simulations and is indicative of the current distribution in Scotland. This highlights how red squirrels currently occupy suitable habitat in the north of Scotland (above the grey squirrel control boundary) as well as in isolated populations in southern and central Scotland. Grey squirrels dominate habitat south of the grey squirrel control boundary as well as habitat in Aberdeen and surrounding areas.

Red squirrel viability when grey squirrels naturally expand their range of occupancy

Fig. 1a–d shows occupancy maps for 150 years of model simulation when squirrels can disperse beyond their current distribution. Findings show that grey squirrels swiftly expand their range into the north-east of Scotland, with consequent loss of red squirrel populations.

The range expansion of grey squirrels into the north-east beyond the initial expansion (through habitats in Moray and along the river Spey) is slow.

There is limited expansion in grey squirrel distribution across the grey squirrel control boundary and into red squirrel occupied regions in central and southern Scotland. In the model this range expansion occurs within the first 30 years and the grey distribution remains relatively fixed thereafter. The mathematical model has been fitted to qualitatively reproduce observed rates of red and grey squirrel expansion through suitable habitat (Jones et al. 2016). Therefore, the absence of grey squirrel expansion north of the grey squirrel control boundary is due to a lack of suitably connected habitat in relation to the assumed and validated grey squirrel dispersal ability.

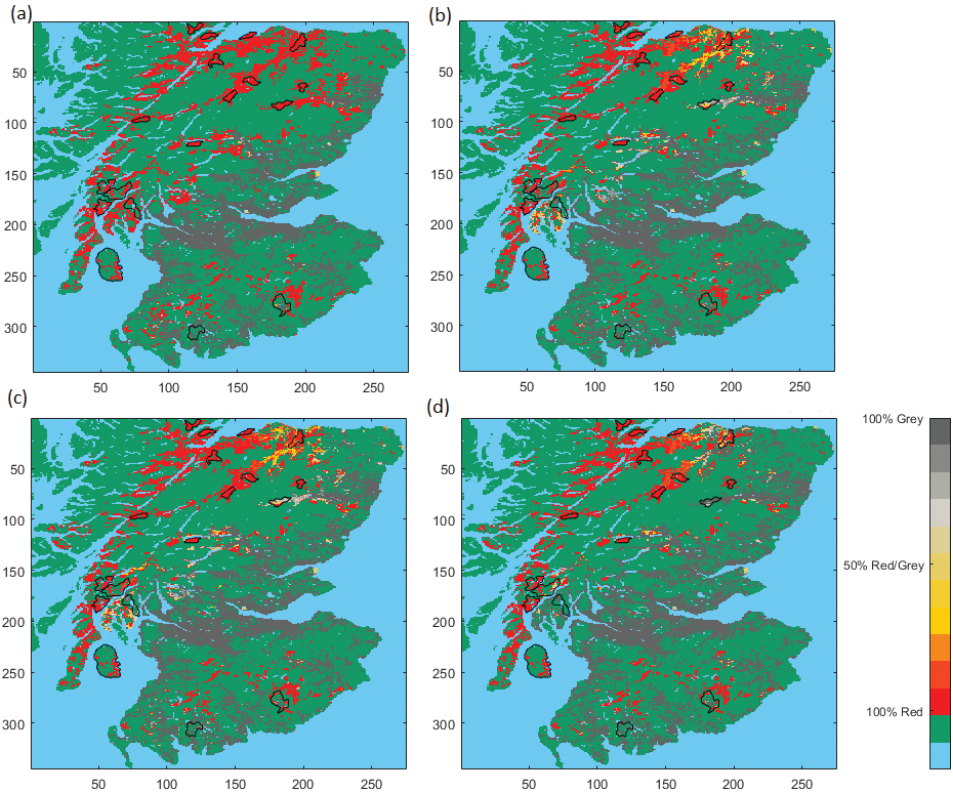

Figure 1. Results showing the relative occupancy of each 1 km grid square. Here (**a**) shows the initial occupancy, defined using the 10-year model spin-up based on observation data, as maintained by trapping at the grey squirrel control boundary, (**b**) shows an average occupancy of the first 30 years of the simulation after grey squirrels are allowed to disperse freely, (**c**) shows the average occupancy results for years 65 to 95 of the simulation where grey squirrels are allowed to disperse freely, and (**d**) shows the long-term occupancy (150 years after the simulation begins) of red and grey squirrels after greys have been allowed to disperse freely. A grid square is classed as being occupied if there are 2 or more individuals of any species present. The scale bar indicates the proportion of the 10 simulations that ended with either red or grey squirrel occupancy. For example, 70% red occupancy in a given grid square indicates that 7 of the 10 simulations that ended in occupancy were occupied by red squirrels. The 19 designated strongholds (Scottish Forestry 2020) are outlined in black.

Much of northern Scotland, above the grey squirrel control boundary, remains occupied by viable red squirrel populations (maps of average squirrel density for these simulations can be found in Suppl. material 1: Fig. S1). This is primarily due to the inability of grey squirrels to disperse to suitable habitat in northern Scotland. Isolated red squirrel populations persist in central and southern Scotland and indicate that these regions are natural strongholds for red squirrels. Here red squirrel populations persist in conifer-dominated regions even though grey squirrels occupy neighbouring habitat.

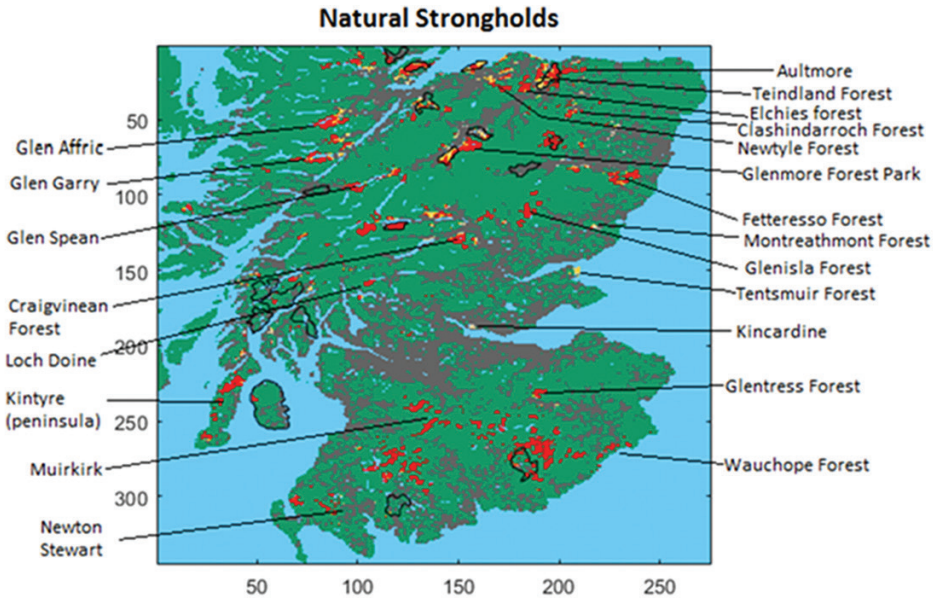

Figure 2. Relative occupancy of red and grey squirrels when grey squirrels are assumed to initially occupy all viable habitat. The names are of the forests that the model predicts can act as natural strongholds. Note, not all natural strongholds have been named due to overlap or close proximity to existing strongholds, which would enable their location to be determined. The 19 designated strongholds (Scottish Forestry 2020) are outlined in black.

Identifying natural red squirrel strongholds in Scotland

Results showing the occupancy of red and grey squirrels at the end of the model simulations, when grey squirrels are assumed to initially occupy all viable habitat, are shown in Fig. 2 (see also Suppl. material 1: Fig. S2). The regions where red squirrels persist in Fig. 2 can be defined as natural strongholds that would support red squirrel populations under a worst-case scenario of grey squirrel expansion across the whole of Scotland. The density of red and grey squirrels for this scenario is shown in Fig. 3a(i)–c(i). There are numerous natural strongholds, but they are often isolated and support low-density red squirrel populations. The qualitative nature of the simulations do not allow us to decide which strongholds would support viable red squirrel populations. The habitat composition for red-dominated regions (natural strongholds) and grey-dominated regions in Fig. 2 is shown in Fig. 4. Natural red squirrel strongholds are predicted for regions composed of conifer and pine species with a general absence of broadleaf and urban habitat. This is typical of large forest plantations in Scotland. Grey squirrel dominated regions contain broadleaf species and urban habitat and grey squirrels can occupy conifer and pine habitats where they are adjacent to broadleaf and urban habitats.

Fig. 3a(ii)–c(ii) shows red and grey squirrel densities, as well as squirrel occupancy, when grey squirrel trapping is applied in grid-squares that contain a grey squirrel population. The amount of trapping applied is equivalent to 18 trap-days per year in each

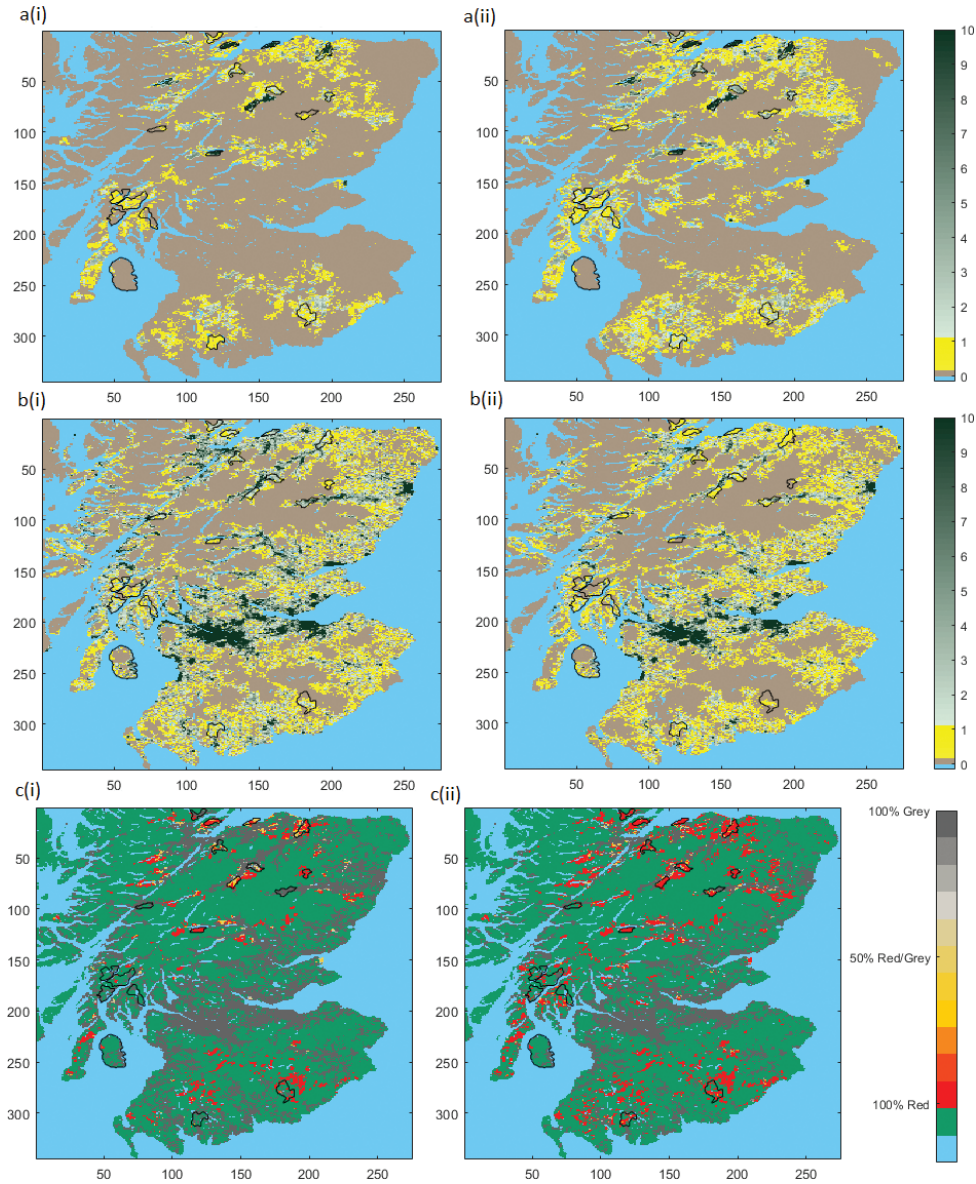

Figure 3. Density and occupancy results when grey squirrels have been introduced everywhere in Scotland. Here (**a**) shows the red squirrel density, (**b**) the grey squirrel density and (**c**) the occupancy results for (**i**) the simulation where no grey squirrel trapping was applied and (**ii**) the simulation where grey squirrel trapping (approx. 18 trap days per year) is applied to each grid square that contains grey squirrels. A grid square is classed as being occupied if there are 2 or more individuals of any species present. The 19 designated strongholds (Scottish Forestry 2020) are outlined in black.

grid cell. The results show that this level of grey squirrel trapping does not reveal new red squirrel strongholds, but does reinforce and enlarge existing natural strongholds. Increases in trapping effort further enhances this effect.

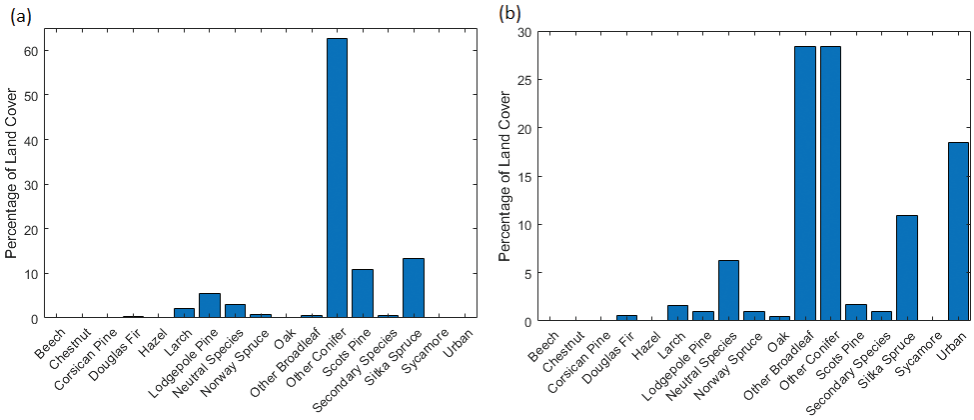

Figure 4. Habitat composition in regions where (**a**) red squirrels have greater than 80% occupancy and (**b**) grey squirrels have greater than 80% occupancy. Named species are from the National Forest Estate 2017 dataset whilst Other broadleaf and Other conifer data is from the National Forest Inventory 2016 dataset.

Discussion

In this study we have developed a spatial mathematical model that includes the competitive and disease interactions between red and grey squirrels in realistic habitats across Scotland to assess the viability of red squirrel populations under several scenarios of grey squirrel expansion. The current strategy for red squirrel conservation in Scotland aims to restrict or reduce the distribution of grey squirrels and to maintain viable red squirrel populations in priority areas for red squirrel conservation (Scottish Natural Heritage 2015). If this conservation strategy is successful (which observations suggest is currently the case (NBN Atlas Partnership 2017)) then the model predicts that there will be large expanses of forest north of the grey squirrel control boundary that support viable red squirrel populations.

The model allowed us to test 'what if' scenarios for red squirrel conservation and predicted that if current levels of grey squirrel control, which aim to restrict grey squirrels to their current distribution, were to end and grey squirrels were allowed to expand their range, then the process of red replacement would be slow. This would allow time for red squirrel conservation management policy to be implemented. Furthermore, during the period of grey expansion there would still remain large expanses of forest in north and north west Scotland that would support viable red squirrel populations. In the absence of control, grey squirrels are predicted to expand northwards, most notably along the north-east and northern coast, with their expansion directly north curtailed by geographical constraints (such as the Cairngorm mountains).

Our results support the current grey squirrel control efforts aimed at containing and removing grey squirrels from Aberdeen and the surrounding area (Scottish Natural Heritage 2015) as this will prevent grey squirrel expansion around the north-east and northern coast of Scotland. Expansion of grey squirrels into central or western Scotland is also impeded by the geography, with the Southern Highland mountains forcing grey squirrels to migrate along the west coast which provides poor habitat.

Since large-scale grey squirrel range expansion beyond their current distribution is prevented by geographical constraints on natural migration, it will be important to provide public information regarding the threat of grey squirrels to prevent artificial dispersal, whether accidental or intentional, across geographical barriers by members of the public. History shows that grey squirrel range expansion was facilitated by humans (e.g. see Signorile et al. 2016). Grey squirrels were introduced into Aberdeen and its surroundings via separate introductions, and considerable resources and effort have been required to keep these populations under control, with the goal of eradication occurring in the future (Scottish Natural Heritage 2015). Further evidence of the risks inherent in the deliberate introduction of grey squirrels can be found in Italy. Grey squirrels were introduced into Piedmont in northern Italy in 1948. Subsequent introductions into Genoa in the 1960s and translocations into Lombardy in the late 1990s have led to further grey squirrel expansion southwards into central Italy as well as northwards across the Po plain, where future expansion into France and Switzerland is a real possibility (Lurz et al. 2001; Martinoli et al. 2010; Signorile et al. 2014). This increase in grey squirrel range in Italy has led to a corresponding reduction in red squirrel density and highlights the need for public information campaigns that detail the risks and consequences of translocations to native wildlife and forestry.

An assumed worst-case scenario where grey squirrels have expanded to all regions in Scotland identified a range of forest regions across Scotland that could support red squirrels under UKFS management practice. We define these forest sites as natural strongholds (see Fig. 2). Several forest sites in the north of Scotland are able to maintain a potentially viable red squirrel population, despite the presence of grey squirrels. However, these sites are relatively small and isolated, with densities of around 2 red squirrels km^{-2}, potentially placing the red squirrel populations resident in the natural strongholds at risk due to stochastic extinction and loss of genetic diversity (Wauters et al. 1994). To overcome this problem, the disparate forests could either be physically connected via forest management, or the genetic viability of the populations could be managed and enhanced by periodic translocations of red squirrels. It should be noted that the density of red squirrels in natural strongholds under UKFS management is in line with that predicted in model studies for the designated strongholds under stronghold forest management (Slade et al. 2020). The study by Slade et al. (2020) also showed that the additional forest management required (above that of UKFS) to satisfy the designated stronghold policy (Forestry Commission Scotland 2012; Scottish Forestry 2020) may have little benefit to red squirrel viability in the absence of grey squirrels. Moreover, it was predicted that some of the designated strongholds could not protect red squirrels from the threat of grey squirrel invasion even when their composition was modified to satisfy designated stronghold policy. The model results presented in this paper also show that the locations of the natural strongholds do not match the locations of the 19 designated forest strongholds in Scotland (the designated strongholds are outlined in black in Fig. 2). Our findings suggests that natural strongholds could offer similar or improved protection to red squirrels, if threatened by grey squirrel invasion, as offered by the designated strongholds.

A potential red squirrel conservation strategy could employ grey squirrel control to reinforce the ability of natural strongholds to sustain a red squirrel population.

Fig. 3 a–c(ii) shows results for squirrel density and occupancy when grey squirrel trapping is applied in all regions where greys are present (see also Suppl. material 1: Section S3). Grey squirrel trapping is applied to all grid squares in which greys are present with approximately 18 trap-days per year per grid square. Although it is unrealistic to apply grey squirrel control across all regions, the model results highlight whether grey squirrel trapping would improve red squirrel viability in natural strongholds. North of the grey squirrel control boundary, grey squirrel trapping leads to an expansion of the regions of red occupancy and an increase in red squirrel density. There is a small expansion of red squirrel occupied regions in southern Scotland. This indicates that grey squirrel control around natural strongholds can lead to red squirrel expansion and improved population viability.

An analysis of the available land-cover data indicated that the forest composition of natural strongholds is comprised predominantly of Sitka spruce in southern Scotland and Sitka spruce, Scots and Lodgepole pine in northern Scotland combined with an absence of broadleaf and urban habitat. This forest composition is already met by forest plantations in the north of Scotland, which consequently act as natural strongholds. Management would still be required to maintain a suitable tree species composition and age structure, in the face of normal timber and harvesting operations, to provide an advantage to red squirrels over greys and to maintain a viable population in the long term. Nevertheless, a review and potential change in management policy for some designated strongholds in northern Scotland may allow current efforts and resources to be reassigned to focus on vulnerable red squirrel populations that are threatened by incursions of grey squirrels. Note, whilst broadleaf and urban habitats are suitable to support viable (high-density) red squirrel populations these habitats favour grey squirrels who out-compete reds. This analysis is supported by a recent statistical occupancy model for red and grey squirrels in Northern Ireland. This study used data collected by citizen science to show that red squirrel occupancy was positively correlated with coniferous woodland and negatively correlated with urban habitat. Likewise, grey squirrels were positively correlated with broadleaved forests and urban habitat and negatively correlated with coniferous habitat (Twining et al. 2020). The study also found a negative correlation between grey squirrels and pine marten (*Martes martes*), a native mustelid whose numbers are increasing, and a positive correlation between pine marten and red squirrels. Theoretical studies using a two prey-one predator ecoepidemic model have also shown that the predator (pine marten) can destabilise the previous grey squirrel dominant equilibria, favouring the native red squirrel (Travaglia et al. 2020). Thus, the impact of a native predator could aid red squirrel survival in the United Kingdom and Ireland via a reduction in grey squirrel density and geographic spread (Twining et al. 2020).

There is consensus that squirrelpox played a key role in the competition and disease mediated invasion of red squirrels when greys squirrels expanded through England and Wales (Tompkins et al. 2003; Bosch and Lurz 2012), where the habitat consisted of broadleaved or mixed stands that could support high squirrel densities. The natural strongholds predicted in this study are dominated by coniferous habitats that generally support low-density red squirrel populations. The impact of squirrelpox on the location

of, and red squirrel density in, natural strongholds was negligible. This is in line with previous studies that show squirrelpox cannot be supported in low-density red squirrel populations (White and Lurz 2014; Jones et al. 2017) and that red squirrels can therefore 'live' with the threat from squirrelpox. Hence, squirrelpox is unlikely to play a key role in grey squirrel invasion in low-density populations in Scotland (Lurz et al. 2015).

In this study we did not consider the impact of climate change on forest composition as it went beyond the scope of this project. However, we recognise that it will be an increasingly important factor in the red/grey squirrel dynamics and therefore red squirrel conservation in the future. Natural strongholds in Scotland largely coincide with managed forest plantations, due to their tree species composition. Thus, incorporating climate change into the model to identify natural strongholds would require knowledge of detailed future forest management plans. Climate change will influence the tree species composition in forests, maturation time and seed mast cycles (Neilson et al. 2005; Bisi et al. 2016) and will likely favour an increase in broadleaf trees through range expansion and species migration (Neilson et al. 2005). Grey squirrels have a competitive advantage in broadleaved habitat and can reach higher densities which may be able to support endemic squirrelpox virus (Tompkins et al. 2003; Rushton et al. 2006). This could make natural strongholds vulnerable to grey squirrel invasion and threaten red squirrel viability. Studies also suggest that climate change may lead to an increase in woodland cover (Ray 2008). This could increase the avenues northwards, above the grey squirrel control boundary, which would facilitate and increase the rate at which grey squirrels can colonise the remainder of Scotland. Conversely, the mountainous habitat that lies directly north of the control boundary could remain resistant to reforestation by climate change. Alternatively, climate change could increase the proportion of land in Scotland that is viable for agricultural use, which in turn could reduce the connectivity of Scottish forests (Gimona et al. 2012). This could aid red squirrels by reducing the ability of grey squirrels to expand their range, but such isolation may reduce genetic diversity and increase the risk of local population extinction. The impact of climate change on red squirrel conservation should therefore be the focus of future research efforts to help sustain viable populations in the long term.

Conclusion

Our findings highlight the existence of forest areas (natural strongholds) north of the grey squirrel control boundary that would currently support viable red squirrel populations without the need for species specific management. Natural strongholds, which often correspond to large commercial forest plantations, are managed according to UKFS policy and are typically composed of Sitka spruce and other conifer and pine species. Our predictions suggest grey squirrel dispersal and expansion will likely be slow even in the absence of grey squirrel control, due to geography in northern Scotland forming a barrier between the current distribution of red and grey squirrels. Together with recently published results (Slade et al. 2020) our findings here support a policy review to better target effort and resources for red squirrel conservation in Scotland.

Acknowledgements

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Supplementary material 1

Details on carrying capacity, habitat and grey squirrel trapping

Authors: Andrew Slade, Andy White, Kenny Kortland, Peter Lurz

Data type: Images, modelling details, extra results

Explanation note: The supplementary information also includes extra results that, whilst informative, were not deemed necessary in the main manuscript.

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The illegal hunting and exploitation of porcupines for meat and medicine in Indonesia

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Abstract

Indonesia is home to five species of porcupines, three of which are island endemics. While all five species are currently assessed as Least Concern by the IUCN Red List of Threatened Species, impacts of harvest and trade have not been factored in. To gain a fuller understanding of the porcupine trade in Indonesia, this study examines seizure data of porcupines, their parts and derivatives from January 2013 to June 2020. A total of 39 incidents were obtained amounting to an estimated 452 porcupines. Various confiscated commodities revealed porcupines are traded for consumption, traditional medicine, trophies/charms as well as for privately run wildlife/recreational parks. Targeted hunting of porcupines for commercial international trade was also evident. Porcupines are also persecuted as agricultural pests and wildlife traffickers take advantage of such situations to procure animals for trade. What clearly emerges from this study is that porcupines are being illegally hunted and exploited throughout their range in Indonesia facilitated by poor enforcement and legislative weakness. Porcupines are in decline due to habitat loss, retaliatory killings and uncontrolled poaching. It is therefore crucial that effective conservation measures are taken sooner rather than later to prevent further depletion of these species. Including all porcupines as protected species under Indonesian wildlife laws and listing them in Appendix II of CITES to improve regulation, enforcement and monitoring of domestic and international trade trends involving porcupines in Indonesia would contribute significantly towards this end.

Keywords

Illegal wildlife trade, seizures, traditional medicine, wildlife consumption

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Introduction

Commercial exploitation of wildlife for meat and medicine has become a significant threat to species globally (Byard 2016; Nijman and Bergin 2017; Ripple et al. 2019; Gomez et al. 2020; WAP 2020). The wildlife meat and traditional medicine industries are worth billions of dollars annually and have little to do with subsistence needs – case in point being the continued demand and illegal trade in tigers, rhinos and pangolins (C4ADS 2020; Four Paws 2020; WJC 2020). For example, the legal bear bile trade in China alone is valued at USD1 billion a year and a variety of bear bile products (pills, powders, ointments, wines, tea, etc) were created to stimulate market demand (WAP 2020). Viewed as highly valuable commodities, species are hunted to the brink of their existence and as one species dwindles, it is replaced by another e.g. with wild tigers in Asia near depletion, lions in Africa, jaguars in South America and leopards worldwide are increasingly targeted as traditional medicine substitutes (Coals et al. 2020; Morgan et al. 2021); similarly, pangolins in Asia have deteriorated significantly due to over harvesting for meat and medicine which has resulted in shifting poaching efforts to African pangolins to meet demand in Asia (Challender et al. 2016; Gomez et al. 2016). Exacerbating the issue is captive breeding of wildlife for commercial trade, such as tigers and bears, which is arguably of little conservation value as wild caught animals are known to be laundered and trafficked through such facilities, and further stimulates demand and trade in highly threatened species (Livingstone et al. 2018; Four Paws 2020; WFFT 2020).

Lesser-known species similarly exploited for the meat and medicine trade in Asia are porcupines. An increasing body of evidence reveals the heavy hunting and trade of porcupines in Asia that are resulting in population declines (Brooks et al. 2010; Lee et al. 2015; McEvoy et al. 2019; Yeung 2019; Loke et al. 2020). Wildlife trade surveys across markets in Southeast Asia have frequently encountered porcupines for sale including dead animals, live animals and body parts such as quills and bezoars (non–digestible food material that forms a stone like mass in the gut of an animal). Their meat is consumed as an alternative and important source of protein in parts of their range and their quills are used for decorative purposes. In traditional Chinese medicine, there is a particularly high demand for porcupine bezoar due to the perception that it has many healing properties (Brown 2015; Lee et al. 2015; Tan et al. 2019). A centuries old practice, the use of porcupine bezoars for medicine appears to have increased significantly in recent years (Lee et al. 2015; Heinrich et al. 2020a).

During a 2019 survey for porcupine bezoars on e-commerce websites in Indonesia, Malaysia and Singapore, 121 adverts selling approximately 680–1332 bezoars were obtained over a three-month period (Heinrich et al. 2020a). The majority of these adverts were located in Indonesia i.e. Kalimantan (Borneo), Java and Sumatra, although most of these occurred in Java. The study revealed the illegal exploitation of porcupines for bezoars both for domestic and international markets. This study takes a closer look at the trade of porcupines in Indonesia to assess the extent of the trade and whether it is a potential conservation concern that needs to be addressed.

Five species of porcupines exist in Indonesia–long-tailed porcupine (*Trichys fasciculata*) and Malayan porcupine (*Hystrix brachyura*) occur on the islands of Borneo and Sumatra, Sumatran porcupine (*H. sumatrae*), endemic to the island of Sumatra, Sunda porcupine (*H. javanica*) endemic to the islands of Java, Bali, Sumbawa, Flores, Lombok, Madura, and Tonahdjampea, and thick-spined porcupine (*H. crassispinis*) endemic to the island of Borneo (Figure 1). All five species are currently assessed as Least Concern by the IUCN Red List of Threatened Species with populations deemed stable with the exception of the Malayan porcupine which is described as having a decreasing population (Amori and Aplin 2016; Aplin 2016; Cassola 2016; Molur 2016; Lunde et al. 2016). That said, no population density details in any range state are provided in these assessments; just a line that describes the species as 'common and widespread' or 'common and prolific' or 'common in suitable habitat'. Further, no major threats to the species nor details on use and trade are listed. While hunting for food is described in parts of their range for the Long-tailed, Malayan and thick-spined porcupines, this is not thought to have a significant impact on populations.

Methods

I collected records of seizures and prosecutions relating to porcupine species in Indonesia for the period January 2013–June 2020. Data were extracted from the Indonesian government website, Sistem Informasi Penelusuran Pekara (SIPP) – an open access information database of the courts for each district in the country and from published online media articles. Searches for related seizures were conducted in both English (search terms: hunting, trapping, trade, illegal trade or wildlife trade in porcupine/*Hystrix*) and Indonesian (search terms: 'BKSDA', 'penyelundupan satwa landak/*Hystrix*', 'perdagangan satwa landak/*Hystrix*', 'polisi satwa landak/*Hystrix*', konservasi landak/*Hystrix*). All reported seizures were carefully checked to avoid duplication.

From each record obtained, I extracted information on date of seizure, species of porcupine seized, commodity (live animals, body parts, etc), quantities of each commodity, purpose of hunting/trade (i.e. for consumption, pets, trophies, etc), location of seizures and trafficking routes, suspects arrested and prosecution outcomes. Using this data, I have also mapped important trade hubs and centres where trade exists. Species identification is based on information extracted from seizure incidents obtained and it is assumed to be accurately reported. Generally, species identified as being seized fell within their distribution range in Indonesia with the exception of one incident where a live Sunda porcupine, which has a restricted distribution to Java and neighbouring islands, was reportedly seized from a 'wildlife recreational park' in West Kalimantan. I have conservatively estimated a minimum number of porcupines recorded in trade from commodities confiscated per seizure incident. Commodities seized generally consisted of live or dead animals and to a lesser extent, bezoar (assuming that one bezoar equates to one animal), and quills (3 incidents involving between 10 and 115 quills which I equate to being from one individual animal in each incident).

Results

From January 2013 to June 2020, I obtained 39 incidents in which porcupines were seized in Indonesia involving an estimated 454 porcupines (Figure 1). At least three species of porcupine were identified in 13 incidents – Malayan porcupine (8 incidents), Sunda porcupine (4 incidents) and Sumatran porcupine (1 incident). In the majority of incidents (67%) however, the species of porcupine involved was not identified/reported (Figure 1).

The most frequent and abundant commodity seized were of live animals (80% of incidents amounting to 429 estimated animals) (Table 1). To a much lesser extent, other commodities seized included quills (7% of incidents), dead specimens (5% of incidents) and in one incident each, meat (attached to skewers), bezoar stones and an internal organ (unspecified).

Of the 39 seizure incidents, 48.7% occurred in Sumatra, followed by Java and surrounding islands (43.6%) (Figure 2). In terms of quantity however, 89.6% of all commodities seized were in Sumatra, specifically involving the provinces of Aceh, North Sumatra and West Sumatra (Figure 2). A large number of incidents (n=12) that occurred in Sumatra involved targeted trading of porcupines (usually involving large numbers of porcupines i.e. on average approximately 32–33 animals per incident) where suspects were reportedly caught in the midst of transporting live porcupines from one location to another predominantly between Aceh, North Sumatra and West Sumatra; or local farmers/plantation owners caught trapping or in possession of porcupines intended to be sold to middlemen. In one incident which occurred in North Sumatra, it was reported that the seized animals (n = 56) were being transported to Aceh, from where they were to be shipped to China. In a few cases, porcupines were seized in general wildlife raids involving multiple species (3 incidents) such as bear and tiger body parts, dead pangolins or pangolin scales, muntjac, mousedeer, etc; or seized from individuals (3 incidents) that were keeping live wild animals without a legal permit including gibbons, sun bears, hawks, eagles, deer, crocodiles, etc.

In Java and surrounding islands, an estimated 44 porcupines were seized predominantly from individuals involved in the trade of live animals and parts (9 incidents, 13 estimated whole animals); or from individuals that were keeping/in possession of a

Table 1. Porcupine commodities seized per species from January 2013 to June 2020 and quantities seized in Indonesia.

Species	Seizures (#)	Estimated whole specimens	Commodity Seized					
			bezoar (piece)	dead (whole)	live (whole)	meat (skewers)	internal organ	quills
Unspecified	26	281	2	18	256	26	1	236
Malayan Porcupine	8	126			126			
Sumatran Porcupine	1	40			40			
Sunda Porcupine	4	7			7			
Total	39	454	2	19	429	26	1	236

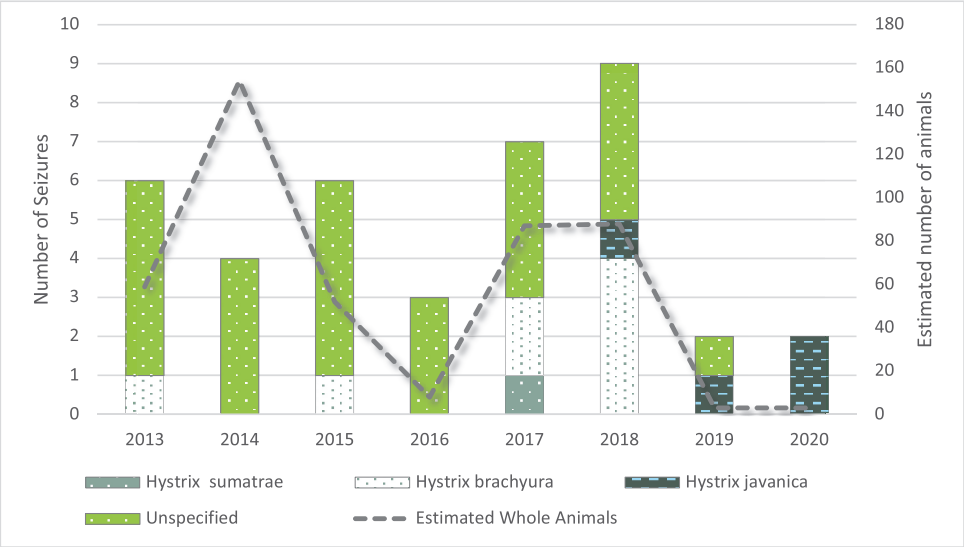

Figure 1. The number of seizure incidents involving porcupines in Indonesia from January 2013–June 2020, including species involved and estimated number of whole animals.

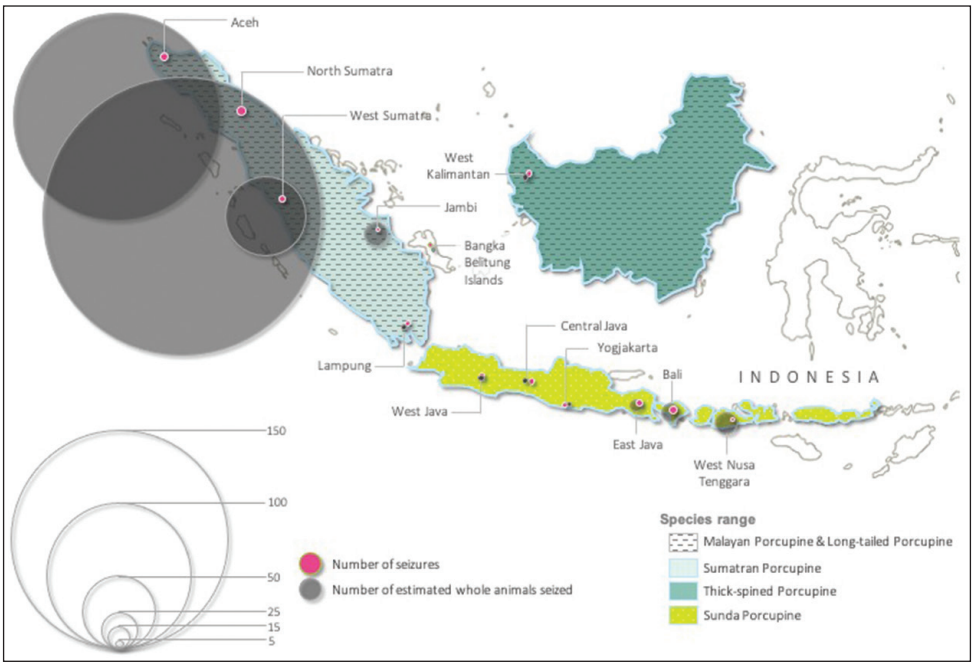

Figure 2. Location of porcupine seizure incidents by provinces in Indonesia including number of seizures at each location and estimated whole porcupines involved from January 2013 to June 2020; as well as the range of the five porcupine species that occur in Indonesia based on data extracted from the IUCN Red List of Threatened Species.

variety of wild animals without a legal permit (8 incidents, 31 estimated whole animals). Only in one of these incidents was it reported that a suspect was caught for illegally trapping and keeping wild porcupines which he subsequently used for breeding purposes.

There were only three incidents reported in Kalimantan (Indonesian Borneo) amounting to an estimated three porcupines (~2 live animals and 111 quills). In two incidents individuals were caught running a wildlife animal park/ mini zoo without a valid permit and involved multiple species including sun bears, birds, deer, slow loris, binturong, crocodiles, etc; and in one incident an accessory shop was raided and found to be illegally trading in protected wildlife parts including orangutan, sun bear, hornbill, muntjac, pangolin, sea turtles, deer and porcupine.

Discussion

Species in trade and legislative loopholes

In the majority of incidents (67%), despite predominantly involving live animals, the species of porcupine seized was not reported making it difficult to determine if specific species are being targeted or whether all species are exploited for trade. However, at least three species were seized which seems to suggest the latter. Further, considering the over-lapping distribution range of the various species (with the exception of the Sunda porcupine), it is safe to assume that trade could potentially encompass all five species. That said, studies have shown that the Malayan porcupine is likely the main species found in trade in Asia primarily due to its wide distribution (Heinrich et al. 2020a, b). The Malayan porcupine was the most frequently identified species confiscated between 2013 and 2018 (in 13 incidents). After 2018, there were fewer porcupine seizures and these incidents either involved the Sunda porcupine or it was unreported (Figure 1).

This can likely be explained by the protection status of different porcupine species in Indonesia. The Act of the Republic of Indonesia No.5 of 1990 concerning conservation of living resources and their ecosystems, widely known as the Conservation Act (No.5) 1990, is the principal legislation pertaining to the regulation of wildlife trade in Indonesia. Under this Act, species are categorized as “Protected” or “Unprotected”. Protected species are prohibited from being caught, injured, killed, kept, possessed, cared for, transported, or traded whether alive or dead, unless permitted by the Government; and are listed under Government Regulation No.7, 1999, Concerning the preservation of flora and fauna. Prior to 2018, the only porcupine species protected in Indonesia was the Malayan porcupine. The only time the protected species list has been revised, since it was gazetted, was in 2018, first in July and then again in September the same year. The amendments included the removal of the Malayan porcupine and the addition of the Sunda porcupine. Since then, seizures of the Malayan porcupine have not been reported. Removal of the Malayan porcupine from the protected species list is highly questionable considering it is the species most frequently confiscated and perhaps this is another indication of the lack of political will to combat the

trafficking of wildlife of commercial value. For example, the Indonesian government revising the protected species list twice within months of release in 2018 was the result of pressure from bird traders to remove certain species that were newly added to the list due to significant population declines (Armstrong and Chng 2020). Calls for stronger protection of otters in Indonesia for similar reasons led to the protection of the Smooth-coated Otter but not the Small–clawed Otter, the species most exploited for domestic and international commercial trade (Gomez and Bouhuys 2018; Gomez and Shepherd 2018).

Technically, the trade and collection of unprotected species in Indonesia is regulated by harvest quotas that are established annually under the Decree of the Minister of Forestry Number 447/Kpts-II/2003 concerning the administration directive of harvest and capture and distribution of the specimens of wild plant and animal species. For the duration of this study period (2013–2020) no harvest quotas were established for the five porcupine species found here and therefore any porcupine in trade are of illegal origins. Having said that, there are no provisions in any of these laws regarding penalties or fines against those violating harvest quotas or/and trading in unprotected species, rendering enforcement action pointless. Notably, porcupine seizures in Indonesia peaked in 2018 with nine seizures (involving an estimated 88 animals) and subsequently dropped to two seizures in 2019 (involving an estimated 3 animals). The inadequate regulation of harvest quotas has been raised countless times with regards to Indonesian species in trade and is seen as a deliberate impediment to conservation of its species (Gomez and Bouhuys 2018; Janssen and Chng 2018; Armstrong and Chng 2020; Latinne et al. 2020; Janssen and Gomez 2021). A clear example of this is the hunting and trade of Tokay geckos (*Gekko gecko*) in Indonesia – whereby studies have shown established harvest quotas being greatly exceeded and fraudulent captive-breeding claims through the years (Caillabet 2013; Nijman and Shepherd 2015). Due to declining populations throughout its range, in 2019 the Tokay Gecko was added to Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in a bid to regulate and monitor international trade. Following this, the Indonesian government significantly increased harvest quotas in 2020, from 50,000 individuals/year prior to 1,800,000 individuals/ year, not only undermining conservation efforts but showcasing the dubious nature in which harvest quotas are determined (Janssen and Gomez 2021).

Porcupine use and trade hotspots

This study revealed a local demand for live porcupines and their parts. In one incident, actual porcupine meat on skewers was seized. Locally, porcupine meat is consumed as an alternative source of protein, as a medicinal cure for asthma and improving one's vitality, and as an aphrodisiac (Farida 2013; Farida 2015; Mustikasari et al. 2019; Nurliani et al. 2020). Quills were seized in three separate incidents in Aceh, Bali and West Kalimantan. These are reportedly used in local traditional medicine, as hairpins by pregnant women, and as souvenirs or talismans against black magic

(Mustikasari et al. 2019). Live porcupines were also confiscated from privately run wildlife/recreational parks or mini zoos along with a host of other protected species due to the lack of a valid permit to either operate such facilities or for possession of protected species. This was particularly evident in Java. Trade of porcupines for traditional medicine use was also evident in two seizure cases in West Sumatra. In one incident, porcupine bezoars were confiscated along with tiger and bear body parts that were reportedly bought from traders around Medan. In another incident, involving 64 live pangolins, the suspect arrested confessed to keeping porcupines in stressful conditions to induce the formation of bezoars which takes place after approximately 6–7 months at which point the porcupines are killed, the bezoar extracted and sold for IDR150K (~USD11). While there were few seizures involving porcupine bezoars, Heinrich et al. (2020a), found a substantial number of porcupine bezoars for sale online in Indonesia. Based on seller location obtained, the majority of these were in Java (31 listings), followed by West Kalimantan (12 listings) and Sumatra (9 listings).

There was also evidence of targeted hunting of porcupines for commercial international trade, predominantly in Sumatra. In an incident that occurred in the province of North Sumatra, 56 live porcupines were seized. According to the suspects arrested, they were hired to transport the animals to Aceh from where they would then be shipped to China. This confirms previous anecdotal information concerning porcupines being shipped to China from Indonesia and other countries in Southeast Asia (C.R. Shepherd, pers. comm). Further, incidents in the provinces of Aceh, North Sumatra and West Sumatra, where the greatest number of porcupines were seized (on average approximately 32–33 animals per incident), have frequently revealed these areas to be important wildlife trade hubs where animals are smuggled out of Indonesia (USAID 2015; Tankandjandji and Sawitri 2016; Gomez et al. 2017). According to USAID (2015), there was a noticeable increase in poaching activities within Kerinci Seblat National Park in Sumatra around 2010 onwards reportedly driven by export markets. Porcupines were among the animals in demand, aside from tigers, Helmeted Hornbills and, pangolins. Heinrich et al. (2020a) also note the international nature of the trade in porcupine bezoars with ~20% of online adverts selling porcupine bezoars, offering international shipping. As none of the porcupine species native to Indonesia are listed in the appendices of the CITES, there is no data available for international trade, making it nearly impossible to monitor trade, regulate international trade and identify trends of concern.

Conservation implications

What clearly emerges from this study is that porcupines are being illegally hunted and exploited throughout their range in Indonesia for local subsistence and commercial trade. While it is difficult to determine the impact this has on porcupine populations, reports in Indonesia indicate the species are in decline due to habitat loss and conversion as well as uncontrolled poaching (Salviana et al. 2017; Farida et al. 2019; Mustikasari et al. 2019). Similar declines in porcupine populations due to over-hunt-

ing have been documented in other parts of their range including Malaysia (Loke et al. 2020), Myanmar (McEvoy et al. 2019), Singapore (Chung et al. 2016) as well as Vietnam and Lao PDR (Brooks et al. 2010). In Cambodia, porcupines are amongst the most frequently confiscated animals (Heinrich et al. 2020b). In Vietnam, porcupines are highly coveted for consumption which has decimated porcupine populations throughout the country (Brooks et al. 2010). Additionally, captive-breeding facilities were found to be laundering and trafficking wild animals causing local extirpations; and shifted poaching efforts to neighbouring Lao PDR to compensate for this (Brooks et al. 2010). Chevallier and Ashton (2006) note that the Cape porcupine in South Africa is widespread and easily adaptable to habitat changes and disturbances, but that this has contributed greatly to its demise as over the years it has come into increasing contact and therefore conflict with humans. Much the same is noted for the Sunda porcupine in Java (Mustikasari et al. 2019). Porcupines are also persecuted as agricultural pests, leaving them further exposed to wildlife traffickers taking advantage of these situations to procure animals for trade. Based on four seizure incidents, farmers or plantation owners were reported to be hunting and trapping porcupines to sell. In one of these incidents, a wildlife trader was arrested for smuggling 20 porcupines he had bought from plantation owners around West Aceh for IDR200K (~USD14)/animal and resold them at IDR450K (~USD32)/animal. He also provided traps for this purpose and asserted that porcupines had becomes pests destroying farmer's; oil palm plantations and would have been killed regardless. In another incident in West Sumatra, an individual was arrested for illegally trading porcupines he caught in his corn field near a forested area using ripe jackfruit as bait. This is concerning as, with the exception of the Malayan porcupine which has a wide range throughout much of Asia, the remaining four species have a more restricted range including three island endemics, the Sumatran porcupine, Sunda porcupine and thick-spined porcupine placing them at higher risk from unsustainable hunting and trade.

Commercialisation of the meat and traditional medicine trade is a key driver of species decline on a global scale (Shairp et al. 2016; Nijman and Bergin 2017; D'Cruze et al. 2020; Stanford et al. 2020; WAP 2020). In Indonesia, this has already resulted in the (near) depletion of several species encompassing tigers, pangolins, reptiles, freshwater turtles, etc (Lyons and Natusch 2011; Auliya et al. 2016; Shepherd et al. 2016; Janssen and Chng 2018; Morgan 2018; Wong and Krishnasamy 2019; Nijman et al. 2019; Rheint et al. 2019; Latinne et al. 2020, Shepherd et al. 2020). Lesser-known species are particularly vulnerable as trade often goes undetected (Alves et al. 2008; Nijman and Bergin 2017; Symes et al. 2018; Janssen and Gomez 2019; Janssen and Gomez 2021). Similarly, the commercial international trade in non-CITES listed species is also poorly documented, regulated or monitored; and this data gap presents a considerable conservation risk as understanding trade dynamics and its impact on these species is extremely difficult. A case in point - a study examining the trade of live reptiles from Indonesia to the US found that more than three quarters of exports were of non-CITES listed species than CITES listed species and encompassed nationally protected and endemic/range restricted species (Janssen and Gomez 2021).

Conclusion

As porcupines face a multitude of threats – habitat loss, retaliatory killings, targeted hunting for commercial trade – it is crucial that all porcupines be listed as protected species under Indonesian wildlife laws to improve regulation and enforcement against illegal trade. Greater resources should also be channelled to improving wildlife regulations, enforcement and scrutiny relating to the uptake of wild animals including species currently without formal protection. A thorough examination of the commercial trade of porcupines is warranted so that appropriate mitigation measures can be developed to protect porcupines from unsustainable and illegal exploitation. More in–depth research is similarly needed to understand porcupine population dynamics in Indonesia to improve understanding of their conservation status, to assess and establish harvest quotas essential for ensuring sustainable trade, and in general to monitor the overall impacts of commercial trade on wild populations. Echoing Heinrich et al. (2020a), greater efforts are also needed to monitor and regulate the international trade in porcupines. Listing these species in Appendix II of CITES should be considered as another means to potentially achieve this as it would require any international trade to take place through a supervisory system which would allow for regulation, and opportunities to track and analyze trends, thus providing an early warning system should wild populations begin to decline.

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RESEARCH ARTICLE

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Genetic diversity and morphological characterisation of three turbot (*Scophthalmus maximus* L., 1758) populations along the Bulgarian Black Sea coast

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Abstract

Turbot (*Scophthalmus maximus* L., 1758) is a valuable commercial fish species classified as endangered. The conservation and sustainability of the turbot populations require knowledge of the population's genetic structure and constant monitoring of its biodiversity. The present study was performed to evaluate the population structure of turbot along the Bulgarian Black Sea coast using seven pairs of microsatellites, two mitochondrial DNA (COIII and CR) and 23 morphological (15 morphometric and 8 meristic) markers. A total of 72 specimens at three locations were genotyped and 59 alleles were identified. The observed number of alleles of microsatellites was more than the effective number of alleles. The overall mean values of observed (Ho) and expected heterogeneity (He) were 0.638 and 0.685. A high rate of migration between turbot populations (overall mean of Nm = 17.484), with the maximum value (19.498) between Shabla and Nesebar locations, was observed. This result corresponded to the low level of genetic differentiation amongst these populations (overall mean Fst = 0.014), but there was no correlation between genetic and geographical distance. A high level of genetic diversity in the populations was also observed. The average Garza-Williamson M index value for all populations was low (0.359), suggesting a reduction in genetic variation due to a founder effect or a genetic bottleneck. Concerning mitochondrial DNA, a

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total number of 17 haplotypes for COIII and 41 haplotypes for CR were identified. The mitochondrial DNA control region showed patterns with high haplotype diversity and very low nucleotide diversity, indicating a significant number of closely-related haplotypes and suggesting that this population may have undergone a recent expansion. Tajima's D test and Fu's FS test suggested recent population growth. Pairwise Fst values were very low. The admixture and lack of genetic structuring found pointed to the populations analysed probably belonging to the same genetic unit. Therefore, a proper understanding and a sound knowledge of the level and distribution of genetic diversity in turbot is an important prerequisite for successful sustainable development and conservation strategies to preserve their evolutionary potential.

Keywords

Black Sea, COIII, CR, microsatellite genotyping, population structure, turbot

Introduction

Over the past few decades, human impacts on wild fish populations have increased drastically worldwide as a result of extensive aquaculture, exploitation of fish stocks for global consumption and human-induced climate changes (Olsson et al. 2007). Excessive fishing and large-scale environmental changes not only affect the spatial distribution and structure of populations, but also introduce extensive modifications into their genetic diversity (Madduppa et al. 2018). The genetic diversity is a fundamental estimation for a population genetic study and crucial to the fishery management and resource utilisation (Xu et al. 2019). Marine fishes generally show low levels of genetic differentiation amongst geographic regions because of higher dispersal potential during planktonic egg, larval or adult history stages, coupled with an absence of physical barriers to the movement between ocean basins or adjacent continental margins (Grant and Bowen 1998; Hewitt 2000). Loss of genetic diversity can reduce the adaptability, lessen the population persistence and lead to a decrease in the productivity of the target species. Such genetic diversity reductions in some of the world's most abundant species may add to the growing long-term impact of fishing on their evolutionary potential, particularly with the abundance staying low and diversity continuing to erode (Pinsky and Palumbi 2014).

The turbot (*Scophthalmus maximus*) is a marine flatfish, with a high commercial value living on the European continental shelf and drawing remarkable attention with respect to fisheries and aquaculture (Iyengar et al. 2000). Despite its economic importance, the turbot is considered vulnerable under the current IUCN Red List Criteria (IUCN 2019). The wild populations of turbot are exposed to a strong anthropogenic pressure as it is considered one of the most valuable commercial species subjected to intensive fishing and is characterised as exploited unsustainably and at a risk of extinction in the Black Sea (Nikolov et al. 2015).

Information on the genetic structure of commercially important fish species is crucial to prevent ecological damage and to ensure sustainable and effective management of exploited stocks and systems (Liu and Cordes 2004; Olsson et al. 2007). Molecular

markers (microsatellites and mtDNA) were applied only to closely-related turbot species in different marine regions in order to assess the genetic diversity (Bouza et al. 2002; Suzuki et al. 2004; Vandamme et al. 2014, 2020; do Prado et al. 2018). Thus, using these molecular markers, limited data on the population structure of *S. maximus* in the Black Sea were obtained (Atanassov et al. 2011; Nikolov et al. 2015; Bessonova and Nebesikhina 2019; Turan et al. 2019b; Firidin et al. 2020; Ivanova et al. 2020).

Accordingly, to avoid depletion of the genetic diversity, fisheries management should be based on a more comprehensive knowledge of the genetic integrity of the populations. The modern molecular methods developed over the past few years offer unique opportunities to rate the genetic population structures; moreover, the subsequent evaluations should be further used to smooth the process of local management and to promote increased harvest under a sustainable fisheries regime.

The aim of the present study is to evaluate the population genetic diversity of three turbot populations along the Bulgarian Black Sea coast and its applicability for the purposes of monitoring and conservation of genetic diversity in terms of sustainable management and rational exploitation of stocks.

Material and methods

Sample collection and DNA extraction

Seventy-two turbot samples were caught by a local fishing vessel in March 2019 and April 2020 at two locations in the Black Sea (Shabla, Shkorpilovtsi and Nesebar) (Fig. 1). For DNA analysis, tissue samples were taken from dorsal fin and stored in 96% ethanol at 4 °C prior to the following analyses. The genomic DNA was extracted by DNeasy Blood & Tissue Kit (QIAGEN). All the DNA extracts were analysed by gel electrophoresis to monitor the DNA quality prior the PCR amplification.

Microsatellite genotyping

Seven microsatellite loci (Sma1-125INRA, Smax-02, Sma3-12INRA, 3/9CA15, B12-IGT14, Sma-E79 and Sma-USC26) were amplified and analysed (Table 1). The polymerase chain reaction (PCR) was performed in a reaction volume of 50 µl containing 2 µl of each primer, 25 µl of Mastermix (MyTaqTM HS Mix) and 2 µl of the target DNA. The reverse microsatellite primers were fluorescently labelled at the 5' end with the 6-FAM dye (Table 1). The PCR amplification was performed under the following conditions: 35 cycles (95 °C for 1 min, 95 °C for 45 sec, 56–60 °C for 50 sec depending on the primer type, 72 °C for 1 min) and 72 °C for 10 min. Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific) was used to carry out the fragment analysis. The size of the fragments was determined with GeneMapper 4.0 (Thermo Fisher Scientific) software package.

Figure 1. Sampling sites (red circles) along the Bulgarian Black Sea coast.

PCR and sequence analysis of mitochondrial DNA (COIII)

The polymerase chain reaction (PCR) using mitochondrial primers (COIII) was carried out in a reaction volume of 50 µl containing 2 µl of each primer, 25 µl of the Mastermix (MyTaqTM HS Mix) and 2 µl of the target DNA. The mitochondrial cytochrome c oxidase subunit III (COIII) was amplified using universal primers (F: AGCCCATGACCTTTAACAGG and R: GACTACATCAACAAAATGTCAGTATCA, according to Valles-Jiménez (2005). The conditions for PCR amplification included the following parameters: 94 °C for 5 min, 95 °C for 30 sec, 49 °C for 1min, 72 °C for 1 min (35 cycles) and 72 °C for 5 min. The mtDNA sequencing was performed by Macrogen Europe B.V. The obtained COIII sequences were deposited in the GenBank under accession numbers MN556886–MN556913 and MW446249–MW446288.

Table 1. Characterization of microsatellite loci for Black Sea turbot genotyping.

Locus	GenBank No	Primer sequences	Repeat motif	Size range
B12-I GT14	AF182086.1	F: GTGATGGAAGATTGTACCAG R: CACAATAAAGGATAGACCAG	(GT)14	113–119
3/9CA15	AF182091.1	F: AGAGTGAAGAACGTACCTGC R: CAATGGAGAGGCAGTATCGG	(CA)15	226–245
Sma1-125INRA	No data	F: CACACCTGACAAAGCTCAAC R:GCTGAACATTTTCATGTTGATAG	(TAGA)11-(TG)4	112–152
Smax-02	No data	F: GGAGGATGTATTGAAAGTGT R: AGAGCAGGTCATTATACAGC	(TG)16	93–141
Sma3-12INRA	No data	F: CACAATTGAATCACGAGATG R: GCCACCACTGCGTAACAC	(TG)21	88–110
Sma-E79	No data	F: GCAGCGACTTGCTTCTTTCT R: GTCAGTTTGTGGTGTGTGGG	(GT)6-(AT)14-(GT)9-(TA)7	227–317
Sma-USC26	No data	F:TCAAACCAACGGACTAACAAACA R:CTTCATTACCAGCCCATCAAAGT	(TATC)12	202–282

PCR and sequence analysis of mitochondrial DNA (CR)

The polymerase chain reaction (PCR) using mitochondrial primers (CR) was carried out in a reaction volume of 50 µl, containing 2 µl of each primer, 25 µl of Mastermix (MyTaqTM HS Mix) and 2 µl of target DNA. The mitochondrial control region (CR) was amplified using universal primers (L15924: 5'AGCTCAGCGCCAGAGCGC-CGGTCTTGTAAA and H16498-5'-CCTGAAGTAGGAACCAGATG, according to Atanassov et al. (2011). The conditions of PCR amplification included the following parameters: 94 °C for 5 min, 95 °C for 45 sec, 50 °C for 1 min, 72 °C for 1 min (35 cycles) and 72 °C for 5 min. PCR product quality control was performed by electrophoresis on 2% agarose gel. The sequencing was performed by Macrogen Europe B.V. The obtained CR sequences were deposited in the GenBank under the accession numbers MN556856–MN556885 and MW446289–MW446330. The new sequences were compiled and analysed with previously deposited sequences extracted from the GenBank database covering all available CR sequences of different populations of *S. maximus* along the Bulgarian Black Sea coast.

Morphology analyses

Eight meristic characteristics were thoroughly investigated: total length (TL), standard length (SL), dorsal fin ray (DFR), pectoral fin ray (PFR), anal fin ray (AFR), ventral fin ray (VFR) back pectoral fin ray (BPFR); caudal fin ray (CFR); plus 15 morphometric characters: M1-Linea-Dorsal height; M2-Linea-Anal height; M3-Beginning dorsal fin origin – end of operculum; M4-Mouth-beginning pectoral fin origin; M5-Mouth-operculum distance; M6-Mouth-end of pectoral fin distance; M7-Mouth-origin of dorsal fin; M8-Length of the dorsal base; M9-Caudal Fin base expanse; M10-Anal base length; M11-Ventral length; M12-Body height; M13-starting-end mouth distance; M14-lateral line; M15-standard length (according to Turan et al. 2019a) that are commonly used to describe Scophthalmidae species.

Statistical analyses

The Hardy-Weinberg equilibrium (HWE) exact tests and loci combinations for linkage disequilibrium with the Markov Chain methods were conducted using GenAlEx 6.5 (Peakall and Smouse 2012). The indices for genetic diversity as Shannon's information index, observed, expected unbiased, expected heterozygosities and inbreeding coefficient were calculated, against that background also using GenAlEx 6.5 (Peakall and Smouse 2012). Polymorphic information content (PIC) and Garza and Williamson index were computed using Arlequin v.3.5.2.2 (Excoffier and Lischer 2010). The analyses of private alleles (alleles observed in only one population), the allelic richness and Mantel test were accomplished by the programme GenAlEx 6.5 (Peakall and Smouse 2012).

The inbreeding coefficient (Fis) was thereupon calculated using Genepop 4.7 (Rousset 2008) and mtDNA sequence data were further analysed by applying MEGA7 (Kumar et al. 2018). The sequence alignment was established, the number of haplotypes, haplotype network and molecular phylogenetic neighbour joining (NJ) tree (Saitou and Nei 1987) and TCS Networks were easily constructed by means of PopArt (Clement et al. 2002). The bootstrap consensus tree was inferred from 1000 replicates. The evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei 1984) and the haplotype (H) and nucleotide (π) diversities were estimated with DnaSP 5.10.01 (Librado and Rozas 2009). The fixation index Fst calculated in Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010) was used to infer the genetic structure. The significance test was obtained from 1023 permutations. Bayesian clustering analysis was performed in STRUCTURE v.2.3.4 (Pritchard et al. 2000) with 10 independent iterations at each K = 1–10 with 20^5 Markov Chain Monte Carlo iterations after a burning period of 20^5 repetitions. The optimum K was estimated using the Structure Harvester (Earl and Von Holdt 2012) following the Evanno method (Evanno et al. 2005) for BIC comparison. Structure graphical results were plotted with CLUMPAK (Kopelman et al. 2015).Correlation analysis was appropriately implemented to identify the specific pattern of linear relationships between morphometric and meristic characters to be further analysed and validated amongst the studied populations, aiming at assessing the similarity/dissimilarity in morphology. The non-parametric statistical test – similarity analysis (ANOSIM) was conducted in order to identify statistically significant differences between samples and hierarchical clustering and NDMS were used to visualise the distribution of samples with respect to their morphology similarities. All statistical analyses and graphic representations were performed using the statistical and programming software R 4.3.8 (R Core Team 2020), packages: 'PerformanceAnalytics' (Peterson and Carl 2014), 'vegan' (Oksanen et al. 2019), 'tidyverse' (Wickham 2017) for data manipulation, 'cluster' (Maechler et al. 2018) for selection and implementation of the proper clustering algorithm and 'factoextra' (Lê et al. 2008) for clustering visualisation, as well as 'dendextend' (Galili 2015), available through the CRAN repository (www.r-project.org).

Results

Genetic variability of microsatellite loci

All analysed microsatellite markers proved to be polymorphic and a total of 59 alleles was discerned within the seven loci. The length of the identified alleles in the investigated loci ranged between 85 and 320 base pairs (bp). The number of alleles per locus ranged between 2 and 13 (Table 2). As a result, Sma-USC26 (13 alleles) was the most polymorphic locus and Smax-02 (two alleles) was the least polymorphic locus. Analysis of allelic frequencies by loci showed that the lowest allelic frequency (0.017) was found at locus Sma-E79 and the highest one (0.717) at locus Smax-02. Reasonable amount of polymorphism in turbot was evident from the allele frequency data, with the mean number of alleles (MNA) being 6.330 ± 0.634.

The Shabla, Nesebar and Shkorpilovtsi populations had the smallest number of alleles per Smax-02 locus in comparison with all Black Sea populations previously investigated (Table 2). For the other loci, the data obtained were similar (Turan et al. 2019b; Bessonova and Nebesikhina 2019; Firidin et al. 2020). The most polymorphic locus in the current study was Sma-USC26 in the Shabla population (Table 2).

Table 2. Number of alleles per locus for Shabla, Nesebar and Shkorpilovtsi turbot populations and comparison with the reference data for turbot from different Black Sea areas and adjacent seas (BG-Bulgaria, GA-Georgia, TR-Turkey). NA – not analysed.

Population	Locus								References
	N of samples	Sma1-125INRA	Smax-02	Sma3-12INRA	B12-IGT14	3/9CA15	Sma-E79	Sma-USC26	
Shabla,BG,BS	**30**	**5**	**2**	**8**	**5**	**5**	**11**	13	Current study
Nesebar,BG,BS	**28**	**4**	**2**	**8**	**5**	**7**	**8**	10	Current study
Shkorpilovtsi, BG, BS	**14**	**6**	**2**	**6**	**4**	**6**	**8**	8	Current study
Varna, BG, BS	10	9	14	9	5	4	NA	NA	Turan et al. 2019b
Trabzon, TR, BS	10	6	4	7	3	10	NA	NA	Turan et al. 2019b
Duzdze, TR, BS	10	8	5	4	3	14	NA	NA	Turan et al. 2019b
Sevastopol, BS	10	11	11	11	9	6	NA	NA	Turan et al. 2019b
Caucasian, BS	63–64	9	13	7	NA	11	11	9	Bessonova and Nebesikhina 2019
Crimean, BS	128–130	7	5	11	NA	10	14	11	Bessonova and Nebesikhina 2019
Sea of Azov	60–61	8	6	6	NA	7	14	11	Bessonova and Nebesikhina 2019; Turan et al. 2019b; Firidin et al. 2020
Marmara Sea, Turkey	10	8	7	5	7	3	NA	NA	
Romania, BS	12	8	NA	12	NA	6	NA	NA	
Istanbul, TR, BS	42	6	NA	7	NA	11	NA	NA	
Kocaeli, TR, BS	34	5	NA	5	NA	7	NA	NA	
Zonguldak, TR, BS	14	4	NA	6	NA	5	NA	NA	
Kastamonou, TR, BS	43	4	NA	9	NA	7	NA	NA	
Sinop, TR, BS	45	5	NA	5	NA	11	NA	NA	
Samsun, TR, BS	51	6	NA	7	NA	9	NA	NA	
Trabzon, TR, BS	25	4	NA	7	NA	8	NA	NA	
Artvin, TR, BS	44	4	NA	7	NA	8	NA	NA	
Abkhasia, GA, BS	8	3	NA	3	NA	4	NA	NA	
Crimean, BS	46	5	NA	5	NA	6	NA	NA	
Sea of Azov	50	6	NA	4	NA	6	NA	NA	

The expected number of alleles varied from 1.529 (Smax-02) to 6.374 (Sma-USC26) (Table 3). Heterozygosity is a major indicator of the level of genetic diversity in a population. With the values reflecting the level of observed heterozygosity (Ho) ranging from 0.107 at locus Smax-02 to 0.929 at locus B12-IGT14 (Table 3), the expected heterozygosity (He) of genetic diversity would vary from 0.406 at locus Smax-02 to 0.843 at locus Sma-USC26, respectively. The total mean of Ho was 0.638 ± 0.470, with maximum and minimum values of 0.684 and 0.582 in Shkorpilovtsi population and Nesebar population, respectively. The overall mean value of He was 0.685 ± 0.026.

The polymorphic information content (PIC) and the values of Shannon-Wiener index (I) provided a relatively broad value range of 0.324–0.825 and 0.596–2.015, respectively. The fixation index (Fis) varied between 0.006 and 0.769, with an average

Table 3. Descriptive statistics of *Scophthalmus maximus* for 7 loci samples from three different populations along the Bulgarian Black Sea coast.

Sampling area		Locus						
		Sma1-125INRA	**Smax-02**	**Sma3-12INRA**	**B12- IGT14**	**3/9CA15**	**Sma-USC26**	**Sma-E79**
Shabla	N	30	30	30	30	30	30	30
	Na	5	2	8	5	5	13	11
	Ne	3.529	1.529	2.913	4.009	3.462	5.172	3.072
	I	1.371	0.596	1.421	1.471	1.340	2.015	1.608
	Ho	0.733	0.233	0.700	0.800	0.800	0.633	0.633
	He	0.717	0.406	0.657	0.751	0.711	0.807	0.674
	uHe	0.729	0.413	0.668	0.763	0.723	0.820	0.686
	Fst	-0.023^{ns}	0.425*	-0.066^{ns}	-0.066^{ns}	-0.125^{ns}	0.215^{ns}	0.061***
	Fis	-0.006	0.439	-0.049	0.078	-0.049	-0.108	0.231
	PIC	67.13	32.35	62.18	70.89	65.89	78.65	65.03
	M	0.23810	0.66667	0.53333	0.15942	0.45455	0.23810	0.23636
Nesebar	N	28	28	28	28	28	28	28
	Na	4	2	8	5	7	10	8
	Ne	3.97	1.813	3.477	4.181	3.862	6.374	2.292
	I	1.383	0.641	1.595	1.492	1.525	2.012	1.296
	Ho	0.643	0.107	0.679	0.786	0.786	0.679	0.393
	He	0.748	0.448	0.712	0.761	0.741	0.843	0.564
	uHe	0.762	0.456	0.725	0.775	0.755	0.858	0.574
	Fst	0.141^{ns}	0.761***	0.047^{ns}	-0.033*	-0.060^{ns}	0.195*	0.303*
	Fis	0.158	0.769	0.066	0.32	-0.015	-0.042	0.213
	PIC	70.1	34.77	68.48	72.57	69.87	82.51	54.44
	M	0.23529	0.66667	0.42105	0.11268	0.45455	0.33333	0.18182
Shkorpilovtsi	N	14	14	14	14	14	14	14
	Na	6	2	6	4	6	8	8
	Ne	3.5	1.849	3.5	3.733	3.267	5.521	3.564
	I	1.412	0.652	1.489	1.352	1.407	1.837	1.609
	Ho	0.857	0.286	0.857	0.929	0.714	0.643	0.500
	He	0.714	0.459	0.714	0.732	0.694	0.819	0.719
	uHe	0.741	0.476	0.741	0.759	0.72	0.849	0.746
	Fst	-0.200^{ns}	0.378^{ns}	-0.200^{ns}	-0.268*	-0.029^{ns}	0.215^{ns}	0.305*
	Fis	-0.164	0.409	-0.164	0.338	-0.234	0.008	0.25
	PIC	66.58	35.37	68.08	68.38	65.13	79.4	68.99
	M	0.28571	0.66667	0.40000	0.10959	0.57143	0.28571	0.13559

N – number of samples, Na – number of different alleles, Ne – number of effective allele, I – Shannon's information index, Ho (observed) and He – (expected) heterozygosities, uHe (unbiased expected heterozygosity), Fst - measures the expected heterozygosity with respect to the total population (p-values < 0.05 *, <0.01**, <0.001***, ns not significant), Fis – fixation index/ inbreeding coefficient, PIC -polymorphic information content, M - Garza and Williamson index (M-ratio index).

value of 0.094 to testify to a slight excess of heterozygotes in the fish group. The Garza-Williamson index was lowest at the B12-I GT14 locus in the Shkorpilovtsi population and highest at the Smax-02 locus in all populations analysed.

All investigated loci differed in terms of the Garza-Williamson index (M), within the range 0.10959 to 0.66667. The M average value in the investigated populations was 0.361 for Shabla, 0.335 for Nesebar and 0.351 for Shkorpilovtsi (Table 3).

The mean values of unbiased expected heterozygosity (uHe) in the analysed populations were similar (0.686±0.049 for Shabla population, 0.701±0.052 for Nesebar and 0.719±0.043 for Shkorpilovtsi), which marked the similar genetic diversity (Bessonova and Nebesikhina 2019).

Pairwise Fst comparisons showed low genetic differentiation between sampling sites (Table 4) and was always not significant. The presence of migrants per generation varied between 15 and 19% (Table 4). The highest rate of gene flow (Nm = 19.498) was observed between Shabla and Nesebar populations.

A Mantel test revealed positive, but not significant relationships between the genetic and geographic distances (R^2 = 0.8273, P = 0.336).

Genetic diversity of mitochondrial DNA

A total of 17 haplotypes for COIII (563 bp) and 41 haplotypes for CR (432 bp) were identified. The sequence analyses of COIII recovered seven haplotypes for Shabla, two haplotypes for Shkorpilovtsi and eight haplotypes for the Nesebar population (Table 5). The majority of the identified COIII haplotypes originated from one prevalent set of haplotypes (Hap 2) following a single nucleotide substitution (Fig. 2). Four haplotypes were unique for the Shabla and Nesebar populations (Table 5) compared to previous data for Black Sea populations (Turan et al. 2019 b).

The COIII haplotype diversity ranged from 0.389 to 0.766 in the three populations, with the highest value presented in the Nesebar population (Table 5). Nucleotide diversity ranged from 0.001 to 0.002. The result of mean COIII haplotype diversity between populations was 0.550, higher than the data presented in Turan et al. (2019b) for five populations in the Black and Marmara Seas (0.380). For the control region (CR), haplotypes (H1, 2 and 4) were common for the three populations analysed (Fig. 3) Unique CR haplotypes were observed in all populations, i.e. Shabla (13), Shkorpilovtsi (7) and Nesrbar (8) (Table 5, Fig. 3).

All three turbot populations analysed showed high levels of haplotype diversity (0.892–0.954), but low nucleotide diversity (0.004–0.006) in the mtDNA control

Table 4. *Scophthalmus maximus* population pairwise FST (below diagonal) and pairwise genetic distances (above diagonal), based on microsatellites.

	Shabla	Nesebar	Shkorpilovtsi
Shabla	–	0.061	0.081
Nesebar	0.013	–	0.067
Shkorpilovtsi	0.016	0.014	–

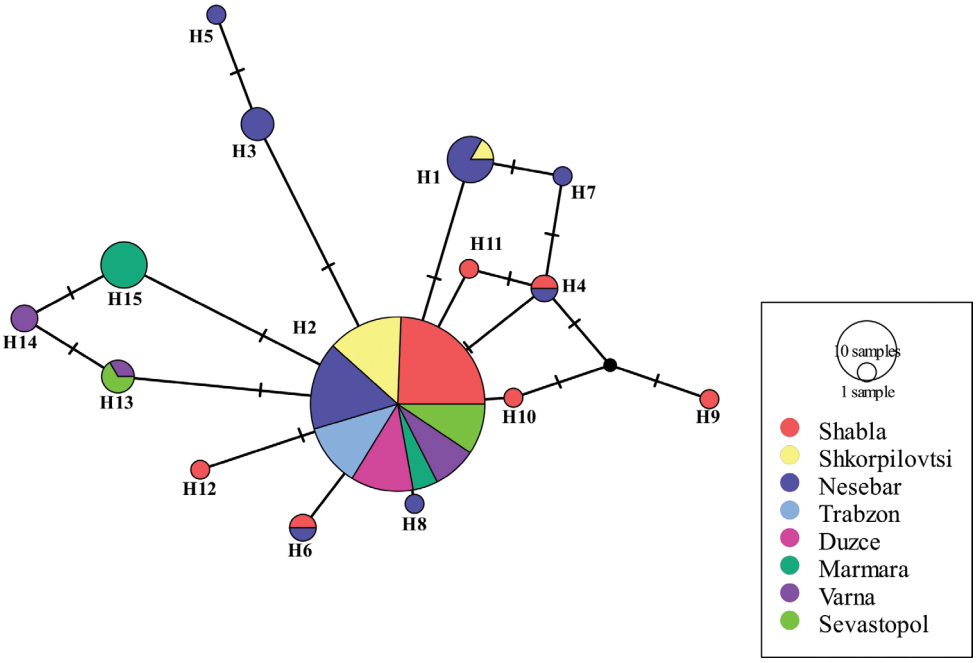

Figure 2. Haplotype network from mtDNA COIII, obtained from the TCS analysis. The size of the circle represents the frequency of each haplotype. Small lines illustrate the substitutions between the respective haplotypes. The small black circle indicates the intermediate haplotypes that are not present in the sample.

Table 5. Genetic diversity parameters of *Scophthalmus maximus* populations, based on mDNA sequence data. Control region reference data from Shabla were included (Ivanova et al. 2020).

	Sampling site	n	H	pHap	Hd	PS	π	D	k	Fs
COIII	Nesebar	26	8	4	0.766	7	0.002	-1.064	1.197	-3.524
	Shabla	28	7	4	0.389	5	0.001	-1.857	0.556	-5.235
	Shkorpilovtsi	14	2	0	0.495	1	0.001	-1.212	0.495	1.139
CR	Nesebar	28	12	8	0.892	10	0.004	-1.097	1.693	-6.925
	Shabla	30	19	13	0.954	18	0.006	-1.713	2.425	-15.924
	Shkorpilovtsi	14	10	7	0.945	10	0.006	-0.832	2.473	-5.520

n: number of sequences, H: Number of haplotypes, pHap: the number of private haplotypes, H_d: haplotype diversity, PS: polymorphic sites, π: nucleotide diversity, D: Tajima's D value; Fs: Fu's Fs value.

region in comparison with Bulgarian Black Sea populations previously analysed (Atanassov et al. 2011) (Table 5).

The population pairwise Fst revealed an overall low level of genetic structure between the turbot populations. Non-significant Fst values were observed except for the comparison between Nesebar and Shabla populations (Table 6).

Genetic structure and phylogenetic trees were unable to detect genetic differentiation between sampling sites due to the low genetic differentiation for the COIII markers (0.002–0.003) and for CR (0.005–0.006) between haplotypes (Table 7). The

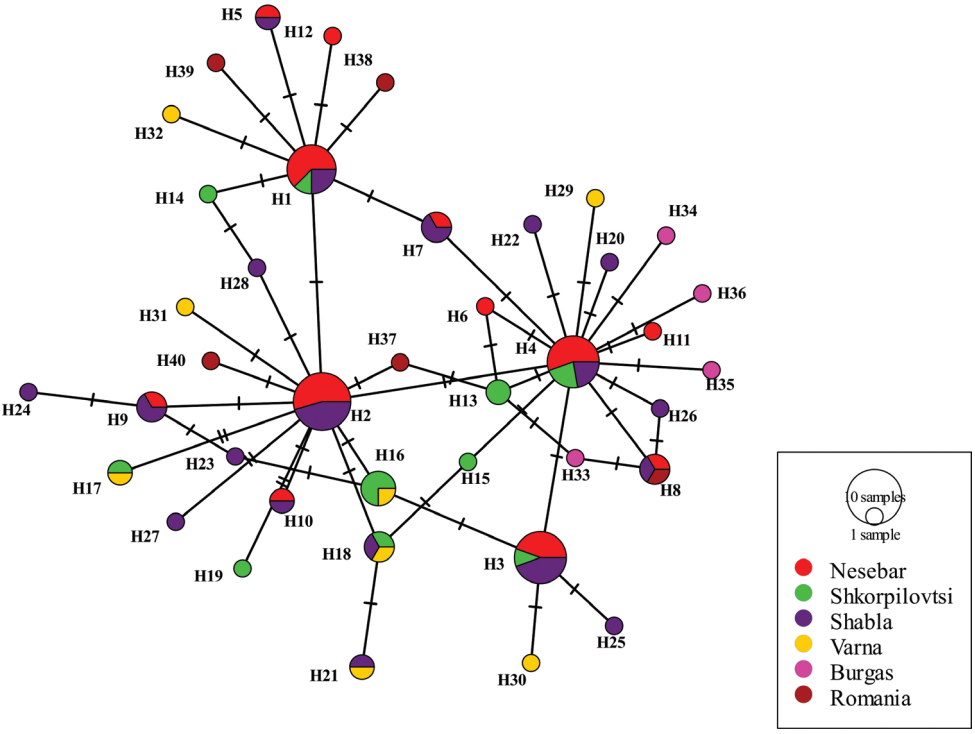

Figure 3. Haplotype network from CR (mtDNA) obtained from the TCS analysis. The size of the circle represents the frequency of each haplotype. Small lines illustrate the substitutions between the respective haplotypes.

Table 6. *Scophthalmus maximus* population pairwise F_{ST} (based on COIII below diagonal) and based on CR (above diagonal), (p-values < 0.05*, < 0.01**, < 0.001***, ns not significant).

	Nesebar	Shkorpilovtsi	Shabla
Nesebar	–	0.004^{ns}	-0.012^{ns}
Shkorpilovtsi	-0.018^{ns}	–	-0.017^{ns}
Shabla	0.117**	0.122***	–

lowest genetic distance (0.002) was detected between Shkorpilovtsi and Shabla populations and the highest value (0.003) was found between Shabla and Nesebar populations, based on COIII analyses. Using CR, the highest genetic distance (0.006) was found between Shabla and Shkorpilovtsi populations (Table 7).

A Mantel test revealed no significant relationship between genetic and geographic distances (R^2 = 0.868 and P = 0.358 for COIII; R^2 = 0.881 and P = 0.324 for CR).

Tajima's D values were negative for all populations (Table 5), but statistically non-significant, with the exception of the Shabla population, indicating an excess of rare nucleotide site variants. The results of Fu's FS test, which is based on the distribution of haplotypes, showed negative values for all regions except Shkorpilovtsi (COIII marker), indicating an excess of rare haplotypes. The overall negative values resulting

Table 7. Pariwise genetic distances between sampling sites, based on COIII (below diagonal) and CR (above diagonal).

	Nesebar	Shkorpilovtsi	Shabla
Nesebar	–	0.005	0.005
Shkorpilovtsi	0.003	–	0.006
Shabla	0.003	0.002	–

Figure 4. Population structure of *Scophthalmus maximus* inferred using the programme STRUCTURE for K = 3 of 72 individuals. Black lines separated individuals from different sampling sites. The structure analyses showed that the samples from three different localities could not be clustered separately as different populations and they are admixed.

from both tests indicate that there is an excess of rare mutations in the populations, but the excess is statistically non-significant.

Based on the mean log likelihood values LnP(K), Bayesian clustering analysis, implemented in STRUCTURE, indicated K = 3 (LnP = -1695.64) as the most likely number of clusters (Fig. 4 and Suppl. material 1: Fig. S7).

Morphological variability

Meristic data analyses provided strong statistically significant correlations only between TL, SL and W (Suppl. material 1: Figs S1, S3, S5), which was rather expected with respect to individual growth theory basics and moderate positive correlation between VFR and BPFR with very close values of correlation coefficient values for Shabla, Nesebar and Shkorpilovtsi populations (r = 0.55, p = 0.00164; r = 0.58, p = 0.00126; r = 0.057, p = 0.0265), (Table 8). Only the Nesebar population showed moderate positive correlation between AFR and VFR (r = 0.58, p = 0.01216). As the age of samples was not determined, to provide a closer look at growth model parameters, the length-weight relationship (LWR) was studied for comparison. In the Shabla population samples, LWR was best approximated with a 5th order polynomial model (R^2 = 0.6732 – non-linear harmonic regression would provide even better approximation); in Nesebar samples, LWR was modelled with the power model: $W = 5e - 06^{*}L^{3.3006}$ (R^2 = 0.0.9608) and in the Shkorpilovtsi population, LWR was best approximated with 2nd order polynomial model (R^2 = 0.9035). These results might highlight either certain differences in food availability, accounting for the weight being a highly variable parameter and/or regional environmental specifics or some background noises as seasonal processes or certain physiological stages, which also may have an impact on the genetic diversity of the studied populations.

Table 8. Morphometric characters' correlations.

Morphometric characters	*Shabla population (n = 30)*	*Nesebar population (n = 28)*	*Shkorpilovtsi population (n = 14)*
M4-M5	*Moderate positive correlation:* $r = 0.52$, $p = 0.003$	*Moderate positive correlation*: $r = 0.68$, $p = 0.00007$	*No correlation*
M4-M6	*Strong positive correlation*: $r = 0.86$, $p < 0.00001$	*Very strong positive correlation*: $r = 0.9$, $p < 0.00001$	*Strong positive correlation*: $r = 0.83$, $p < 0.000129$
M5-M6	*Moderate positive correlation:* $r = 0.62$, $p = 0.0003$	*Moderate positive correlation:* $r = 0.67$, $p = 0.00001$	*Moderate positive correlation:* $r = 0.60$, $p = 0.018$
M6-M10	*Moderate positive correlation:* $r = 0.55$, $p = 0.0003$	*Moderate positive correlation:* $r = 0.57$, $p = 0.0003$	*No correlation** Correlation coefficient $r = 0.51$ is significant at significance level $a = 0.1$
M6-M11	*No correlation*	*No correlation*	*Strong positive correlation*: $r = 0.71$, $p < 0.004$
M2-M12	*Moderate positive correlation:* $r = 0.54$, $p = 0.0021$	*Moderate positive correlation:* $r = 0.62$, $p = 0.0003$	*No correlation*
M6-M12	*Moderate positive correlation:* $r = 0.66$, $p = 0.00007$	*Moderate positive correlation:* $r = 0.7$, $p = 0.00003$	*Moderate positive correlation:* $r = 0.61$, $p = 0.021$
M10-M12	*Moderate positive correlation:* $r = 0.61$, $p = 0.0003$	*Moderate positive correlation:* $r = 0.63$, $p = 0.0003$	*Moderate positive correlation:* $r = 0.55$, $p = 0.042$
M10-M14	*Moderate positive correlation:* $r = 0.7$, $p = 0.00002$	*Moderate positive correlation:* $r = 0.61$, $p = 0.00057$	*Strong positive correlation:* $r = 0.73$, $p = 0.003$
M10-M15	*Very strong positive correlation:* $r = 0.9$, $p < 0.00001$	*Strong positive correlation:* $r = 0.83$, $p < 0.00001$	*Very strong positive correlation:* $r = 0.91$, $p = 0.00001$
M14-M15	*Strong positive correlation:* $r = 0.74$, $p = 0.00001$	*Moderate positive correlation:* $r = 0.67$, $p = 0.000096$	*Strong positive correlation:* $r = 0.77$, $p = 0.00013$

Correlation analysis applied on morphometric characters revealed a certain linear functional pattern evident with slight differences amongst the samples, taken from the populations under investigation. The morphometric characters' correlation pattern in Shkorpilovtsi samples was slightly different from the pattern identified for Shabla and Nesebar samples (although it has to be noted that the correlation coefficient p values are sensitive to the number of samples n).

Analysis of similarity (ANOSIM) was carried out to identify statistically significant differences between the samples. The results showed that there was no statistically significant differences between Shkorpilovtsi and Shabla and Nesebar and Shabla samples ($Significance_{Shabla–Shkorpilovtsi} = 0.359$; $Significance_{Shabla–Nesebar} = 0.8869$, with an even distribution of high and low dissimilarity ranks in and between populations). However, there were statistically significant differences between the samples taken from Shkorpilovtsi and Nesebar populations ($Significance_{Shkorpilovtsi–Nesebar} = 0.063$). In addition, the hierarchical clustering outcome (Suppl. material 1: Figs S2, S4, S6) showed that the clusters formed are mixed and specimens of all three populations are represented evenly and, furthermore, no specific outliers were identified. The latter is also visible from the non-metric multidimensional scaling (NMDS) plot (Fig. 5). No specific clusters were formed with respect to morphology and only single specimens are dispersed close to the major cluster formed due to specific morphometric characters deviation (TL, SL and W), which are generally considered to vary in more broad ranges.

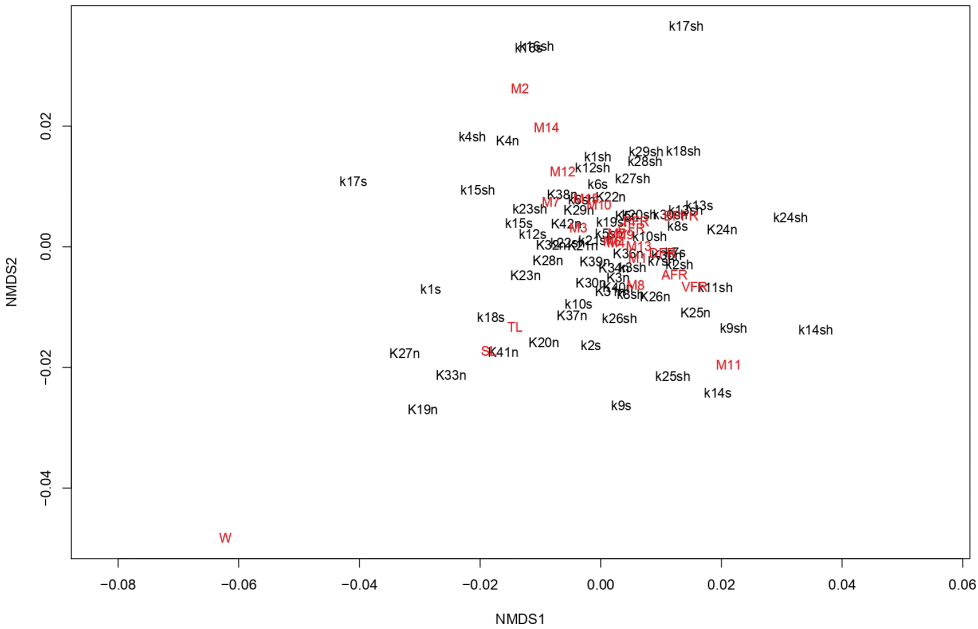

Figure 5. Bray-Curtis-based non-metric multidimensional scaling of *Scophthalmus maximus* meristic and morphometric data (all samples).

Discussion

The results obtained in this study correspond to the creation of a database of the genetic diversity for turbot populations along the Bulgarian Black Sea coast. A comprehensive analysis of the acquired morphological and molecular data will enable a subsequent assessment of the impact of fishing on the structure of turbot populations. By knowing the genetic characteristics of valuable populations, we can monitor relatively easy changes in heritable traits and in the level of average genetic diversity (Olsson et al. 2007).

Apparently, the selection of microsatellites with a range of polymorphism has led to a reduction in the risk of overestimating genetic variability, which might occur with the selective use of highly polymorphic loci. On the whole, the allelic diversity (mean number of observed alleles per locus) for populations along the Bulgarian Black Sea coast (3.559) is higher than that reported by Bessonova and Nebesikhina (2019) for the same loci in turbot populations from the Crimean Black Sea (3.430) and Azov Sea (3.420) and lower than species from the Caucasian Black Sea coast (3.623). The mean values for the obtained and expected heterozygosity (Ho = 0.638 and He = 0.685) are close to those indicated by previous studies for the same loci in turbot populations (Estoup et al. 1998; Iyengar et al. 2000; Bouza et al. 2002; Bessonova and Nebesikhina 2019; Turan et al. 2019b; Firidin et al. 2020). The relatively high heterozygosity and allelic diversity of these populations suggest that local gene flow takes place amongst them. The observed heterozygosity for Smax-02 and Sma-E79 loci (Shabla population), for Smax-02, Sma-USC26 and Sma-E79 (Nesebar) and Sma-E79

(Shkorpilovtsi) are significantly lower than the expected value in turbot populations ($P < 0.05$) (Table 3). There are several reasons that can explain this significant reduction in Ho compared with He, as high rate of migration, errors in reading alleles and inbreeding (Skaala et al. 2004; Li et al. 2009). The unbiased expected heterozygosity (uHe) showed a mean value of 0.702 ± 0.027, similar for those described by Bessonova and Nebesikhina (2019) for the Black Sea turbot (0.622 ± 0.086), which marked an equal level of the genetic diversity in the three populations investigated.

Nevertheless, a significant deviation was detected, from the HWE at a 0.05 α-level at all of the investigated loci with the exception of Smax-02 locus (Shabla population), Smax-02, B12-I GT14, Sma-USC26 and Sma-E79 loci (Nesebar population) and B12-I GT14 and Sma-E79 loci (Shkorpilovtsi population) ($P < 0.05$). Departures from HWE at other loci may be the result of founder and/or bottleneck effects followed by a high rate of inbreeding (Kaczmarczyk and Wolnicki 2016). A marker with a low Fis value will show the genetic diversity of a group more accurately, which means that this specific marker has a high discrimination power (Han et al. 2018). The positive and high value for Fis for the three populations was recovered for Smax-02 locus, based on the small number of the recorded alleles (Table 3) and indicates heterozygote deficiency that could be caused by inbreeding, presence of null alleles and admixture of distinct populations (Guo et al. 2013). The Smax-02 locus is also the locus with the least information (PIC is 0.324) and the lowest Shannon index (0.596).

The studies of the turbot populations, characterised by genetic diversity parameters (PIC and I), indicate that these values were high for six of the loci analysed (PIC and I higher than 0.6 and 1.3, respectively) indicating, thereby, high genetic diversity (Froufe et al. 2004; Fopp-Bayat 2010) (Table 3). The PIC values from each loci varies between populations analysed (0.622–0.825) and were comparable with the data for turbot population from the Black Sea (0.713–0.853) (Firidin et al. 2020) and from Turkey, France and South Korea (PIC 0.3–0.9) (Han et al. 2018) indicating the random loci were under independent selection pressure and seem to be ideal to be used as a divergence index. For detection of the bottleneck events, the Garza-Williamson index (M) was successfully employed. The data acquired (ranging from 0.335 to 0.361) across the studied populations are comparable with the data found for the Varna population (western Black Sea), based on the same loci (G-W index 0.4 on average) (Turan et al. 2019b), which suggests a sustainable reduction of genetic variation in this population as a result of founder and/or bottleneck effects (Kaczmarczyk and Wolnicki 2016). It should be pointed out that, in all studied populations, M indices were lower than 0.68 indicating a recent reduction in population size due to overfishing. The results of the Mantel test did not detect a clear correlation between geographic distance and the size of genetic differences existing between populations' pairs.

Generally, genetic differentiation based on microsatellites is considered as very weak. The mean value of Fst was 0.014 and the AMOVA results revealed that it was mostly related to a within-population variation (99.4%) rather than variation amongst populations (0.6%). The highest Fst value was observed between Shabla and Shkorpilovtsi = 0.016). The population pairwise Fst revealed an overall low level of genetic

structuring between the turbot populations (Table 4). Based on the Fst values, it can be concluded that the rate of differentiation between populations is low and a high rate of migration (Nm =17.484, average) can be the main factor underlying this low differentiation. The Fst results were lower when compared to other Black Sea and Marmara populations (Fst = 0.249 according to Turan et al. 2019 b; Fst = 0.138 according to Firidin et al. 2020) and evidence of genetic sub-structuring along the Bulgarian Black Sea coast was not detected.

Mitochondrial DNA polymorphism is widely used to determine population structure, species differences and evolutionary relationships (Avise et al. 1987). The COIII gene of mtDNA is a genetic marker used for species identification in the genus *Scophthalmus* in the Black Sea (Turan et al. 2019a). The *S. maximus* populations displayed a genetic pattern typical of a recent population expansion due to its one common COIII haplotype (Hap 2) present across the range and the high number of unique haplotypes (Fig. 2). The Shabla population was characterised by the low levels of haplotype diversity (0.389) and nucleotide diversity (0.001) which is probably a result from a founder event or population bottleneck followed by rapid population growth (Xu et al. 2019). Population expansion in Shabla was also supported by highly significant negative values for both Tajima's D (–1.857, $P < 0.05$) and Fu's FS (–5.235 $P < 0.05$). The values of genetic diversity, as well as the distribution of private alleles are comparable amongst the populations, in spite of the low number of samples from Shkorpilovtsi.

The mean haplotype diversity and nucleotide diversity indices calculated as 0.550 and 0.001 for COIII and 0.930 and 0.005 for CR are similar to the data for COI and Cyt-b for the Black Sea turbot populations presented by Firidin et al. (2020). The genetic distances calculated, based only on COIII, present clear structure/correlation between populations according to the geographic distances (Table 7), but they are not significant according to the Mantel to test.

Studying more variable regions such as mtDNA CR to investigate the genetic variability across the Black Sea would give more valuable information about population structuring, based on mtDNA analyses (Firidin et al. 2020; Ivanova et al. 2020). The high levels of genetic diversity and low levels of nucleotide diversity observed in *S. maximus*, based on the mtDNA CR sequences, are in agreement with earlier studies of turbot populations from the Black Sea (Suzuki et al. 2004; Atanassov et al. 2011; Ivanova et al. 2020). A similar spatial pattern of distribution of genetic variability in mtDNA control region was also reported for other fishes (Xiao et al. 2008, 2014). High haplotype diversity in CR suggests large, stable, effective population sizes over time in the continental shelf fishes (Stepien 1999) and, in concurrence with low nucleotide diversity, it has been linked to population growth after a period of low effective population size (Grant and Bowen 1998). The inference of population expansion is further supported by the star-like patterns in Figure 3. The low nucleotide diversity in CR indicate a high number of closely-related haplotypes and suggest that this population may have undergone a recent expansion (Slatkin 1993; Mendez-Harclerode et al. 2007), supported also by non-significant and negative Tajima's D and Fu's Fs (Alcaraz and Gholami 2020).

Pairwise Fst values were very low, with the highest value for COIII observed between Shabla and Shkorpilovtsi populations and for CR between Shkorpilovtsi and Nesebar populations. Therefore, no significant genetic differentiation was evident between any populations and showed that *S. maximus* within the examined range constitutes a non-differential mtDNA gene pool. Results from STRUCTURE analysis (Fig. 4) also revealed some degree of admixture in the studied populations. In this regard, the observed heterozygote deficiency could also be caused by inbreeding depression, as a result of wild populations experiencing considerable reduction mainly because of overfishing and increased level of pollution.

The present mtDNA and microsatellite analyses of turbot populations along the Bulgarian Black Sea coast using seven microsatellites and two mtDNA markers give no evidence for genetic subdivision of this species in comparison with population genetic structuring found between the north and south Black Sea populations using microsatellites (Firidin et al. 2020). Based on both microsatellite and mtDNA analyses, Turan et al. (2019b) found one genetic unit of turbot in the Black Sea (Trabzon, Duzdge, Varna and Sevastopol populations). In the present study, *S. maximus* populations were considered to be a single stock, which probably could be attributed to this genetic unit.

A lack of correlation between genetic and geographic distances along the Bulgarian Black Sea coast was recorded. This result may be evidence of the hydrodynamic factors that have an effect on the dispersal potential of larvae phases and subsequently affect genetic differentiation (Vandamme et al. 2014, 2020). *S. maximus* spawn with pelagic eggs (Vasil'eva 2007) and perform compensatory migration to the north against taking the caviar to the south of the currents along the Bulgarian coast (Karapetkova and Zhivkov 2006). Considering the lack of long active migration of species, the ocean currents might be responsible for this homogeneity in examined groups. In the North Sea, the turbot larvae drift on average 102 km (Barbut et al. 2019). Seawater temperature, food availability and coastal currents explain a significant component of geographically distributed genetic variation, suggesting that these factors act as drivers of local adaptation (Ruggeri et al. 2016; Diopere et al. 2017). The strength of local adaptation is dependent on the interaction between connectivity, population size and environmentally and human induced pressures (Bernatchez 2016).

Fishing pressure on turbot stock affects the size of the catches; however, there is no evidence of the impact of fishing on population genetic diversity. Genetic monitoring, in addition to the stock assessment, should also be carried out to track and identify all the potential population-genetic changes. Placing a restriction on the maximum catch size of 45 mm prevents the loss of rare alleles from older and larger individuals. It could also be an effective tool for protecting the genetic diversity. The regular monitoring and quota determination of the *S. maximus* populations are necessary to control the turbot population status. Close collaboration between molecular geneticists and fisheries biologists would be required to undertake extensive research into the recruiting processes of the marine populations and their possible implications for fisheries management and conservation (Hauser et al. 2002).

Conclusions

The DNA barcoding has proved to be an invaluable tool for tracking and monitoring of endangered populations, thus giving a sharper focus on the strategic conservation of distinct genetic stocks and mitigation on human impacts along their range. The molecular characterisation and analysis of the genetic structure in turbot populations along the Bulgarian Black Sea coast contributes to the considerable knowledge about the levels and genetic diversity distribution patterns. Microsatellite and mitochondrial markers both indicated close genetic relationships between populations. It should, however, be pointed out that, in the examined populations, a high level of genetic diversity was observed. The lack of strong population genetic structure was probably due to the small geographic distances and high gene flow between them. The derived low values of the G-W index are specific for reduced populations and may indicate a dramatic decrease in the population size in the past. The applied molecular approach proves critical for any rigorous monitoring of the impacts of overexploitation and genetic management of threatened fish species along the Bulgarian Black Sea coast. The results confirmed the high effectiveness of the use of different types of markers for performing genetic analysis and relevant provision of reliable information with regard to the genetic diversity within the turbot populations. The genetic characteristics of turbot populations, revealed in this study, will provide useful information for continuous and effective resource management. Moreover, constant monitoring may be needed to maintain the high level of genetic diversity in natural populations.

Finally, the underlying rationale behind the adoption of a more integrated approach (genetic and morphological) to the study of *S. maximus* populations in the Bulgarian Black Sea coast will provide more accurate assessment of the population structure as well as it will facilitate detection of any probable changes in gene pools of the wild populations in connection with their more effective management. Therefore, a proper understanding and a sound knowledge of the level and distribution of genetic diversity in turbot is an important prerequisite for successful sustainable development and conservation strategies to preserve their evolutionary potential.

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Supplementary material 1

Genetic diversity and morphological characterization of three turbot (*Scophthalmus maximus* L., 1758) populations along the Bulgarian Black Sea coast

Authors: Petya Ivanova, Nina Dzhembekova, Ivan Atanassov, Krasimir Rusanov, Violin Raykov, Ivelina Zlateva, Maria Yankova, Yordan Raev, Galin Nikolov

Data type: molecular data

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Potential changes in the distribution of *Delphinium bolosii* and related taxa of the series *Fissa* from the Iberian Peninsula under future climate change scenarios

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Abstract

A particular threat posed by climate change for biodiversity conservation, one which has scarcely been studied, is the overlap of the potential distribution areas in phylogenetically closely related species. In this study, Species Distribution Modelling (SDM) was used to investigate the potential changes in the distribution of *Delphinium bolosii* and *D. fissum* subsp. *sordidum* under future climatic scenarios. These two closely related and endangered endemic species from the Iberian Peninsula do not have complete reproductive barriers between them. The two models selected different predictors with a similar effect in the biological cycle. Both taxa need low winter temperatures to break seed dormancy and sufficient rainfall to complete the flowering and fruiting stages. The current potential distribution areas of both taxa do not currently overlap. However, the results showed that potential changes may take place in the species' distribution range under future climate scenarios. The models predict a reduction of the potential distribution area of *D. bolosii* while, conversely, the potential distribution area of *D. fissum* subsp. *sordidum* increased. In both cases, the predicted contraction in range is very high, and loss of habitat suitability in some current localities is worrying. Notwithstanding, the models do not predict overlaps of potential areas under climate change scenarios. Our findings can be used to define areas and populations of high priority for conservation or to take action against the impacts of climate change on these endangered species.

Keywords

changes in range, endangered species, larkspur, MaxEnt, range overlap, species distribution models

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Introduction

Recent studies suggest that climatic change may well be one of the most important challenges that humanity will face in forthcoming decades to avoid precisely what models forecast, in the worst-case scenarios – namely the sixth mass extinction in the history of the Earth (Barnosky et al. 2011). Climate change effects on biodiversity involve many components, such as: (1) decreased genetic diversity and/or populations; (2) alterations to interspecific relationships (e.g. plant-animal systems); and (3) changes in plant communities (Bellard et al. 2012).

Rare species are more susceptible to environmental changes (e.g. climate warming) than common species (Lomba et al. 2010). The International Union for Conservation of Nature (IUCN) recommends developing new quantitative methods to determine species ranges, potential suitable habitats and potential shifts in distribution ranges when faced with climate change, especially for rare and threatened species (Fordham et al. 2012). By means of species distribution models (SDMs), a technique that is being increasingly employed in the 21st century and uses environmental variables together with species occurrences (Guisan and Zimmermann 2000), it is possible to obtain this information; e.g. ecological drivers of species' ecological niches and geographic distributions and predicting how future climatic change will affect biodiversity (Araújo et al. 2011; Thuiller et al. 2011). SDMs are crucial for anticipating the ecological impacts of climate change in more susceptible areas, and for devising adequate conservation planning and decision making (Riordan and Rundel 2014).

The interpretation and usefulness of SDMs have been discussed in several works (e.g. Sinclair et al. 2010; Austin and Van Niel 2011). It has been found that SDMs almost never consider interspecific interactions, species' genetic and plastic ability and temporal and physiological responses, despite some exceptions (such as Lavergne et al. 2010). Likewise, species are considered static elements, but their dynamism has been documented (Schröder and Seppelt 2006). However, Araújo and Luoto (2007) confirmed that models based only on environmental variables on a regional scale (e.g. the Iberian Peninsula) can adequately quantify the impact of climatic change on species distributions. For species with generalist requirements, such as heterogeneity of habitats and plant communities, broad spectrum of pollinators and non-specific seed dispersal adaptations, SDMs can offer good results to address several ecological and biodiversity conservation issues (Sinclair et al. 2010). This is the case with the endangered endemic Iberian species of the *Delphinium* L. series *Fissa* B. Pawl.

The origin of *Delphinium* series *Fissa* (Delphinieae, Ranunculaceae) lies in Central Asian mountains and extended westward throughout the Mediterranean Basin in the Late Miocene, namely during the Messinian Salinity Crisis (6–5.3 Ma) (Blanché 1991). At present, *Delphinium* series *Fissa* is represented in the western Mediterranean region by three taxa: *D. fissum* Waldst. and Kit. subsp. *fissum* is distributed from northern Italy and the French Maritime Alps to the Pyrenees, whereas two other taxa, *Delphinium bolosii* C. Blanché and Molero and *D. fissum* subsp. *sordidum* (Cuatrec.) Amich, E. Rico and J. Sánchez, are differentiated in the Iberian Peninsula (Ramírez-

Rodríguez et al. 2019, 2020). According to Bosch (1999), the reproductive barriers during this differentiation process are still incomplete, and the formation of fertile hybrids is possible through interbreeding. These two taxa have undergone an allopatric speciation process to currently give rise to non-overlapping distribution areas. One interesting application of SDMs is to study changes in the potential distribution areas of phylogenetically closely related species as the overlap of the potential areas induced by climate change might lead to hybridisation and competition processes, which could threaten species' persistence. However, these biological impacts of climate change have scarcely been studied (Krosby et al. 2015).

Recently, Rus et al. (2018) generated potential distribution models according to current climate conditions for *D. fissum* subsp. *sordidum*, and identified the environmental factors to explain its distribution, which are related to the taxon's biological and ecological characteristics. The selected predictors and its percentage contribution were: mean temperature of wettest quarter (26.2%), temperature annual range (25.7%), precipitation of warmest quarter (22.1%), slope (21%), precipitation of coldest quarter (2.7%), and hillshade (2.3%).

The present study aims to: (1) generate the current model for *D. bolosii* with the same methodology used for *D. fissum* subsp. *sordidum*, and compare the predictors of both models; (2) determine and evaluate the potential impacts of climate change on the distribution range of the series *Fissa* species in the Iberian Peninsula, as an example of closely related species without complete reproductive barriers; and (3) provide information for the medium and long-term conservation of these taxa under climate change scenarios.

Material and methods

Study area and test species

The study area corresponds to the Iberian Peninsula, which is located to the southwest of the European continent (Fig. 1). This territory covers an area of 582918 km^2 and is characterised by its asymmetrical distribution of main mountainous systems and a strong climatic gradient. Most of the Iberian Peninsula has a Mediterranean climate, characterised by a summer with at least two very dry and hot months. Moreover, it has high levels of biodiversity due to its great bioclimatic and biogeographic heterogeneity determined by geological and historical factors (Rivas-Martínez et al. 2002). Consequently, the Iberian Peninsula is one of the floristically richest areas of Europe and the Mediterranean Basin (Aedo et al. 2013).

According to the phylogenetic results obtained by cpDNA (Ramírez-Rodríguez et al. 2019), field observations and reviewed herbarium material (Ramírez-Rodríguez et al. 2020), the populations located in the Pre-Pyrenean and Catalan Mediterranean System correspond to *D. bolosii* (Blanché and Molero 1983; López-Pujol et al. 2015), as well as the cited populations on the eastern slope of the Iberian System (Mateo and Pisco 1993, sub *D. fissum* subsp. *sordidum*; Pitarch 2002, sub *D. mansanetianum*).

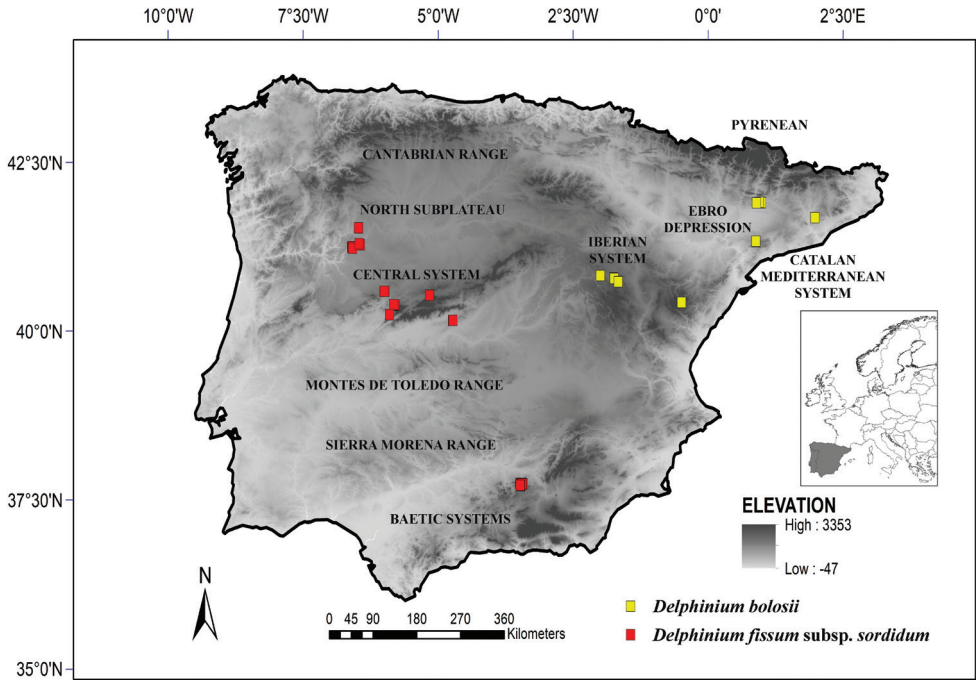

Figure 1. Location of the Iberian Peninsula in the south-western Europe. The main Iberian mountain ranges are pointed out as well as the known localities for *D. bolosii* and *D. fissum* subsp. *sordidum*.

Delphinium bolosii is a perennial rhizomatous larkspur endemic of the north-eastern Iberian Peninsula (Fig. 1). This species lives in *Quercus rotundifolia* Lam. forests on schists on the edge of communities with *Buxus sempervirens* L., *Jasminum fruticans* L., and *Acer monspessulanum* L., and in open clearings in *Quercus humilis* Mill. forests (Fig. 2A, B). It is catalogued as EN [B1ab(iii,iv,v)+2ab(iii,iv,v); C1] on the Red List of Spanish Vascular Flora (Moreno 2008).

Delphinium fissum subsp. *sordidum* is a rhizomatous hemicryptophyte endemic of the central-western and southern Iberian Peninsula (Fig. 1) that lives in chestnut forests, oak forests, holm-oak forests and open areas between shrubs (Fig. 2C, D). The subspecies is included in the Red List of Spanish Vascular Flora (Moreno 2008) under the category EN [B2ab(v)c(iv); C2b]. For further details, see Rus et al. (2018) and Ramírez-Rodríguez and Amich (2019).

Occurrence data and environmental variables

Fourteen occurrences for *D. bolosii* were obtained, but those that occurred in the same 1 × 1 grid cell were removed to reduce spatial correlation (Benito et al. 2013). This left 12 valid occurrences at the defined grid resolution. Although the number of occurrences is low, they represent the whole distribution range of *D. bolosii* after decades of study of the species (Blanché and Molero 1983; Mateo and Pisco 1993; Bosch et al. 1998,

Figure 2. Habitats and flowering individuals of the studied species: *D. bolosii* (**A, B**) and *D. fissum* subsp. *sordidum* (**C, D**).

2019; Pitarch 2002; Orellana et al. 2007; Blanché et al. 2014; López-Pujol et al. 2015; Ramírez-Rodríguez et al. 2019, 2020). As potential predictors, 19 bioclimatic variables and one topographic variable (elevation) were considered and downloaded from the WorldClim database (Hijmans et al. 2005, available at http://www.worldclim.org). Three additional topographic variables (aspect, hillshade and slope) were obtained from the elevation variable by ArcMap 10.3.1 (ESRI, Redlands, California, USA). A list of all the used variables is available in Suppl. material 1: Table S1. These variables were the same as those used by Rus et al. (2018) to generate the potential model of *D. fissum* subsp. *sordidum*. No edaphic, phytocenotic, land use and landscape features variables were used due to the study taxa develop in a broad variety of soils and habitats.

For the future predictions, the CCSM4 model was used, which was downloaded from the Fifth Assessment Report of the International Panel on Climate Change (IPCC AR5 WG1 2013) for the years 2050 and 2070 with two IPCC Representative Concentration Pathways: "stabilisation" (RCP 4.5) and "high increase" (RCP 8.5). They were selected as they represent contrasting scenarios to predict potential climate change effects. Thus, the emissions in RCP 4.5 peak around 2040 before declining. In RCP 8.5, emissions continue to rise throughout the 21st century (Meinshausen et al. 2011). CCSM4 is an efficient global climate projection that predicts the influence of future climatic changes on the distribution of plant species in the Mediterranean Basin (Abdelaal et al. 2019).

Spatial modelling

The model for *D. bolosii* was created using Maxent v. 3.3.3 (Phillips et al. 2006; Merow et al. 2013), following the same methodology used for *D. fissum* subsp. *sordidum* by Rus et al. (2018). For details about data preparation, Maxent configuration, model fitting, model evaluation and jack-knife procedure, see Rus et al. (op. cit.). This methodology is adequate for modelling species with a low number of occurrences (Pearson et al. 2007). To test the fit of model, the area under the curve (AUC) of the receiver-operating characteristic (ROC) was used (Fielding and Bell 1997). Although this metric has been criticised in some recent works (Jiménez-Valverde 2012), it is still the most widely used metrics, and is a good tool to evaluate models for the same species and within the same geographical scope.

To determine the potential distribution areas, binary outputs of presence/absence were generated by setting thresholds in the logistic models. Firstly, the 10th percentile training presence logistic threshold (10P) was used to transform habitat suitability, as estimated by the models, into a binary prediction. This method is well recognised for distinguishing suitable regions from unsuitable ones (Hughes 2017). Then the current distribution maps were overlaid on those obtained for all the four future climate scenarios selected to identify any potential changes in range: contraction, expansion, refuge, non-suitable areas (Hatten et al. 2016). Secondly, more restrictive thresholds were set to differentiate three classes of potential habitats (Yang et al. 2013): low potential (10P-0.6), medium potential (0.6–0.8) and high potential (0.8–1). After taking into account these thresholds, the extent of the potential distribution areas (both current and future) were calculated in all the potential zones (see Fig. 1) as the studied species showed a markedly disjunct distribution. For the known localities, the current values of habitat suitability were compared with those obtained under the climate change scenarios.

Results

Current model for *Delphinium bolosii*

The AUC value was 0.962 ± 0.032 for the *D. bolosii* model, indicating that the model performed well at predicting the current distribution of the species. The current potential distribution area was 35814.9 km^2 for threshold 10P = 0.4585, of which 14127.1 km^2 (39%) showed a medium-high suitable habitat probability (HS ≥ 0.6). This potential distribution was found mainly in two areas of the north-eastern Iberian Peninsula: the Pre-Pyrenean, along with Catalan Mediterranean System, and the south-eastern Iberian System (Fig. 3).

For the *D. bolosii* model, the mean temperature of the driest quarter had the strongest relative influence on habitat suitability (82.3%). Other predictors for this model and their contribution were: precipitation of the coldest quarter (5.2%), temperature seasonality (4.7%), precipitation seasonality (4.2%), slope (3.5%) and Isothermality (0.2%). The

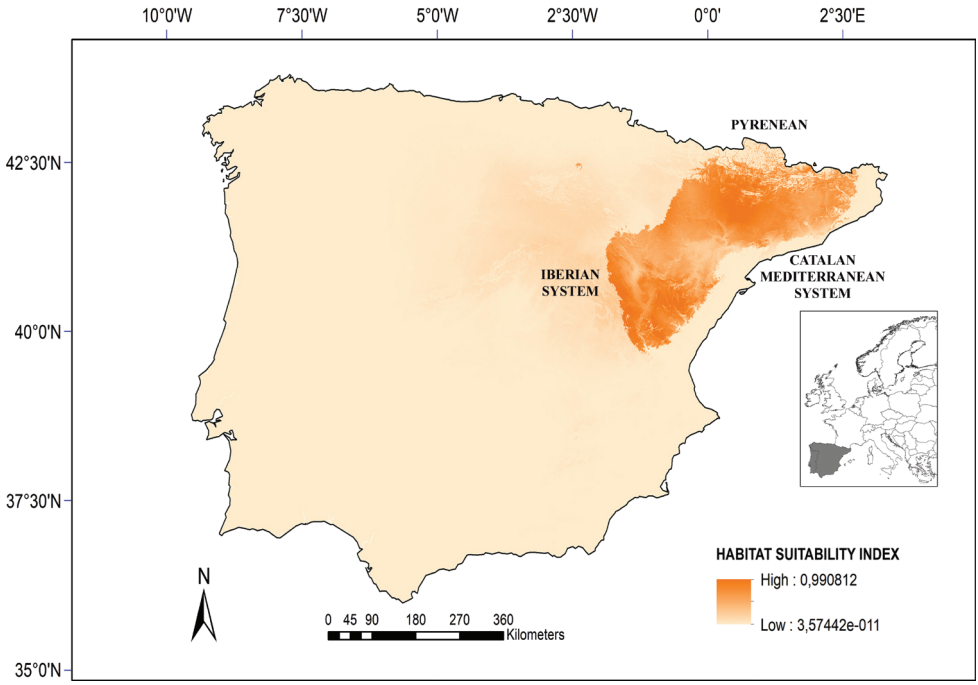

Figure 3. Predicted habitat suitability for *D. bolosii*.

effects of these environmental variables on habitat suitability for *D. bolosii* are shown in Fig. 4. For example, the probability of presence was maximum when the mean temperature of the driest quarter went close to 0 °C. The selected variables and its percentage contribution to the *D. bolosii* and *D. fissum* subsp. *sordidum* models are compared in Table 1.

Potential effects of climate change on the series *Fissa*

Our results showed that potential changes could take place on the distribution range of the series *Fissa* species, i.e., when comparing the extent and quality of the present and future suitable habitats for *D. bolosii* (Table 2) and *D. fissum* subsp. *sordidum* (Table 3).

For *D. bolosii*, the total potential distribution area reduced for all the studied climate change scenarios, except for one (2070 RCP 4.5) (Table 2), mainly in the outer areas of its potential distribution (Fig. 5). The predicted contraction in range for this taxon was 55.5% in the harsher scenario (2070 RCP 8.5) (Table 4). In contrast, the potential distribution area of *D. fissum* subsp. *sordidum* increased in all the climate change scenarios (especially for the harsher scenario). This expansion of potential areas occurred mainly towards inland areas of the northern Sub-Plateau, the eastern Central System, the western Iberian System and the north-eastern area of the Baetic Systems (Fig. 6). Despite this expansion, the models forecast relatively high loss or contraction percentages (47.3% for 2070 RCP 4.5) (Table 4), especially to the east of the northern Sub-Plateau and the Sierra Morena range (Fig. 6). However for both taxa, the potential

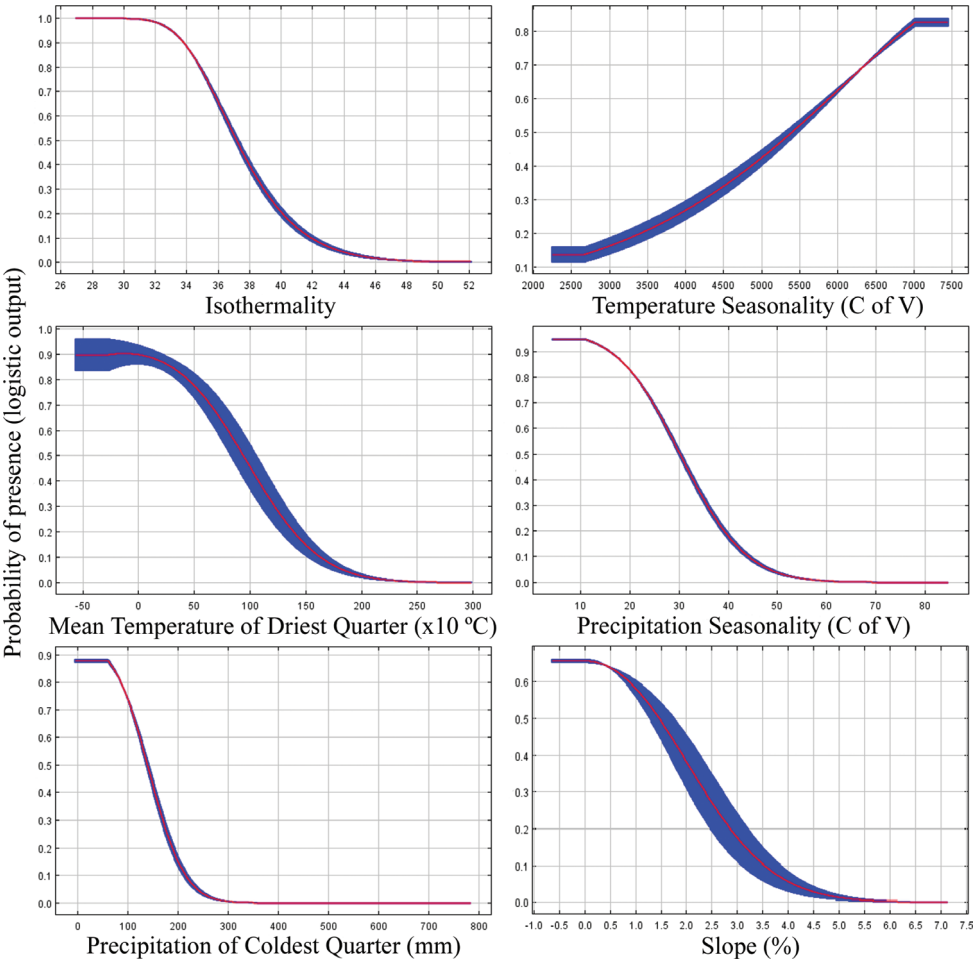

Figure 4. Response curves for environmental variables selected as predictors in the ecological niche model for *D. bolosii*.

Table 1. Environmental variables used and its percentage contribution to the *D. bolosii* (this study) and *D. fissum* subsp. *sordidum* (Rus et al. 2018) models.

Code	Environmental variables	% Contribution	
		D. bolosii	***D. fissum* subsp. *sordidum***
Bio3	Isothermality	0.2	
Bio4	Temperature seasonality	4.7	
Bio7	Temperature annual range		25.7
Bio8	Mean temperature of wettest quarter		26.2
Bio9	Mean temperature of driest quarter	82.3	
Bio15	Precipitation seasonality	4.2	
Bio18	Precipitation of warmest quarter		22.1
Bio19	Precipitation of coldest quarter	5.2	2.7
Slo	Slope	3.5	21
Hil	Hillshade		2.3

Table 2. Extent of current and future potential distribution areas for *D. bolosii* (km^2), relative to the areas and Habitat Suitability (HS). L, Low (10th Percentile=10P-0.6); M, Medium (0.6–0.8); H, High (0.8–1). Listed are the estimated range sizes (% change in range size) in current and future (2050, 2070) projections under the optimistic (RCP 4.5) and the pessimistic (RCP 8.5) models for climate change.

		HS	EIS	PP-CMS	ST	T	ST/T
Current		L	7416.3	14271.5	14127.1	35814.9	0.39
		M	6848.6	7158.9			
		H	107.7	11.9			
RCP 4.5	2050	L	2139.3	3018.7	27790.8	32948.8	0.84
		M	6077.7	7070.3	(96.7)	(–8.0)	
		H	3716.8	10926.0			
	2070	L	1886.9	2081.7	39060.1	43028.7	0.91
		M	4485.0	5971.8	(176.5)	(20.1)	
		H	10002.3	18601.0			
RCP 8.5	2050	L	281.2	565.8	31654.5	32501.5	0.97
		M	1562.3	1898.4	(124.1)	(–9.3)	
		H	11481.2	16712.6			
	2070	L	61.5	163.4	24989.1	25214.0	0.99
		M	1036.9	443.3	(76.9)	(–29.6)	
		H	10838.0	12670.9			

EIS: Eastern Iberian System; PP-CMS: Pre-Pyrenean and Catalan Mediterranean System. ST: Sub-Total = M+H; T: Total = L+M+H.

Table 3. Extent of current and future potential distribution areas for *D. fissum* subsp. *sordidum* (km^2), relative to the areas and Habitat Suitability (HS). L, Low (10th Percentile=10P-0.6); M, Medium (0.6–0.8); H, High (0.8–1). Listed are the estimated range sizes (% change in range size) in current and future (2050, 2070) projections under the optimistic (RCP 4.5) and the pessimistic (RCP 8.5) models for climate change.

		HS	NSP	CS	MTR	SMR	BS	WIS	ST	T	ST/T
Current		L	7122.5	7872.2	1108.3	890.0	3773.7	709.3	2126.0	23602.1	0.09
		M	80.1	760.3	108.5	95.3	275.9	-			
		H	-	427.5	58.9	133.0	186.6	-			
RCP 4.5	2050	L	8011.2	9529.8	3285.3	1106.4	4949.5	3769.0	9173.8	39825.0	0.23
		M	52.9	1343.3	478.4	42.3	783.5	308.4	(331.5)	(68.7)	
		H	-	648.5	1344.6	29.1	3817.3	325.6			
	2070	L	4817.2	7299.9	434.7	25.8	3137.8	5653.6	7073.5	28442.4	0.25
		M	25.1	841.0	53.6	-	581.0	601.5	(232.7)	(20.5)	
		H	0.7	1265.2	39.7	-	3022.6	643.2			
RCP 8.5	2050	L	11870.2	11428.2	1047.5	232.9	4774.8	11364.0	20379.6	61097.3	0.33
		M	1171.2	1846.1	103.2	7.3	1173.9	1613.9	(858.6)	(158.9)	
		H	1370.4	7352.8	261.4	2.0	3976.8	1500.7			
	2070	L	10552.1	12966.6	2057.6	275.9	3301.9	6276.9	22879.5	58310.5	0.39
		M	1382.9	2990.2	272.0	15.9	441.4	641.2	(976.2)	(147.1)	
		H	2819.5	8952.1	575.0	7.3	4301.7	480.4			

NSP: North Sub-Plateau; CS: Central System; MTR: Montes de Toledo Range; SMR: Sierra Morena Range; BS: Baetic Systems; WIS: Western Iberian System. ST: Sub-Total = M+H; T: Total = L+M+H.

area with a medium-high suitability habitat increased. In both cases, habitat quality also significantly increased, i.e. the proportion with a medium-high suitability habitat in relation to the total potential habitat (Tables 2, 3).

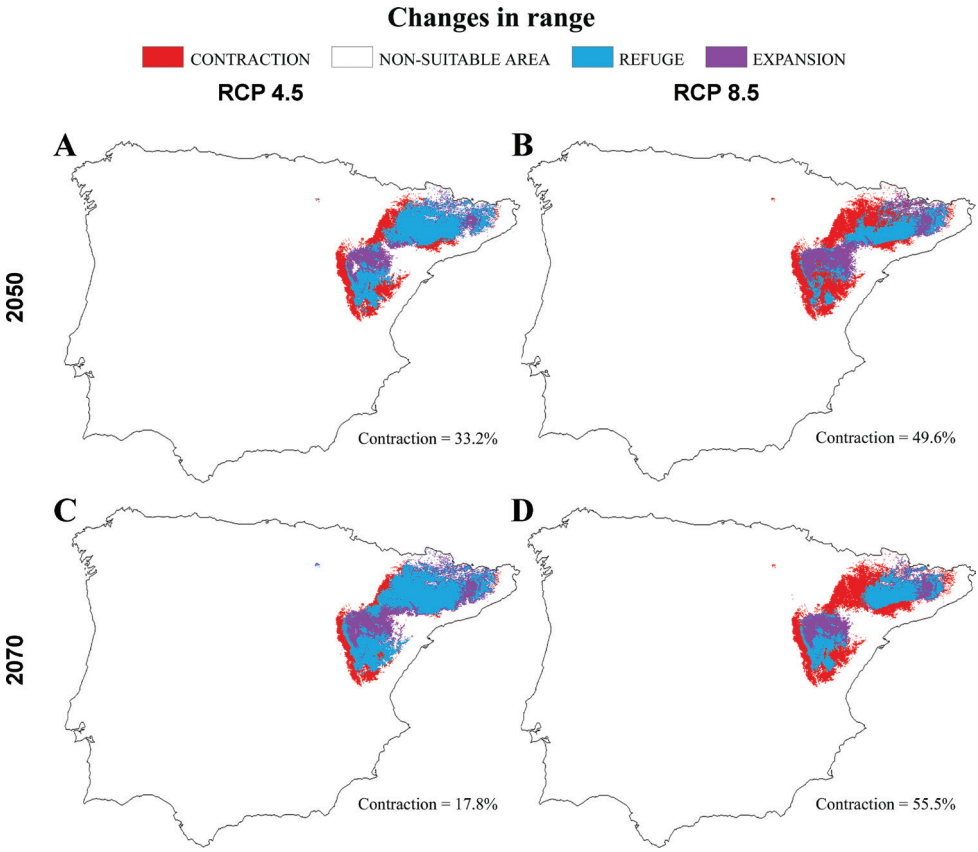

Figure 5. Projected changes in range for *D. bolosii* under future climate change scenarios for 2050 (**A, B**) and 2070 (**C, D**).

Table 4. Changes in range (percent) for *D. bolosii* and *D. fissum* subsp. *sordidum* between current time period and future climate change scenarios.

Taxon	Change in range	2050 RCP 4.5	2070 RCP 4.5	2050 RCP 8.5	2070 RCP 8.5
D. bolosii	Refuge (Persistence)	66.8	82.2	50.4	44.5
	Contraction (Loss)	33.2	17.8	49.6	55.5
	Expansion (Gain)	24.7	37.8	40.2	25.4
D. fissum subsp. *sordidum*	Refuge (Persistence)	77.9	52.7	70.9	80.9
	Contraction (Loss)	22.1	47.3	29.1	19.1
	Expansion (Gain)	91.6	67.3	189.2	167.6

Regarding the habitat suitability of both taxa in currently known populations, models forecast some variations under climate change scenarios (Table 5). For *Delphinium bolosii*, habitat suitability would reduce, generally in the populations located in the Catalan Mediterranean System and the eastern Iberian System, whereas habitat suitability would remain or increase in the Pre-Pyrenean populations. For

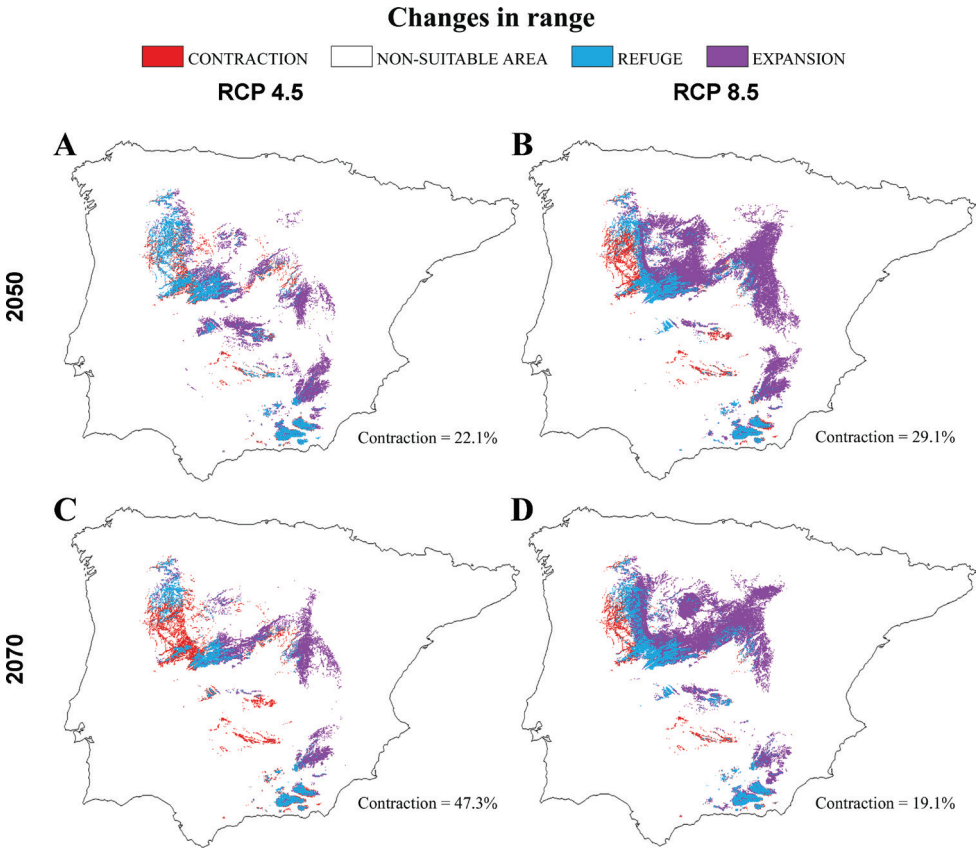

Figure 6. Projected changes in range for *D. fissum* subsp. *sordidum* under future climate change scenarios for 2050 (**A, B**) and 2070 (**C, D**).

Table 5. Habitat suitability values by areas of the current occurrences points of *D. bolosii* and *D. fissum* subsp. *sordidum* under future climate change scenarios.

Taxon	Areas	Current	2050 RCP 4.5	2070 RCP 4.5	2050 RCP 8.5	2070 RCP 8.5
D. bolosii	Pre-Pyrenean – CMS	0.43–0.68	0.02–0.94	0.04–0.96	0.04–0.99	0.04–0.99
	Eastern Iberian System	0.16–0.81	0.11–0.16	0.16–0.98	0.18–0.31	0.12–0.31
D. fissum subsp. *sordidum*	Baetic Systems	1	1	0.99–1	1	1
	Central System	0.07–0.94	0.07–0.96	0.02–0.74	0.24–1	0.21–1
	North Sub-Plateau	0.04–0.51	0.01–0.14	0–0.03	0–0.03	0.02–0.61

CMS: Catalan Mediterranean System.

D. fissum subsp. *sordidum*, the main changes would be increased habitat suitability for some populations of the Central System in the RCP 8.5 scenario, and major loss of suitability for almost all the populations in the northern Sub-Plateau. In the Baetic Systems, habitat suitability would remain very high.

Discussion

The models obtained for the series *Fissa* species reinforce the hypothesis of the differentiation of one eastern taxon (*D. bolosii*) and another western taxon (*D. fissum* subsp. *sordidum*) in the Iberian Peninsula following the migratory patterns proposed by Bocquet et al. (1978). The Iberian System may act as a geographic barrier during this differentiation process.

Apparently, the *D. bolosii* model selected different predictors that the model provided by Rus et al. (2018) for *D. fissum* subsp. *sordidum*. However, when we contemplated the differences between the climate features of the north-eastern Iberian Peninsula and the rest of the study area, a remarkable similarity was observed in the effect of these variables on the biological cycle of both taxa. Such similarities, which are discussed later, would be in accordance with the niche conservatism theory (i.e. retention of fundamental niche characteristics over evolutionary time) in phylogenetically closely related taxa (Peterson et al. 1999).

Firstly, the mean temperature of the driest quarter had the strongest influence on the habitat suitability of *D. bolosii*. In contrast, the variable that contributed the most in the model of *D. fissum* subsp. *sordidum* was the mean temperature of the wettest quarter (Rus et al. 2018). The driest quarter in the north-eastern Iberian Peninsula (corresponding to potential distribution of *D. bolosii*) was winter, and the wettest quarter in the western Iberian Peninsula (corresponding to potential distribution of *Delphinium fissum* subsp. *sordidum*) was also winter (Rodríguez-Ballesteros 2015). Thus habitat suitability was maximum for both taxa when the average winter temperature was 0–4 °C. According to Blanché et al. (2014), low temperatures are required to break seed latency in *D. bolosii*.

Secondly, both models' selected variables were related to the continentality of climate: with *D. bolosii* it was temperature seasonality, but was temperature annual range for *D. fissum* subsp. *sordidum* (Rus et al. 2018). A marked contrast between low winter temperatures and high summer temperatures increased habitat suitability for the two taxa. Both are hemicryptophytes with a rhizome that remains in a latent state during the cold season. Thus subsequently during a period of suitable conditions, the rhizome can develop a rosette of palmatisect leaves and a dense inflorescence with a height that equals 1 m or more.

Both models selected the precipitation of the coldest quarter (i.e. winter season), although the optimal values for *D. bolosii* (approximately 100 mm) were lower than those indicated for *D. fissum* subsp. *sordidum* (approximately 200 mm). This difference can be explained because when *D. bolosii* restarts its biological cycle at the end of winter, it depends less on winter water reserves than *D. fissum* subsp. *sordidum* as spring is generally rainier in the north-eastern than in the western Iberian Peninsula.

Regarding the precipitation of the warmest quarter (i.e. summer season), *D. fissum* subsp. *sordidum* requires approximately 50 mm to complete blooming and fruiting stages (Rus et al. 2018). In contrast, the *D. bolosii* model did not select this variable, despite having a similar biotype and biological cycle. This may be due to summer

being relatively rainy from storms in the north-eastern Iberian Peninsula. In such territories, spring and autumn are the rainiest seasons, followed by summer, and finally by winter (Rodríguez-Ballesteros 2015). The habitat suitability for *D. bolosii* is greater when precipitation seasonality is low. In this context, it should be pointed out that the distribution of rainfall over the year is more important than the annual global rainfall in the distribution of many species (Del Río et al. 2018).

Our models showed potential impacts of climate change on the series *Fissa* species in the Iberian Peninsula, a result that is consistent with previous climate change analyses conducted with mountain species (Lenoir et al. 2008; Engler et al. 2011) and threatened taxa (Mendoza-Fernández et al. 2021). Studies about the effects of climate change on biodiversity have predicted range contractions for many species, but range expansion has also been documented (see e.g. Berry et al. 2003; Hamann and Wang 2006). For *D. fissum* subsp. *sordidum*, our results indicated that the areas with an oceanic climate in the central-western Iberian Peninsula and mountain ranges with lower elevations, such as Sierra Morena, are more sensitive to changes and, thus, face marked habitat suitability reductions. Conversely, the models forecast range expansion in the areas with a continental climate in the central Iberian Peninsula, as well as the mountain ranges with a bigger scope and higher elevation. For *D. bolosii*, the outside areas of mountain ranges showed more marked habitat suitability losses given their lower elevation. The habitat suitability loss around the Pyrenees for the most pessimistic scenario (RCP 8.5) was remarkable. Engler et al. (2011) indicated that the flora of the Pyrenees appears especially sensitive to climate change due to increased temperature, but mainly due to reduced precipitation. However, the inner areas of the Eastern Iberian and the Pre-Pyrenean Systems showed good persistence and an increase in potential areas. These results can be explained by the complex meteorological processes indicated by López-Moreno et al. (2008), in which several variables take part, such as wind speed and direction, surface temperature and relative humidity.

Both taxa have limited dispersal ability since seed dispersal occurs by boleochory. These limitations can prevent species from successfully tracking climate to the potential areas of future overlaps (Engler et al. 2009; Krosby et al. 2015). However, sporadic events of long-distance seed dispersal by herbivores seem to be possible in *D. fissum* subsp. *sordidum* (Melendo et al. unpublished data). Anyway, no overlapping was found between either the potential distribution areas in current climatic conditions or the potential areas of the projected models. The possible range expansion of *D. fissum* subsp. *sordidum* towards the Iberian System, especially in the most pessimistic scenario, coincided with the retraction of the potential areas of *D. bolosii* towards the Eastern Iberian System.

From the conservation point of view, loss of habitat suitability seems worrying for some known populations of these two endangered endemic taxa. In particular, the *Delphinium fissum* subsp. *sordidum* populations located on the northern Sub-plateau, and some *Delphinium bolosii* populations located in the Catalan Mediterranean and Eastern Iberian Systems. According to Wiens (2016), climate change-induced contraction in range sizes poses a local extinction threat to many species. However, other factors must be considered. For example, some highly threatened populations have only

a few individuals and a high inbreeding rate (Orellana et al. 2007; Bosch et al. 2019; Ramírez-Rodríguez and Amich 2019), while others have many individuals (Ramírez-Rodríguez et al. 2017; Ramírez-Rodríguez and Amich op. cit.). Some populations are also affected by other threats, such as too many herbivores (Ramírez-Rodríguez et al. 2016; Ramírez-Rodríguez and Amich op. cit.).

During the modelling process of the studied species, biotic variables like vegetation cover, species interactions or dispersal ability were not used. Biotic interactions were implicitly considered. Thus the realized niche of the studied species was modelled, which partly incorporates these interactions. In this way, models were simplified, but provided a static representation of the biotic interactions. This implies a source of uncertainty for models if interactions between species were modified, which could occur with climate change (Suttle et al. 2007). For instance, Lepidopterans are an important group of pollinators for the studied species (Bosch et al. 1998; Ramírez-Rodríguez and Amich 2017), which might shift northward as a future response to climate change (Parmesan et al. 1999). Although continuous efforts are being made to explicitly integrate biotic interactions into distribution models, the pitfall of predicting how such interactions will evolve under future environmental conditions still needs to be overcome.

Another source of uncertainty in modelling species distribution stemmed from our inability to predict how species will be able to genetically adapt or express their phenotypic plasticity when faced with changing environmental conditions (Theurillat and Guisan 2001). In this context, long-life species and those species with limited dispersal ability better conserve the realized niche (Pearson and Dawson 2003). Indeed, the studied species have these features: a rhizome that can remain for decades and short-distance seed dispersal. Given such a slow migration rate, it is unlikely that the studied species could reach all its recent suitable habitats (Engler et al. 2009). Accordingly, the projections that forecast a large gain in the distribution range might be overestimated.

The changes in vegetation cover associated with climate change might also be another source of uncertainty. However, we think that this might not be relevant because the studied species are able to live in wide-ranging plant communities. However, the selected spatial scale may prove more significant because, in some cases, it might not reflect the more suitable microhabitats for the studied species (Randin et al. 2009).

Conclusions

In the Iberian Peninsula, the series *Fissa* of the genus *Delphinium* has diversified into two endemic and endangered taxa, *D. bolosii* and *D. fissum* subsp. *sordidum*, which do not have complete reproductive barriers and need similar climatic requirements. Despite their ecological similarities, climate change would cause different effects in the distribution area of each taxon: while for *D.bolosii* the total potential area would decrease, it would increase for *D. fissum* subsp. *sordidum*. In both cases, the potential distribution area would shift towards areas of higher continentality at present. The

aforementioned orographic barriers may play an important role in the maintenance of non-overlapping potential areas. However, some of the populations would face a high risk of local extinction; therefore, monitoring efforts would be conducted for these populations as well as a joint conservation plan which includes in situ and ex situ conservation measures for both taxa.

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Supplementary material 1

Table S1. Environmental variables used in this study

Authors: Rubén Ramírez-Rodríguez, Manuel Melendo-Luque, Juan Diego Rus-Moreno, Francisco Amich

Data type: environmental variables

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RESEARCH ARTICLE

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Genetic variability and conservation of the endangered Pannonian root vole in fragmented habitats of an agricultural landscape

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Abstract

The distribution of the endangered glacial relict subspecies, the Pannonian root vole *Alexandromys oeconomus mehelyi* Éhik, 1928, is restricted to scattered localities in south-western Slovakia, which belong to the north-eastern zone of its range. Human-induced changes and fragmentation of the landscape have led to the gradual loss of suitable habitats and threaten its long-term survival. The study area in the Danubian Lowland is characterised by small habitat fragments and temporal fluctuations of the habitat area. Root voles were sampled at nine sites to study the level of genetic variability and structure of local subpopulations by scoring 13 microsatellite loci in 69 individuals. Genetic differentiation varied amongst local populations and we did not find a significant isolation-by-distance pattern. Bayesian clustering analysis suggested that dispersal effectively prevents marked genetic subdivision between studied habitat fragments. Significant pairwise differentiation between some subpopulations, however, may be the result of putatively suppressed gene flow. Low genetic diversity in the recent populations probably reflects the isolated location of the study area in the agricultural landscape, suggesting that long-term survival may not be assured. In order to maintain genetic diversity, it is essential to preserve (or even restore) habitats and ensure the possibility of gene flow; habitat protection is, therefore, recommended. Continuous assessment is necessary for effective conservation management and to predict the long-term survival chances of the Pannonian root vole in the study area.

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Keywords
Alexandromys oeconomus mehelyi, Danubian Lowland, landscape change, microsatellite analysis

Introduction

The root vole *Alexandromys* (*Microtus*) *oeconomus* (Pallas, 1776) is the only extant Holarctic species of the Microtini tribe with twenty-five known subspecies distributed in the Northern Hemisphere (Pardiñas et al. 2017). The widespread species is categorised as 'Least Concern' in the IUCN Red List of Threatened Species (Linzey et al. 2016).

As a result of glacial and postglacial changes in its distribution range, the root vole is currently represented by three subspecies in Western and Central Europe (Musser and Carleton 2005; Pardiñas et al. 2017), *A. oeconomus stimmingi* (Nehring, 1899) (Nehring 1899), *A. oeconomus arenicola* (de Sélys-Longchamps, 1841) (de Sélys-Longchamps 1841) and *A. oeconomus mehelyi* Éhik, 1928 (Éhik 1928), all of which belong to the Central European phylogroup (Brunhoff et al. 2003). As a protected species of the European fauna, the root vole is included in Appendix III of the Bern Convention (Council of Europe 1982). Two subspecies, *A. o. mehelyi* and *A. o. arenicola*, are endangered and need a feasible conservation action plan with special management strategy to maintain populations (Pardiñas et al. 2017). The Pannonian root vole *A. o. mehelyi*, an isolated subspecies present in the Carpathian Basin, occurs in the southern part of Slovakia in the Danubian Lowland (Miklós et al. 2014; Ambros et al. 2016), locally in north-eastern Austria (Thissen et al. 2015a) and in some isolated localities in Hungary (Horváth and Herczeg 2013; Lanszki et al. 2015; Kalmár and Riezing 2017). This subspecies is considered to be a glacial relict in Central-Europe (Brunhoff et al. 2003) with populations inhabiting refugial areas including freshwater marshes, swamps, floodplains or watersides (Baláž and Fraňová 2013; Kalmár and Riezing 2017). Based on intensive research in the past, all studies defined the suitable habitats of the Pannonian root vole as wet sites with dense vegetation cover, typically dominated by sedge (*Carex* sp.) or hummocks of sedge and common reed (*Phragmites australis*) mosaics (Kratochvíl and Rosický 1955; Stollmann and Ambros 1998; Hulejová Sládkovičová et al. 2016). Due to its special habitat requirements and rarity, apart from some larger known populations, it is common that merely one or a few specimens are found sporadically or only indirect evidence indicates its occurrence (Miklós et al. 2011; Purger 2014; Thissen et al. 2015a, b). The subspecies is sensitive and exhibits rapid response to natural and anthropogenic disturbances, especially to their additive effects and it was shown that individuals could cross barriers to reach suitable areas (Horváth and Herczeg 2013). Habitat loss, fragmentation and degradation due to changes in land-use, like drainage or mowing, are amongst the well-known risk factors that threaten the Panonian root vole (Thissen et al. 2015a; Gubányi et al. 2009). As this rare, habitat specialist subspecies is present exclusively in scattered populations in these remnant habitats, it appears amongst the priority species of communities in Annexes II and IV of the Habitats Directive (European Commission 1992).

The distribution of Pannonian root vole in the territory of Slovakia is determined by the change of the landscape and its natural conditions in the postglacial era. Over the past 130 years, the Danubian Lowland in southern Slovakia has experienced significant landscape modifications, such as extensive flood protection interventions and construction of a channel network in agricultural lands. As a consequence, habitats preferred by the root vole are currently fragmented and isolated by agricultural lands in southern Slovakia (Ambros et al. 2016). Furthermore, flooding events, the oscillation of ground water level and dry periods have a substantial effect on the connectivity between remnant patches of suitable habitat, determining gene flow amongst them. Landscape fragmentation and loss of habitats represent a significant risk factor in the Danubian Lowland for this rare subspecies which occurs only in scattered populations in south-western Slovakia, thus, the Pannonian root vole is strictly protected in the country (Miklós et al. 2014; Ambros et al. 2016).

Habitat loss and fragmentation caused by landscape modification affect wildlife populations worldwide (Wilson et al. 2016). The negative impact of edge effects, higher sensitivity to environmental and demographic stochasticity and the change of different characteristics of small, fragmented populations in reduced habitats may lead to increased risk of local extinction (Willi and Hoffmann 2009). Simultaneously, as the isolation of remnant patches becomes larger, dispersal and thus gene flow amongst local populations may become hampered (Storfer et al. 2010). Consequently, subdivided and isolated populations undergo changes in their genetic structure and variability (Lino et al. 2019).

The response of small mammalian populations to habitat fragmentation has been widely studied (e.g. Kozakiewicz et al. 1999; Mortelliti et al. 2010). Wetland and marshland associated species are particularly threatened by the negative effects of fragmentation (Rushton et al. 2000; Pita et al. 2010), as the available habitats are already fragmented and isolated to varying degrees as a result of human activities, such as infrastructure development, water management or agricultural drainage (Čížková et al. 2013). The consequences of habitat fragmentation on genetic diversity in subdivided small mammal populations have been in the focus of comprehensive research (e.g. Gaines et al. 1997; Fietz et al. 2014). A number of papers aimed to study Microtine rodents as appropriate model organisms (Redeker et al. 2006; Marchi et al. 2013) in the investigation of habitat fragmentation which poses substantial threat to endemic and relict vole species or subspecies that are especially sensitive to its negative effects (Buzan et al. 2010; Pita et al. 2014). Rare species and subspecies, including voles inhabiting wetlands, have been the subject of studies that investigated the genetic characteristics of populations occurring in fragmented or isolated habitats in the Nearctic (List et al. 2010; Neuwald 2010; Parmenter et al. 2015) and Palearctic regions (Van De Zande et al. 2000; Telfer et al. 2003; Centeno-Cuadros et al. 2011) and, based on the levels of genetic diversity and revealed genetic patterns, many authors stressed implications or proposed measures for conservation.

To ensure the viability of root vole populations or subpopulations, it is reasonable to study genetic variability and genetic structure as factors affecting the adaptive traits

and future persistence of populations. Wetland habitats experienced fragmentation and significant shrinkage in south-western Slovakia and only a few habitat fragments are left for root voles. In the present survey, we used microsatellite analyses to study the genetic diversity and structure of Pannonian root vole subpopulations inhabiting remnant habitat fragments in the Danubian Lowland, where the greatest threat to their long-term survival is habitat loss, fragmentation and degradation (Gubányi et al. 2009; Horváth et al. 2012; Horváth and Herczeg 2013; Miklós et al. 2014; Thissen et al. 2015a; Ambros et al. 2016). Understanding the spatio-temporal dynamics and population genetic patterns of this subspecies could be valuable for developing and improving an appropriate and effective conservation action plan and to derive recommendations for the conservation management of this endangered subspecies.

Materials and methods

Study area and samples

Located in the south-western part of Slovakia, the study area lies in the Danubian Plain (Podunajská rovina), part of the Danubian Lowland, belonging to the Pannonian biogeographical region. The landscape was formed by the tributaries of the Danube (Little Danube and others) and by the southern Váh, Nitra and Žitava Rivers. Soil properties and climatic conditions make this region ideal for agriculture. In the area, wheat, sugar beets, sweet corn, vegetables, fruits and tobacco are grown. In the late 19th century, the study area was located in the centre of wet meadows interlaced by meanders of Žitava River, as shown by the Third Military Survey of the Habsburg Empire (1869–1887) (2018). Over the past 130 years, extensive flood protection measures and construction of the channel network fragmented the area of natural meanders of the Žitava River, which changed the direction and hydrodynamics of their flow and led to the aridification of the surrounding ecosystems. By 1926, the original wet meadows had dried up and a network of meanders had formed into a compact wetland habitat crossed by two channels. The consequences of these interventions were still visible in 1964, but the size of wetland habitats had become further reduced by 1990 (Topographic Maps of Czechoslovakia 2018). Today, remnants of the original meander system exist only as a few patches, where the Pannonian root vole still survives. The extension, shape and connectivity of recent wetland habitats scattered across the agricultural landscape are determined by water levels and temporary floods. In the summer and winter of 2010, the extreme precipitation raised water levels and caused the flooding of the Danube (Slovak Hydrometeorological Institute 2011). The last highest water level rise of the river was recorded in spring 2013 (Jakubcová et al. 2014; Matoková and Smrtík 2014), which also influenced our study area. The localities included in this study are situated in the area interlaced by three channels between Patince, Chotín and Marcelová Villages (Fig. 1).

The research was carried out in different types of habitat fragments like waterlogged areas overgrown by *Carex* spp. and *Phragmites* spp. at the edges of channels, oxbow lakes and remnants of former tributaries intersecting large areas of agricultural

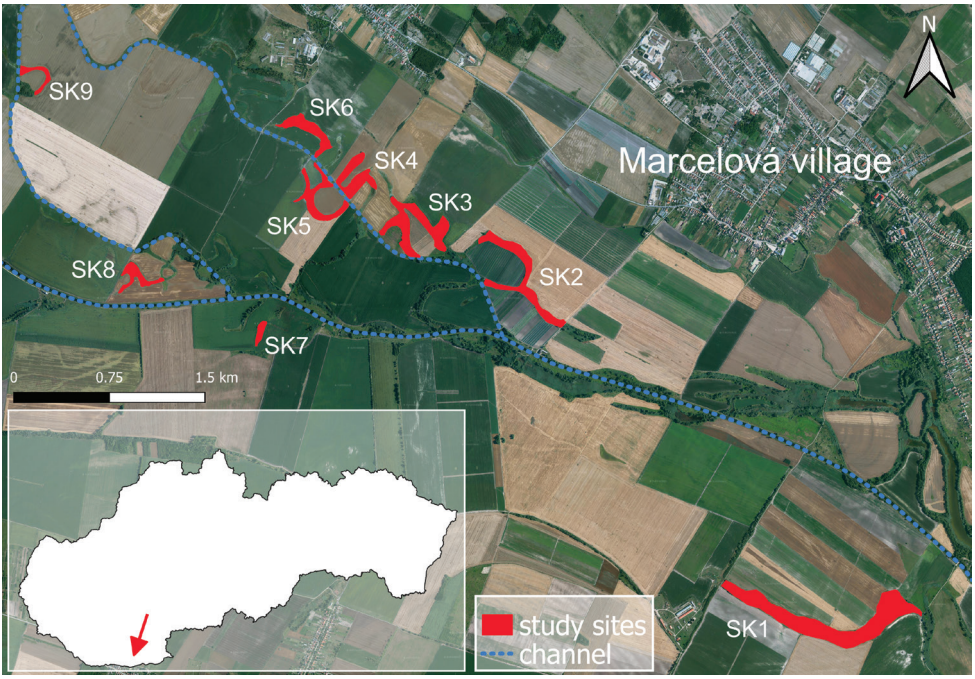

Figure 1. The distribution of the sampled localities of root vole subpopulations in the Danubian Lowland. The sampling sites found in the habitat fragments are indicated by letter and number codes. The insert shows the location of the study area in Slovakia.

lands. Animals were captured with live traps baited with apple and cereals. One line transect of 25 traps with 10 m intervals was established for five consecutive nights in each trapping site. Trapping was conducted three times a year (spring, summer, autumn), from 2014 to 2017. Traps were inspected once a day. Upon capture, each vole was investigated for body weight, age and reproductive status before release. At the first capture, the tail tip of each vole was clipped for genetic analyses. The clipped tail tips were put immediately in 96% ethanol and preserved at -85 °C in the laboratory until DNA extraction. Trapping and sampling methods were realised in agreement with the rules of State Nature Protection of Slovak Republic "Species and habitats monitoring of European importance within the Habitats Directive and the Birds Directive" project. Due to the rarity and endangered status of the Pannonian root vole, sample sizes were low in some trapping transects and, therefore, a total of 69 tissue samples from nine sites were used in molecular analyses.

Changes in habitat fragment patterns and estimation of subpopulation sizes

Five characteristics related to wetland habitat fragments were measured or calculated in six time periods between 2004 and 2019 (January 2004, March 2011, April 2014, March 2017, August 2017 and March 2019): 1) number of all habitat fragments presumably suitable for the Pannonian root vole; 2) number of temporarily

suitable habitat fragments appearing occasionally, depending on water levels; 3) total area of all habitat fragments measured in hectares; 4) average size of habitat fragments (ha); and 5) overall connectivity of our region of interest. Suitable habitat fragments were delimited based on the subspecies' known habitat preference for humid, densely vegetated areas (see Introduction), which markedly differed from the vegetation of agricultural parcels in the study area. The size of particular habitat fragments (ha) was calculated in QGIS software 3.4.12-Madeira (QGIS Development Team 2019). To calculate the connectivity of the whole area (total size: 2873.8 ha), the equivalent connectivity (EC) of PC (probability of connectivity) probabilistic index was used (Saura et al. 2011a, b), where Euclidian edge-to-edge distances with 700 metres' maximum dispersal distance threshold parameter (5% probability for a species to exceed 700 m) were set as a connection between habitat fragments. This distance was set as the average dispersal distance observed on the root vole by Steen (1994). All connectivity indices were calculated in Conefor 2.6 (Saura and Torné 2009). The maps of habitat fragments were created as digitalised topographic maps of Google Earth in map scale 1:5000. Individuals from the same locality were treated as a putative subpopulation (hereafter referred to as subpopulation).

Due to different trapping efforts at each study location, the size of each subpopulation was evaluated as the relative abundance (rA) of individuals captured at the location per 100 trap-nights (C/100TN), based on data without recaptures. The number of captured specimens (N) was recalculated into the transformed rA index assuming a random (Poisson) distribution (rA = (–ln (1-N/100) 100) of small mammals to remove the saturation effect caused by single traps (Caughley 1977). Relative abundance values were averaged over trapping occasions.

We tested the correlation between average rA of Pannonian root vole and average habitat fragment size using Pearson correlation analysis, where both data were log-transformed due to non-normal distribution.

Genetic analysis

DNA extraction

DNA extractions were performed using commercial Isolate II Genomic DNA Kit (Bioline) according to the manufacturer's protocol with the following modifications: during pre-lysis, samples were incubated overnight at room temperature; after adding preheated Elution Buffer G (70 °C), the elution step covered the incubation of samples at room temperature for 30 minutes and after that at 70 °C for 5 minutes before elution.

Analysis of genetic markers and genotyping

The thirteen microsatellite loci included in the analyses were developed for *Microtus arvalis*: Mar003, Mar016, Mar049, Mar063, Mar076 (Walser and Heckel 2008) and for *Alexandromys oeconomus*: Moe1, Moe2, Moe3, Moe4, Moe5, Moe6, Moe7 and Moe8

(Van de Zande et al. 2000). Microsatellite markers were grouped into four multiplex sets (Set1A: Moe1, Moe2, Moe3; Set1B: Moe4, Moe5, Moe6; Set2: Moe7, Moe8; Set3: Mar003, Mar016, Mar049, Mar063, Mar076).

Multiplex PCR reactions were performed in 12 µl volumes containing 2 µl (~80 ng) of DNA and a volume of 10 µl of the following mixture: 3.9 µl of KAPA2G Fast Multiplex Mix (KAPA Biosystems), 0.8 µl of BSA, 0.5 µl (concentration of 10 pm/ng) of each primer and RNase-free water to fill the volume to 10 µl. Amplification of DNA was carried out using peqSTAR 96X Universal thermal cycler (Peqlab).

To amplify microsatellites in Set 1A, Set 1B and Set 2 the PCR reaction consisted of the initial step at 94 °C for 7 minutes, 30 cycles including: denaturation at 94 °C for 1 minute, annealing at 55 °C in case of Set 2 and 60 °C in case of Set 1A and Set 1B for 2 minutes and extension at 72 °C for 90 seconds, followed by a final step at 72 °C for 10 minutes. Microsatellites in Set 3 were amplified in a PCR reaction including the following steps: 7 minutes at 95 °C, 35 cycles of 30 seconds at 94 °C, 1 minute at 57 °C and 90 seconds at 72 °C and after the cycles a final step of 10 minutes at 72 °C. To prepare the genotyping procedure 1 µl PCR product of each sample was mixed with 12 µl formamide and 0.3 µl GeneScan 500-LIZ size standard (Applied Biosystems). After a denaturation step of 5 minutes at 95 °C, a cooling step was implemented. Genotyping was carried out using ABI PRISM 310 Genetic Analyser (Applied Biosystems) and microsatellite genotypes were examined using GeneMapper software v.4.0 (Life Technologies).

Clustering analysis of samples

We successfully genotyped 69 individual samples and the amplification success varied amongst markers (94.2–100%). The presence of null alleles may cause significant heterozygote deficit and deviation from the HWE. We therefore estimated the proportion of null alleles (NA) at each locus in each subpopulation using the programme FREENA (Chapuis and Estoup 2007). All genotypic distributions were in accordance with HWE expectations for all loci and, as we did not detect null alleles based on FREENA, consequently, all loci were included in the analyses.

Bayesian clustering of microsatellite genotypes was performed using STRUCTURE v.2.3.2 (Pritchard et al. 2000). Ten independent runs were performed for each value of K ranging from one to ten under a model assuming admixture and correlated allele frequencies (Falush et al. 2003). Each run comprised a burn-in period of 100,000 replications followed by a run length of 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. The results of replicated runs for each value of K from one to ten were combined using STRUCTURE HARVESTER v.0.6.94 (Earl and vonHoldt 2012) and the optimal value of K was assessed by the inspection of log-likelihood values and according to the ΔK method developed by Evanno et al. (2005). Twenty independent runs were conducted with K fixed at the estimated optimal number of clusters where a burn-in of 100,000 and 1,000,000 MCMC iterations were used. The outputs of replicated runs were combined using the Greedy algorithm in CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007) and DISTRUCT v.1.1 (Rosenberg 2004) was used to visualise cluster assignments.

Genetic variability in subpopulations

The mean number of alleles (A), observed (H_O) and expected (H_E) heterozygosity (Nei 1978) and inbreeding coefficients (F_{IS}) were calculated using GENETIX v.4.05.2 (Belkhir et al. 2004). The allelic richness (AR) was estimated using the rarefaction procedure for the lowest sample size (n = 10) in the programme FSTAT v.2.9.4 (Goudet 2003). Deviation of subpopulations from Hardy-Weinberg equilibrium (HWE) was calculated with exact tests assessing heterozygote deficiency and excess in GENEPOP v.4.2 (Rousset 2008). The basic level of significance was set to $P = 0.05$ and for multiple comparisons, we applied a Bonferroni procedure compensating for the risk of an inflating type 1 error.

Genetic variation between subpopulations

The programme FREENA was used to estimate global F_{ST}, by performing 10,000 permutations. In addition, a Monte Carlo test of likelihood ratio G-statistic (Goudet et al. 1996) was performed using package *hierfstat* v.0.04-22 (Goudet and Jombart 2015) in R v.3.5.0 (R Core Team 2018) to test the presence of genetic structuring. Estimations of pairwise F_{ST} were implemented in FSTAT v.2.9.4 according to Weir and Cockerham (1984). Significant differences of F_{ST} estimators from zero were tested using 100,000 permutations without the assumption of Hardy-Weinberg equilibrium and significance level was set to $P = 0.05$ and adjusted using the Bonferroni correction. In addition, isolation by distance was tested by assessing the correlation between the geographical distance matrix (given in kilometres) and pairwise $F_{ST}/(1-F_{ST})$ estimates using Mantel's Test with 10,000 permutations in the programme ISOLDE in GENEPOP v.4.2. The GENETIX v.4.05.2 package was used to investigate genetic relationships amongst all genotyped individuals of the nine subpopulations by factorial correspondence analysis (FCA), a method that identifies the linear combination of variables (allele frequencies at different loci) that captures the most variation between observations (individuals or populations) and visualises genetic relationships in 2D space determined by FCA axes. Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) was implemented in GENALEX v.6.5 (Peakall and Smouse 2006, 2012) to test the proportion of genetic variance amongst individuals and subpopulations. Statistical significance of the variance components was assessed with 999 permutations.

Results

Habitat fragment patterns and subpopulation sizes

Between 2004 and 2019, we identified 26 permanent habitat fragments as suitable habitats for root voles, in 14 of which their presence were confirmed. We have also identified several temporarily suitable fragments, the number of which varied season-

ally and annually. All measured characteristics of potential habitats (number of fragments and temporary fragments, total area of all habitat fragments, mean fragment size and connectivity of the whole area) changed over the six time periods of study (see Table 1). In general, connectivity had higher values in periods when the number and size of habitat fragments were higher. In March 2004 and March 2011, the number of temporary fragments increased, filling the matrix of agricultural land between permanent fragments with new suitable habitat (see Fig. 2). The highest values of parameters were observed in 2011, except for the average fragment size. After this period, the total area of habitat fragments, the number of temporary fragments and the connectivity decreased and permanent fragments were thus more isolated. Values of parameters remained relatively similar in the following years. Partial changes in habitat fragment characteristics and connectivity are detectable in seasonal comparison between March and August 2017, with higher values in spring. In a detailed view of the study sites in Fig. 2, it can be seen that, while in August 2017, sites SK3, SK4, SK5 and SK6 were relatively small and isolated from each other, in March 2004, 2011 and 2017, the same sites were larger and only a channel interrupted their direct connection.

The average occupancy of fragmented habitats by the Pannonian root vole varied spatially (Fig. 3). While some fragments had a high relative population size, others had very low. The analysis did not confirm a positive correaltion between average rA of Pannonian root vole and average size of fragmented habitats ($r = 0.15$, $N = 9$, $P = 0.74$).

Clustering analysis

Genetic structuring inferred from STRUCTURE analysis is presented in Fig. 4 for K values ranging from 2 to 5. The ΔK method indicated that the optimal number of clusters was 5. However, this method can evaluate ΔK only for $K > 1$ and the inspection of log-likelihood scores revealed that $K = 1$ had a relatively high likelihood, similar to other values of K. Generally, assignment probabilities were roughly symmetric, which indicated that none of the clustering analyses captured the real population structre. Notably, when K was fixed at 5, SK1 individuals sampled in 2014 (individuals 3, 6, 7 and 8) and an additional individual from SK5 (individual 26 sampled in 2015) had relatively high assignment probabilities to the same cluster and the same for individuals 57 and 60 sampled in SK9 in 2017. However, not all individuals sampled in 2014 and 2015 were assigned to this cluster (indicated with grey in Fig. 4).

Table 1. Changes in patch characteristics during the six time periods between 2004 and 2019.

Time period	Area of all patches (ha)	Mean size of patches (ha)	No. of all patches	No. of temporary patches	Connectivity*
2004 March	179.26	3.51	51	25	840705.8
2011 March	210.19	3.28	64	38	975203.3
2014 April	144.49	3.80	38	12	669245.9
2017 March	149.73	3.56	42	16	679802.1
2017 August	140.92	4.14	34	8	627252.9
2019 March	143.26	4.21	34	8	642355.8

* calculated in a total study area of 2873.8 ha

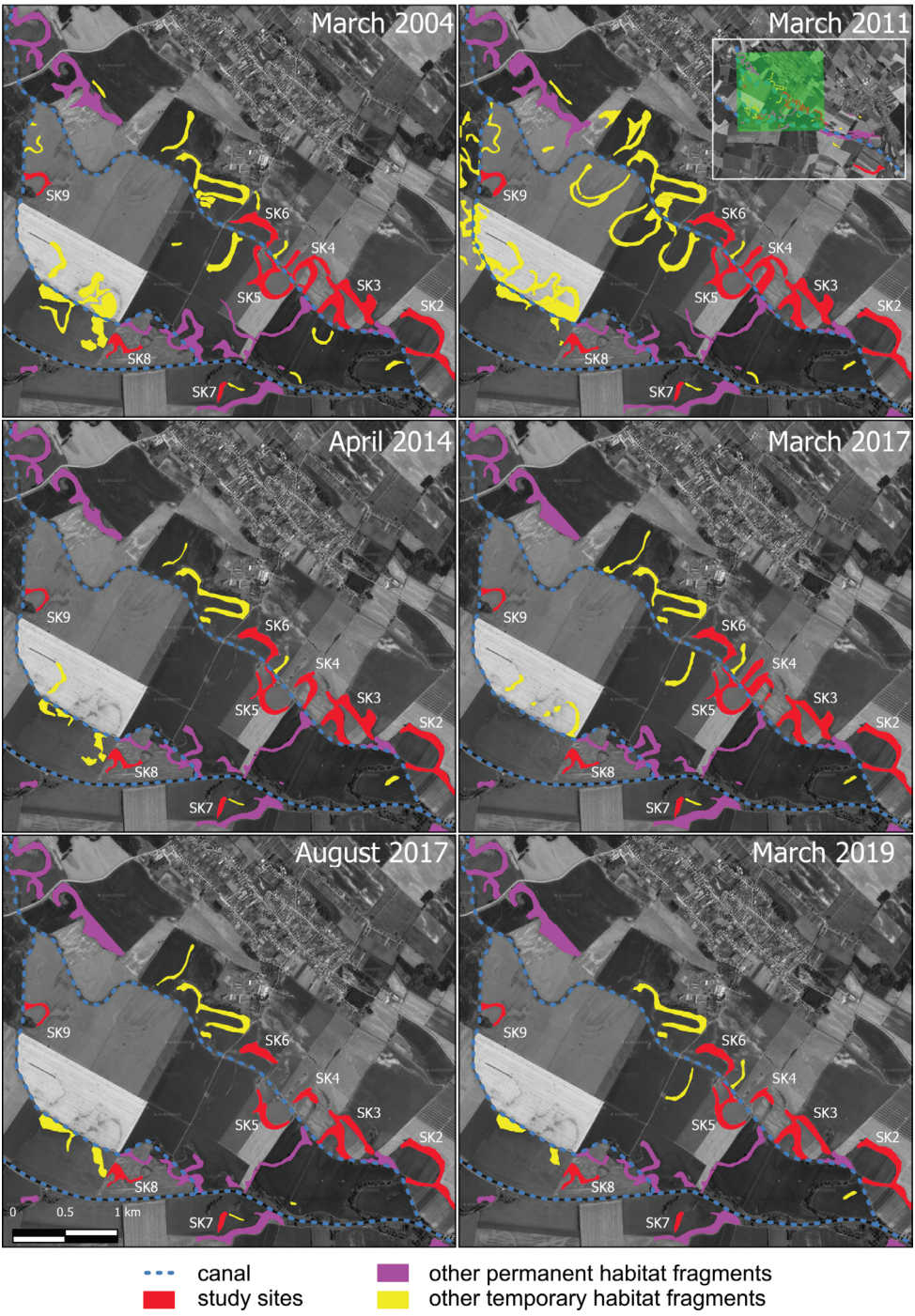

Figure 2. Change in the number of suitable habitat fragments for the Pannonian root vole in the north-western part of the study area over time. The green box in the insert shows the boundaries of the larger maps.

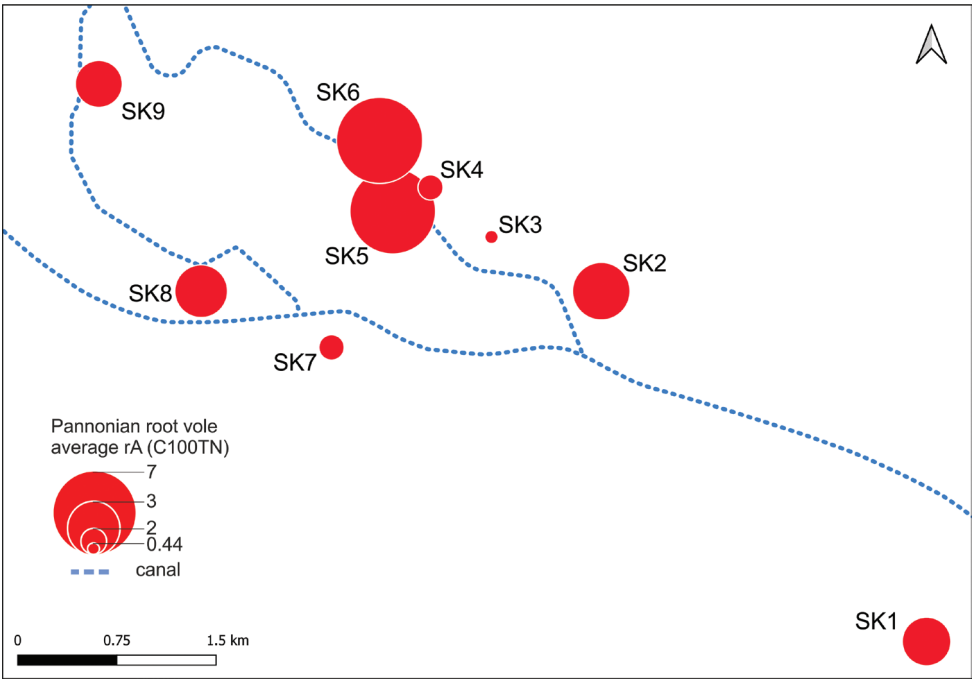

Figure 3. The relative abundance (rA) of Pannonian root vole subpopulations in sampled habitat fragments. Relative abundance values were averaged over trapping occasions and average rA was visualised on the map as graduated symbol size.

Hardy-Weinberg equilibrium and genetic diversity in subpopulations

Clustering analysis did not give a strong evidence of structuring; therefore, measures of genetic diversity were calculated for all subpopulations separately and for all samples pooled together. Genetic diversity and HWE were not calculated for locations SK3, SK4 and SK7 due to their small sample size (N = 3).

The number of alleles per locus in subpopulations ranged from 2 (locus Moe4) to 16 (locus Moe7), and the mean number of alleles per locus (A) ranged from 4.31 to 5.62 (Table 2). Allelic richness (AR) across subpopulations ranged from 4.22 to 5.46, being the highest in the SK1 subpopulation and lowest in the SK8 subpopulation. H_O (range between 0.562 and 0.669) was the highest in subpopulations SK8 and SK9, while the lowest value was shown for SK5. In contrast, H_E (range between 0.624 and 0.657) showed another pattern, with the highest value for SK1 and the lowest value observed for SK8. Two subpopulations, SK8 and SK9 had heterozygosity excess, based on observed and expected heterozygosity values. Subpopulations SK1, SK2, SK5 and SK6 showed significant heterozygote deficiency on the basis of F_{IS} (significant positive values). Subpopulations SK1, SK2 and SK5 deviated from HWE according to heterozygote deficiency exact tests in GENEPOP (P = 0.05) after Bonferroni correction

(Table 2). Heterozygote excess exact tests were not significant in any of the cases (data not shown). Mean number of alleles and expected heterozygosity indicated higher genetic diversity and the exact test showed a significant deficiency of heterozygotes when all samples were pooled together.

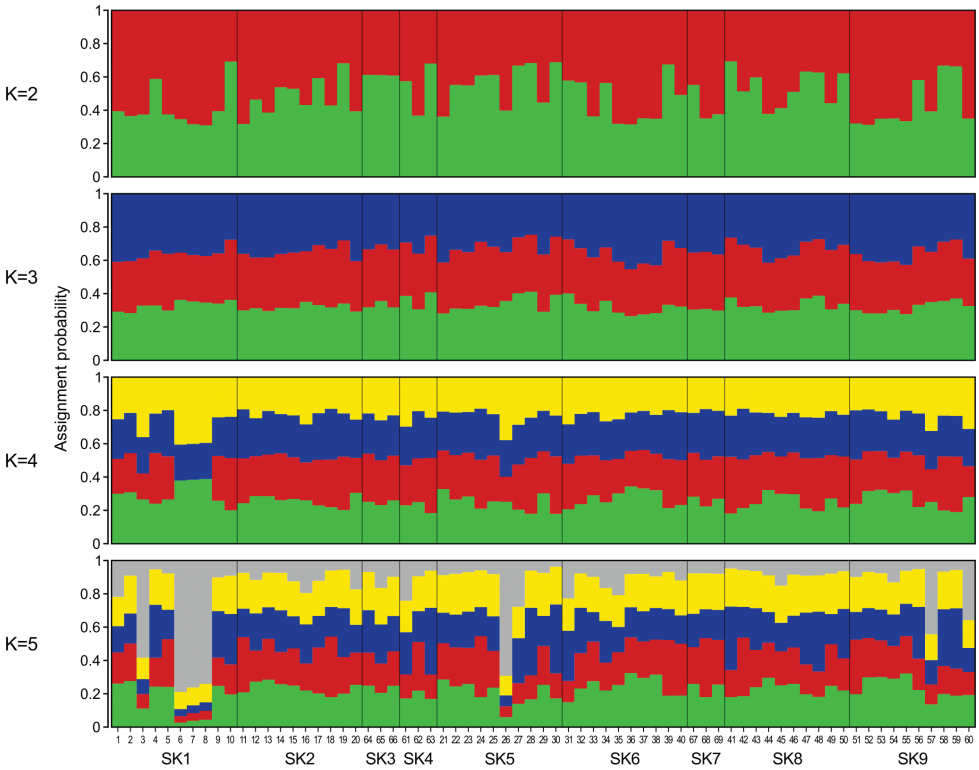

Figure 4. Genetic structure of the sampled root vole subpopulations in the Danubian Lowland. The graph is based on STRUCTURE runs when K was fixed at 2–5. Each individual is represented by a line proportionally divided into colour segments corresponding to its membership in certain clusters. Black lines separate the individuals from different habitat fragments.

Table 2. Genetic diversity in root vole subpopulations and in the total population based on 13 microsatellite loci.

Location	A	AR	H_E	H_O	HWE		F_{IS}
					P	±SE	
SK1	5.62	5.46	0.657	0.586	< 0.001*	0.0000	0.161*
SK2	5.08	5.00	0.654	0.574	< 0.001*	0.0002	0.176*
SK5	4.62	4.49	0.631	0.562	< 0.001*	0.0003	0.161*
SK6	5.15	5.02	0.651	0.619	0.006	0.0010	0.103*
SK8	4.31	4.22	0.624	0.665	0.161	0.0059	-0.013
SK9	4.62	4.50	0.655	0.669	0.228	0.0115	0.030
Total	7.23	7.23	0.694	0.614	< 0.001*	0.0000	0.122*

A: mean number of alleles per locus, *AR*: allelic richness, H_E: expected heterozygosity, H_O: observed heterozygosity, F_{IS}: inbreeding coefficient, *HWE*: *P* values and standard errors of Hardy-Weinberg equilibrium exact tests; * significant values of *HWE* (after Bonferroni correction) and F_{IS}. (In case of the total population, A = AR).

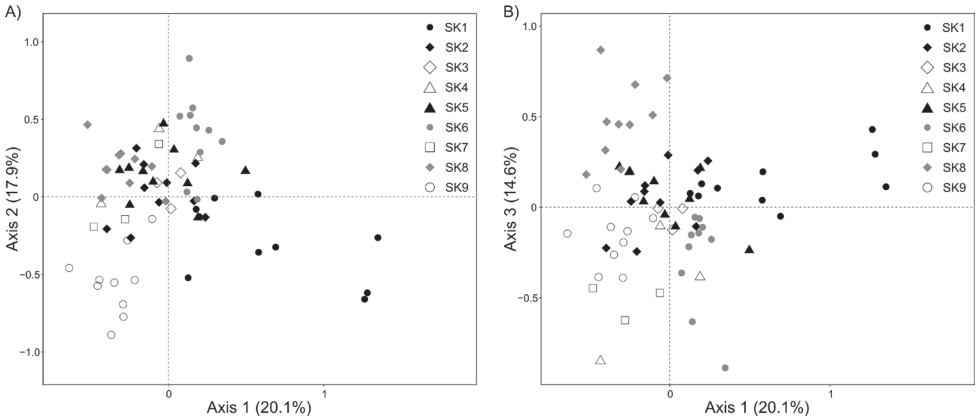

Figure 5. Two-dimensional plots of FCA performed for nine subpopulations showing the 1st and 2nd (**A**) and the 1st and 3rd (**B**) axes. The proportion of explained variance is written in parentheses on each axis.

Table 3. Tests for genetic differentiation between nine root vole subpopulations in the Danubian Lowland. Below diagonal: pairwise F_{ST} values. Above diagonal: *P* values of G-tests implemented in FSTAT.

Location	SK1	SK2	SK5	SK6	SK8	SK9
SK1		0.234	0.307	0.185	< 0.001**	0.010
SK2	0.015		0.344	0.099	0.064	0.020
SK5	0.013	0.004		0.479	0.137	0.012
SK6	0.010	0.009	-0.002		0.001*	< 0.001**
SK8	0.071	0.021	0.016	0.045		< 0.001***
SK9	0.033	0.013	0.014	0.052	0.048	

* Significant *P* values on the nominal level of 0.05 (*), 0.01 (**) and 0.001 (***) after Bonferroni correction.

Genetic differentiation between subpopulations

Subpopulations SK3, SK4 and SK7 were not included in F_{ST} analyses because of their small sample size (N = 3). The global F_{ST} for six subpopulation samples was 0.025 (95% CI: 0.01–0.041). The overall G-test was significant ($P < 0.001$), indicating genetic structuring amongst locations. Pairwise F_{ST} values were relatively low, although variable. The highest F_{ST} values were observed for SK8 and SK9. Pairwise genetic differentiation was not significant in most of the comparisons, except in cases where one subpopulation of the pair was always SK6, SK8 or SK9 (Table 3). Significant F_{ST} was observed between SK1 and SK8, but when the individuals of SK1 sampled in 2014 were excluded from pairwise F_{ST} analyses, this difference became non-significant ($F_{ST} = 0.048$). Microsatellite-based genetic distances did not correlate with geographical distances between subpopulations (a = 0.0254, b ~ 0, $P = 0.6$), thus spatial separation (IBD) was not confirmed.

The FCA plot, based on individual genotypes, clearly separated SK1 along the first factorial axis (explaining 20.1% of variation) from all other subpopulations. The second axis (explaining 17.9% of variation) mainly separated the individuals from SK1 and SK9, while individuals from SK6 showed only a weak segregation

(Fig. 5A). Subopulation SK8 was separated along the third axis (explaining 14.6% of variation, Fig. 5B).

In the Analysis of Molecular Variance, significant genetic variation was attributed to the differences between subpopulations (4.4%, $P < 0.001$) and most of the variability occured within subpopulations (95.6%).

Discussion

Our results show that genetic variation and differentiation in subpopulations of the Pannonian root vole is in good agreement with connectivity between habitat fragments, with temporary fragments playing an important role in vole migration between flood events.

Habitat connectivity

The number, size and shape of habitat fragments in the studied region varies in time as the result of exogenous factors (precipitation, surface water levels, agricultural activities). These dynamic changes have an effect on fragment connectivity, suggesting that connectivity was positively influenced by the number of habitat fragments. As we have also noted, permanent habitat fragments, relatively distant from each other at one time, can change size and shape and become neighbouring habitats at another time. In addition, the temporary fragments can play the role of stepping stones during vole movements. Thus, despite the constant presence of habitat fragments and channel-side vegetation, fragment connectivity can vary seasonally and yearly, as can change the possibility of individuals' replacement between the studied subpopulations. In Norway, root voles increased dispersal distance as a response to fragmentation, but it was less affected by connectivity (Bjørnstad et al. 1998). Detailed data on the dispersal ability of the Pannonian root vole are still missing, although they have been shown to be able to cross barriers when the quality of original habitats deteriorated and they may follow stepping stones when searching for new habitats (Horváth and Herczeg 2013). Moreover, Kratochvíl and Rosický (1955) observed that Pannonian root voles in sedge (*Carex* sp.) survived on hummocks when the water level increased (50–70 cm). When the water level decreased, voles spread across the lower parts of these hummocks, while some individuals dispersed to new suitable fragments. In view of this, a possible explanation for the observed genetic patterns could be that the animals found shelter on hummocks during floods in 2010 and probably 2013, thus opening the way for admixture. Later, when water levels fell, presumably admixed individuals resettled lower areas.

Genetic differentiation of the Pannonian root vole

Levels of genetic differentiation between the habitat fragments varied, but were mostly non-significant and we found no support for isolation by distance between subpopula-

tions. Bayesian clustering in STRUCTURE did not reveal pronounced genetic structuring, indicated by approximately equal assignment probabilities to different clusters in all cases of K from 2 to 5. This result suggested that dispersal effectively prevents marked genetic subdivision between studied habitat fragments, which can be additionally confirmed by the lack of isolation by distance between fragments. Given the small geographical scale and landscape pattern of the study area, we would expect gene flow between localities to maintain very low or no differentiation between subpopulations. In a study conducted in the Netherlands, Mauritzen et al. (1999) concluded that ditches are likely to favour linear movements of root voles and may enhance connectivity in an agricultural landscape. Most habitat fragments sampled in our study were directly connected by reed- and sedge-lined channels that are likely to serve as dispersal corridors and permanent or temporary habitat fragments in the study area also likely to facilitate dispersal (Ambros et al. 2016). Although the longest geographical distance along the channels separating our sampling sites from their nearest neighbours was nearly 6 km, as for SK1 and SK2, the presence of root vole was confirmed in other permanent habitat fragments between these two sampling sites (which could not be included in our study due to the small number of samples) and most of the habitat fragments sampled were relatively close to each other. Movement distances of male root voles can exceed several hundred metres in a short time or more than two kilometres within a few days (Steen 1994; Andreassen et al. 1996).

Consistent with the changing possibility of individuals' replacement between the studied fragments, AMOVA results also showed a low, but significant, percentage of variability between subpopulations. In addition, signs of genetic differentiation were detected between subpopulations SK8, SK9 and SK6, based on significant pairwise F_{ST} values and the FCA analysis confirmed the separation of these samples. Results may therefore indicate that dispersal is not unhindered between all subpopulations and root vole individuals in the network of studied habitat fragments may not be viewed as a panmictic population.

Genetic diversity of the Pannonian root vole

In a detailed study, Hulejová Sládkovičová et al. (2018) already noted that, despite the high genetic variability of the subspecies, local populations of Pannonian root vole may show signs of genetic depletion. Although our analyses were limited by small sample sizes due to the rarity of the Pannonian root vole, our results indicate lower genetic diversity in subpopulations (in terms of allelic richness, mean number of alleles per locus and expected heterozygosity) than what was measured in other populations from south-western Slovakia (Hulejová Sládkovičová et al. 2018). Similarly, reduced genetic diversity (based on the mean number of alleles per locus and the expected heterozygosity) was observed compared to the populations of another isolated Western European root vole subspecies, namely *A. o. arenicola* (Van de Zande et al. 2000). Expected heterozygosity and mean number of alleles in the total studied population indicated decreased variability compared to other Slovakian populations (Hulejová Sládkovičová et al. 2018). Low genetic diversity of the studied root vole

population putatively reflects a pronounced geographical isolation of the surviving population and the decrease in effective population size that has likely occurred. In addition, flood events may have reduced the size of the vole population (Kratochvíl and Rosický 1955), which may have created a bottleneck, although its influence on the genetic diversity of the studied population cannot be clearly confirmed on the basis of our results alone. The reduced genetic diversity is in accordance with the view of Hulejová Sládkovičová et al. (2018) who suggested that the Danube River and associated branch network could facilitate vole dispersal, but local populations embedded in the agricultural landscape and further from the Danube are likely to remain isolated. Moreover, based on the evaluation of the distribution of Pannonian root vole, our study area is close to the eastern boundary of the subspecies' range (Gubányi et al. 2009; Ambros et al. 2016) and populations occurring here are likely isolated by the distance from other populations to the west.

Subpopulations SK8 and SK9 tend to have lower levels of allelic richness, which is consistent with the possibly lower probability of dispersal through the agricultural land matrix compared to other sites. However, we did not find significant deviation from the Hardy-Weinberg equilibrium in these subpopulations. We observed deviations from the Hardy-Weinberg equilibrium in SK1, SK2 and SK5 and significant positive F_{IS} coefficients in the same subpopulations, together with SK6, which may result from the social structure of root voles. Matriline-based groups in root vole populations (Tast 1966) give the possibility of sampling kin individuals, which may result in departures from the Hardy-Weinberg equilibrium (Aars et al. 2006). On the other hand, Stewart et al. (1999) argue that genetic composition (and departures from the Hardy-Weinberg equilibrium) of water vole (*Arvicola amphibius*) colonies is under the influence of yearly fluctuation and suggested that genetic drift is a plausible cause resulting in that pattern. They interpreted high levels of genetic diversity as a sign of gene flow between colonies. Similarly, despite the relatively large number of analysed individuals, Pilot et al. (2010) observed yearly changes in departures from the Hardy-Weinberg equilibrium in a root vole population in Poland; nevertheless, a high level of genetic diversity was maintained over the years. In addition, they found no genetic differentiation between years regardless of changes in density, kin structure and deviations from HWE, indicating that genetic composition is stable over time in large, demographically-stable populations. In contrast, studies of water vole metapopulations revealed temporal genetic differentiation, but genetic variability remained high as a result of gene flow (Stewart et al. 1999; Aars et al. 2006).

Possible migration from Danube River

Regarding individuals 3, 6, 7 and 8 (sampled in 2014 and 2015) in SK1, highlighted by clustering analyses, their high assignment probability to a separate cluster may reflect their distinct origin. Habitat fragment SK1 is the closest to the Danube River amongst the studied fragments and the floods in 2010 or 2013 potentially facilitated dispersal from further areas and the aforementioned individuals might be immigrants or their

descendants. Other individuals that had relatively high assignment probabilities to the same cluster were captured in SK5 (individual 26 from 2015) and SK9 (individual 57 and 60). These can be found at a few kilometres distance from SK1, but given the small spatial scale, it is not unlikely that these specimens may be the offspring of dispersing individuals. Alternatively, it is also possible that genetic drift over time changed the genetic composition of subpopulations in the fragmented landscape; hence, some (but not all) samples collected in 2014 and 2015 were highlighted by STRUCTURE clustering. However, we are not able to declare which possibility is more plausible without genotyping individuals from other areas and without temporal analysis of samples.

The genetic diversity of the local subpopulations in the study area is relatively low; although the studied subpopulations probably have connections with each other due to the effect of extensive floods and the network of fragments and channels in the agricultural landscape, their reduced genetic variability is detectable compared to the pooled genetic variability of other populations of Pannonian root vole occurring closer to the more uninterrupted marshlands in Szigetköz, Hanság and Neusiedlersee Regions (Hulejová Sládkovičová et al. 2018). Based on the pattern of remnant habitat fragments, it is likely that root vole subpopulations in our study area form a metapopulation system and the observed genetic patterns do not contradict this possibility. However, studies on extinction and recolonisation patterns and migration rates would be essential to support this view (Gaggiotti 2004; van der Merwe et al. 2016).

Conclusions and management implications for conservation

Landscape changes and habitat destruction resulted in the fragmented distribution of root vole habitats in the study area and fluctuating surface water levels induce considerable changes in habitat size, quality and connectivity to this day. Only one fragment (SK2) in our study area is protected as a Special Protection Area. However, for the long-term persistence of root vole populations, it would be critical to ensure legal protection of habitats. The importance of protected core areas has been demonstrated for water vole metapopulations (MacPherson and Bright 2011) and mainland or source population demography has been shown to shape habitat use of root voles (Glorvigen et al. 2013). Based on the low differentiation, it seems unlikely that the studied vole subpopulations formed a mainland-island type metapopulation system (Stewart et al. 1999), but the signs of admixture highlight the vital role of habitat fragments and gene flow in the maintenance of genetic diversity. On the other hand, Van de Zande et al. (2000) proposed that weak differentiation may be an indicator of progressive isolation of local populations and accordingly, the prevention of fragmentation should be considered in the conservation management of *A. o. arenicola*. It has been suggested for other vole species and subspecies that persistent gene flow between habitat fragments is crucial for the maintenance of genetic variability (Telfer et al. 2003). Therefore, it is necessary to protect not only habitat fragments with current occurrences of the Pannonian root vole, but also other potentially-suitable habitat fragments, to form a habitat network and to ensure the possibility of gene flow (Neuwald 2010). Water levels in

the study area and, as we demonstrated, total size of permanent habitat fragments and number of temporary habitats are in dynamic change, which further emphasises the contribution of these fragments to the survival of the subspecies in the agricultural landscape (Kalmár and Riezing 2017).

It is expected that the overall genetic diversity of the subpopulations will decrease as a result of their small size and isolated location in the agricultural matrix. This implies that the restoration of habitats and corridors is indispensable for the long-term preservation of diversity, as has been stressed earlier (Thissen et al. 2015a; Hulejová Sládkovičová et al. 2018). As a habitat specialist, the Pannonian root vole is sensitive to habitat quality changes, which are likely to be the key factors determining habitat use (Glorvigen et al. 2013; Horváth and Herczeg 2013). Thus, it is important to preserve optimal water level conditions for the root vole, for example, by controlled construction of infrastructure that may cause aridification and the disappearance of the subspecies (List et al. 2010; Kalivodová et al. 2018). For this reason, it is necessary to take the needs of the subspecies into consideration when infrastructural projects are planned and managers should be involved in landscape and land-use planning. Finally, long-term monitoring is recommended to provide further information about population and subpopulation parameters (Hayes et al. 2017), genetic diversity and structure (Proença-Ferreira et al. 2019) and on the changes of habitats (Martensen et al. 2017) to elaborate an effective conservation management action plan that ensures the long-term survival of the Pannonian root vole.

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