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Predicted impacts of transport infrastructure and traffic on bird conservation in Swedish Special Protection Areas

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Abstract

The ecological impacts of roads and railways extend into the surrounding landscape, leading to habitat degradation and reduced wildlife densities within an area that is considerably larger than the actual road or railway corridor. For birds, an extensive meta-analysis has identified an average of 20% density reduction within 1 km from the infrastructure. I investigated to what extent this density reduction can be expected to compromise the habitat quality and conservation value of Swedish Natura 2000 areas designated for the protection of birds (Special Protection Areas; SPAs). The majority (63%) of Swedish SPAs are, to some extent, found within this 1 km road/railway effect zone (REZ). The total overlap between SPA and REZ is approximately 126,000 ha or 4.2% of the country's SPA area. There are, however, large differences amongst bio-geographical regions. In the southern (continental) and coastal regions combined, 25.8% of the total SPA area fall within REZ, representing an estimated 4–7% reduction in bird abundance within SPAs. The probability of overlap with REZ is higher for larger SPAs. However, the proportion of overlap is higher for smaller SPAs and, accordingly, smaller sites can be assumed to experience a greater impact from transport infrastructure and traffic. The impacts on Natura 2000 sites are particularly concerning as this network of protected areas is a cornerstone for maintenance and restoration of biodiversity within the EU. I recommend placing a stronger emphasis in the management of Natura 2000 sites on the threats to wildlife conservation caused by transport infrastructure and traffic. Special attention should be paid to sites with a large overlap with the REZ and sites hosting particularly vulnerable taxa or habitats. Infrastructure owners and managers should make their best efforts to minimise and compensate for the negative impacts of roads and railways and associated traffic in SPAs and other protected areas.

Keywords
Effect zone, Natura 2000, railway, road, Sweden

Introduction

Ecological impact of transport infrastructure

Infrastructure development is recognised as one of the significant drivers of global biodiversity loss and, with increasing traffic and expanding infrastructure networks worldwide, the pressure on biodiversity is expected to increase in coming decades (EEA-FOEN 2011, EEA 2012, OECD 2012). The impacts of transport infrastructure on wildlife are well described (Forman et al. 2003, van der Ree et al. 2015) and include loss of habitat, traffic casualties, creation of physical barriers, disturbance by noise, light and other visual cues, spread of chemicals, dust and alien species, changes in hydrology and microclimate and accidental spills. Most of these impacts extend into the surrounding landscape, leading to degradation and fragmentation of habitats and, for some species, to restricted movements, increased mortality and avoidance of a zone around the infrastructure (Forman et al. 2003, EEA-FOEN 2011, van der Ree et al. 2015, Tulloch et al. 2019).

Due to these impacts, the population densities of many wildlife species are reduced within a distance from larger infrastructures (Rytwinski and Fahrig 2015). For example, the population densities may be reduced up to about 1 km distance for birds (Forman and Deblinger 2000, Forman et al. 2002, Benítez-López et al. 2010) and anurans (Eigenbrod et al. 2009) and up to 5 km for mammals (Benítez-López et al. 2010). Not only large infrastructure but also minor and unpaved roads may have a considerable impact on some species (e.g. van Langevelde and Jaarsma 2009, Benítez-López et al. 2010, Shanley and Pyare 2011). Based on an extensive meta-analysis, Benítez-López et al. (2010) showed that the mean bird and mammal abundance in an effect zone around infrastructures is reduced by 20–30% and with an increasing reduction with proximity to the infrastructure (Fig. 1).

Accordingly, in regions with dense infrastructure networks, large natural areas may be situated within this effect zone and therefore may be impoverished in species sensitive to traffic and transport infrastructure. For example, in the United States, the road effect zone covers 15–22% of the total land area and more than 60% of some particularly exposed biomes, such as coastal regions and river basins (Forman 2000, Riitters and Wickham 2003). In Spain, a country with intermediate road density with European standards, it is expected that reduced bird densities, due to transportation infrastructure, will affect 49% of the country and lead to reduced mammal densities in as much as 96% of the country (Torres et al. 2016). Some habitats of particular importance to biodiversity in Europe, such as wetlands, semi-natural grasslands and temperate broad-leaved forest, may be disproportionately affected by roads because of the landscape structure (Helldin et al. 2013, Karlson and Mörtberg 2015, Torres et al. 2016). Disturbances (noise and visual cues) tend to be stronger and extend further

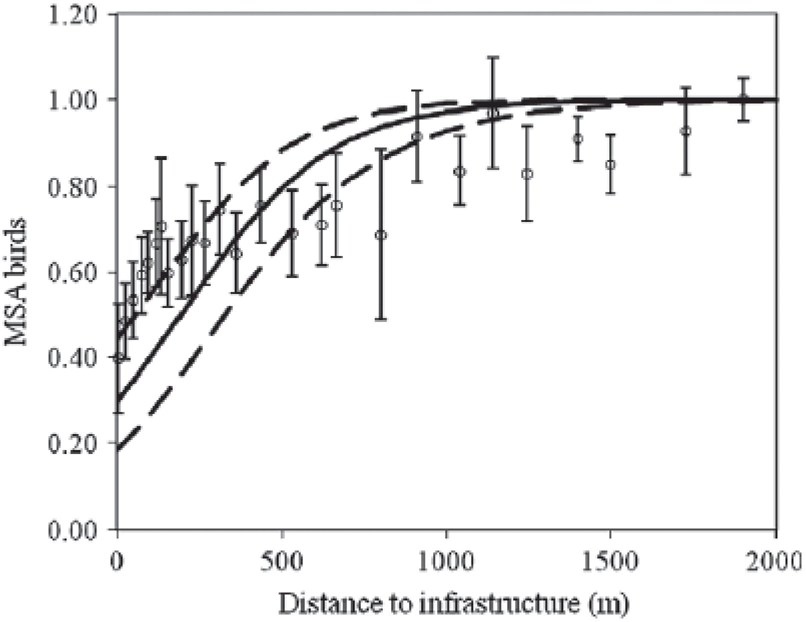

Figure 1. Mean species abundance (MSA) of birds as a function of distance to infrastructure (logistic regression). Open dots represent the pooled results of a meta-analysis per distance interval ±SE. The solid black line denotes the estimated curve for the decline of MSA in proximity to infrastructure; dashed lines are the 95% upper and lower limits of the confidence bands of the curve. Figure from Benítez-López et al. (2010).

from the road in open habitats as compared to forest (Forman and Deblinger 2000, Reijnen and Foppen 2006). There is growing concern globally about the impact of roads and traffic on wildlife populations in protected areas and other biodiversity hotspots (Forman and Deblinger 2000, Ament et al. 2008, Selva et al. 2011, Laurance and Balmford 2013, Bager et al. 2015, Gadd 2015, Seshadri and Ganesh 2015).

Integrity of the Natura 2000 network

The Natura 2000 network of protected areas is a key tool in the maintenance and restoration of biodiversity within the European Union (EU). The network consists of Special Protection Areas (SPAs), designated according to the Birds Directive and Special Areas of Conservation (SACs), designated according to the Habitats Directive (EEC 1992, EEC 2010). Under these directives, the network is supposed to provide protection for vulnerable wildlife and habitats. One important motivation for designating Natura 2000 sites, particularly in coastal and other lower-elevation areas, is the protection against negative impacts of urbanisation and infrastructure development (EEA 2012). There are currently more than 26,400 sites in the Natura 2000 network, accounting for ca. 18% of EU's land territory.

The network is, however, biased toward highland areas, and lowland areas are under-represented (Oldfield et al. 2004, Maiorano et al. 2007). In addition, the average size of the Natura 2000 sites is quite low and particularly so in lowlands (Maiorano et al. 2007, Gaston et al. 2008, EEA 2012). As smaller sites are more susceptible to pressure from land use and human activities surrounding them, major concerns are expressed about the capacity of existing protected areas to maintain their biodiversity values (Chape et al. 2005, Gaston et al. 2008, Maiorano et al. 2008, Kati et al. 2015).

Though many assessments for the effectiveness of the Natura 2000 network in protecting species have been reported in recent years, a vast majority of these relied on rather static population data, such as species' geographic distribution, species presence/absence or habitat suitability (e.g. Araújo et al. 2007, Maiorano et al. 2007, 2015, Sánchez-Fernández et al. 2008, Iojă et al 2010, López-López et al. 2011, Gruber et al. 2012, Albuquerque et al. 2013, D'Amen et al. 2013, Lison et al. 2013, Rubio-Salcedo et al. 2013, Trochet and Schmeller 2013) and, accordingly, were designed to assess the ecological representation of the network rather than tracking changes in population densities due to environmental impacts. In view of the many negative population trajectories reported for both avian and non-avian species in the EU (EEA 2015), it appears necessary to analyse the ecological functionality of the Natura 2000 network with regard to pressures both within and outside the designated areas, but few studies have taken this course.

Frequency of transport infrastructure within Natura 2000 sites was investigated by Tsiafouli et al. (2013), showing that as a European average, roads are present in 29% of the sites, with a higher frequency in the countries in the south. A preceding study of Greek Natura 2000 sites, by Votsi et al. (2012), showed that 85% of sites are bisected by roads. Insufficient functional connectivity of the Natura 2000 network has been reported by Gurrutxaga et al. (2011) and Opermanis et al. (2012), suggesting that dispersal barriers exist between sites, for example in the form of large roads (Gurrutxaga et al. 2011). With regard to disturbance, the European Environmental Agency estimated that almost 20% of Natura 2000 areas are presently adversely affected by high levels of environmental noise, largely owing to major transport infrastructure (EEA 2016). In an assessment of the impact on Natura 2000 sites of major traffic arteries, planned or under construction as part of EU's TEN-T framework, Byron and Arnold (2008) estimated that 379 SPAs (8% of sites) and 953 SCIs (4% of sites) would be adversely affected by these new traffic arteries, with potential effects also on the coherence of the Natura 2000 network.

Aim of the study

Sweden is one of the European countries that is least fragmented by transport infrastructure and built-up areas (EEA-FOEN 2011) and the physical impact of infrastructure is generally not well acknowledged in Swedish nature conservation. It was not until recently that national status reports for biodiversity have described the threats from infrastructure and traffic on species conservation (Bernes 2011, Naturvårdsverket 2015a) and the current national conservation action plan contains few requirements for infrastructure managers (Naturvårdsverket 2015b). Biodiversity is generally insufficiently described in impact assessments for transportation infrastructure plans and projects (Wärnbäck 2013, Karlson et al. 2014). Few, if any, management plans for protected areas address the full array of potential ecological impacts of transport infrastructure on the areas' conservation status or management (Helldin and Tytor 2017). This ignorance is not unique for Sweden, as it appears to be largely similar in most EU member states (Tsiafouli et al. 2013, EEA 2015; but see, for example, Selva et al. 2011, Votsi et al. 2012).

In order to illustrate and highlight the impacts of infrastructure on protected areas in particular, I estimated the frequency and proportion of Swedish SPAs situated within the predicted effect zone for birds around existing larger transport infrastructure (roads and railways) and hence cannot be expected to reach their full conservation potential due to infrastructure impacts. I included only SPAs in the estimation, i.e. areas designated specifically for the protection of birds, because the effects of roads and railways on birds are well described in literature and apparently can impact the majority of bird species (Reijnen and Foppen 2006, Benítez-López et al. 2010, Rytwinski and Fahrig 2015). I assessed the conservation value of SPAs that is lost due to infrastructure in terms of reduced predicted bird abundance. Due to the large geographic variation over the country in density of the infrastructure network and proportion of area within an SPA, I separated the analyses between biogeographical regions. I tentatively explored the association between the infrastructure impact on an individual SPA and its size and dominating habitats. In this paper, I present the results of these analyses and propose improvements for the management of Swedish SPAs.

Methods

A map of the effect zone for birds around the existing larger Swedish roads and railways (REZ in the following) was produced. I used a standardised effect distance of 1 km from the infrastructure, following the results from a meta-analysis presented by Benítez-López et al. (2010). To my knowledge, this is the most comprehensive analysis of infrastructure impacts on bird densities, including 49 bird datasets and 201 bird species. As most studies in the meta-analysis were conducted in biomes that occur in Sweden, i.e. taiga, temperate broadleaf forest or alpine/tundra (39 of the 49 datasets) and on species that occur in Sweden (105 of the 201 species), I judged the results to be relevant to a Swedish perspective. The results have also previously been applied to assess the impacts of the road network on birds in Sweden (Karlson and Mörtberg 2015) and Europe (Torres et al. 2016). I consider the assumption of a 1 km effect distance to be conservative because i) individual studies in the meta-analysis indicated reduced bird populations at larger distances and ii) impacts at greater distances may not necessarily result in direct population declines, but yet be of ecological significance. Infrastructure data were obtained in December 2015 from Open Street Map (http://openstreetmapdata.com), using only the following road classes: primary road, secondary road, tertiary road, motorway, trunk road and railway (thus excluding minor roads for which ecological effects are less well known).

The REZ map was overlaid with all Swedish SPAs to calculate the area and proportion of each site situated within REZ. The area and habitat distribution of SPAs were obtained in December 2015 from European Environment Agency's Natura 2000 database (http://www.eea.europa.eu/data-and-maps/data/natura-2000-eunis-database). SPAs were separated depending on biome (based on a combination of the Natura 2000 database and Global Biomes data from the CIESIN; http://sedac.ciesin.columbia.edu/data/set/nagdc-population-landscape-climate-estimates-v3/maps?facets=theme:climate) and proximity to coast (data on coastline obtained in December 2015 from Open Street Map; http://openstreetmapdata.com) on the following terms:

- *continental region:* sites with >50% of the area within EU continental region,
- *mixed-forest region:* sites with >50% of the area within EU boreal region and in CIESIN broadleaf and mixed-forest region,
- *boreal region:* rest of sites within EU boreal region but with no part within EU alpine region or
- *alpine region:* sites with at least some part of the area within EU alpine region

in combination with

- *coastal:* mainland sites with at least some part within 20 km from coast of mainland Sweden (including mainland islands Öland and Gotland) or
- *inland:* the rest of mainland sites.

The alpine region in Sweden is only inland, i.e. no alpine coastal sites exist. As only two continental sites are inland, all continental sites were pooled in one region. In addition, an off-coastal region was formed including all sites with no contact with mainland Sweden, irrespective of terrestrial biome. Hereby, a total of seven biogeographical regions were obtained (Table 1 and Fig. 2).

To assess the bird conservation value of SPAs that is lost due to infrastructure, I used an average of 20% (C.I. 12–33%) reduction of bird abundance within the 1 km effect distance from the infrastructure, as indicated by the results presented by Benítez-López et al. (2010; Fig. 1). GIS analyses were conducted using ArcGIS version 10.2 and QGIS version 2.6. To explore the differences between biogeographical regions in the proportion of SPA area within REZ, an ANOVA with Tukey´s post-hoc test was conducted, with region as explanatory variable. To explore how the degree of impact on an SPA is associated with its size and dominating habitat, two different analyses were performed for each region. For the probability for an SPA to overlap to any degree with REZ (response variable either 0 or 1), a generalised linear model with a logit link function (logistic regression) was used, and for the proportion of overlap with REZ (i.e. a response variable from >0 to 1), a beta-regression model (Ferrari and Cribari-Neto 2004) was used, including only sites with overlap. In both types of models, explanatory variables were 1) SPA size, 2) proportion of forest habitat, 3) proportion of wetlands and 4) proportion of agricultural land and grasslands. SPA size was log-transformed to improve normality and all variables were standardised to make parameter estimates

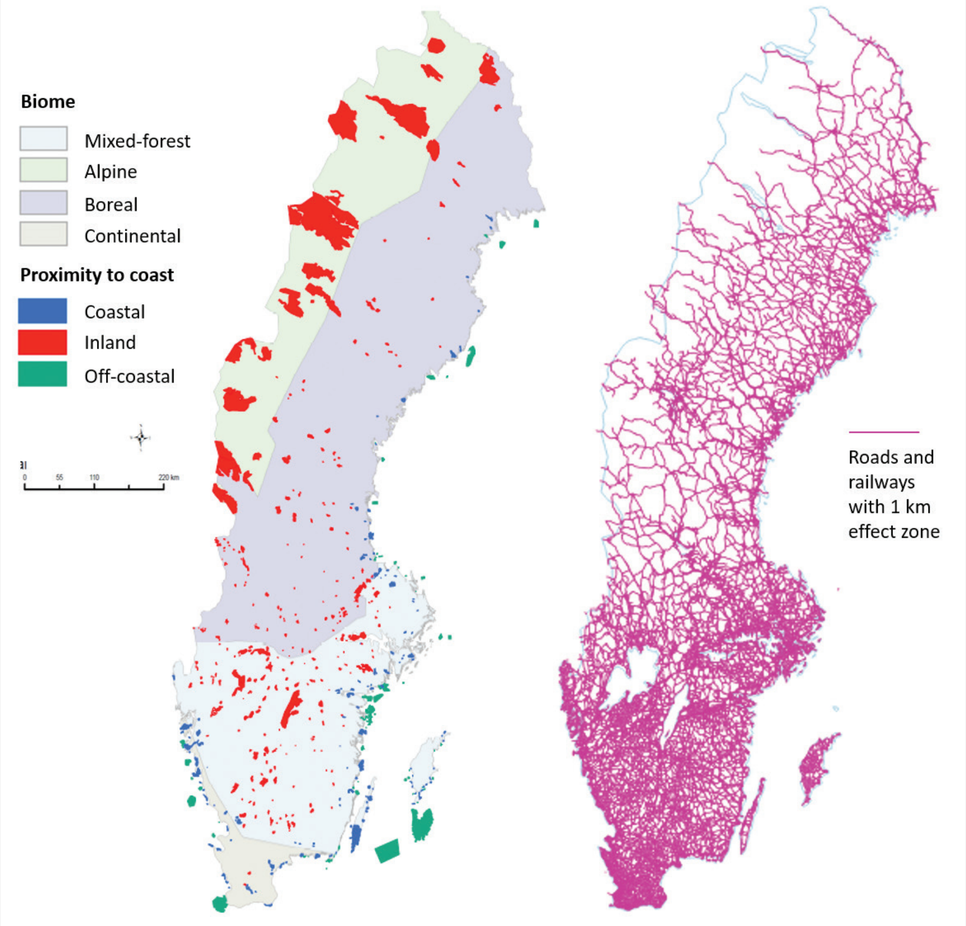

Figure 2. Special Protection Areas (SPAs) in Sweden divided by biogeographical region (left map) and larger roads and railways with predicted effect zone (REZ; right map).

comparable. Model selection was based on AIC and the final models were the ones with a combination of explanatory variables resulting in the lowest AIC. Statistical analyses were conducted using programme R version 3.4.2.

Results

The overlay of SPAs and REZ showed that 339 of Sweden´s 538 SPAs (63%) have at least some part and 123 (23%) have most of their area within REZ (Table 1). In terms of area, a total of approximately 126,000 ha or 4.2% of the total SPA area in the country lies within REZ.

However, national level figures on the impacted area give a crude picture, as the results pointed to large differences amongst the biogeographical regions (Table 1). The proportion of SPA within REZ differed among regions (F = 13.8, p <0.001), with SPAs in the alpine and off-coastal regions having a significantly smaller proportion of their area in REZ than the other regions, and boreal inland SPAs having a significantly smaller proportion than continental SPAs. Alpine and off-coastal areas have a number of large SPAs and hold most of Sweden´s total SPA area, but have sparse networks of large (terrestrial) transport infrastructure and, accordingly, they reduce the national average. Continental, mixed-forest coastal and boreal coastal regions, however, are comparably more impacted; in these three regions combined, a total of 46,046 ha or 25.8% of the total SPA area fall

Table 1. Predicted impacts on SPAs. Number, area and proportion of Swedish SPAs within an effect zone of 1 km from larger transport infrastructure (REZ) and predicted total reduction in bird abundance (with 95% confidence interval) due to the effects. Results are given for the entire country and by biogeographical region. Detailed results for each SPA are provided in appendix available online at http://triekol.se/earlier-work/infrastructure-impacts-on-protected-areas/.

Region	Total no. of SPAs	Total area of SPA (ha)	No. of SPAs affected to		Total area of SPA in REZ (ha)	Proportion of total SPA in REZ (%)	Reduction in bird abundance (% with C.I.)
			>0%	>50%			
Continental	41	53,331	41	18	19,202	36.0	7.2 (4.3–11.9)
Mixed-forest coastal	94	103,064	71	24	20,865	20.2	4.0 (2.4–6.7)
Mixed-forest inland	161	258,804	119	43	34,622	13.4	2.7 (1.6–4.4)
Boreal coastal	22	21,712	15	8	5,968	27.5	5.5 (3.3–9.1)
Boreal inland	130	162,924	63	30	13,122	8.1	1.6 (1.0–2.7)
Alpine (only inland)	26	1,984,005	16	0	28,242	1.4	0.3 (0.2–0.5)
Off-coastal	64	415,308	14	0	3,913	1.0	0.2 (0.1–0.3)
All Sweden	**538**	**2,999,149**	**339**	**123**	**125,946**	**4.2**	**0.8 (0.5–1.4)**

Table 2. Variables associated with road/railway effect zone (REZ) overlap with Swedish SPAs divided by biogeographical region. Values given are mean estimates of coefficients of logistic regressions and beta-regressions with standard errors (SE) and probabilities (P). Values are only given for variables that were included in the final model. The right column gives the variation explained by the final model.

Region	Log(size)		Forested area		Wetland		Agri & grassland		
	Estimate (SE)	*P*	Estimate (SE)	*P*	Estimate (SE)	*P*	Estimate (SE)	*P*	
Logistic regression (probability of overlap)									**Explained deviance**
Continental †	–	–	–	–	–	–	–	–	–
Mixed-forest coastal	0.45(0.27)	0.094							0.03
Mixed-forest inland					0.49(0.21)	0.019	0.91(0.38)	0.016	0.07
Boreal coastal			-1.06(0.57)	0.065					0.16
Boreal inland			-2.61(0.75)	<0.001	-2.06(0.70)	<0.001			0.21
Alpine (only inland)	1.31(0.64)	0.039			1.23(0.64)	0.056			0.23
Off-coastal	1.07(0.40)	0.008			0.79(0.32)	0.015			0.18
Beta-regression (proportion of overlap)									**Pseudo R^2**
Continental ‡	–	–	–	–	–	–	–	–	–
Mixed-forest coastal	-0.56(0.15)	<0.001					0.44(0.15)	0.002	0.26
Mixed-forest inland	-0.64(0.12)	<0.001					0.47(0.11)	<0.001	0.40
Boreal coastal	-0.85(0.34)	0.013							0.36
Boreal inland	-0.56(0.16)	<0.001	-0.48(0.16)	0.004			0.43(0.16)	0.009	0.41
Alpine (only inland)	-0.78(0.11)	<0.001			-0.38(0.17)	0.022			0.50
Off-coastal	-1.09(0.24)	<0.001					0.40(0.19)	0.036	0.38

† Could not be analysed as all sites had value 1, i.e. overlap to some degree with REZ.

‡ No variable contributed to the final model; i.e. the model including only the intercept had the lowest AIC.

within REZ. In terms of bird conservation value, present infrastructure impacts are predicted to cause a 4–7% reduction in bird abundance in SPAs in the three most impacted regions (continental, mixed-forest coastal and boreal coastal) and an average of 1% reduction when all SPAs in the country are taken into consideration (Table 1).

The overlap models with the lowest AIC included SPA size or habitat composition in all regions except the continental (Table 2). As the most strongly emergent pattern, larger SPAs appear to have a higher probability of overlap with REZ (in three regions), but a lower proportion of overlap with REZ (in all regions except continental). Another pattern, however less emergent, is that the overlap with REZ is larger in SPAs with more agricultural land and grasslands (in four regions) and smaller in SPAs with more forest habitat (in two regions). The pattern regarding overlap with SPAs with more wetland is inconclusive, and in general, only a minor part of the variation in overlap could be explained by SPA size and dominating habitat.

Discussion

The results indicate that a significant proportion of Swedish SPAs, both in terms of area and number of sites, lies within a predicted effect zone for birds around present larger transport infrastructure (REZ) and therefore can be expected not to reach their full potential as a bird habitat. The reduction due to transport infrastructure impacts may not be dramatic when seen in the country as a whole, with only around 4% of the total SPA area affected, corresponding to ca. 1% reduction in predicted bird abundance within SPAs. However, for more urbanised parts of the country, with a denser infrastructure network, the predicted impact and reduction is nearly an order of magnitude larger and may well be one of the main factors determining bird abundance in protected areas. This is the case in the southern (continental) and coastal regions of the country, where the urbanisation and landscape fragmentation is at a similar level to that of most western and central European countries (EEA-FOEN 2011).

At the level of individual SPAs, smaller sites tend to have a higher proportion of overlap with REZ and, accordingly, can be assumed to experience a greater impact than larger sites. This pattern emerges in all biogeographical regions, except the continental where most SPAs are small and indeed impacted to a large degree. In effect, the larger impact on smaller sites amplifies the bias against area protection in the lowlands, i.e. the southern and coastal regions. This is in line with general concerns previously expressed about the small size of many protected areas in Europe and about the impacts from transport infrastructure, traffic and other urban development in the landscape surrounding them (Shafer 1995, Gaston et al. 2008, Maiorano et al. 2008, Kati et al. 2015). However, as indicated by Helldin and Tytor (2017), these concerns are not well expressed in the management plans for Swedish SPAs and appear particularly underestimated in the regions where the impacts are the largest.

The functions of predicted bird abundance and distance to infrastructure described by Benítez-López et al. (2010) have previously been applied twice to assess the ecological impact of an infrastructure network at a larger geographical scale. Karlson and Mörtberg (2015) presented an assessment of the impacts of roads on habitats of high diversity value in Sweden (irrespective of their protection status), concluding that natural grasslands and southern broadleaved forest are likely to be particularly impacted; on a national level, 13–19% and 16–24%, respectively, of the total areas of these habitats are found within the predicted road effect zone for birds. Torres et al. (2016) estimated a 19.0% (CI: 9.6–25.6%) reduction in national bird numbers due to transport infrastructure for Spain, when considering all land, not only protected areas. They too concluded that some habitats (most notably farmland and maritime wetlands) might be disproportionately affected by transport infrastructure. In relation to these previous assessments, the present study is unique in that it points out the impacts specifically on protected areas, i.e. areas where nature conservation should be a top priority.

The assessment was aimed at providing a general picture and was therefore simplified in several respects. A fixed-width REZ is less realistic, as the actual effect depends on the local context, such as the habitat distribution, topography, species and ecological processes involved (Forman and Deblinger 2000, Riitters and Wickham 2003, Biglin and Dupigny-Giroux 2006), or the road characteristics (e.g. Reijnen and Foppen 2006, Rytwinski and Fahrig 2015). Additionally, the greater reduction in bird densities near the infrastructure within REZ (Fig. 1) provides an opportunity to assess the decline in bird abundance within the effect zone in individual sites in greater detail than conducted here (e.g. Torres et al. 2016, Tulloch et al. 2019).

Furthermore, the analysis of impacts on different habitats within SPAs was rather coarse, since the Natura 2000 database does not provide habitat maps. Therefore, I could not explore to what degree EU priority habitats (habitat types of community interest; EEC 1992) are distributed disproportionately within REZ.

Because the study was focused on transport infrastructures in terrestrial environments (roads and railways), the low predicted impact on off-coastal protected areas is not surprising. A corresponding analysis of the frequency of shipping and proximity to marine fairways would be of relevance to assess traffic impacts in marine reserves. Such an analysis would, however, need a different approach than the one here described, as the ecological effects of shipping are not yet comprehensively described and effect distances are less well established (Pirotta et al. 2019).

Implications for the management of protected areas

The present study underlines the concern about the impact of transport infrastructure on wildlife in protected areas in general (Forman and Deblinger 2000, Ament et al. 2008, Selva et al. 2011, Tsiafouli et al. 2013) and in the smaller areas in particular (Shafer 1995, Maiorano et al. 2008). Following article 4 of the Birds Directive, EU Member States must take appropriate steps to avoid habitat deterioration and significant species disturbance within SPAs (EEC 2010). Accordingly, a stronger emphasis on keeping natural areas free from the impacts of heavy traffic and new roads and railways has been proposed for conservation and transport planning (Selva et al. 2011, 2015, Laurance and Balmford 2013, IENE 2015).

Management plans for Natura 2000 sites should better acknowledge and consider the threats to wildlife conservation caused by both present transport infrastructure and new development projects in and near sites (Cortina and Boggia 2014, Helldin and Tytor 2017). Assessments of the effectiveness of individual Natura 2000 sites in maintaining biodiversity should include monitoring of population density and demography of species of special conservation concern (Gaston et al. 2008).

In order to correctly address the impacts from transport infrastructure, managers of Natura 2000 sites should conduct more detailed assessments of REZ in the respective site, based on habitat maps, species occurrences, and local road or railway characteristics (traffic, corridor width, etc.), to serve as a basis for setting priorities in conservation planning and action. Such site specific assessments could take into account the greater reduction in population density in close proximity to the transport infrastructure, and impacts on some wildlife species extending further than the 1 km used in the current study (Reijnen and Foppen 2006, Benítez-López et al. 2010). For example, Tulloch et al. (2019) describe a method to produce site- and taxon-specific REZs, incorporating local biodiversity distribution and expert opinion on the impacts on different taxa. Special attention to road effects should be paid in protected areas with a large overlap with REZ, in areas hosting particularly vulnerable taxa, and in areas with pronounced impacts on EU priority habitats.

Conservation authorities should ensure that infrastructure owners and managers make their best efforts to minimise the negative impact of nearby roads and railways and related traffic. Technical mitigation of impacts of transport infrastructure on birds could include preventing bird-vehicle collisions (e.g. with flight diverters), planning the timing of infrastructure maintenance and construction work to avoid particularly sensitive periods, providing crossing structures, and reducing noise and visual impacts through walls, berms or adapted paving. Such road mitigation measures for birds have been implemented on a few sites in Sweden, but are still lacking over the vast majority of roads and railways (Trafikverket 2017), and moreover, are not always considered in new construction projects. Traffic calming, speed reduction, or road closure (permanent or temporary) would also provide reductions in road mortality, disturbance, and barrier effects. Finally, compensatory measures such as habitat improvement or additional area protection could reinforce remnant populations and restore vital ecological processes.

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RESEARCH ARTICLE

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Popularity of pet otters on YouTube: evidence of an emerging trade threat

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Abstract

In response to growing reports of otters in the pet trade, and suggestions that the popularity of pet otters on social media may be driving demand, we collated YouTube videos of pet otters to test for trends in the number of videos published, their exposure (number of views) and popularity. We used English-language search terms to provide a global overview, as well as local language search terms for four South East Asian countries identified as being of potential importance in the pet otter trade (Indonesia, Malaysia, Thailand and Vietnam), and Japan. We found that not only had the number of videos depicting pet otters increased in the last two to three years (2016–2018), but that their popularity and/or engagement had also increased. Notwithstanding some country-level differences in the details of effects observed, the greatest increases in both the number of videos produced and their popularity occurred in Indonesia and Japan. At a global-level, commercial "viral" video sites appeared to be influential in terms of posting highly popular pet otter videos. At a national level, potentially influential videos tended to be produced by four or five individual otter owners. The appearance of phrases such as "I want one" in the comments section of the English-language videos, although not necessarily a statement of actual intent, suggests that these videos may be driving demand amongst their viewers and followers; similar analyses of video comments in each local language are warranted. Our results show an increase in social media activity that may not only be driving the apparent increase in popularity, but also amplifying awareness of the availability of these animals as pets, as well as creating and perpetuating the (erroneous) perception of otters as a suitable companion animal. At a global level, there are welfare concerns associated with otters in the pet trade, and, in South East Asia specifically, there are serious conservation concerns. We recommend increased regulation of these activities on social media, increased public awareness of the negative impacts of the pet trade on otters, and increased international protection. Specifically, we suggest the need to uplist both small-clawed and smooth-coated otters (*Aonyx cinereus* and *Lutrogale perspicillata*, respectively) to CITES Appendix 1.

Keywords
animal welfare, *Aonyx cinereus*, conservation, *Lutrogale perspicillata,* social media, wildlife trade

Introduction

People have been domesticating wild animals (Driscoll and Macdonald 2010) and keeping exotic species for centuries (Mitchell 2009; Grigson 2016); but recent decades have seen a notable increase in the keeping of exotic (non-domesticated) companion animals (Grant et al. 2017) and global demand for these species is a significant driver of contemporary wildlife trade, accounting for almost a fifth of recent wildlife trade reports (Baker et al. 2013). Animals kept as pets range from parrots, songbirds and falcons, to turtles, lizards, snakes and fish, to small primates and big cats (Bush et al. 2014; Gallagher 2017), and involve vertebrate and invertebrate taxa (e.g. stag beetles in Japan, New 2005). The popularity of specific species appears to be changeable, influenced by cost and availability (Stringham and Lockwood 2018), rarity (and perceived "prestige", Tournant et al. 2012), and fashion fads (Rehfeld 2005) driven by external factors such as celebrity interest. For example, news reports of Paris Hilton and her pet kinkajou (*Potos flavus*) in 2007 were closely followed by increases in exports of kinkajous from their native Guyana to the US (L. Harrington, unpub. data; trade.cites.org). Exotic pets have special requirements in captivity, which owners may not be aware of, or are unable to provide (Grant et al. 2017; Warwick et al. 2018); consequently, exotic pets often end up abandoned or in rescue centres (e.g. Cheyne 2009). Some argue that it is unethical to keep these animals as pets (e.g. on animal welfare grounds, Warwick 2014), and for individuals/species that are sourced from the wild, excessive trapping can endanger wild populations (e.g. Duckworth et al. 2012 and references therein). For some species, the sheer volume of trade means that even non-threatened species may suffer rapid declines (Nijman et al. 2018). Where captive breeding is possible, poor facilities and inadequate management can pose serious welfare concerns (D'Cruze et al. 2014), and can (where corruption and illegality is involved) provide a mechanism for laundering illegally captured wild individuals (e.g. Nijman and Shepherd 2015). The challenge for wildlife protection efforts is being able to predict 1) which species will become the next popular pet, and 2) what impact consumer demand will have on the survival of wild populations and the welfare of the individuals involved. This study addresses the first of these, focusing specifically on otters in the pet trade, and the use of social media to assess current trends.

Pets (cats and dogs) are hugely popular on social media (Kotenko 2013; Porter 2016; O'Connor 2017) and, in parts of the world where there is ready access to computers and the internet, there is a similarly vast online culture of exotic pet videos and posts. Three billion people access, and are exposed to, content on social media every day (as of 2018, wearesocial.com), and the power of social media to influence public attitudes, consumer behaviour and lifestyle choices is well-recognised (Nekaris et al. 2013 and references therein; Diehl et al. 2016). As such, observations of social media activity provide unique and valuable insight into not only the activities of those that post on social media, but also the influence that they might exert on their audiences (followers, subscribers, etc.) – in particular, the (potential) desire to carry out the behaviours (e.g. purchasing, or otherwise obtaining, an exotic pet) shown in the post. Because much social media activity takes place in the public domain, posts and the responses to posts provide a readily available data source reflecting the activities and views of (potentially) very large numbers of particular groups of people (in this case, those interested in pet otters). Collation and analysis of social media data can therefore be used to assess current interests and influences and to detect trends that, for pets (and other products), may be indicative of emerging markets and potential associated arising threats to the animals involved.

Otters (Mustelidae, subfamily Lutrinae) have long been a popular species amongst the general public, particularly in the western world (for example, as illustrated by popular novels and media productions such as "Tarka the Otter" [Williamson 1927] and "Ring of Bright Water" [Maxwell 1960]). However, whilst there are historical accounts of people taming otters and using them to catch fish (Gudger 1927; an activity still practised by fishermen in Bangladesh, Feeroz et al. 2011), their semi-aquatic habits have largely precluded widespread adoption as "house" pets. Recently, this seems to have changed; most notably (but not exclusively) in South East (SE) Asia. An assessment of online markets in Indonesia in 2012 recorded 63 live otters (small-clawed otters, *Aonyx cinereus*, and smooth-coated otters, *Lutrogale perspicillata*) listed for sale by 46 sellers (Aadrean 2013), and 800 pet otter owners are known in Jakarta alone (IOSF 2014). Following the seizure of 11 live (small-clawed and smooth-coated) otters at Bangkok International Airport, in January 2013 (Shepherd and Tansom 2013), a series of investigative reports by TRAFFIC revealed a total of 13 seizures in four SE Asian countries (Indonesia, Malaysia, Thailand and Vietnam) between 2015 and 2017, involving 59 live otters (predominantly small-clawed otters) and an extensive online market place, selling juvenile otters, operating through Facebook (Gomez and Bouhuys 2018). All four of the otter species that occur in SE Asia (small-clawed otter, smooth-coated otter, hairy-nosed otter, *Lutra sumatrana*, and Eurasian otter, *L. lutra*) face a number of serious potentially interacting and compounding threats associated with increasing human populations and their activities (habitat loss and degradation, over-fishing, and aquatic pollution) as well as poaching for their pelts in parts of their range (Wright et al. 2015; de Silva et al. 2015; Aadrean et al. 2015; Roos et al. 2015). Here, we are concerned predominantly with small-clawed and smooth-coated otters.

Otters have extensive home ranges in the wild, extending over tens of kilometres (depending on species), and spend a proportion of their time in water (Kruuk 2006). In captivity, development of abnormal repetitive behaviours (potentially indicative of stereotypy and poor animal welfare) are common amongst North American otters (*Lontra canadensis*) – the precise reasons for which are unknown but may be associated with predictable feeding times and an inability to actively forage for live prey (Morabito and Bashaw 2012). Individuals of social otter species (e.g. small-clawed otters) should not be kept alone (Heap et al. 2008), and the recommendations for zoos and aquaria housing otters are that they have a pool at least three times the otters' body length (Conley

2009). These, and other considerations (such as dietary requirements, Maslanka and Crissey 1998, and adequate enrichment, Nelson 2009), present challenges that most domestic owners of otters are unlikely to be able to meet, with potential welfare implications for otters kept in unregulated situations (Reed-Smith and Larson 2017).

There is some evidence that trade in live otters in SE Asia is increasing: for example, seizures in Japan increased from two in 2007 to 32 in 2017 (Kitade and Naruse 2018; see also Siriwat and Nijman 2018 for evidence of increasing online sales in Indonesia), which has raised significant concern amongst conservationists, and animal welfare groups. One suggestion is that this apparent trend may be driven by the popularity of otter pets on social media (e.g. Kitade and Naruse 2018; see also Morgan and Chng 2017) but currently there are no temporal data on social media activity associated with pet otters (as distinct from sales of otters via social media, specifically) to demonstrate potential growing influence. Here, we test the hypothesis that social media activity, globally and specifically in SE Asia, (as well as interest in, and popularity of, this activity) has increased over time, using YouTube videos of pet otters as an indicator of social media activity. YouTube is currently the largest and most popular video sharing website (Malik and Tian 2017). We focus on four SE Asian countries where online trade in live otters was reported by Gomez and Bouhuys (2018) to be high – Indonesia, Malaysia, Thailand and Vietnam – and Japan, where recent findings suggest pet otters are becoming increasingly popular (Kitade and Naruse 2018). For each set of videos, we asked the following questions:

1) Has the number of videos portraying otters as pets increased?
2) Has the exposure (“reach”), popularity of, and engagement with, these videos increased?
3) Who is posting the most popular (and potentially influential) videos?
4) Does the response to videos (as illustrated by viewer comments) suggest that viewers want their own otter as a pet?

Our aim was to provide evidence for the potential role of social media as a driver of the otter pet trade, and, more broadly, to demonstrate how social media data may be used to provide insights into social interests and trends, pointing to potential emerging markets.

Methods

Video selection

YouTube videos portraying pet otters were initially collated using the search term “pet otter”, and manually screening all hits for relevance (filtered by view count first and then by upload date to ensure that no recent videos with low view counts were missed). Inclusion criteria were videos showing otters in contact with humans, being touched or picked up, in buildings or other artificial surroundings, such as swimming pools or gardens, or otters restrained in any way, for example, in cages, or on leads. Videos showing otters in rehabilitation centres or zoos, people playing or interacting with (but not touching) wild otters, people swimming with captive otters through established organisations (e.g. centres that offer interactions with nature to terminally ill children), video compilations, clips from films, animations, or educational videos advising against keeping otters as pets, were excluded. Selected screened videos were saved as a playlist in YouTube. For SE Asian countries we used the following local language search terms: "berang-berang" in Indonesian, "memerang" in Malay, "con rái cá" in Vietnamese, and "นาก" in Thai (translations of the word otter), and for Japan "ペットのカワウソ" (translation of pet otter). Preliminary searches of Japanese videos using the translation of the word "otter" alone identified a large number of irrelevant videos that did not conform to our inclusion criteria (which was not the case for searches for other countries); therefore, we adopted a more restrictive search term for Japanese videos. The difference will have affected only the number of videos screened, not the number of videos selected for analysis. We included reposted videos (even if the original was also selected) because we were interested in the number of videos available to potential viewers rather than the number of unique videos per se. For the same reason, all videos from YouTube channels showing frequent updated videos of the same otter were also included but only if they came up in the original search. Selected videos under each language search term were saved as separate playlists. All videos were retrieved in November 2018.

Data extraction and analysis

All statistical analyses were carried out in R (version 3.5.1, R Core Team 2018). For each playlist created, we extracted publication date, number of views, "likes", "dislikes", and comments, for all videos in the playlist, using the "tuber" package (Sood 2018). To characterise each video, we defined three parameters: "exposure" (or "reach", views), "popularity" (likes) and "engagement" (comments), quantified as the number of total and daily views, the ratio of likes:views, and the ratio of comments:views, respectively (the latter two expressed as ratios to account for the effect of different number of views). To provide context for parameter values, we refer throughout to published benchmark figures for YouTubers (referred to as marketing "metrics of success"): 10,000 total views (the minimum requirement for carrying advertisements, https://youtube-creators.googleblog.com/2017/04/introducing-expanded-youtube-partner.html), a like: view ratio of 0.04 (or 4 likes per 100 views) and a comment:view ratio of 0.005 (5 comments per 1,000 views), and a percentage of dislikes no greater than 40% (Robertson 2014). We used descriptive statistics, and chi-square tests, to characterise the three parameters (exposure, popularity and engagement), and to test for associations between parameters and countries, using simulated p values (based on 2000 replicates) for tests with low expected values. Pairwise post hoc chi-squared tests were performed with the package "fifer" (Fife 2017), p values adjusted for multiple comparisons. Because the data were heavily left-skewed, we used non-parametric statistical tests to compare average (median) parameter values among countries, with Bonferroni corrections for multiple tests where appropriate.

For each playlist, we also identified a subset of potential "influencer" videos, defined as those that were both popular (liked; > median like:view ratios) and reached a large number of people (views; > median views). To assess who was posting these potentially influential videos, we manually checked the description of the video, and of the YouTube channel posting the video (including the profile details) and recorded the channel, the type of video (i.e. an original video posted by the owner of the otter, or a reposted video posted by, for example, a "viral" video clips channel), the number of channel subscribers, and the location of the channel (where available). In accordance with ethical research practices (see e.g. Zook et al. 2017), to protect the identity of the individuals posting the videos, collated data files were anonymised by removing user URL (web address), and neither channel names or video titles were reported.

To test for trends over time (in exposure, popularity, engagement and relative dislikes) we considered successive publications of videos by upload date as a time series (with frequency equal to the average number of posts per year), and used the tslm function in the "forecast" package (Hyndman 2017) in R to quantify and test the significance of any underlying trend once cyclical and random components had been removed. Note that because the number of posts per year varied, cyclical components of the time series were meaningless and were not included in the Results (for the purpose of this analysis we were interested only in underlying trends); also, that "trends" in this sense refers to trends in recently published versus older videos, rather than trends over time per se (i.e. the observed response to a particular video could have occurred at any time since its publication and not necessarily at the time of publication). For trends in exposure we used views per day to account for the differing lengths of time since publication, popularity and engagement were quantified as likes:views and comments:views, respectively (as above), and relative dislikes as the number of dislikes as a percentage of the number of dislikes and the number of likes. We excluded videos posted in November 2018 to reduce any possible effect (or lack of effect) due to recent publication (although these videos were counted in the total number of videos published).

Comment content analysis

Finally, we selected all (English-language) ("pet otter") influencer videos that had a minimum of 1,000 comments, to explore the content of comments and thus to assess the extent to which viewers expressed a desire to own a pet otter themselves. For each of the videos selected, we extracted the full text of all comments (also using the tuber package) and exported the text for each video to a text file for further analysis. Comment text was cleaned prior to analysis by removing symbols, numbers and common English stopwords, and transforming the text to lower case. Additional stopwords and frequent words that had little meaning out of context or that added little to the understanding of the text were identified as part of an iterative process and also removed: this resulted in the removal of the stopwords "actually", "aren't", "can", "didn't", "doesn't", "don't", "isn't", "I've", "just", "know", "probably", "really", "shouldn't", "that's", "think", "que", words used as search terms ("otter/s" and "pet/s") and those appearing in the title of the video, as well as profanities, slang acronyms (e.g. fef), and words related to YouTube ("amp", "com", "href", "http/s", "quot", "video", "watch", "www", "youtube"). Within the cleaned comment text, we identified the ten most frequently appearing words as an indicator of comment content and a reflection of the sentiments of commenters. Where "want" (or other similar words identified post hoc) appeared in the most frequent words, we identified significant word associations (i.e. those words that most often occurred alongside "want"), and verified the context within which the word was originally expressed by searching manually through the text for each occurrence of the term, recording the number of times that the word appeared in a phrase suggesting desire by the commenter to have their own pet otter (e.g. "I want one", as in Nekaris et al. 2013). All text manipulation and analysis was carried out in R using the txt mining package "tm" (Feinerer et al. 2008; Feinerer and Hornik 2018), and comment content visualised using the "wordcloud" package (Fellows 2018).

Results

Number of videos

We identified a total of 64 videos on pet otters using (English-language) search terms, and 173, 35, 115, 58, and 338 for Indonesian, Malaysian, Thai, Vietnamese and Japanese videos, respectively. English-language pet otter videos were first published in 2009 (although the next English-language video was not published until August 2011), SE Asian videos were first published in 2010 or 2011 (depending on country), and Japanese videos in 2012; for all language videos, annual numbers were relatively low before 2016 but (with the exception of those in Thailand) increased thereafter at an annual rate of 1.1–4.3, most notably in Indonesia (where the number of videos doubled each year between 2016 and 2018) and Japan (where it quadrupled), reaching peaks of at least 83 and 215, respectively, in 2018 (Fig. 1).

We did not formally categorise videos based on content but most could be described broadly as showing an otter: being "petted" (touched but not held) by a person or group of people, eating, swimming (indoors in a bath or outdoors in a small plastic pool), interacting with other pets, walking or playing outside (sometimes restrained on a lead), or doing something considered to be amusing (Fig. 2). Many of the videos showed pups or juvenile otters; we did not attempt to identify the species, but most appeared to be small-clawed otters.

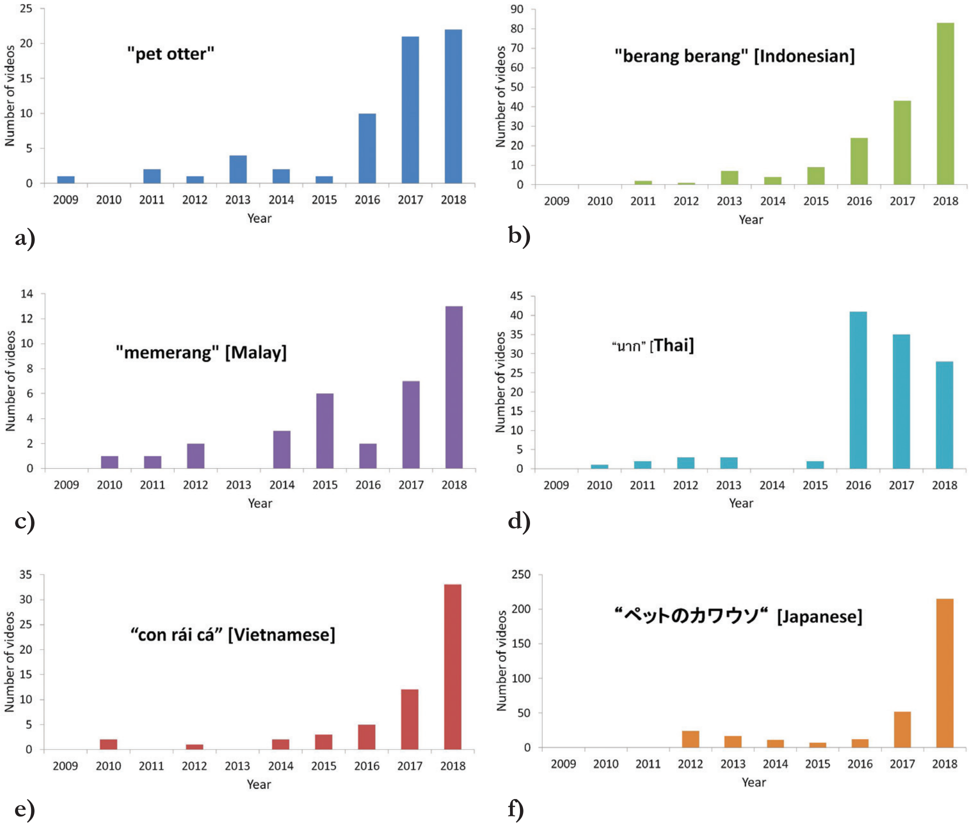


Figure 1. Number of pet otter videos (including reposted videos) published per year for **a** English language **b** Indonesian **c** Malaysian **d** Thai **e** Vietnamese and **f** Japanese search terms. Note that some videos appeared under more than one search term (e.g. many of the Japanese videos appeared under both English and Japanese translations of "pet otter"), and that the numbers published in 2018 are an underestimate since they did not include videos published in December of that year.

Exposure, popularity and engagement

Detailed analyses of SE Asian videos focused on Indonesian and Thai videos as these comprised the largest sample sizes and represented both increasing and decreasing numbers of videos; these were compared with those in English and Japanese languages.

For all four sets of videos, indices of exposure, popularity and engagement were strongly left-skewed, with the vast majority of videos scoring relatively low on all three measures (see Appendix 1). For example, 84.4% (n=54) of English-language pet otter videos received < 400,000 views (exposure), less than one twelfth of the maximum views recorded (5.7 million), and 93.6% (n=162) of Indonesian otter videos received <50,000 views, less than one thirtieth of the maximum views recorded (1.5 million). Compared with benchmark values (see Methods), however, exposure was high (>10,000

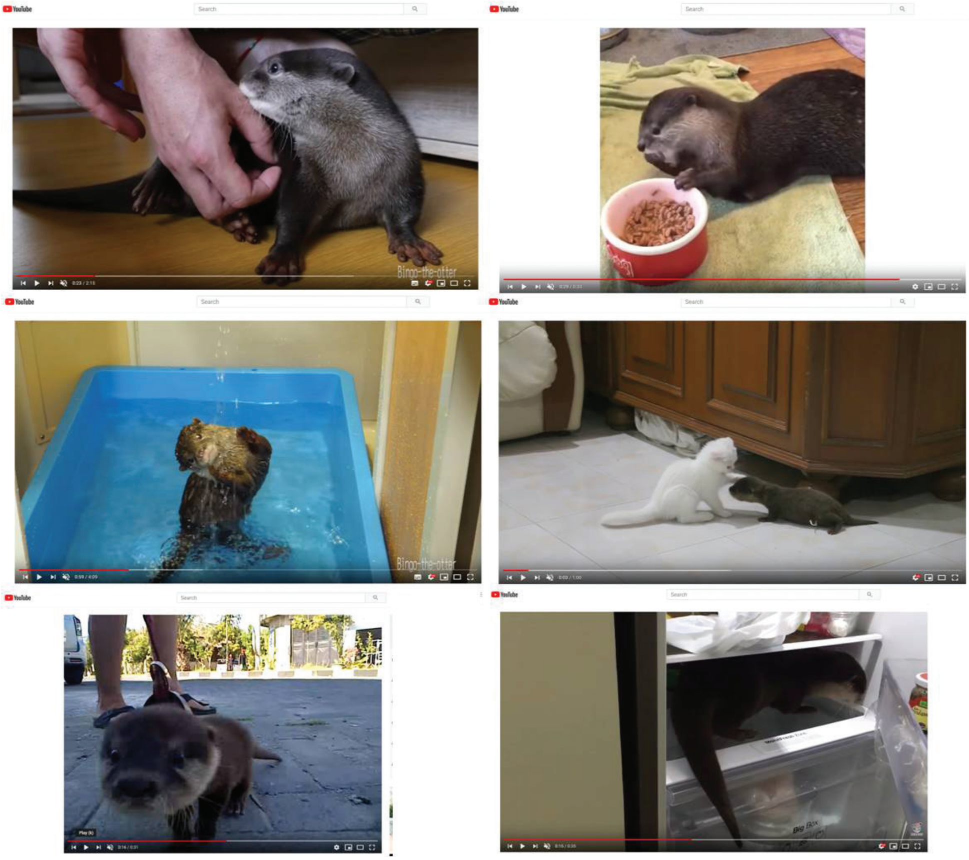


Figure 2. Screenshots of YouTube videos portraying pet otters, under the search terms "pet otter", "berang-berang" (otter in Indonesian) and "ペットのカワウソ" (pet otter in Japanese). Downloaded from www.youtube.com (individual videos anonymised to protect the identity of users).

views) for over half (53.1%, n=34) of English-language videos and between a fifth and a half of local language videos (Indonesian: 18.5%, n=32; Thai: 40%, n=46; and Japanese: 52.1%, n=176). Five English-language pet otter videos and fifteen Japanese pet otter videos received over one million views, as did two Indonesian and two Thai videos. Maximum views per day reached 16,127 for English-language pet otter videos (median = 42) and 2,818 (but with an average – median – of only 1) for Indonesian otter videos, 2,332 (median 11) for Thai otter videos, and 34,091 (median 22.5) for Japanese videos.

In contrast with exposure rates, popularity and engagement indices for English-language pet otter videos were relatively low in comparison with benchmark levels (see Methods). Most (84.1%, n=53) like:view ratios (popularity) were <0.02 (two likes per 100 views). The maximum like:view ratio recorded (0.5) was based on very small sample size (1 like, 2 views) and considered to be unrepresentative, only two (3.2%) other videos had like:view ratios ≥0.04. Most of the local language otter videos also had like:view ratios <0.02 (Indonesian: 82.1%, n=142; Thai: 89.6%, n=103; Japanese:

76.3%, n=238) and the proportion of videos scoring 0.04 or more was relatively low (11.2, 4.8 and 5.4%, respectively, for Indonesian, Thai and Japanese videos). However, there was a statistically significant difference in the proportion of "popular" videos among countries(chi-squared test χ^2 = 8.81, df=3, simulated p value = 0.025) and a slight (non-statistically significant) tendency for more Indonesian videos to score highly on popularity indices than other pet otter videos (pairwise comparisons: English-language vs. Indonesian, p=0.072, Indonesian vs. Thai, p=0.050, and Indonesian vs. Japanese, p=0.029, critical value with Bonferroni correction for 6 tests = 0.008), although popularity overall was significantly lower for Indonesian (median likes:views = 0.003 i.e. 3 likes per 1,000 views) and Thai videos (0.004) than for either Japanese (0.007) or English-language (0.009) videos (pairwise Wilcox tests, adjusted $p<0.01$) and lower for Indonesian videos than Thai videos (pairwise Wilcox test, adjusted p=0.009). There was no statistical difference between the popularity of English-language and Japanese videos (pairwise Wilcox test, p=0.259; overall difference among countries: Kruskal-Wallis χ^2 = 29.1, df = 3, $p<0.001$). Similarly, most (74.6%, n=47) English-language videos, and most local language videos (Indonesian videos 72.8%, n=126; Thai videos 73.9%, n=85; Japanese videos 65.0%, n=202) received less than one comment per thousand views (engagement). No English-language video received 5 comments or more per 1,000 views (comment:view = 0.005), but 10.4% (n=18) of Indonesian videos, 7.7% (n=24) of Japanese videos and 3.5% (n=4) of Thai videos did (albeit, for Indonesian videos, based on <1,000 views). On average, engagement (comments:views) was significantly lower for Indonesian videos (median = 0) than for all others (medians = 0.0004 for Thai and Japanese videos, and 0.0005 for English-language videos; Kruskal-Wallis χ^2 = 22.0, df = 3, $p<0.001$, pairwise Wilcox tests: Indonesian videos vs. Japanese $p<0.001$, Indonesian vs. English-language videos p=0.017 and Indonesian vs. Thai videos p=0.002, all others $p > 0.05$) but the likelihood of a video receiving high engagement scores differed among countries (chi-squared test χ^2 = 10.49, df=3, simulated p value = 0.019) being significantly higher for Indonesian videos than for English-language videos (pairwise comparison, p=0.008; critical value with Bonferroni correction for 6 tests = 0.008).

Only one each of the English-language, Thai or Japanese videos exceeded the benchmark value of 40% dislikes, whereas 16 (11.7%) of Indonesian videos exceeded the benchmark (chi-squared test χ^2 = 37.5, df=3, simulated p value < 0.001). In all cases, median percentage dislikes were less than 3%.

Influencer videos

Amongst the English-language pet otter videos, we identified 19 influencer videos (those with > median views and > median like:view ratios). Three were original videos published by two otter owners (in 2017 and 2018), both from Japan. A fourth also appeared to be an original post by an otter owner (in 2014), from an unknown location. Most influencer videos (n=14) appeared to be reposted videos; six of these were posted by three different commercial US-based YouTube channels that buy and sell, or licence, video clips, and one by a Philippine YouTube channel posting trending video clips. One of the US channels reposted three videos from an original Instagram account of an otter owner in Bangkok, Thailand; another reposted the same video of one of the Japanese otters twice in two successive years. Other videos were posted on various animal channels from unknown locations. The five (most viewed) pet otter videos with over a million views comprised two original videos posted by one of the Japanese otter owners (with 147,000 subscribers), and three reposted videos posted on one of the US-based commercial YouTube channels (with >300,000 subscribers), a personal YouTube channel (with a single playlist of funny videos and 0 subscribers) and a dedicated cat channel (with 4,800 subscribers), the latter two both from unknown locations.

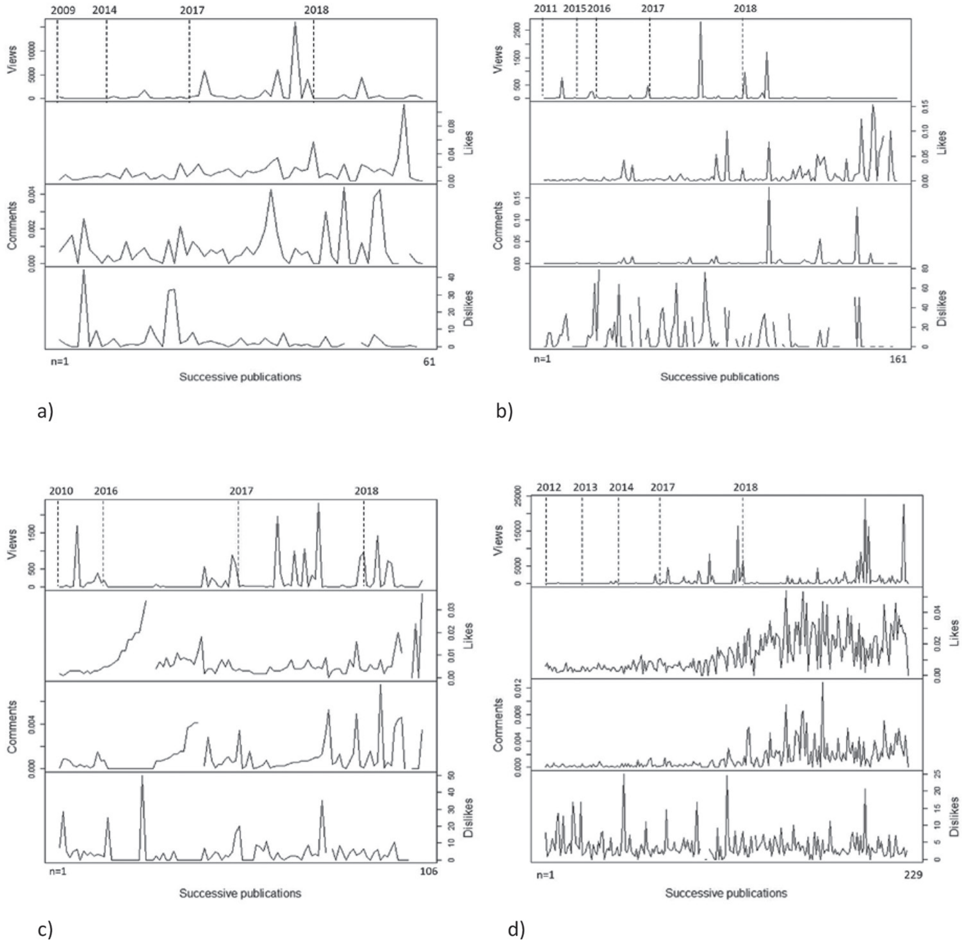
Of 30 Indonesian influencer videos, at least six appeared to be original videos by four different Indonesian otter owners, two were selling otters, at least three appeared to be local online TV channels featuring local people with pet otters, and one was an Indonesian educational channel. One of the Indonesian TV channels provided a link to the “Otter lover Indonesian Community” (or “musang lovers”, which means civets but also includes otters, and refers to a community group that keep civets and otters as pets and meet socially with their pets). Five Indonesian videos had more than 10,000 views – these included two YouTubers with their own pet otter (although neither was a dedicated otter channel, with 858 and 68,000 subscribers, respectively) and one seller (with 166 subscribers); the only Indonesian video with more than a million views portrayed an Indonesian actress with her pet otter, posted on an Indonesian celebrity channel (with 172,000 subscribers).

Thai influencer videos portrayed predominantly (20 of 29) original videos posted by three otter owners from Thailand on their own dedicated otter channels (16 of which, including one with over a million views, were posted on a single channel, with 13,000 subscribers); three videos were posted on local TV/news and entertainment channels. One other Thai video that received over a million views (but with a relatively low popularity score of 0.003) was posted on a local entertainment channel and differed from most other otter videos in portraying a large adult smooth-coated otter interacting with, and being handled by, its apparent owner (most other videos showed juvenile otters, and often very small pups that were not yet weaned).

We did not attempt to assess the source of all Japanese influencer videos (n=96) due to time constraints but the top 28 (those with > 100,000 views and > median like:view ratios) were posted exclusively by four different Japanese otter owners (two of which also appeared amongst the English-language influencer videos). Eight of these videos received over one million views, and above-median popularity scores.

Trends

Views per day of English-language pet otter videos peaked at 16,127 for a video published in December 2017 but declined in 2018 to levels comparable to those before 2017 (Fig. 3a) and there was no overall trend (Table 1). However, there was a statisti-



a)

b)

c)

d)

Figure 3. Time series plots showing successive publications of pet otter videos under **a** English language (n=61, 2009-2018) **b** Indonesian (n=161, 2011–2018) **c** Thai (n=106, 2010–2018), and **d** Japanese (n=229, 2012–2018) search terms (see Fig. 1). Views are views per day, Likes and Comments are like:view, comment:view ratios, respectively, and Dislikes are % dislikes relative to the total number of likes and dislikes (see Methods). Searches were carried out in November 2018, and each time series shown includes all YouTube videos published 30 days or more prior to the search date. Note that the time represented in each time series differs because the date of the first video published differed among search terms, and that the timeline is non-linear because increasing numbers of videos were published in each consecutive year (as shown in Fig. 1) – the year of the beginning of each time series, 2017 and 2018, and some of the earlier intervening years, are marked along the top of each graph. Missing values depicted in the graphs are due to the exclusion of peak values based on small sample size (counts of < 5 for likes, comments, or dislikes), or, for Dislikes, videos with 0 likes and 0 dislikes.

cally significant positive trend in like:view ratios over time, which increased over successive postings by an average of two additional likes per 10,000 views (Fig. 3a, Table 1). There was no comparable trend in comment:view ratio (Fig. 3a). Percentage dislikes

Table 1. Trend statistics for exposure, popularity and engagement indices for English-language (n=61), Indonesian (n=161), Thai (n=106) and Japanese pet otter videos (n=229), 2009–2018. In all cases, 2018 includes videos posted up until the end of October (videos published in November were excluded, see Methods). The trend is the average increase between successive video publications; F, df, and p values are based on ANOVA of the trend component of the time series. Statistically significant trends are shown in bold.

Videos	Index	Trend	F	df	P
pet otter (English)	views per day (exposure)	14.9	0.74	1, 59	0.394
	likes:views (popularity)	**0.0002**	**6.31**	**1, 59**	**0.015**[1]
	comment:views (engagement)	$5.9e^{-06}$	0.51	1, 58	0.477
	% dislikes	**-0.13**	**4.13**	**1, 56**	**0.047**[2]
berang berang (Indonesian)	views per day (exposure)	-0.28	0.34	1, 159	0.560
	likes:views (popularity)	**0.0002**	**28.04**	**1, 158**	**<0.001**
	comment:views (engagement)	$5.04e^{-05}$	2.77	1, 158	0.098
	% dislikes	-0.068	2.63	1, 125	0.107
นาก (Thai)	views per day (exposure)	1.41	1.15	1, 104	0.285
	likes:views (popularity)	$1.8e^{-05}$	0.67	1, 100	0.416
	comment:views (engagement)	**$1.25e^{-05}$**	**7.93**	**1, 102**	**0.006**
	% dislikes	-0.012	0.22	1, 101	0.640
ペットのカワウソ (Japanese)	**views per day (exposure)**	**10.6**	**14.54**	**1, 227**	**<0.001**
	likes:views (popularity)	**0.0001**	**158.8**	**1, 227**	**<0.001**
	comment:views (engagement)	**$1.47e^{-06}$**	**83.5**	**1, 227**	**<0.001**
	% dislikes	-0.006	2.65	1, 222	0.105

[1] the positive trend in likes:views for English-language videos remains statistically significant if the two outliers (see Fig. 3a) are removed (trend=0.0001, F=4.11, df=1, 57, p=0.047); [2] conversely, the negative trend in % dislikes is dependent on the three videos published with % dislikes > 30%

showed a statistically significant negative trend, although this was driven by three videos with high percentage dislikes (30–40%) posted in 2013 and 2016 (Fig. 3a).

Trends in Indonesian videos were broadly similar to those of English-language pet otter videos with no trend over successive postings for views per day (but a few outstanding videos posted between August 2017 and February 2018, Fig. 3b, Table 1), a statistically significant positive trend in like:view ratios over time (that increased by an average of two additional likes per 10,000 views with each successive posting, Fig. 3b), and no apparent trend in comment:view ratio. In this case, however, there was also no trend in percentage dislikes (Fig. 3b). Thai videos also showed no trend in views per day (with several videos viewed over 1,000 times per day throughout the time series) (Fig. 3c) but, in contrast with both English-language and Indonesian videos, showed a significant increase in comment:view ratios over successive postings but no trend in like:view ratios or in percentage dislikes (Fig 3c). Japanese pet otter videos showed significant increases in views per day, like:view and comment:view ratios; percentage dislikes showed a slight tendency to decline but the trend was not statistically significant (Fig. 3d, Table 1).

Comment analysis

Five English-language pet otter videos were selected for further analysis of comments. In all cases, comments were characterised (based on the ten most frequently appearing

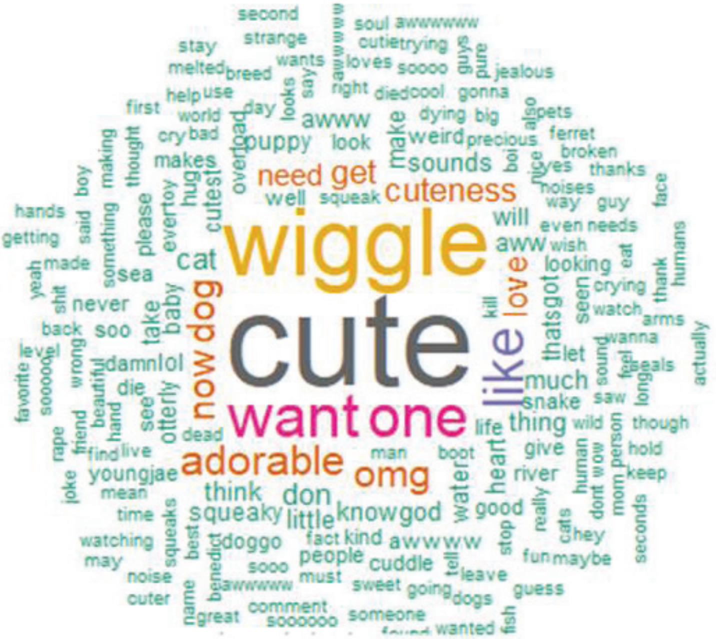


Figure 4. Wordcloud depicting the nature of comments for one of the pet otter influencer videos (defined as the most viewed and the most popular videos; > median views, and > median like:view ratios) selected for comment content analysis (those with > 1,000 comments). The wordcloud shown here is for Video 2 (see Table 2; wordclouds for the other four videos are given in Appendix 3). Font size is relative to the frequency at which each of the words appears in the comments; stopwords and profanities have been removed (see Methods).

Table 2. Most frequently cited words in potential influencer English-language pet otter videos. n is the number of comment lines analysed; freq is the number of times the word occurred in the entire document and was not necessarily the same as the number of comments that the word occurred in. Specific words mentioned in the text are highlighted.

Video 1 (n=3,922)		Video 2 (n=3,254)		Video 3 (n=1,114)		Video 4 (n=945)		Video 5 (n=1,366)	
Word	freq	Word	freq	Word	freq	Word	freq	Word	freq
cute	491	**cute**	600	**cute**	126	**cute**	97	**cute**	146
one	160	**wiggle**	408	**like**	114	**one**	54	**like**	123
want	145	**want**	244	**cage**	113	**like**	53	**love**	70
omg	139	**one**	238	animal	93	water	52	**one**	64
like	131	**like**	204	baby	56	**want**	48	animals	56
dog	124	**adorable**	140	little	51	**get**	33	**get**	55
animal	102	omg	117	**one**	48	people	29	water	45
adorable	95	dog	115	wild	48	sea	27	**adorable**	45
little	86	now	109	**sad**	46	animals	27	best	43
cat	86	**love**	101	water	44	wild	25	good	41

words) by words such as “cute”, “like”, “love” and “adorable”, as well as words such as “want” and “get” (Table 2; Fig. 4), although potentially negative words – “cage” and “sad” – also appeared frequently in Video 3 (see Table 2) that depicted an otter in a cage at night with a stuffed toy. The word “want”, “get” and “one” appeared in the ten most frequently appearing words for three, two and five of the five videos, respectively. Want and one were associated where they occurred together (r=0.36-0.45) and (with the exception of Video 3) approximately 40–50% of the comments containing one or all of these words included phrases such as “I want one”, “I need one”, “I want a pet otter”, “where can I get one?” (full list in Appendix 2). For Video 3, only 8.7% of 115 comments containing the word “one” included such phrases.

Discussion

Our analysis revealed an increasing number of pet otter videos published under both English, SE Asian (with the exception of Thai), and Japanese, search terms that (for English-language, Indonesian and Japanese videos) were also increasingly popular, and (for Thai and Japanese videos) increasingly engaged with (i.e. more recently published videos were more popular, and more engaged with, than older videos). This increase in social media activity, and response, suggests growing interest in, and popularity of, pet otters, both globally and nationally in some countries in east and SE Asia. Increasing trends were most notable for Japanese pet otter videos (see Fig. 3d), perhaps due to larger sample size, but nevertheless indicating a clear increase in popularity (from 5 likes per 1,000 views in early publications to 2 – 4 per 100 views for videos published in 2018) as well as a statistically significant increase in exposure not seen in other language videos (see Table 1). As in Nekaris et al.’s (2013) study, most comments of the five videos for which we analysed comment content responded to endearing characteristics of the otters shown (most of which were young animals) and appeared to echo the presumed affection of the owner. Whilst the comments overall were variable both among and within videos, the frequent appearance, and relatively high ranking, of words and phrases associated with an apparent desire to purchase a pet otter suggest that some highly popular videos might incite desire to own these animals as pets.

Welfare concerns for pet otters

The short snap-shots of captive otters in domestic settings shown in all YouTube videos viewed created the appearance of an animal that behaves much like a domestic dog – and this was particularly the case in SE Asian and Japanese language videos, where otters were often shown walking unrestrained along the bank of a river accompanied by a person, or (in Japan) outside on a lead (leash), walking amongst crowds of people in a town or city. Almost all videos (regardless of original language or country of origin) portrayed a picture of an affectionate "easy to handle" animal in the house. The increasing numbers of these types of videos create the (erroneous) perception that otters are suitable animals to be kept as pets (see Ross et al. 2011) but complex habitat and dietary requirements of otters, combined with the potential to cause harm to people (by biting), mean that otters are actually extremely difficult to keep as pets (Warwick et al. 2014). Recent reports by local animal welfare groups of abandoned or rescued otters in poor states of health, having suffered weight loss and malnutrition (see e.g. https://www.facebook.com/petotters/), demonstrate some of the most serious animal welfare issues that can be involved, but the portrayal of otters swimming in very small water containers (often less than the body length of the otter in width) in a number of the videos viewed also raises further questions about whether it is acceptable on animal welfare grounds to keep a wild animal in these (perhaps well-intentioned, but limited) conditions. For animals captured in the wild, there are opportunities for suffering at all stages of capture and transport (Baker et al. 2013) and adults (upon which unweaned young are still dependent) are probably killed (IOSF 2014).

Conservation concerns for otters in SE Asia

We focused on four SE Asian countries and Japan to assess national-level interest in pet otters on social media because an apparent emerging interest in keeping pet otters (predominantly small-clawed otters, but occasionally also smooth-coated otters) had been highlighted in these countries (Gomez and Bouhuys 2018; Kitade and Naruse 2018). For both species (both of which are native to SE Asia) there is a paucity of data on population size but populations of both are believed to be declining and both are listed as Vulnerable on the IUCN Red List of Threatened Species (Wright et al. 2015; de Silva et al. 2015). Because the source of individuals observed as pets is unknown (Gomez and Bouhuys 2018; Kitade and Naruse 2018), it is not currently possible to assess the actual impact of the pet trade on wild otter populations in the region. Nevertheless, the precarious nature of wild populations of all otter species in this region, means that any additional, or increasing, pressure on wild populations, is a serious cause for concern, and, for small-clawed otters specifically the pet trade is considered likely to pose a significant impact (CITES 2019).

Smooth-coated otters are protected in all SE Asian countries considered in this study (enforceable by a prison sentence of up to 4 years for possession and trade – depending on the country and specific national legislation); small clawed otters are similarly protected in Thailand, Malaysia and Vietnam, but not in Indonesia (Gomez and Shepherd 2018). Whilst there is some level of protection for small-clawed otters in Indonesia under national forestry regulations because there are no harvest quotas for otters (which are otherwise required for hunting/capture), it is difficult to enforce because there are no associated punishments (Gomez and Bouhuys 2018). At an international level, both species are protected under CITES Appendix 2, which requires regulation of commercial trade, but because there are no restrictions on domestic trade of CITES Appendix 2 species in Japan (only Appendix 1 species imported into Japan are protected under the Law for the Conservation of Endangered Species of Wild Fauna and Flora, https://www.env.go.jp/en/nature/npr/ncj/section6.html), and neither small-clawed or smooth-coated otters are native to Japan, once these species arrive in Japan, there are no regulatory means by which laundering can be prevented or traceability established (Kitade and Naruse 2018).

There are reports of otter breeding facilities in Thailand and in Indonesia (Gomez and Shepherd 2018) but these reports are unverified and, if the facilities do exist, they are either operating illegally (Thailand) or contain illegally-obtained animals (Indonesia) (Gomez and Bouhuys 2018). Indeed, Siriwat and Nijman (2018) suggest that online traders in Thailand are aware of the illegality of selling pet otters, but appear not to be concerned about the consequences of selling one or two.

Who are the social media "influencers"?

Amongst the global, English-language pet otter videos, most potentially influential videos (defined here as those that were both popular and reached large numbers of people) were reposted videos, several of which were posted by commercial US-based YouTube channels purporting to host viral or trending videos. As in Bakshy et al.'s (2011) analysis of Twitter users, and contrary to expectation, not all videos posted by users (or on channels) with large numbers of subscribers appeared to be influencers, and, in some cases, potential influencers had very small or non-existent social networks.

Across all local language videos, potentially influential videos tended to be posted by a very small number of individual otter owners, suggesting that whilst the source of influence might be *a priori* unpredictable, it is somewhat limited. Further, and in accordance with other studies of social media activity (e.g. Bakshy et al. 2011; Morgan et al. 2014; Harrington et al. 2018), most YouTube videos in this study actually received very little attention. However, the continually increasing number of videos available (particularly in Japan) raises concerns of multiplier effects, and the possibility that the combined effect of many non-influential videos may together create an effect greater than the sum of their individual parts. In other fields, individual adoption of behaviours has been found to be more likely when participants receive reinforcement from multiple neighbours in the social network (Centola 2010). Increasing numbers of videos showing potentially desirable exotic pets could be considered analogous to "big seed marketing" (Watts et al. 2007), which is extremely efficient with respect to rate of spread (in this case, of interest in pet otters and potentially the desire to own one). If this were the case for exotic pet ownership, everyone posting or reposting pet otters on social media could be considered to be an influencer.

Media influence and links with demand

Apparent surges in the popularity of other exotic pets (clownfish [*Amphiprion* spp.], Fennec foxes [*Vulpes zerda*] and snowy owls [*Bubo scandiacus*]), have previously been attributed to demand driven by children's films. In these cases, however, scientific studies have failed to find clear evidence of a causative link. For example, the "Nemo effect" (the suggested effect of the children's film Finding Nemo on purchases of clownfish representing the main character of the film Nemo) was more likely linked to media hype referring to the scarcity of the species and the anthropogenic allee effect (Militz and Foale 2015) than the film character itself. Similarly, increases in the trade of fennec foxes pre-dated the release of the Zootopia film with which fennec foxes were associated (Verissimo and Wan 2016), and a study comparing exposure to the Harry Potter films and owl trade in the UK failed to detect evidence of the link that had been widely reported in the media (Megias et al. 2017). At a local level, however, the sale of owls in bird markets in Java and Bali increased massively after the release of the Harry Potter films and books in Indonesia (Nijman and Nekaris 2017), and Macdonald et al. (2017) describe how raccoons became established as an invasive species in Japan as a result of people releasing unwanted pet raccoons purchased during the airing of a Japanese animated TV series "Rascal the raccoon". It is possible that the influence of videos on social media portraying "real" people with "real" pets may differ from that of animated or fictional characters and species, insofar as they are tangible and represent a certain reality; in this context, the links between actual demand and social media interest, and the scales at which they might function, warrant further scrutiny.

In Japan, where the increase in social media activity associated with pet otters was particularly great, the popularity of otters as pets, and the perception that they are easy to keep, is likely also perpetuated by otter cafés (McMillan 2018), a recent phenomenon that itself generates social media coverage by news reporters and visitors. The first otter café reportedly opened in Japan in 2016, followed by a further seven Japanese cafés exhibiting otters in 2017 (McMillan 2018).

Social media insights as a wildlife protection tool

Whilst social media (through driving demand for, and facilitating online sales of, threatened wildlife species) poses a significant risk to conservation and animal welfare, it also offers opportunities as a wildlife protection tool (through the vast repository of data available, and the insight provided into people's interests and activities, Malik and Tian 2017; see also Siriwat and Nijman 2018). This study is one of very few scientific studies of which we are aware examining either the portrayal of exotic pets on social media or the social media response to such posts. Nekaris et al. (2013) - who presented an in-depth analysis of the response to a single particularly influential video of a pet slow loris (*Nycticebus* spp.) being tickled – is one exception, but despite the success of Nekaris et al's study in undertaking, and tracking the response to, educational campaigns, there have been few follow-ups. The use of social media in conservation science is still a relatively underutilised tool (Di Minin et al. 2015). There are data limitations associated with publicly-available data; for example, in this study we were not able to account for potential changes in response to a video over time. Post hoc tests found no evidence of a linear correlation between any of the indices used and time since publication (Pearson's correlation coefficient < 0.3 in all cases) but this does not fully exclude potential bias due to recency (i.e. the possibility that likes or comments per views is higher in the first weeks or months following publication), and these characteristics of YouTube videos and other social media posts warrant further scrutiny (see e.g. Chatzopoulou et al. 2010). Trawling social media and retrieving associated metadata is also a time consuming process, which can make such studies (particularly where large sample sizes, or multiple search terms, are needed) prohibitive. We used an automatic metadata retrieval process using freely available packages implemented in R (R Core Team 2018) that allowed rapid metadata retrieval once a playlist had been created. Although we manually screened and selected individual videos for each playlist (which added considerably to the time required for processing) this step was necessary to ensure that all videos included in the dataset were relevant. There are a number of automated approaches for the search and retrieval of social media posts (see e.g. Malik and Tian 2017), which, with the employment of machine learning techniques (Di Minin et al. 2018), may further increase the efficiency of similar analyses in the future.

Harris et al. (2015) suggest that species that are being over-exploited through trade can be identified on the basis of market data and observations of increasing prices alongside decreasing supply – notwithstanding data limitations, the use of social media data to detect increasing activity and/or popularity associated with a specific species or product offers a significant advantage over a market based approach by detecting and highlighting trends in interest well ahead of any more serious effects such as decreasing supply. We did not attempt to quantify the number of otters shown in the videos, nor do we suggest that everyone that comments "I want one" on a YouTube video of a pet otter necessarily intends to buy one, rather we suggest that our results are indicative of an emerging threat. Social media analysis in this context can thus provide an early-warning system highlighting potentially problematic wildlife trade issues and enabling targeted mitigation measures to be put in place before they become a problem.

Recommendations

For otters in SE Asia, there is an urgent need to identify where pet otters are being sourced from, via what route, and to clarify the (il)-legality of the situation. Further research is needed on the drivers for the current trend in keeping pet otters, and to assess the role of social media in driving demand (in SE Asia and Japan, as a priority, but also globally). We did not attempt to translate and analyse comments posted in response to the local language SE Asian videos, but such analyses would be useful to assess local level, culturally-influenced, responses, and may be warranted to predict local-level emerging trade risks, detect national differences, and identify geographical priorities. More broadly, the role of social media in driving demand for new products warrants attention. Does social media instigate and create desire for owning a particular product (whether a pet otter, other exotic pet species, or any other illegal or unethical wildlife product), or does it simply respond to an existing desire and further amplify it?

Beyond the use of social media to detect trends and identify potential trade threats, there is a need for targeted interventions by social media companies and users. The commitment by a coalition of technology, e-commerce and social media companies (including Facebook and Instagram), in 2018, to reduce the trade in wildlife products by 80% by 2020 (Global Coalition to End Wildlife Trafficking Online; Bale 2018; see www.worldwildlife.org/pages/global-coalition-to-end-wildlife-trafficking-online) and Instagram's initiative to alert viewers to search terms (hashtags) that might be associated with "posts that encourage harmful behaviour to animals .." (i.e. animal selfies; Daly 2017) provide models for the type of actions that can be taken. These actions should be targeted specifically at key entities (influencers) identified in this, and other similar studies. At the user level, social media provides opportunities for education and awareness raising (Nghiem et al. 2012) - and to encourage behaviour change (e.g. Nekaris et al. 2013; Waters and El-Harrad 2013), which is most likely to be achieved (see Moorhouse et al. 2017) by highlighting the illegality of possessing an otter in many SE Asian countries, and drawing attention to the fact that wild otters bite, have sharp teeth, and are potential carriers of zoonotic diseases (e.g. Hsu and Mathura 2018).

The current proposal to move small-clawed and smooth-coated otters to CITES Appendix 1 (proposed by India, Nepal and the Philippines, and Bangladesh, India and Nepal, respectively, and backed by the IUCN Otter Specialist Group, www.otterspecialistgroup.org; CITES 2019), due to be voted on at the CITES CoP in 2019, will (if upheld) provide protection for these species exported to non-range states where there is no national-level protection (i.e. Japan), and effectively place them on the list of species for which trade is prohibited under the Wildlife-Friendly Online Trade Policy (www.worldwildlife.org/publications/wildlife-friendly-online-trade-2017-a-harmonized-policy-for-e-commerce-and-social-media-companies) adopted by the Global Coalition (above). We urge policy-makers, practitioners and researchers to take these steps.

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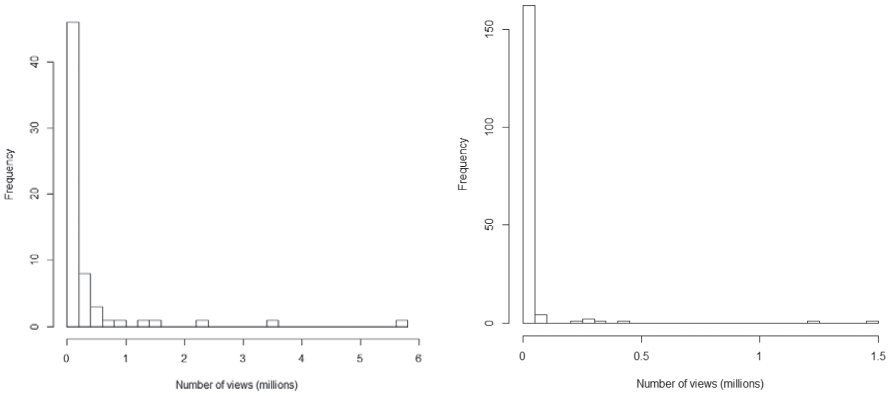
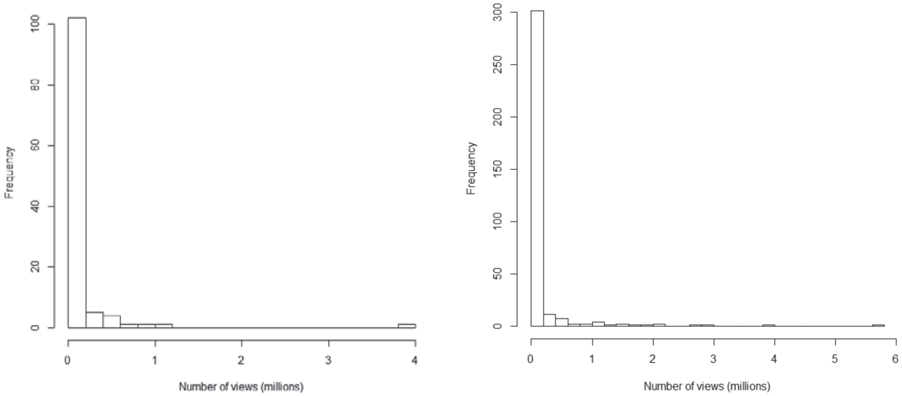
Appendix 1

Data distributions – exposure (number of views), popularity (like:view ratios) and engagement (comment:views ratios)



a) “pet otter”

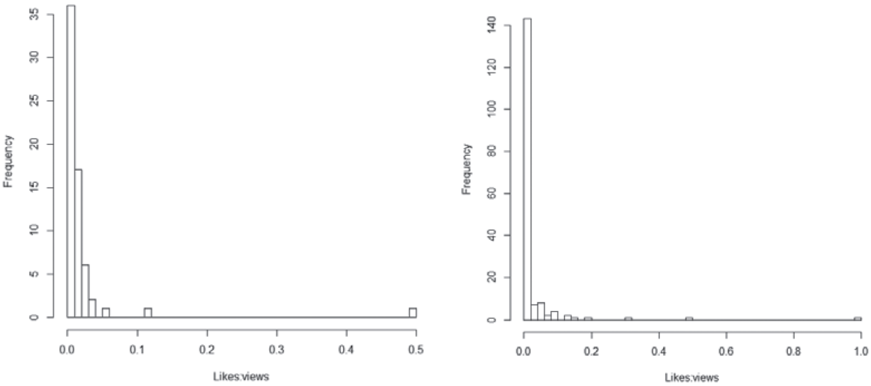
b) “berang berang” (Indonesian)



c) “นาก” (Thai)

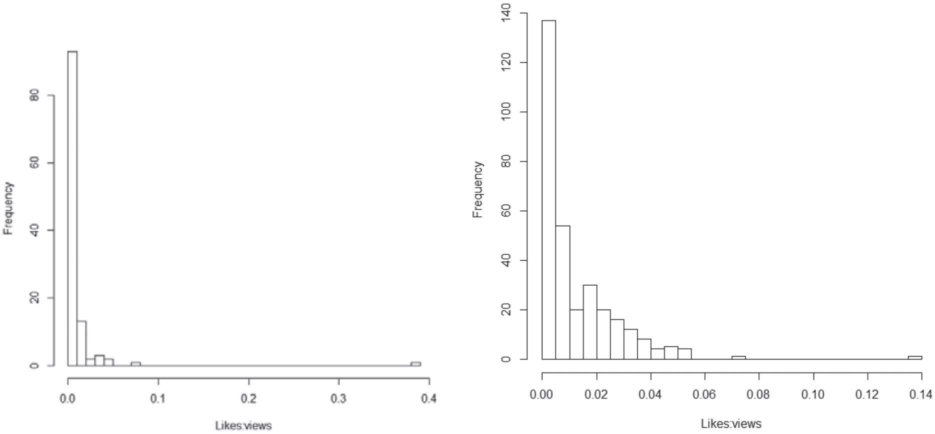
d) “ペットのカワウソ“ (Japanese)

Figure A1. Total number of views.



a) “pet otter”

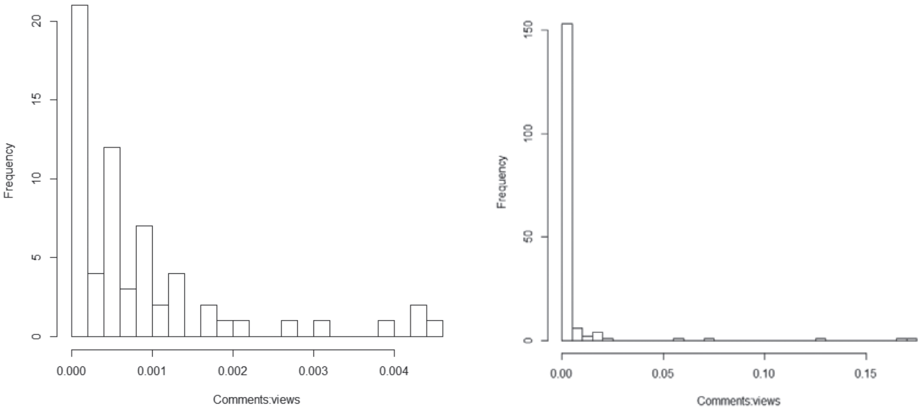
b) “berang berang” (Indonesian)



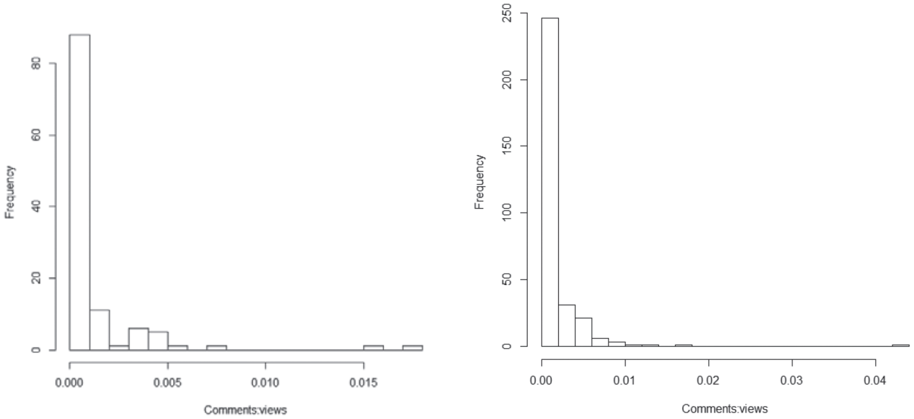
c) “นาก” (Thai)

d) “ペットのカワウソ“ (Japanese)

Figure A2. Like:view ratios.



a) “pet otter” b) “berang berang” (Indonesian)



c) “นาก” (Thai) d) “ペットのカワウソ“ (Japanese)

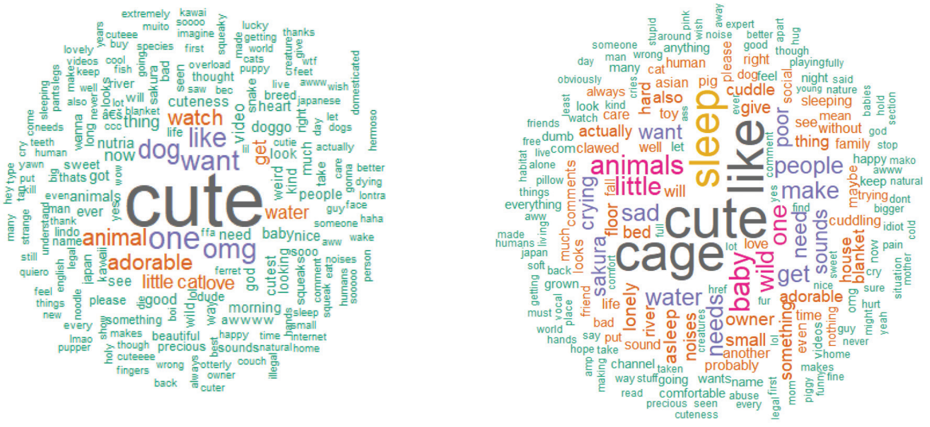
Figure A3. Comment:view ratios.

Appendix 2

Phrases containing the term one, interpreted as indicating some desire for obtaining a pet otter

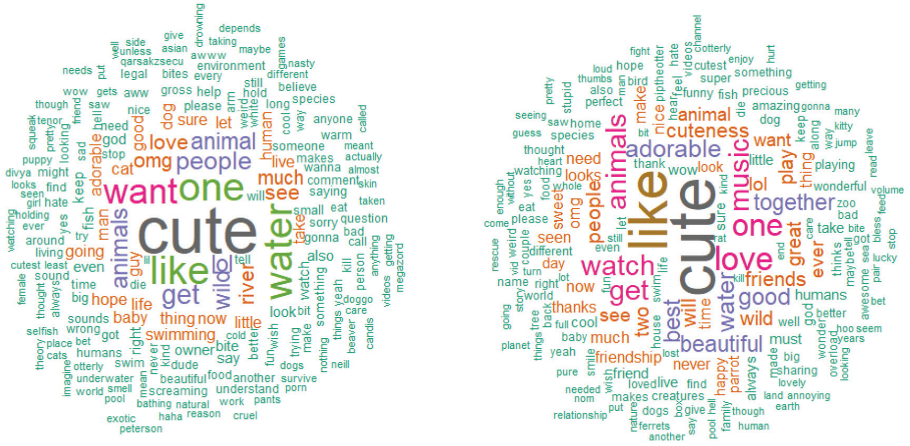
I want one, where do I get one, how can I get one, I need one, wish I had one, how do I go about retrieving one, can I have one, why doesn’t everyone have an otter, how does one go about getting an otter in Japan, how do you own one of these guys, can you have one of those as a pet, I’m gonna get one, I’d get one [if I could].

Appendix 3



a) Video 1

b) Video 3



c) Video 4

d) Video 5

Figure A4. Wordclouds depicting the nature of comments for four pet otter influencer videos (Videos 1, 3, 4, and 5 in Table 2). "Influencer video' defined as the most viewed and most popular videos with > median views, and > median like:view ratios. Font size is relative to the frequency at which each of the words appears in the comments; stopwords and profanities have been removed (see Methods).

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Remediation potential of early successional pioneer species *Chenopodium album* and *Tripleurospermum inodorum*

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Abstract

Remediation with plants is a technology used to decrease soil or water contamination. In this study we assessed the remediation potential of two weed species (*Chenopodium album* and *Tripleurospermum inodorum*) in a moderately metal-contaminated area. Metal concentrations were studied in roots, stems and leaves, in order to assess correlations in metal concentrations between those in soil and plants. Furthermore, we calculated bioaccumulation factor (BAF), bioconcentration factor (BCF) and translocation factor (TF) values to study the accumulation of metals from soil to plants and translocation within plants. We found correlation in metal concentrations between soil and plants. The metal accumulation potential was low in both species, indicating low BAF and BCF values. In contrast, high TF values were found for Mn, Ni, Sr, Zn, Ba, Fe, Cu and Pb in *C. album*, and for Fe, Mn, Ni, Zn and Sr in *T. inodorum*. Our results demonstrated that the potential of *C. album* and *T. inodorum* might be limited in phytoextraction processes; however, when accumulated, metals are successfully transported to aboveground plant organs. Thus, to achieve the efficient remediation of metal-contaminated soils, removal of the aboveground plant organs is recommended, by which soil disturbance can also be avoided.

Keywords
bioaccumulation, trace elements, phytoextraction, pollution, translocation

1. Introduction

Unsustainable land use leads to the qualitative and quantitative deterioration of soils, which is an urgent worldwide problem (Mills et al. 2006; Luo et al. 2009; Hoefer et al. 2015). This usually induces the alteration of the macro- and microelemental composition and concentrations of elements in the soil (Ishikawa et al. 2014). Elevated concentrations of potentially toxic metals are known to alter several physical and chemical properties of the growing media and also pose a high risk to organisms (e.g. plants) that are associated with it (Bes et al. 2010; Mahar et al. 2016; Ren et al. 2018a, b). In response, plants could also have elevated metal concentrations in their organs (Del Río-Celestino et al. 2006). The method that utilizes the inherent potential of plants to reduce environmental risk is called phytoremediation (Cunningham and Berti 1993; Flathman and Lanza 1998; Pilon-Smits 2005). Previous studies have revealed that several plants are able to accumulate metals in uncommonly high concentrations. Utilizing this physiological feature, the most successful metal accumulator species are used to extract metals from the contaminated media, thus decreasing the degree of contamination. This type of remediation is called phytoextraction (Dickinson et al. 2009; Zimmer et al. 2009). Moreover, there are other methods of phytoremediation, depending on plant-contaminant interactions (Mahar et al. 2016). Compared to conventional remediation methods, phytoremediation is a low-cost and cost-effective method with a low negative impact on the environment and which establishes long-term green cover in the contaminated area (Vangronsveld et al. 2009).

Field application for phytoextraction purposes usually involves fast growing species characterized by high biomass production (French et al. 2006; Van Nevel et al. 2011; Kacálková et al. 2015; Salam et al. 2019). The metal accumulation potential of plants from natural vegetation was also studied in previous experiments (Brekken and Steinnes 2004; Petřík et al. 2009; Irshad et al. 2015; Bandiera et al. 2016). In natural successions, herbaceous species are reported to play an important role as initial vegetation in terms of phytoremediation, mainly due to their good extraction and degradation rates (Olson et al. 2003). In the later periods of remediation Olson et al. (2003) suggested the use of trees as successfully applicable plants in soils pre-remediated by grasses. Leewis et al. (2013) also demonstrated that natural herbaceous vegetation has good remediation potential and even more significant importance than species used in later remediation phases. Additionally, Malik et al. (2010) highlighted that certain grasses can be used more successfully in phytoremediation processes than trees due to their high growth and biomass production rate. Girdhar et al. (2014) also found that numerous weeds exhibit high levels of inherent metal tolerance. Due to their high colonization ability they are often abundant even in contaminated areas without having harmful effects on the local plant diversity.

Among weeds with these characteristics, information on metal accumulation in Chenopodiaceae species has been widely reported. In this family, annual, stress tolerant *Chenopodium album* (L.) (Lamb's quarters) is one of the most studied species (Hu et al. 2012). Parisien et al. (2015) demonstrated that *C. album* had low bioaccumulation factor (BAF) values regarding soil Cd concentrations. Gupta and Sinha (2008) highlighted that *C. album* had remarkable Fe, Mn, Cd, Cr, Cu, Ni, Pb and Zn accumulation potential, in some cases with bioconcentration factor (BCF) values over 100. Bhargava et al. (2008) also reported that BCF values were high for Fe, Cd and Cu in 13 different accessions of *C. album* and thus recommended application for phytoextraction purposes. Remediation potential can be further increased by adjusting the most optimal row spacing. For *C. quinoa*, Bhargava et al. (2007) found that the highest biomass and thus the highest rate of metal uptake could be reached by sowing at 20–25 cm spacing. In plantations with a low number of individuals manual removal of plants is an efficient harvesting method; in addition, Präger et al. (2018) suggested the use of a combine harvester as a practical method on extensive *C. quinoa* cultivars. Furthermore, when aiming to maximize yield and extraction efficiency by *Chenopodium* species, sowing time is also a major factor to consider (Isobe et al. 2016).

Tripleurospermum inodorum (L.) Sch. Bip. (Scentless mayweed) is an annual, and in some regions overwintering species (Kay 1994). The Cu-binding capacity of *T. inodorum* root exudates was analyzed under hydroponic conditions (Dousset et al. 2001). This species has not yet been used in comprehensive studies assessing metal accumulation potential on contaminated soils. Several studies are available on the related *Matricaria* species, primarily on *Matricaria chamomilla* and *Matricaria recutita* (Armendaríz et al. 2014; Stanojkovic-Sebic et al. 2015). With regards to applicability in phytoextraction, *M. recutita* was found to be a useful metal accumulator species (Radulescu et al. 2013). Unfortunately, the taxonomical classification of species within the *Tripleurospermum* and *Matricaria* genera was controversial and could be used differently by different authors (Applequist 2002). For instance, *Tripleurospermum inodorum* (L.) Sch. Bip. was often referred to as *Matricaria inodora* L. (Samatadze et al. 2014).

The aim of this study was to analyze the metal accumulation and translocation potential of two common, early successional pioneer weeds, *Chenopodium album* and *Tripleurospermum inodorum*, grown in moderately metal-contaminated soils. We explored the difference between the species in terms of their metal concentrations among plant organs and among the differently contaminated parts of the study area. Moreover, accumulation and translocation factors were used to evaluate whether the species, or any of their plant organs (root, stem, shoot (= stem + leaf) and leaf) were capable of accumulating metals in high concentrations. We hypothesized that *C. album* would show excellent remediation potential (high BAF, BCF and TF values). Based on the metal accumulation characteristics of related species, *T. inodorum* was also expected to have good remediation potential.

2. Materials and methods

2.1. Study area

The study area was in the suburban area of Debrecen, Hungary. For the period between 1971 and 2000 the average annual temperature was 10.0 °C, the average annual rainfall was 549 mm and the average annual sunshine duration slightly exceeded 2000 hours (OMSZ n.d.). The 26 ha study area (Lovász-zug, 47°29.0'N; 21°47.3'E) used to function as a series of settling ponds in the communal wastewater treatment process of the city. Secondary biological purification was performed in the area from the 1930s until the 1950s, which was later supplemented by physical treatment. In the initial years of operation, earth deflector walls were formed to facilitate the wastewater stream; thus, the efficiency of purification was greatly increased. From the early 1970s, secondary treatment remained as the only function of the pond system, due to the establishment and continuous development of a modern wastewater treatment plant. The pond system had ceased to operate by the early 2000s (Tőzsér et al. 2018). During the last decades of operation, the study area had been loaded with variable amounts of sewage sludge, contaminated with several macro- and microelements, such as toxic metals.

2.2. Soil sampling and analysis

Soil samples were collected with a 50-mm Dutch soil auger from the three differently contaminated parts of the study area (northern – moderately contaminated part 1; middle – strongly contaminated part 2; southern – moderately contaminated part 3, after Tőzsér et al. (2018); contamination levels were determined by Nagajyoti et al. (2010) and Tóth et al. (2016)) in late September 2015. We selected the soil sampling date to be in alignment with plant sampling near the end of the vegetation period. To spatially arrange the samples, a stratified random design was used. Within each soil core, eight subsamples were collected from increasing depth ranges (0–10, 11–20, 21–30, 31–40, 41–50, 51–60, 61–70, 71–80 cm). Samples (N = 216) were put into plastic packages and stored in them at + 4 °C until laboratory work.

To measure the pH of soil solutions, soil samples (5 g) were put into plastic tubes, complemented with 20 ml of deionized water, shaken and left to settle overnight. Then, pH values were determined with a Hach HQ 40d portable multimeter. For the elemental analysis, we homogenized air-dried soil samples (0.2 g with accuracy of 0.005 g) with agate mortar, put into 100-ml glass beakers and dried at 105 °C overnight. Samples were digested in 4 ml 65% (m/m) HNO_3 and 0.5 ml 30% (m/m) H_2O_2 on hot plates until total evaporation of the chemicals. Then, 5 ml of 3×-deionized water was added to the dried samples. Prior to pouring the solution into plastic tubes, we put glass beakers into an ultrasonic water bath to yield the sample residues which adhered to beaker walls. Then, samples were diluted to 10 ml using 1% (m/m) nitric acid. The following elements were analyzed with MP-AES (Microwave Plasma-Atomic Emission Spectrometry): Al, Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Sr and Zn. These elements are of various levels of environmental concern; we refer to them as "metals" throughout the study, based on Ali and Khan (2018). All concentration values refer to dry weight basis.

2.3. Plant sampling and analysis

Plant individuals were collected from the differently contaminated parts of the study area during early September 2015. We selected the plant sampling date to assess the metal accumulation potential of the species by the end of the vegetation period. Five individuals of *C. album* and *T. inodorum* were collected from a radius of 10 meters around each soil core. We put all samples into plastic packages and stored them at +4 °C until the laboratory process. In the laboratory, individuals were washed and plant organs such as roots, stems and leaves were separated. Each plant organ was air-dried in a paper bag for 24 hours. After this, plant organs were dried at 60°C for 48 hours.

Prior to elemental analyses, 0.2 g of plant samples (with an accuracy of 0.005 g) were homogenized with agate mortar. Then, plant samples were digested in 4 ml 65% (m/m) HNO_3 and 0.5 ml 30% (m/m) H_2O_2. We put the solutions into glass beakers and supplemented them with 3×-deionised water to a quantity of 25 ml. After this, the solutions were put into plastic centrifuge tubes. The following metals were analyzed with MP-AES: Al, Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Sr and Zn.

2.4. Bioaccumulation factor (BAF), bioconcentration factor (BCF) and translocation factor (TF)

We used the bioaccumulation factor (BAF), bioconcentration factor (BCF) and translocation factor (TF) as widely applied indicators in assessing the remediation potential of plant species. For these factors, calculations were made for Al, Fe, Mn, Ba, Cr, Cu, Ni, Pb, Sr and Zn. The bioaccumulation factor refers to the ratio of metal concentration in shoots (C_{shoot}) and metal concentration in soil (C_{soil}) (Li et al. 2007; Rezvani and Zaefarian 2011):

$$BAF = C_{shoot}/C_{soil}$$

The bioconcentration factor refers to the ratio of metal concentration in selected plant organs (also calculated for stem and leaves separately) ($C_{plant\ organ}$) and metal concentration in the soil (C_{soil}) (Ndeda and Manohar 2014):

$$BCF = C_{plant\ organ}/C_{soil}.$$

The translocation factor refers to the ratio of metal concentration in selected aboveground plant organs ($C_{aboveground\ plant\ organ}$) and metal concentration in roots (C_{roots}) (Cui et al. 2007; Mellem et al. 2009; Malik et al. 2010):

$$TF = C_{aboveground\ plant\ organ}/C_{roots}.$$

2.5. Statistical analysis

For the statistical analyses the natural logarithms (ln) of the concentration values were used. Levene's Test was used for analyzing the homogeneities of variances. The Generalized Linear Model (GLM) was used to test significant differences ($p < 0.05$) between the metal concentrations (I) of plants, (II) of plant organs and (III) of parts of the study area with different contamination levels. To study the differences in metal concentration between plant organs, we used a principal component analysis (PCA). To analyze the correlation in metal concentrations between soil samples and plant organs, Pearson's correlation coefficient (r) was calculated and significance was assessed at the 0.05 level.

3. Results

3.1. Metal concentrations in plants among the differently contaminated parts of the study area, plant organs and plant species

The Generalized Linear Model showed that there were significant differences ($p < 0.05$) in Al, Ca, Fe, K, Mg, Mn, Na, Ba and Zn among the differently contaminated parts of the study area. Soil pH did not differ significantly between the three parts of the study area (Table 1). We found significant differences in Al, Ca, Fe, K, Mg, Mn, Na, Ba, Cr, Cu, Ni, Pb, Sr and Zn concentrations among plant organs. Regarding the differences between *C. album* and *T. inodorum*, concentrations of K and Mg were significantly higher in *C. album*, while concentrations of Al, Fe, Mn and Na were significantly higher in *T. inodorum*. Furthermore, mean concentrations of Ba, Cr, Cu, Ni and Pb were higher in *T. inodorum*, while Sr and Zn concentrations were slightly higher in *C. album*; however, these differences were not significant (Suppl. material 1: Tables S1, S2).

3.2. Metal concentrations in *C. album*

There were significant differences in metal concentrations in plant organs of *C. album* among the differently contaminated parts of the study area (Table 2 and Suppl. material 1: Table S3). Metal concentrations were also different among the plant organs of *C. album* based on principal component analysis (PCA) (Fig. 1).

Table 1. Soil pH and metal concentrations in samples from the study area (mean ± SE).

	Part of the study area		
	Northern	**Middle**	**Southern**
pH	7.7 ± 0.1	7.7 ± 0.1	7.8 ± 0.1
Al	5.3 ± 0.2	5.6 ± 0.2	5.7 ± 0.2
Ba	70.1 ± 5.6	65.6 ± 4.1	42.6 ± 1.7
Ca	31.4 ± 2.3	28.2 ± 2.9	19.5 ± 2.2
Cd	0.7 ± 0.1	1.4 ± 0.7	0.7 ± 0.1
Cr	129 ± 21	303 ± 143	63.3 ± 41.8
Cu	35.3 ± 7.2	49.3 ± 13.4	17.6 ± 4.8
Fe	10.6 ± 0.4	11.6 ± 0.6	11.2 ± 0.6
K	1.1 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
Mg	6.2 ± 0.4	5.9 ± 0.3	5.2 ± 0.6
Mn	396 ± 26	380 ± 11	339 ± 20
Na	362 ± 27	363 ± 61	244 ± 24
Ni	23.8 ± 1.6	32.1 ± 5.5	25.8 ± 1.6
Pb	27.4 ± 4.1	44.3 ± 17.7	8.0 ± 4.3
Sr	88.9 ± 7.0	86.4 ± 7.6	55.8 ± 6.3
Zn	153 ± 23	192 ± 45	60.8 ± 15.3

Note: Concentrations of Ba, Cd, Cr, Cu, Mn, Na, Ni, Pb, Sr and Zn are shown in mg kg^{-1}, while concentrations of Al, Ca, Fe, K and Mg are shown in g kg^{-1}.

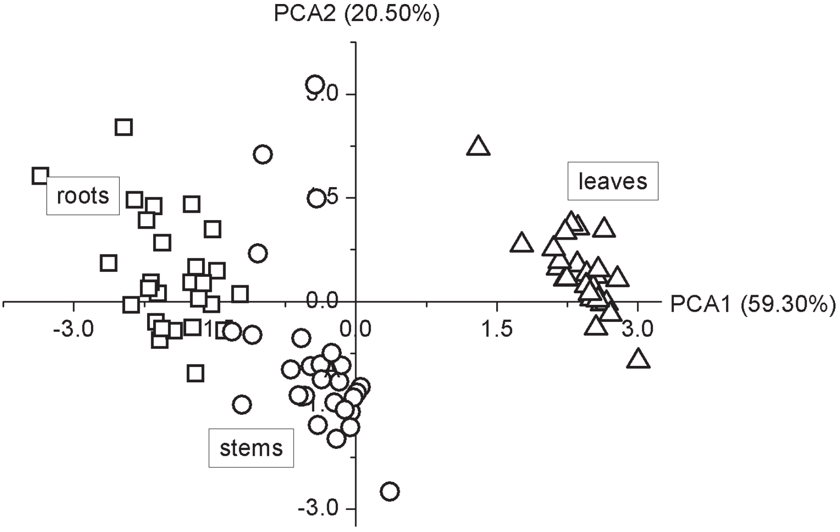

Figure 1. Principal component biplot of metal concentrations (mg kg^{-1}) of *Chenopodium album* in roots, stems and leaves. Notations: square – roots, circle – stems, triangle – leaves.

In the cases of Mg, Mn and Zn, leaves accumulated metals in significantly higher concentrations than roots and stems in all the three parts of the study area. In the cases of Fe and Cu, leaves accumulated significantly higher concentrations of metals than roots and stems only in the strongly contaminated middle part. In the cases of Ca, K and Sr,

Table 2. Metal concentrations in plant organs of *Chenopodium album* among the three parts of the study area (mean ± SE).

	Part of the study area								
	Northern			Middle			Southern		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Al	5.0 ± 0.6	2.0 ± 0.54	3.5 ± 0.6	6.4 ± 2.1	1.1 ± 0.2	2.9 ± 0.3	17.6 ± 3.9	1.2 ± 0.2	5.2 ± 1.8
Ba	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.02	0.1 ± 0.01	0.3 ± 0.02	0.2 ± 0.03	0.1 ± 0.01	0.2 ± 0.02
Ca	0.01 ± 0.01	0.2 ± 0.02	0.9 ± 0.03	0.08 ± 0.01	0.3 ± 0.1	1.0 ± 0.06	0.07 ± 0.01	0.1 ± 0.02	0.9 ± 0.09
Cr	0.1 ± 0.01	0.03 ± 0.01	0.1 ± 0.02	0.1 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	0.1 ± 0.03	0.02 ± 0.01	0.03 ± 0.01
Cu	0.4 ± 0.02	2.0 ± 1.7	0.5 ± 0.02	0.4 ± 0.01	0.3 ± 0.01	0.5 ± 0.02	0.4 ± 0.01	0.4 ± 0.01	12.2 ± 11.7
Fe	4.9 ± 0.6	5.4 ± 3.3	6.6 ± 0.5	5.9 ± 1.8	2.1 ± 0.2	6.5 ± 0.5	15.0 ± 3.4	2.0 ± 0.2	29.6 ± 23.6
K	0.9 ± 0.04	2.2 ± 0.1	2.9 ± 0.09	1.0 ± 0.03	2.6 ± 0.2	3.1 ± 0.08	1.0 ± 0.05	2.6 ± 0.2	3.0 ± 0.1
Mg	0.1 ± 0.01	0.1 ± 0.01	1.0 ± 0.04	0.1 ± 0.01	0.1 ± 0.03	0.9 ± 0.03	0.1 ± 0.01	0.1 ± 0.02	1.2 ± 0.06
Mn	0.5 ± 0.03	0.5 ± 0.03	1.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.04	1.7 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	4.9 ± 1.5
Na	0.04 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	0.1 ± 0.1	0.01 ± 0.0	0.04 ± 0.02	0.03 ± 0.02	0.01 ± 0.01
Ni	0.03 ± 0.01	0.02 ± 0.01	0.1 ± 0.1	0.04 ± 0.01	0.1 ± 0.04	0.04 ± 0.01	0.1 ± 0.02	0.03 ± 0.01	0.04 ± 0.01
Pb	0.1 ± 0.01	0.1 ± 0.01	0.03 ± 0.01	0.1 ± 0.04	0.1 ± 0.01	0.03 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
Sr	0.9 ± 0.04	1.6 ± 0.1	2.8 ± 0.1	0.8 ± 0.04	1.8 ± 0.2	3.0 ± 0.2	1.0 ± 0.1	1.6 ± 0.1	3.2 ± 0.2
Zn	1.7 ± 0.1	1.5 ± 0.1	5.9 ± 0.3	1.6 ± 0.1	1.4 ± 0.1	5.7 ± 0.5	1.3 ± 0.1	1.1 ± 0.1	2.8 ± 0.2

Note: Concentrations of Al, Ba, Cr, Cu, Fe, Mn, Ni, Pb, Sr and Zn are shown in mg kg^{-1}, while concentrations of Ca, K, Mg and Na are shown in g kg^{-1}.

leaves accumulated the highest concentrations of metals, as well; however, we found significant differences between leaves and stems and also between stems and roots. In the cases of Al and Ba, leaves and roots accumulated metals in significantly higher concentrations than stems. In the cases of Cr and Ni, accumulations in leaves and roots were comparable. Cd concentrations were always below the detection limit; thus, this metal was excluded from further analyses. In the cases of Na and Pb, significant differences were not found among plant organs within the parts of the study area (Suppl. material 1: Table S3).

We also studied metal concentrations in selected plant organs among the differently contaminated parts of the study area. We found higher metal concentrations in the southern part compared to the northern and middle parts of the study area (Table 2 and Suppl. material 1: Table S4). In the southern part of the study area, root Al, Fe, Mn and Ni concentrations were significantly higher than in the northern and middle parts of the area. Root Mg and Ba concentrations were significantly different between the southern and the middle, and between the southern and the northern parts. We observed a significantly higher concentration of Zn in all plant organs in the northern than in the southern part. The middle part did not differ significantly from the other two parts of the study area. Root Ca, K, Na, Cr, Cu and Sr concentrations did not differ significantly among the three parts of the study area.

3.3. Metal concentrations in *T. inodorum*

We also found significant differences in metal concentrations in plant organs of *T. inodorum* among the differently contaminated parts of the study area (Table 3 and Suppl.

Table 3. Metal concentrations in plant organs of *Tripleurospermum inodorum* among the three parts of the study area (mean ± SE).

	Part of the study area								
	Northern			Middle			Southern		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Al	19.5 ± 2.8	7.2 ± 2.0	11.0 ± 1.3	37.4 ± 6.9	7.5 ± 1.3	6.8 ± 0.9	28.3 ± 3.4	6.6 ± 0.8	16.2 ± 5.1
Ba	0.4 ± 0.03	0.2 ± 0.02	0.2 ± 0.01	0.7 ± 0.1	0.3 ± 0.02	0.2 ± 0.01	0.5 ± 0.1	0.3 ± 0.02	0.2 ± 0.02
Ca	0.2 ± 0.02	0.1 ± 0.01	0.8 ± 0.3	0.2 ± 0.02	0.2 ± 0.02	0.6 ± 0.01	0.2 ± 0.03	0.1 ± 0.02	0.6 ± 0.06
Cr	0.2 ± 0.04	0.2 ± 0.04	0.1 ± 0.02	0.4 ± 0.1	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.03	0.1 ± 0.01	0.1 ± 0.02
Cu	0.6 ± 0.04	0.3 ± 0.02	0.8 ± 0.1	0.6 ± 0.1	0.3 ± 0.01	0.7 ± 0.03	0.5 ± 0.04	0.3 ± 0.04	22.2 ± 21.5
Fe	17.3 ± 2.5	7.1 ± 1.9	12.2 ± 1.2	32.6 ± 6.0	7.0 ± 1.1	9.2 ± 1.0	22.6 ± 2.6	5.8 ± 0.9	60.4 ± 47.8
K	0.7 ± 0.07	0.6 ± 0.05	1.5 ± 0.1	0.9 ± 0.04	0.6 ± 0.05	1.8 ± 0.05	0.7 ± 0.04	0.7 ± 0.04	1.6 ± 0.1
Mg	0.1 ± 0.01	0.03 ± 0.01	0.3 ± 0.06	0.1 ± 0.01	0.04 ± 0.01	0.2 ± 0.03	0.1 ± 0.01	0.04 ± 0.01	0.2 ± 0.01
Mn	1.5 ± 0.2	1.4 ± 0.2	3.6 ± 0.6	2.5 ± 0.3	3.2 ± 0.6	4.9 ± 0.5	3.1 ± 0.4	3.8 ± 0.7	9.6 ± 1.3
Na	0.2 ± 0.01	0.1 ± 0.01	0.3 ± 0.3	0.1 ± 0.01	0.03 ± 0.01	0.1 ± 0.07	0.2 ± 0.02	0.1 ± 0.01	0.02 ± 0.01
Ni	0.1 ± 0.01	0.1 ± 0.1	0.1 ± 0.01	0.2 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.02
Pb	0.2 ± 0.1	0.1 ± 0.01	0.1 ± 0.03	0.1 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.1 ± 0.01	0.03 ± 0.01	0.1 ± 0.01
Sr	1.7 ± 0.1	1.2 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.9 ± 0.2
Zn	2.6 ± 0.2	1.9 ± 0.2	2.6 ± 0.4	2.9 ± 0.2	2.7 ± 0.3	2.9 ± 0.3	1.4 ± 0.1	1.4 ± 0.1	1.7 ± 0.2

Note: Concentrations of Al, Ba, Cr, Cu, Fe, Mn, Ni, Pb, Sr and Zn are shown in mg kg-1, while concentrations of Ca, K, Mg and Na are shown in g kg-1.

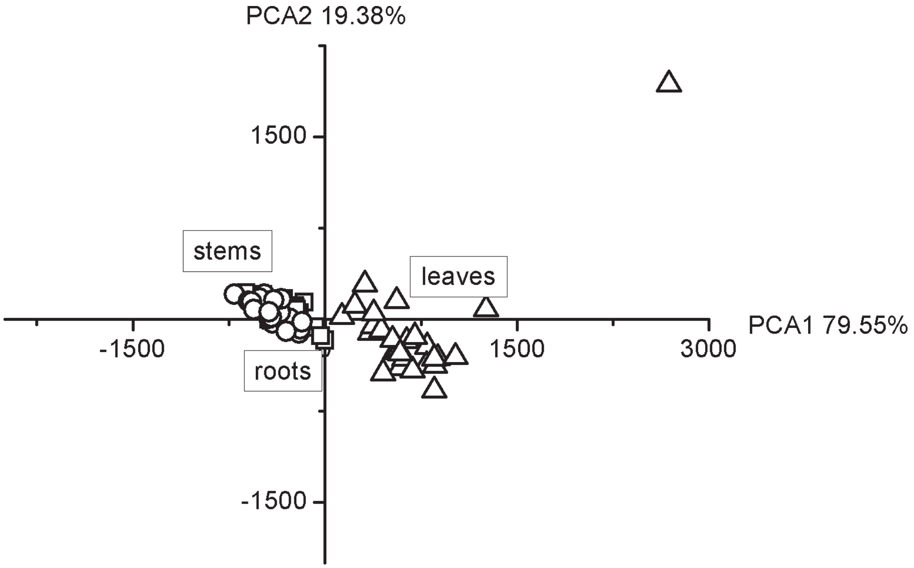

Figure 2. Principal component biplot of metal concentrations (mg kg^{-1}) of *Tripleurospermum inodorum* in roots, stems and leaves. Notations: square – roots, circle – stems, triangle – leaves.

material 1: Table S5). Based on PCA scores, the metals in leaves differed from those in roots and stems of *T. inodorum* (Fig. 2).

Concentrations of Al, Ba, Cr and Pb were significantly the highest in roots, while concentrations of Ca, K, Mg and Mn were the highest in leaves. In the cases of Fe, Na, Cu, Ni and Sr, the highest concentrations were accumulated in roots and leaves. We found the lowest concentrations of these metals in stems. The concentration of Zn was comparable in all the plant organs, with only negligible differences among them. The concentration of Cd was below the detection limit in all of the cases; thus, this metal was excluded from further analyses.

Generally lower concentrations were found in the northern part compared to the middle and southern parts of the study area (Table 3 and Suppl. material 1: Table S6). In the middle part of the study area, roots accumulated the highest concentrations of Al, K and Ba, whereas leaves accumulated the highest concentrations of Zn. We found the highest Mn concentration in the southern part of the study area regarding all plant organs. Root concentrations of Na and Ni were significantly the highest in the northern and the southern, and in the southern and the middle parts of the study area, respectively. Based on the selected plant organs, Ca, Mg, Cr, Cu, Pb and Sr concentrations did not differ significantly between the differently contaminated parts of the study area.

3.4. Correlations between soil metal concentrations and their accumulation in plants

3.4.1. Correlations between soil metal concentrations and their accumulation in *C. album*

In the northern part of the study area, no significant correlation was found in metal concentrations between the soil and the plant organs of *C. album* (Suppl. material 1: Table S7). In the middle part of the study area, we did not find any negative correlation in the concentrations of Al ($r = -0.700$, $p = 0.036$) and Fe ($r = -0.683$, $p = 0.042$) between the soil and roots and for Pb between the soil and stems ($r = -0.798$, $p = 0.010$). In the southern part of the study area, positive correlations were found in the cases of Cr ($r = 0.733$, $p = 0.025$) and Ni ($r = 0.733$, $p = 0.025$) between the soil and roots. We found negative correlations in the concentrations of Al ($r = -0.783$, $p = 0.013$), Mg ($r = -0.717$, $p = 0.030$) and Ba ($r = -0.731$, $p = 0.025$) between the soil and stems.

3.4.2. Correlations between soil metal concentrations and their accumulation in *T. inodorum*

In the northern part of the study area, Fe concentrations of soil and stems were positively correlated ($r = 0.683$, $p = 0.042$) (Suppl. material 1: Table S8). In the middle part of the study area we found negative correlations in Cu concentrations ($r = -0.883$, $p = 0.002$) between the soil and roots and in Ca concentrations ($r = -0.717$, $p = 0.030$) between the soil and stems. Leaf and soil Sr ($r = 0.733$, $p = 0.025$) concentrations were positively correlated. In the southern part of the study area, soil and root Al concentrations were negatively correlated ($r = -0.667$, $p = 0.050$).

3.5. Metal accumulation and translocation potential in plants

3.5.1. Bioaccumulation factor (BAF), bioconcentration factor (BCF) and translocation factor (TF) values for *C. album*

Bioaccumulation factor (BAF) values were lower than 1 for all metals, which indicates that accumulation was not found in *C. album* (Suppl. material 1: Table S9). Likewise, bioconcentration factor (BCF) values were low in all plant organs (Suppl. material 1: Table S10), which indicated that metal accumulation from soil was also restricted. Some metals had high translocation potential between roots and aboveground plant organs of *C. album* (Suppl. material 1: Table S11). TF values were higher than 1 for Fe, Mn, Ba, Cu, Ni, Pb, Sr and Zn. Very high TF values (> 3) were also demonstrated in leaves in the northern (4.7 for Ni, 3.6 for Zn, 3.4 for Sr), in the middle (3.8 for Sr, 3.7 for Zn, 3.5 for Mn) and in the southern parts of the study area (4.1 for Mn, 3.4 for Sr). In general, compared to roots, accumulation was higher in leaves than in stems. The Pb accumulation in stems was higher than in leaves in all the three parts of the study area, and Ni and Cr in the middle part of the area. We did not observe major differences in TF values among the differently contaminated parts of the study area.

3.5.2. Bioaccumulation factor (BAF), bioconcentration factor (BCF) and translocation factor (TF) values for *T. inodorum*

Corresponding to *C. album*, bioaccumulation factor (BAF) values and bioconcentration factors (BCF) were lower than 1 for *T. inodorum* (Suppl. material 1: Table S9, S10). Translocation factors (TF) were higher than 1 for several metals between roots and aboveground plant organs of *T. inodorum* (Suppl. material 1: Table S11). TF values were higher than 1 for Fe, Mn, Cu, Ni, Sr and Zn. Very high TF values (> 3) were only found in leaves in the southern part of the study area (5.0 for Fe, 3.3 for Mn). Compared to roots, accumulation was slightly higher in leaves than in stems in the middle and southern parts of the study area, while in the cases of Al, Fe, Ba, Cr, Ni and Pb, higher TF values were found in stems than in leaves in the northern part. We did not find significant differences in TF values among the three parts of the study area in the cases of stems, while TF values in leaves were only slightly different among the differently contaminated parts of the study area (southern > middle > northern).

4. Discussion

4.1. Metal concentrations in plants

In the literature discussing environmental pollution, metals are usually classified as (i) trace metals which strongly exceed critical values and (ii) toxic metals which are known as strong contaminants (Kabata-Pendias and Mukherjee 2007). To determine the level of soil contamination, the threshold values of the Hungarian Governmental Regulation 10/2000. (VI.2.) (2000), Ministry of the Environment Finland (2007) and Tóth et al. (2016) are available for each metal. Cr and Zn concentrations were higher than the threshold values. Cd concentrations were below the detection limit; thus, the metal was not a contaminant in the study area. The Pb and Sr could be potentially risky due to their inherent toxic characteristics; however, their level of contamination was moderate. Based on these, we focused on Cr, Pb and Zn concentrations in soil and plants. Other metals were either not contaminants or their concentrations were too low to be contaminants.

4.1.1. Metal concentrations in *C. album*

We demonstrated that metal concentrations were very low in plant organs of *C. album*. In a previous study Gupta and Sinha (2007) reported higher Cr, Pb and Zn concentrations in plant organs of *C. album* than we did in our study. Similarly to our findings, the authors also demonstrated higher Cr and Pb concentrations in leaves than in roots and stems, but the highest Cu and Zn concentrations were in roots. Studying metal accumulation in *Chenopodium botrys*, Nouri et al. (2009) indicated higher metal concentrations than in our study and found higher Zn concentration in roots compared to shoots. Malik et al. (2010) reported higher Cr, Pb and Zn concentrations in *C. album* than those in this study. They observed higher Cr concentrations in roots than in shoots, but higher Zn concentrations in shoots than in roots. We observed insignificant differences in Cr accumulation among plant organs. As presented in our study, Malik et al. (2010) found similar Pb concentrations in the two plant organs. Nazir et al. (2011) found similar Zn concentrations in soil to concentrations found in this study, but their results showed higher Zn concentrations in the shoots than in the roots of *C. album*. Similarly, we detected significantly higher Zn concentrations in leaves than in roots. To sum up, we found generally lower metal concentrations in organs of *C. album* than reported in the relevant literature, while the distribution of metals within plants was quite varied, as in previous papers.

4.1.2. Metal concentrations in *T. inodorum*

We found that metal concentrations were also very low in plant organs of *T. inodorum*. However, in some cases, we observed significant differences among plant organs even in low concentration ranges. Geneva et al. (2014) found higher Pb and Zn concentrations in roots and shoots of *M. recutita* plants than presented in our paper for *T. inodorum*. A higher Pb concentration was also reported by Lydakis-Simantiris et al. (2012), who also demonstrated that roots accumulated much higher concentrations of the metal than leaves of *M. recutita*, which is a specific characteristic of Pb. In the cases of Pb and Zn, we did not find significant differences in metal concentrations among plant organs. Low concentrations of Pb could arise from the low mobility and insoluble Pb-complexes in the root zone (Brennan and Shelley 1999). In contrast to our results, the authors found higher Cr concentrations in roots than in stems in each of the five studied species. As a general finding, metal concentrations in *T. inodorum* were lower than presented in the literature for related species.

4.2. Correlations between soil metal concentrations and their accumulation in plants

We found a significant negative correlation in Pb concentrations between the soil and stems of *C. album*. Yoon et al. (2006) also demonstrated a negative correlation in Pb concentrations between the soil and several species of natural vegetation, which was attributed to the inherent low mobility of the metal and basic pH conditions. In contrast to our findings, Jung (2008) found that there were positive correlations in Pb concentrations between the soil and in the plant organs of six crop plant species. Further, by increasing soil concentrations Liang et al. (2016) demonstrated increasing Pb concentrations in plants. We demonstrated significant positive correlations in Cr concentrations between the soil and the roots of *C. album*. In accordance with our findings, Samantaray et al. (2001) demonstrated a significant positive correlation in Cr concentrations between the soil and the roots of four tree species. In a study involving several plant species, including *C. chenopodioides*, Galfati et al. (2011) indicated positive correlations in the concentrations of Cr and Zn between the soil and the accumulation of these metals in plant organs. Despite being quite insoluble, Cr can enter plants easily via ion channels, while essential Zn is readily taken up in concentrations proportional to soil contamination levels.

In the case of *T. inodorum* we found a significant positive correlation in Sr concentrations between the soil and leaves. Lydakis-Simantiris et al. (2012) reported that increasing soil Pb concentrations resulted in elevated Pb concentrations in the roots of *M. recutita* individuals, which was explained by the generally good accumulation potential of the family and by the restricted translocation potential of Pb from roots to aboveground plant organs. In contrast, we did not find significant correlations in Pb concentrations between the soil and plant organs. Greger (1999) found no correlation between soil and *Arrhenatherum pratensis* shoots for Pb and Zn concentrations, mainly due to soil conditions and the formation of complexes that did not favor good bioavailability of metals. Grejtovský et al. (2006) reported that increasing soil Zn concentrations (control, 50, 150 and 300 mg kg^{-1}, respectively) increased Zn concentrations in *M. chamomilla* shoots significantly. Our results indicated that soil metal concentrations did not have any major influence on metal concentrations in plant organs of *C. album* and *T. inodorum*, induced primarily by the relatively high pH values. Nouri et al. (2009) presented similar results, highlighting that plant metal concentrations were basically not influenced by those in the soil, which is the result of the detoxification and exclusion mechanisms developed by certain species. The poor contamination-indicating potential of plant species was also emphasized by Kabata-Pendias and Pendias (1992). According to the Yoon et al. (2006), metal bioavailability, plant-bacteria interactions and several soil parameters could influence metal accumulation jointly. In addition, we found that the multi-contaminated soil of our study area could trigger the uptake of contaminants via interactions between metals (Grejtovský et al. 2006).

4.3. Metal accumulation and translocation potential in plants

4.3.1. Metal accumulation and translocation potential in *C. album*

Bioaccumulation factor (BAF) and bioconcentration factor (BCF) values were simultaneously very low in *C. album*, while translocation factor (TF) values were high in several cases. We found high (> 1) translocation factor (TF) values for Fe, Mn, Ba, Ni, Pb and Sr in the stems and for Fe, Mn, Ba, Cu, Ni, Pb, Sr and Zn in the leaves of *C. album*. In a study on the phytoextraction capacity of *C. album* Gupta and Sinha (2007) reported that leaves accumulated Fe in higher concentrations than roots and stems; thus, the highest TF values appeared for leaves. Similarly to these findings, we found significantly higher Fe concentrations in leaves than in stems, resulting in the highest TF values for leaves. We attributed this to the accumulation-favoring effect of sewage sludge found in deeper soil layers in the study area (Gupta and Sinha 2007). Malik et al. (2010) highlighted that the TF value for Zn was 1.3 for the leaves of *C. album*, which was lower than the values in our study (TF for Zn 2.2–3.7). In addition, Farrag et al. (2013) indicated that the TF values for Mn, Ni and Pb were higher than 1 in the aboveground plant organs of *Chenopodium ambrosioides*, which is similar to our findings. In contrast to our observations they demonstrated that the TF value for Cr was higher than 2. Similarly to what was presented in our study, Zehra et al. (2009) found higher Pb concentrations in aboveground plant organs than in roots (TF > 1) in the cases of three weed species. In contrast, Nazli Alipour et al. (2014) concluded that the accumulation potential of Pb in *C. album* was restricted; the authors demonstrated low (< 0.2) shoot TF values in uncontaminated and also in heavily Pb contaminated soils. It is likely that low mobility and soil organic matter could inhibit Pb accumulation, resulting in low concentrations and mobility in *C. album*. Manan et al. (2015) showed major differences in TF values for Cu, Pb and Zn in two medicinal plants; *Centella asiatica* had TF values higher than 1 for the three metals, which was similar to the results for *C. album* in our study. Furthermore, *Orthosiphon stamineus* had TF values lower than 1 for the three metals. The reported high TF values for Zn in our study could be explained by the fact that this metal is an essential nutrient for plants (Yoon et al. 2006). We detected a high TF value for Mn (4.1) in *C. album*, which is comparable to that presented by Lorestani et al. (2011). However, we found much higher TF values for Cu (5.5) and Fe (6.5) than reported by Lorestani et al. (2011). In comparison with nine other weeds Irshad et al. (2015) reported that *C. album* had similar or higher TF values for Cr and Zn. Furthermore, Gupta and Sinha (2008) demonstrated that among the five studied weeds *C. album* was the species that showed the highest translocation potential for Zn (TF 3.6) and Ni (2.7), while this species also had a very high translocation potential for Mn (8.6).

4.3.2. Metal accumulation and translocation potential in *T. inodorum*

Bioaccumulation factor (BAF) and bioconcentration factor (BCF) values were also very low for *T. inodorum*, while translocation factor (TF) values deserved attention. We found high (> 1) TF values for Mn, Cu, Ni and Zn in the stems and for Fe, Mn, Cu, Sr and Zn in the leaves of *T. inodorum*. Geneva et al. (2014) presented TF values higher than 1 for Zn (1.3) in *M. recutita* shoots, while lower than 1 for Cu (0.2–0.7) and Pb (0.05–0.30). Compared to these results, TF values for Zn were lower than those shown in our study (TF = 0.8–1.3). In accordance with our results, Lydakis-Simantiris et al. (2012) found low leaf TF values for Ni (0.2–0.3) and Pb (0.1–0.2) in *M. recutita*, indicating low translocation potential for the two metals. Similarly to data presented for stems and leaves in this paper, the average translocation factor value was the highest for Mn (5.3) in a study on Mn, Cr, Ni and Pb accumulations in five weeds conducted by Nwaedozie et al. (2015). They demonstrated higher TF values for Mn than we did in the case of *T. inodorum*; furthermore, they found TF values for Cr, Ni and Pb comparable to those in our paper. Average TF values for Mn (1.1–3.3) were similar to those found in several natural vegetation species by other authors. Studying *Cynodon dactylon*, Kumar et al. (2015) indicated TF values between 1 and 2 for Mn. In a study involving *Cannabis sativa*, *Rumex dentatus* and *Silybum marianum*, Zehra et al. (2009) presented highly varying TF values for Cu, Mn and Zn; however, these values regularly exceeded 1, thereby showing good remediation potential. Based on these findings, we stress that *T. inodorum* showed high levels of Cu-, Mn- and Zn-tolerance and good accumulation of these metals; this was also reported by Liu et al. (2006) for several plant species. In general, comparing the two species studied in this paper, we indicated that *C. album* had a more favorable translocation potential for the studied metals than *T. inodorum*.

4.3.3. Effects of metal interactions on translocation of metals in *C. album* and *T. inodorum*

Translocation factor (TF) values in *C. album* and *T. inodorum* are influenced by several soil parameters. Interactions between metals are typical not only in soils but also in plants. As a common phenomenon, based on their ionic radius, Cd and Zn are in competition for the binding sites located in the transport proteins; thus, they can hinder the accumulation and translocation of each other into and within plants (Hart et al. 1998; Papoyan et al. 2007). This statement was confirmed in our study, since translocation of Zn was much more intensive than that of Cd in both species. Sharma and Dubey (2005) and Pourrut et al. (2011) observed the same mechanism for both Pb^{2+} and K^+, indicating a competing interaction between the two cations for the same K-channel. It is also in accordance with our findings, since we found high TF values for Pb, while that for K remained very low in *C. album*. Furthermore, Fe^{2+}, Mn^{2+} and Zn^{2+} were also found to have identical transporters (Korshunova et al. 1999; Solti et al. 2011), which explains the equally high TF values for these metals presented for *C. album* and *T. inodorum*. It was previously found that Fe cations have the same transportation pathway within plants as Cr^{3+}, resulting in similar concentrations of the two metals (Solti et al. 2011). In contrast, we highlighted the great translocation potential for Fe and low TF values for Cr at the same time. Llugany et al. (2000) reported that Sr^{2+} interferes with K^+-metabolism, while there is an inhibiting interaction between Sr^{2+} and Ca^{2+}. We observed high TF values for Sr and low TF values for K, assuming an antagonist effect between the two metals within the two species. Similarly to Llugany et al. (2000), we demonstrated contrasting levels of translocation for Sr and Ca for both species. Based on these we found that ionic radius had a major influence in determining the translocation characteristics observed for *C. album* and *T. inodorum*.

5. Conclusions

The results of this study indicated that both *Chenopodium album* and *Tripleurospermum inodorum* showed low metal accumulation potential in the moderately contaminated study area. Comparing the two species, *T. inodorum* appeared to be a better accumulator of Al, Fe, Mn, Na, Ba, Cr and Ni, while *C. album* was a better accumulator of K, Mg and Sr. Metal concentrations in the two species were generally low. Bioaccumulation factor (BAF) and bioconcentration factor (BCF) values for metals were also low (BAF and BCF < 0.1). In contrast, translocation factor (TF) values were high (> 1) for Fe, Mn, Ba, Cu, Ni, Sr, Pb and Zn in *C. album* and for Fe, Mn, Cu, Sr and Zn in *T. inodorum*. We found that several factors, such as metal interactions and soil characteristics, could influence metal accumulation in plant organs causing a lower accumulation potential of the studied species than reported by the previous studies. Based on the high TF values, aboveground plant organs, especially leaves, could be metal-rich depositories. Summarizing, *C. album* and *T. inodorum* are capable of indicating and accumulating several soil metals, and thus have good potential in the early stages of phytoremediation, assisting the further remediation characterized by woody species.

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Supplementary material 1

Tables S1–S11

Authors: Dávid Tőzsér, Béla Tóthmérész, Sándor Harangi, Edina Baranyai, Gyula Lakatos, Zoltán Fülöp, Edina Simon

Data type: statistical data

Explanation note: **Table S1.** Results of General Linearized Model analysis. **Table S2.** Mean element concentrations of *Chenopodium album* and *Tripleurospermum inodorum*. **Table S3.** Differences in element concentrations among plant organs of *Chenopodium album* within each part of the study area by *p* significance values. **Table S4.** Differences in element concentrations in selected plant organs of *Chenopodium album* among the parts of the study area by *p* significance values. **Table S5.** Differences in element concentrations among plant organs of *Tripleurospermum inodorum* within each part of the study area by *p* significance values. **Table S6.** Differences in element concentrations in selected plant organs of *Tripleurospermum inodorum* among the parts of the study area by *p* significance values. **Table S7.** Correlations in element concentrations between soil and plant organs of *Chenopodium album* in the three parts of the study area. **Table S8.** Correlations in element concentrations between soil and plant organs of *Tripleurospermum inodorum* in the three parts of the study area. **Table S9.** Bioaccumulation factor (BAF) values of *Chenopodium album* and *Tripleurospermum inodorum*. **Table S10.** Bioconcentration factor (BCF) values of *Chenopodium album* and *Tripleurospermum inodorum*. **Table S11.** Translocation factor (TF) values of *Chenopodium album* and *Tripleurospermum inodorum*.

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Phylogeographic patterns and demographic history of *Pomacea canaliculata* and *Pomacea maculata* from different countries (Ampullariidae, Gastropoda, Mollusca)

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Abstract

Invasive species of *Pomacea* snails are of growing concern when it comes to the conservation of global biodiversity. *Pomacea canaliculata* has been listed among the world's 100 worst invasive species. In this work, phylogeographic patterns and the demographic history of *P. canaliculata* and *P. maculata* from different countries were analyzed using mtDNA cytochrome c oxidase subunit-I (COI) sequences. The results showed that *P. canaliculata* and *P. maculata* had high genetic diversity, significant genetic differentiation, limited gene flow and stable population dynamics among different countries. Genetic diversity of *P. canaliculata* was higher than *P. maculata*. Our study will also provide important information for the effective prevention and control of the spread of *Pomacea* snails.

Keywords

phylogeographic patterns, demographic history, Pomacea canaliculata, Pomacea maculata

Introduction

Biological invasions are considered one of the highest causes of biodiversity loss on a global scale (Vitousek 1997; Ricciardi 2007; Thomaz et al. 2015). They are not only a major threat to the loss of native biodiversity and the structure and function of ecosystems, but also seriously affect social and economic development and human health. Freshwater ecosystems are especially vulnerable as a result of widespread human disturbance (Havel et al. 2005; Turak et al. 2016). Invasive bivalves are among the most successful invasive species in freshwater ecosystems (Ricciardi 2007; Oliveira et al. 2011; Paschoal et al. 2015; Ng et al. 2018), which typically comprise r-selected species which adapt to colonize a wide range of aquatic environments and exhibit high fecundity, rapid growth, and broad physiological tolerance to several abiotic factors (Hayes et al. 2015; Nakano et al. 2015). Such characteristics allow them to become dominant species (Karatayev et al. 2007).

Pomacea (Gastropoda: Ampullariidae) is an edible large freshwater snail native to the Amazon River basin of South America (Cowie 2002). It was introduced as the apple snail to many countries of east and southeast Asia (China, Japan, Philippines, Korea, Vietnam, Thailand, Cambodia, Singapore, etc.; Cowie 2002; Joshi and Sebastian 2006), the continental USA (Alabama, Arizona, California, Florida, Georgia, Texas; Rawlings et al. 2007), partial areas of Oceania (Papua New Guinea, New Zealand; Hayes et al. 2008), parts of Europe (Spain; Andree and López 2013), and some Pacific islands, notably the Hawaiian Islands (Tran et al. 2008) due to it having a high nutritional value of rich protein, carotene, vitamins, and minerals. However, *Pomacea* snails' economic potential was over-estimated because consumers disliked the snails' taste and texture, but of the many, mostly small, aquaculture operations that arose, relatively few persisted and the local market failed to sell this snail (Cowie 2002; Hayes et al. 2008; Lv et al. 2013) and led people to give up farming *Pomacea* snails. Subsequently, these snails became pests of wetland rice and other crops, causing massive economic losses due to their wide adaptability to the freshwater habitat combined with a high fecundity (Cowie 2002; Rawlings et al. 2007; Hayes et al. 2008). However, their ecological impacts are more difficult to estimate as they also continue to spread into nonagricultural wetlands of many countries in the current period (Levin 2006; Wood et al. 2006; Rawlings et al. 2007). At the same time, *Pomacea* snails have been associated with the decline of native snails, and the loss of macrophytes and freshwater bryozoans in natural wetlands, resulting in shifts in ecosystem state and function (Carlsson et al. 2004; Carlsson and Lacoursière 2005; Wood et al. 2006). Additionally, *Pomacea* snails can affect human health, having driven the emergence of human eosinophilic meningitis in China: they have been identified as a major intermediate host of *Angiostrongylus cantonensis*, the rat lungworm (Lv et al. 2008, 2009a, b). *Pomacea canaliculata* was therefore listed among the world's 100 worst invasive species (Lowe et al. 2000).

There have been many studies on *Pomacea*, such as distribution (Rawlings et al. 2007), disease (Lv et al. 2009a, b; Lv et al. 2011), phylogenetic (Rawlings et al. 2007; Hayes et al. 2008; Hayes et al. 2009; Hayes et al. 2012; Lv et al. 2013), and taxonomy (Hayes et al. 2012). However, studies on phylogeographic patterns and the demographic history of *Pomacea* ssp have been minimal. The aim of this study is to analyze genetic diversity and structure of *P. canaliculata* in 17 countries and *P. maculata* from 13 countries based on mtDNA COI sequences, which will provide a basis for effectively preventing and managing the spread of these two species.

Materials and methods

Data source

Pomacea belongs to the family Ampullariidae and is the largest of nine extant genera (Rawlings et al. 2007; Hayes et al. 2008). Due to their morphology there is considerable intraspecific variation and it is difficult to determine the true number of species (Cazzaniga 2002; Hayes et al. 2008). For example, the common name 'golden apple snail' also suggests that more than one species was recognized in Asia (e.g., Keawjam and Upatham 1990; Mochida 1991; Yipp et al. 1991). At the same time, it had even been referred to several species such as *Pomacea insularum* (Orbigny, 1835) and *P. canaliculata* (Lamarck, 1819) (Simpson et al. 1994; Roger 1996). Until recently, this snail was classified as two species, *Pomacea canaliculata* and *P. maculata* (as *P. insularum*, which is now a junior synonym of *P. maculata*) based on molecular, anatomy, and morphological data (Hayes et al. 2008; Hayes et al. 2012; Matsukura et al. 2013; Lv et al. 2013).

Due to the widespread issues with misidentification of *Pomacea* species, 146 sequences or haplotype sequences of *P. canaliculata* and 164 sequences or haplotype sequences of *P. maculata* were downloaded from GenBank (Suppl. material 1) and were classified as two species *P. maculata* and *P. canaliculata*, according to Rawlings et al. (2007), Deaton et al. (2007), Hayes et al. (2008), Tran et al. (2008), Matsukura et al. (2008), Jørgensen et al. (2008), Hayes et al. (2009), Song et al. (2010), Collier et al. (2011), Andree and López (2013), Lv et al. (2013), Matsukura et al. (2013), Mu et al. (2015), Ng et al. (2016), Letelier et al. (2016), Perez et al. (2017), Bocxlaer et al. (2017) (Suppl. material 1). COI sequences of *P. maculata* and *P. canaliculata* included 13 countries and 17 countries, respectively (Fig. 1).

Data analysis

The Clustal X1.81was used to align the mtDNA COI sequences of *P. canaliculata* and *P. maculata*, respectively (Thompson et al. 1997). DNASP 5.0 was used to analyze nucleotide composition, haplotype diversity (Hd) and nucleotide diversity (π) of the *P. canaliculata* and *P. maculata* for each country (Librado and Rozas 2009).

To test the phylogenetics of *P. canaliculata* (59 haplotypes) and *P. maculata* (42 haplotypes) COI haplotypes, MRBAYES v.3.2.2 was used to analyze a phylogenetic study using Bayesian inference (Ronquist et al. 2012). A comparison of 24 models of evolution was used to determine the initial model of evolution (HKY+G) in MRMODELTEST v.2.2. MRBAYES was run using 3,000,000 generations and six concurrent Markov Chains and 2 hot chains sampled at intervals of every 100 generations for a total of 30,000 trees. The stationarity of the log likelihood values was ensured using a 25% burn-in (7500 trees; Nylander 2004). Phylogenetic analyses included COI sequences for *Pomacea paludosa* (GenBbank accession numbers EU528477

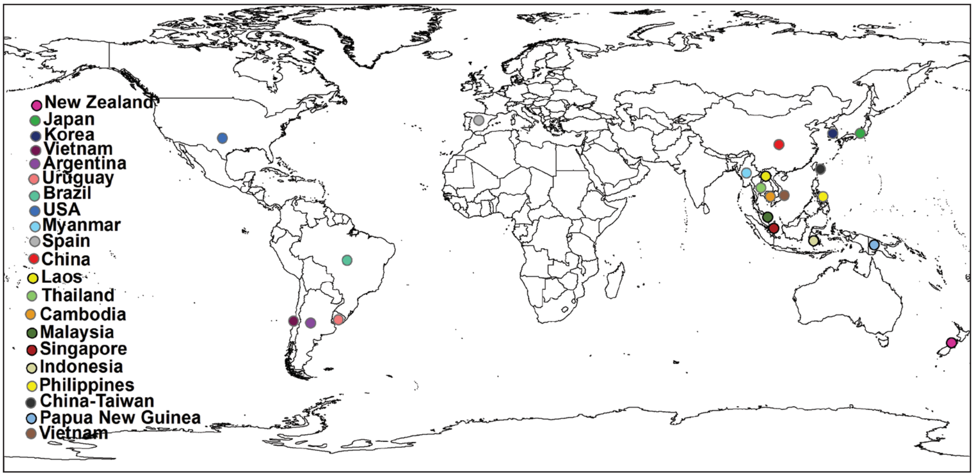

Figure 1. Distribution for *P. canaliculata* and *P. maculata* from 17 countries and 13 countries, respectively.

and EF514960) and *Pomacea diffusa* (GenBank accession numbers EU528564 and MF462141). As outgroups for the *Pomacea* dataset, COI sequences for *Pila conica* were used (GenBank accession numbers EU528588 and EU274570). NETWORK 4.5 was used to construct a haplotype network of the *P. canaliculata* (59 haplotypes) and *P. maculata* (42 haplotypes) COI haplotypes based on a 95% connection limit with gaps defined as missing data (Leigh and Bryant 2015).

A hierarchical analysis of molecular variance (AMOVA) was used to evaluate patterns of genetic structure in the COI dataset based on Arlequin 3.5 (Excoffier and Lischer 2010). The AMOVA was used to partition variance components to populations and to individuals within each collection location, where 1000 permutations were performed to test the significance of each pairwise *P. canaliculata* and *P. maculata* population comparison, respectively. Arlequin 3.5 was used to conduct a test for isolation by-distance by testing the significance of a correlation between pairwise Nei's D and geographic distance among country (Excoffier and Lischer 2010). A Mantel test was used to analyze the correlation computations between pairwise genetic and geographic distances between countries (Jensen et al. 2005). ArcMap GIS (ESRI) was used to measure the geographic distances within countries.

To examine deviations from neutrality of *P. canaliculata* and *P. maculata* for each country, Arlequin 3.5 was used to conduct Tajima's D and Fu's Fs tests (Excoffier and Lischer 2010). DNASP 5.0 was used to analyze mismatched distribution analysis (MDA) of *P. canaliculata* and *P. maculata* for each country. BEAST 1.4.7 (Drummond and Rambaut 2007) was used to compute the Bayesian Skyline Plot (BSP) (Drummond et al. 2005) analysis. BSP was used to reconstruct the effective population size fluctuations since the time of the most recent common ancestor (TMRCA). MCMC was run for 500 million steps, with sampling every 1000 generations and following a burn-in of the initial 10% cycles. TRACER 1.5 was used to conduct inspections of the results and construction of the BSP (Rambaut and Drummond 2007). The model comparison function in TRACER 1.5 was used to assess the fit of the constant size population model and Bayesian Skyline coalescent models to the dataset.

Results

Phylogeographic patterns

Fifty-nine and 42 unique COI haplotypes of *P. canaliculata* and *P. maculata* were identified from 17 and 13 countries globally, respectively. The China-mainland and Brazil had the greatest variation with 24 haplotypes and 26 haplotypes in *P. canaliculata* and *P. maculata*, respectively (Table 1). Both haplotypes' diversity values of *P. canaliculata* and *P. maculata* at each population varied between 0 and 1.000 (Table 2). The greatest haplotype diversity of *P. canaliculata* occurred in Thailand, Philippines, Korea, Vietnam and Uruguay and *P. maculata* occurred in Vietnam and Thailand. Nucleotide diversity values of *P. canaliculata* ranged from 0 to 0.093 and *P. maculata* ranged from 0 to 0.029 (Table 2). The greatest nucleotide diversity of *P. canaliculata* occurred in Uruguay and *P. maculata* occurred in Brazil. In addition, the haplotype diversity of *P. canaliculata* and *P. maculata* of South America was greater than other continents (Table 2).

Phylogenetic analysis (Fig. 2a) showed that the clade of *P. canaliculata* had strong support (1.00). The 59 haplotypes of *P. canaliculata* were divided into four clades: 27 haplotypes of *P. canaliculata* from Argentina, USA, Papua New Guinea, Myanmar, Korea, Philippines, Vietnam, Malaysia, Japan, Chile, Thailand, Indonesia, China-Taiwan, and China formed one clade. Thirteen haplotypes of *P. canaliculata* from Philippines, Japan, Laos, Vietnam, and China formed a second clade. Eight haplotypes of *P. canaliculata* from Argentina, Japan, China-Taiwan, Uruguay, and Singapore formed a third clade, and eleven haplotypes of *P. canaliculata* from Argentina, Myanmar, Korea, China-Taiwan, Uruguay, Japan, and China formed the last clade.

Phylogenetic analysis (Fig. 2b) showed the clade of *P. maculata* had strong support (1.00). The 42 COI haplotypes of *P. maculata* were divided into two clades: 32 haplotypes of *P. maculata* from Brazil, Argentina, USA, Spain, Singapore, New Zealand, Vietnam, Malaysia, Thailand, Cambodia, and China formed one clade and ten haplotypes of *P. maculata* from Brazil, Argentina, USA, Japan, Korea, Thailand, and China formed a second clade.

The single haplotype network was produced based on COI haplotypes of *P. canaliculata* and *P. maculata* (Fig. 3a, b). The most frequent haplotypes of *P. canaliculata* and *P. maculata* were Hap4 and Hap17, which had 43 and 37 individuals. Forty-eight and 18 haplotypes were rare haplotypes in both *P. canaliculata* and *P. maculata* and just a single individual occurred in a country. As seen in the phylogeny of *P. canaliculata* and *P. maculata* (Fig 2a, b), the results of the haplotypes network also showed a similar geographic structure.

Table 1. Haplotype distributions of *Pomacea canaliculata* and *Pomacea maculata* from 17 countries and 13 countries, respectively.

Continent	Country(code)	*Pomacea canaliculata*		*Pomacea maculata*	
		Number of haplotype sequences or sequences	Haplotype distribution	Number of haplotype sequences or sequences	Haplotype distribution
Asia	China-mainland (CM)	31	Hap4, Hap30, Hap31, Hap33, Hap34, Hap35, Hap42, Hap43, Hap44, Hap45, Hap46, Hap47, Hap48, Hap49, Hap50, Hap51, Hap52, Hap53, Hap54, Hap55, Hap56, Hap57, Hap58, Hap59	6	Hap17, Hap27, Hap28
	China-Taiwan (CT)	4	Hap4, Hap34, Hap36	–	–
	Japan (JA)	22	Hap4, Hap5, Hap25, Hap30, Hap33, Hap34, Hap35, Hap36	6	Hap17, Hap37
	Philippines (PH)	13	Hap4, Hap20, Hap21, Hap22, Hap23, Hap24, Hap25, Hap26, Hap27, Hap28, Hap29, Hap30, Hap31	–	–
	Vietnam (VI)	2	Hap4, Hap30	2	Hap17, Hap42
	Thailand (TH)	2	Hap37, Hap38	2	Hap17, Hap37
	Myanmar (MY)	3	Hap4, Hap33	–	–
	Korea (KO)	2	Hap4, Hap34	1	Hap38
	Indonesia (IN)	3	Hap40, Hap41	–	–
	Laos (LA)	1	Hap30	–	–
	Malaysia (MA)	2	Hap4	1	Hap17
	Cambodia (CA)	–	–	1	Hap17
	Singapore (SI)	1	Hap36	1	Hap17
Oceania	Papua New Guinea (PNG)	2	Hap4	–	–
	New Zealand (NZ)	–	–	1	Hap17
Europe	Spain (SP)	–	–	9	Hap17
North America	United States (USA)	20	Hap4	66	Hap17, Hap37, Hap38, Hap39, Hap40, Hap41
South America	Uruguay (UR)	2	Hap32, Hap39	–	–
	Chile (CH)	4	Hap4	–	–
	Brazil (BR)	–	–	54	Hap1, Hap2, Hap3, Hap4, Hap5, Hap6, Hap7, Hap8, Hap9, Hap10, Hap11, Hap12, Hap14, Hap15, Hap16, Hap17, Hap18, Hap19, Hap20, Hap21, Hap22, Hap23, Hap24, Hap25, Hap26
	Argentina (AR)	32	Hap1, Hap2, Hap3, Hap4, Hap5, Hap6, Hap7, Hap8, Hap9, Hap10, Hap11, Hap12, Hap14, Hap15, Hap16, Hap17, Hap18, Hap19	15	Hap29, Hap30, Hap31, Hap32, Hap33, Hap34, Hap35, Hap36, Hap37, Hap38

The AMOVA results showed that 23.84% and 37.77% of the total genetic variance among countries was significant (F_{ST} =0.23836 and 0.37772, p<0.001, Table 3), respectively. Pairwise F_{ST} of *P. canaliculata* and *P. maculata* all ranged from -1.00 to 1.00 among countries (Table 4, Table 5). Pairwise gene flow of

Table 2. Genetic diversity of *Pomacea canaliculata* and *Pomacea maculata* from 17 countries and 13 countries of global based on COI sequences, respectively. Key: H = number of haplotypes, Hd = haplotype diversity, π = mean nucleotide diversity.

Continent	Country	*Pomacea canaliculata*			*Pomacea maculata*		
		H	Hd	Π	H	Hd	π
Asia	China-mainland	24	0.968	0.030	3	0.700	0.023
	China-Taiwan	3	0.833	0.032	–	–	–
	Japan	8	0.835	0.029	2	0.600	0.009
	Philippines	13	1.000	0.028	–	–	–
	Vietnam	2	1.000	0.044	2	1.000	0.002
	Thailand	2	1.000	0.002	2	1.000	0.016
	Myanmar	2	0.667	0.029	–	–	–
	Korea	2	1.000	0.038	1	0	0
	Indonesia	2	0.667	0.001	–	–	–
	Laos	1	0	0	–	–	–
	Malaysia	1	0	0	1	0	0
	Cambodia	–	–	–	1	0	0
	Singapore	1	0	0	1	0	0
	Total	42	0.924	0.030	6	0.695	0.023
Oceania	Papua New Guinea	1	0	0	–	–	–
	New Zealand	–	–	–	1	0	0
	Total	1	0	0	1	0	0
Europe	Spain	–	–	–	1	0	0
	Total	–	–	–	1	0	0
North America	United States	1	0	0	5	0.708	0.006
	Total	1	0	0	5	0.708	0.006
South America	Uruguay	2	1.000	0.093	–	–	–
	Chile	1	0	0	–	–	–
	Brazil	–	–	–	26	0.970	0.029
	Argentina	19	0.924	0.023	10	0.914	0.018
	Total	21	0.933	0.031	36	0.978	0.030
Global Total		**59**	**0.853**	**0.029**	**42**	**0.893**	**0.022**

P. canaliculata and *P. maculata* all ranged from 0 to 249.75 among the countries (Table 4, Table 5).

Genetic differentiation as represented by pairwise genetic distance values of *P. canaliculata* (P = 0. 816) and *P. maculata* (P = 0. 527) among countries was not correlated with geographic distance, indicating that more geographically distant site combinations did not produce higher levels of genetic differentiation.

Demographic history

The mismatch distribution of analysis of pairwise differences was not significantly different from the expected distribution of the expanding population model based on COI sequences of *P. canaliculata* and *P. maculata* (Fig. 4a, b). Similarly there was a lack of statistical significance of Tajima's D test ($p < 0.01$), and non-significant Fu's FS ($p < 0.01$). Moreover, when all samples were pooled together, Tajima's D and Fu's FS test were again not significant ($p < 0.01$, Table 6). Additionally, the BSPs showed that *P. canaliculata* and *P. maculata* have had a stable historical population size with a small

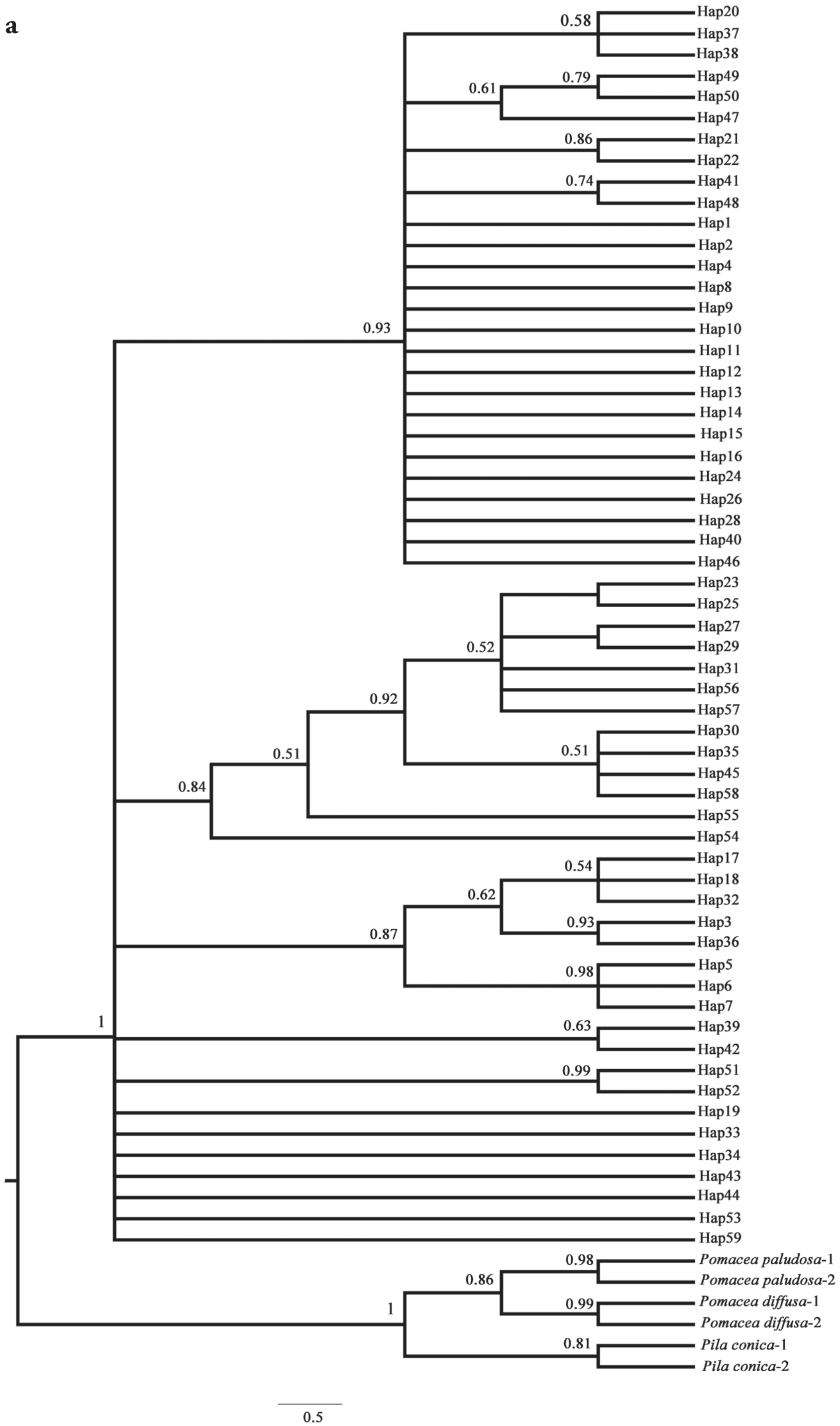

Figure 2. Phylogenetic analysis of the COI sequences for *P. canaliculata* (**a**) and *P. maculata* (**b**) from 17 countries and 13 countries using Bayesian Inference (BI), respectively.

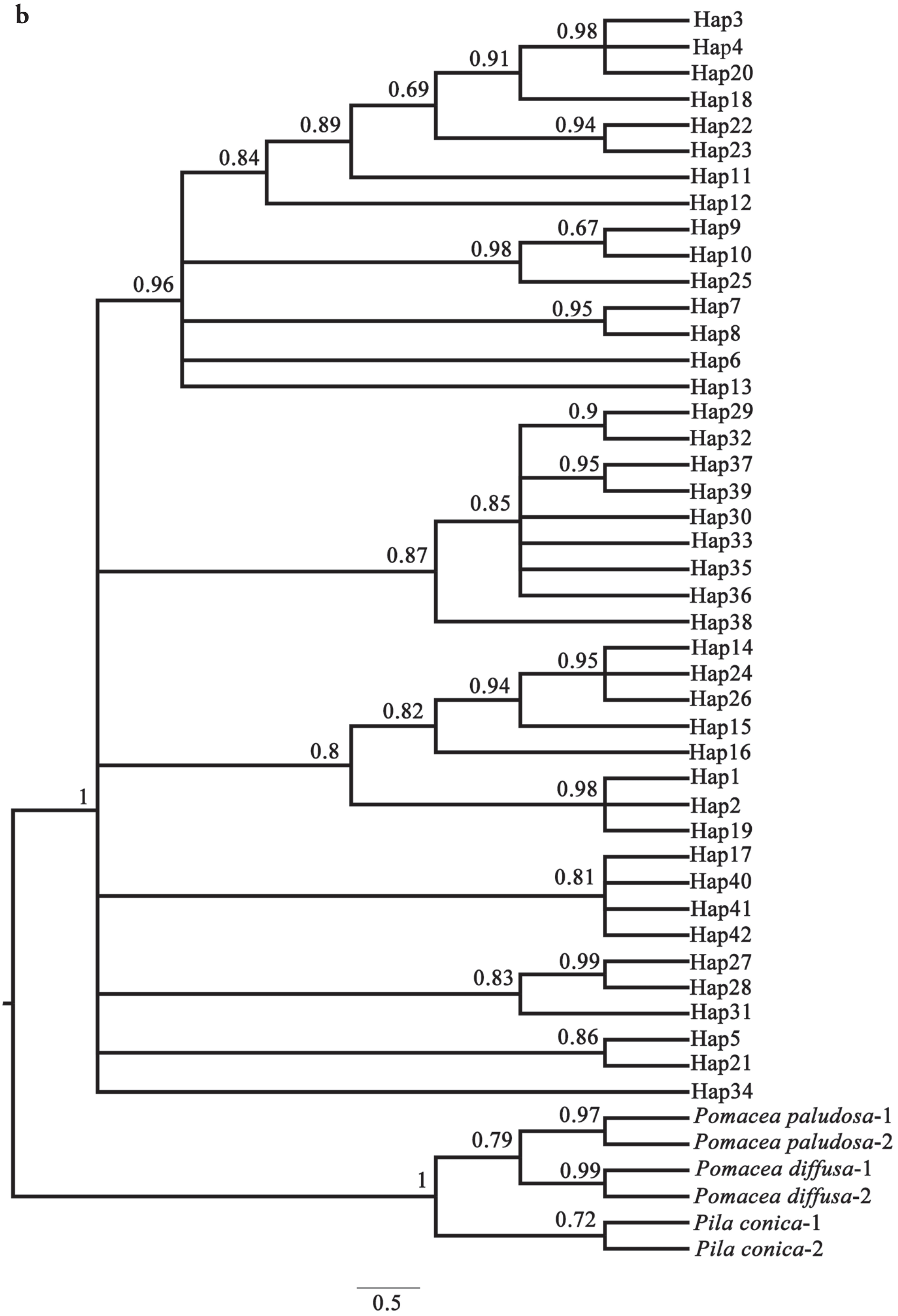

Figure 2. Continued. Phylogenetic analysis of the COI sequences for *P. canaliculata* (**a**) and *P. maculata* (**b**) from 17 countries and 13 countries using Bayesian Inference (BI), respectively.

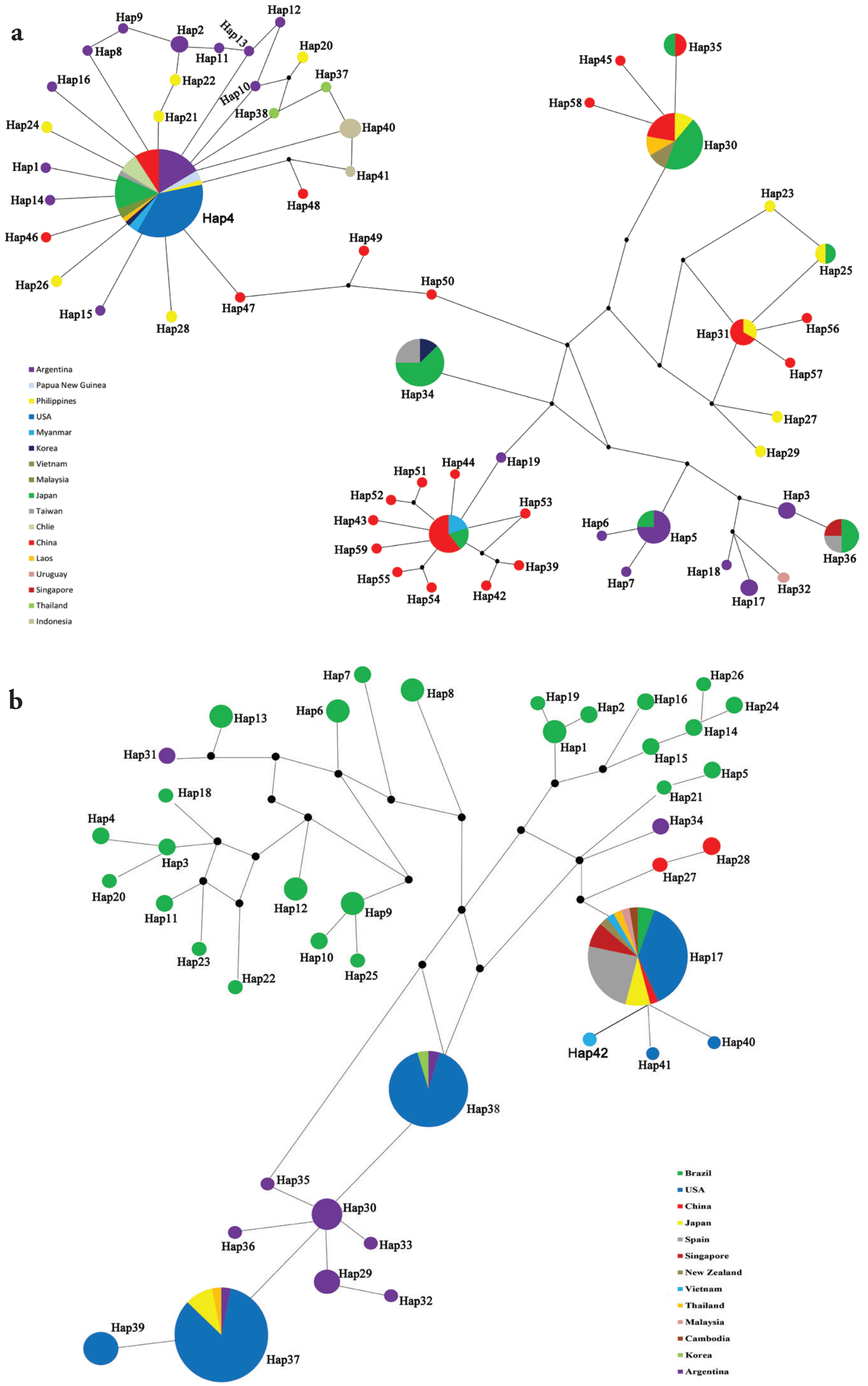

Figure 3. Haplotype network for *P. canaliculata* (**a**) and *P. maculata* (**b**) from 17 countries and 13 countries of global based on COI sequences. Each cross-hatched line represents one base-pair.

Table 3. Analysis of molecular variation (AMOVA) calculated from mtDNA COI sequences for *Pomacea canaliculata* and *Pomacea maculata* from 17 countries and 13 countries, respectively. All *F*-statistics were statistically significant (p<0.001). Key: F_{ST}=0.23836; F_{ST}=0.37772.

COI sequences	***P. canaliculata***				***P. maculata***			
Source of variance	**df**	**Sum of squares**	**Variance components**	**Percentage of variation**	**df**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among populations	17	322.04	1.78	23.84	12	380.34	2.72	37.77
Within populations	129	733.73	5.69	76.16	153	686.62	4.49	62.23
Total	146	1055.77	7.47	–	165	1066.95	1.73	–

Table 4. Analysis of genetic differentiation coefficient (F_{st}) (below diagonal) and gene flow (N_m) (above diagonal) calculated using COI mtDNA sequence data among 13 countries of *Pomacea maculata* in global. Bold type indicates statistical significance (α = 0.05). Country codes as in Table 1.

	CM	**JA**	**VI**	**TH**	**KO**	**MA**	**CA**	**SI**	**NZ**	**SP**	**USA**	**BR**	**AR**
CM		249.750	0.194	11.114	8.371	0.190	0.194	0.189	3.481	249.750	0.496	249.750	0.524
JA	0.001		0.084	49.750	20.583	1.284	0.077	0.071	0.444	31.000	0.762	249.750	0.796
VI	**0.563**	**0.749**		0.256	NA	NA	NA	NA	NA	NA	NA	0.534	NA
TH	0.022	0.005	**0.494**		3.917	0.255	0.254	0.251	1.474	49.750	0.633	249.750	0.615
KO	0.029	0.012	**1.000**	**0.060**		1.523	NA	NA	0.290	5.432	NA	0.406	0.454
MA	**0.568**	**0.163**	**1.000**	**0.495**	**0.141**		NA	NA	0.738	2.177	NA	0.159	NA
CA	**0.563**	**0.765**	**-1.000**	**0.496**	**1.000**	**1.000**		NA	NA	NA	NA	0.549	NA
SI	**0.570**	**0.779**	**-1.000**	**0.499**	**1.000**	**1.000**	**-1.000**		NA	NA	NA	0.534	NA
NZ	**0.067**	**0.360**	**-1.000**	**0.145**	**0.463**	**0.253**	**-1.000**	**-1.000**		NA	0.372	1.962	0.380
SP	0.001	0.008	**-1.000**	0.005	**0.044**	**0.103**	**-1.000**	**-1.000**	**-1.000**		1.139	249.750	1.124
USA	**0.335**	**0.247**	**1.000**	**0.283**	**1.000**	**1.000**	**1.000**	**1.000**	**0.402**	**0.180**		0.079	NA
BR	0.001	0.001	**0.319**	0.001	**0.381**	**0.611**	**0.313**	**0.319**	**0.113**	0.001	**0.759**		8.083
AR	**0.323**	**0.239**	**1.000**	**0.289**	**0.355**	**1.000**	**1.000**	**1.000**	**0.397**	**0.182**	**1.000**	0.030	

Table 5. Analysis of genetic differentiation coefficient (F_{st}) (below diagonal) and gene flow (N_m) (above diagonal) calculated using COI mtDNA sequence data among 17 countries of *Pomacea canaliculata* in global. Bold type indicates statistical significance (α = 0.05). Country codes as in Table 1.

	CM	**CT**	**JA**	**PH**	**VI**	**TH**	**MY**	**KO**	**IN**	**LA**	**MA**	**SI**	**PNG**	**USA**	**UR**	**CH**	**AR**
CM		24.750	0.004	2.002	27.528	0.059	0.212	NA	0.025	NA	10.620	0.235	0.422	0.640	41.417	0.581	0.059
CT	0.010		0.004	0.902	1.869	0.034	0.048	NA	NA	NA	0.244	0.146	0.194	0.391	249.750	0.411	0.000
JA	**0.986**	**0.984**		7.815	0.125	0.508	1.000	NA	0.330	NA	0.114	1.006	1.498	3.596	NA	3.596	0.496
PH	**0.111**	**0.217**	0.031		24.750	0.377	0.261	0.982	0.982	1.000	0.119	0.240	0.994	0.373	249.750	0.375	0.378
VI	0.009	**0.118**	0.666	0.010		NA	0.113	0.052	0.142	0.049	22.477	0.578	0.074	4.136	249.750	3.782	NA
TH	**0.809**	**0.881**	**0.330**	**0.399**	**1.000**		NA	NA	NA	NA	NA	NA	NA	NA	2.497	NA	NA
MY	**0.541**	**0.839**	**0.200**	**0.489**	**0.689**	**1.000**		0.505	0.256	0.499	NA	NA	0.164	NA	4.958	NA	NA
KO	**1.000**	**1.000**	**-1.000**	**0.203**	**0.829**	**1.000**	**0.331**		NA	NA	NA	0.496	0.164	0.496	NA	0.494	NA
IN	**0.910**	**1.000**	**0.431**	**0.203**	**0.638**	**1.000**	**0.494**	**1.000**		NA	NA	0.243	0.267	0.385	1.630	0.377	NA
LA	**1.000**	**1.000**	**-1.000**	**0.200**	**0.836**	**1.000**	**0.334**	**-1.000**	**1.000**		NA	0.499	0.168	0.499	NA	0.490	NA
MA	0.023	**0.506**	**0.686**	**0.678**	0.011	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**		NA	0.038	NA	249.750	NA	NA
SI	**0.515**	**0.632**	**0.199**	**0.510**	**0.302**	**1.000**	**1.000**	**0.335**	**0.507**	**0.334**	**1.000**		NA	NA	5.185	NA	NA
PNG	**0.372**	**0.563**	**0.143**	**0.201**	**0.772**	**1.000**	**0.604**	**0.604**	0.484	0.598	0.869	1.000		0.291	83.083	0.289	0.093
USA	**0.281**	**0.390**	**0.065**	**0.401**	**0.057**	**1.000**	**1.000**	**0.335**	**0.394**	**0.334**	**1.000**	**1.000**	0.462		62.250	NA	NA
UR	0.006	0.001	**-1.000**	0.001	0.001	0.091	0.048	**-1.000**	**0.133**	**-1.000**	0.001	0.046	0.003	0.004		62.250	2.438
CH	**0.301**	**0.378**	**0.065**	**0.400**	**0.062**	**1.000**	**1.000**	**0.336**	**0.399**	**0.338**	**1.000**	**1.000**	**0.464**	**1.000**	0.004		NA
AR	**0.809**	**1.000**	**0.335**	**0.398**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**0.729**	**1.000**	0.093	**1.000**	

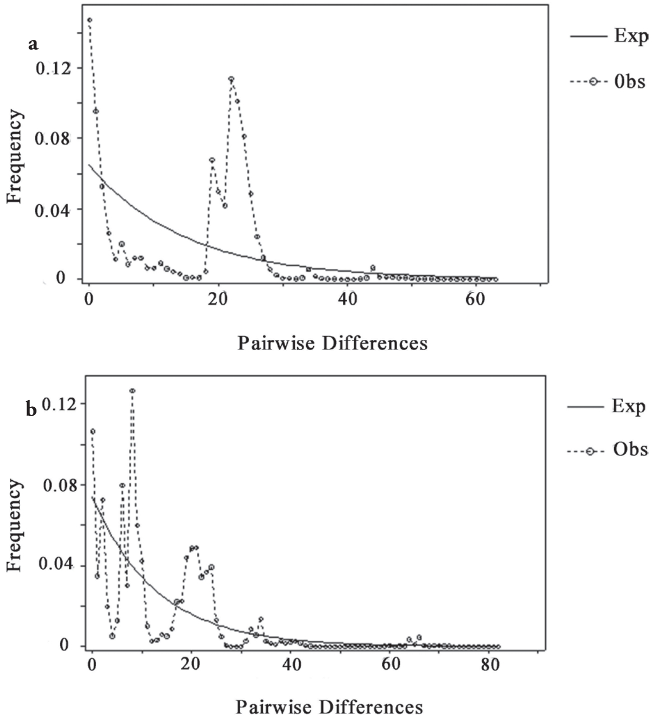

Figure 4. Mismatch distribution analysis (MDA) for *P. canaliculata* (**a**) and *P. maculata* (**b**) from 17 countries and 13 countries based on COI sequences.

recent expansion event occurring between 100,000 – 150,000 and 150,000 – 200,000 years ago, respectively (Fig. 5a, b). However, the model comparison analysis showed that constant population size was the best fit for the model of the data set, suggesting that there was not much support for the recent expansion trend.

Discussion

Phylogeographic patterns

Genetic diversity is an important basis for evaluating the status of population resources, which plays an important role in adaptations to habitat changes and maintenance of long-term survival and evolution (Freeland et al. 2011; Liu and Yao 2013; Liu et al. 2017). Invasive bivalves and *Pomacea* snails are typically r-selected species, which means they are adapted to colonize a wide range of aquatic environments and exhibit high fecundity, rapid growth, and broad physiological tolerances to several abiotic factors. In this study, *P. canaliculata* and *P. maculata* had high genetic diversity (0.853 and 0.893), which indicates adaptations to enable colonization of a wide range of

Table 6. Neutrality tests for *P. canaliculata* and *P. maculata* from 17 countries and 13 countries based on mtDNA COI sequences, respectively. Bold type indicates statistical significance (P < 0.01).

Country	*P. canaliculata*			*P. maculata*		
	Pi (%)	Tajima's D	Fu's FS	Pi (%)	Tajima's D	Fu's FS
China-mainland	15.13	0.16	-3.98	13.40	-1.15	4.50
China-Taiwan	16.33	0.34	3.47	–	–	–
Japan	14.54	0.70	6.33	5.40	2.20	5.78
Philippines	13.98	1.38	-4.39	–	–	–
Vietnam	22.00	0	3.09	1.00	0	0
Thailand	1.00	0	0	9.00	0	2.20
Myanmar	14.67	0	4.86	–	–	–
Korea	19.00	0	2.94	0	0	0
Indonesia	0.67	0	0.20	–	–	–
Laos	0	0	0	–	–	–
Malaysia	0	0	0	0	0	0
Cambodia	–	–	–	0	0	0
Singapore	0	0	0	0	0	0
Papua New Guinea	0	0	0	–	–	–
New Zealand	–	–	–	0	0	0
Spain	–	–	–	0	0	0
United States	0	0	0	3.74	1.72	4.73
Uruguay	47.00	0	3.85	–	–	–
Chile	0	0	0	–	–	–
Brazil	–	–	–	16.80	0.44	-0.46
Argentina	11.53	-0.02	-2.30	10.65	-2.37	0.17
Total	14.46	-0.79	-13.49	12.93	-1.24	-2.61

habitats. However, genetic diversity of ancestral populations (Brazil, Argentina, etc.) was higher than the recently established populations (Singapore, Papua New Guinea, New Zealand, Spain, etc.). The low haplotype diversity may be attributed to:

1) possible genetic exchange, genetic bottlenecks and genetic drift (Matsukura et al. 2013; Shirk et al. 2014), which could be the result of the initial founder event that led to speciation in *P. canaliculata* and *P. maculata* (Freeland et al. 2011);
2) the introduction of alien species to a non-native location may not be directly from the native range, but from a successful invasive population elsewhere, which could be the result of the bridge-head effect (Eric et al. 2010);
3) sample numbers of *Pomacea* for each country were significantly different for this study, so further genetic analysis is needed to clarify this (Hayes et al. 2008);
4) growth and reproduction of *P. canaliculata* and *P. maculata* are closely related to water temperature: many studies showed that the snail was not adapted to low temperatures (Andree and López 2013; Byers et al. 2013; Hayes et al. 2015; Bernatis et al. 2016). In addition, climate change (Byers et al. 2013) and environment factors such as pH (Byers et al. 2013), calcium carbonate (White et al. 2007; Perlman 2016), dissolved oxygen (Seuffert and Martín 2009), salinity (Verbrugge et al. 2012; Martin and Valentine 2014) may affect their life history.

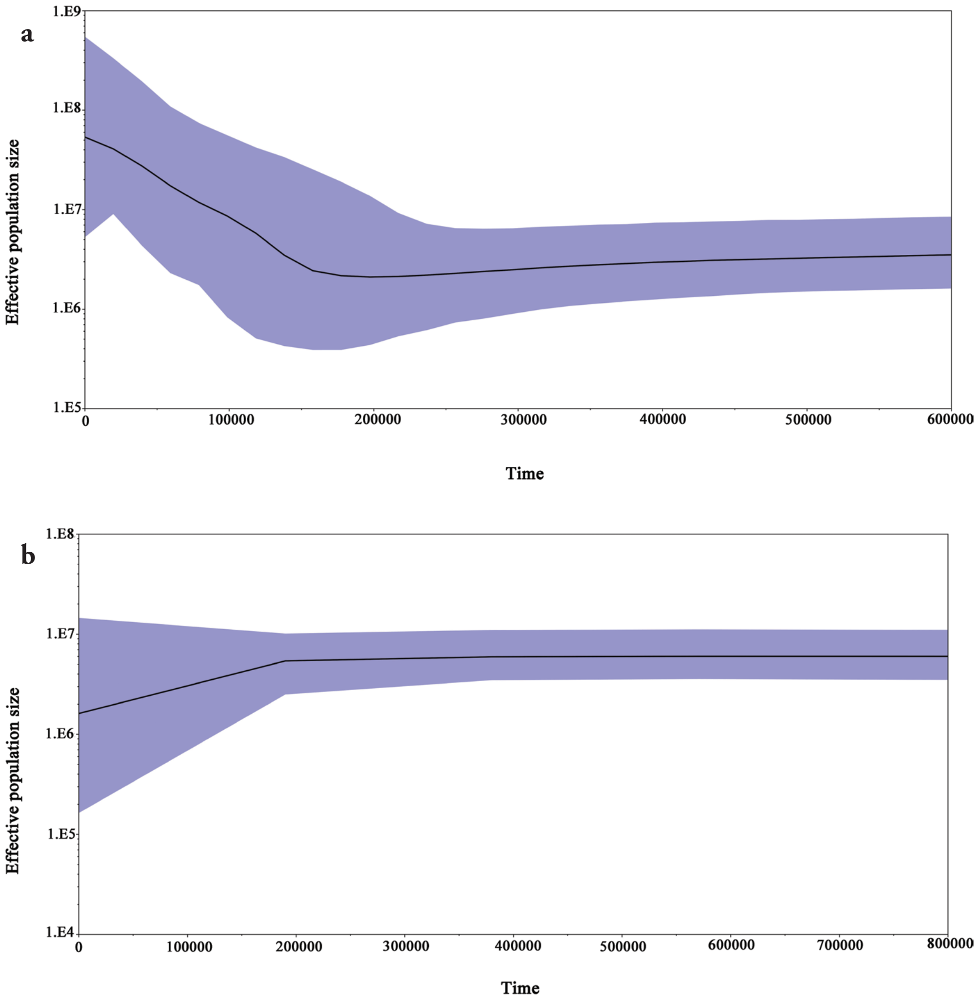

Figure 5. Bayesian skyline plot for *P. canaliculata* (**a**) and *P. maculata* (**b**) from 17 countries and 13 countries reconstructing the population size history using an evolutionary rate of 2.0 × 10^{-8} substitutions/site/year.

5) genetic diversity of *P. canaliculata* and *P. maculata* is related to human factors. *Pomacea* was introduced to many countries as food, but its economic potential was overestimated. Some regions, such as North America and Europe, attached great importance to the prevention and management of invasive species, which made it difficult for them to survive (Hayes et al. 2015; Joshi et al. 2017).

The geographic structure among countries using the mtDNA COI dataset was somewhat ambiguous. The phylogenetic analysis showed 59 haplotypes of *P. canaliculata* divided into four clades. These results also indicated a complicated pattern of introduction into non-native countries. The somewhat ambiguous genetic structures that may be attributed to *Pomacea* were introduced to many areas where they are consumed as food (Lv et al. 2009a; Hayes et al. 2008; Lv et al. 2013; Joshi et al. 2017).

Genetic differentiation was high in different countries hosting *P. maculata* and *P. canaliculata*. In addition, estimates of gene flow (N_m) were generally low (i.e., N_m <1). The higher level of genetic differentiation and low gene flow may be attributed to:

1) geographical isolation as an important factor that affects distribution patterns and genetic structure of species (Hayes et al. 2008; Lv et al. 2013). *Pomacea* are amphibious and show a preference for shallow ditches and ponds surrounding human settlements in which the water habitat is relatively isolated (Cowie 2002; Hayes et al. 2012; Joshi et al. 2017);
2) the dispersal ability of *Pomacea* is relatively low and its activity range is limited (Cowie 2002; Hayes et al. 2012; Joshi et al. 2017), which may lead to a gradual reduction of gene flow, and resulting in genetic differentiation among populations;
3) introduced populations usually experience a bottleneck if founded by a few individuals, and their genetic variability is expected to decrease in the newly colonized range (Dlugosch and Parker 2008; Hayes et al. 2008; Lv et al. 2013; Joshi et al. 2017).

In theory, an increase of geographic distance should correlate with a gradual reduction of gene flow, resulting in genetic differentiation among populations, i.e., isolation-by-distance (Hurtrez-Boussès et al. 2010; Husemann et al. 2012). However, genetic differentiation as represented by pairwise genetic distance values of *P. canaliculata* (P = 0. 816) and *P. maculata* (P = 0. 527) among countries was not correlated with geographic distance, which indicated more geographically distant site combinations did not produce higher levels of genetic differentiation.

Demographic history

The mismatch distribution analysis and neutrality tests showed that *P. maculata* and *P. canaliculata* across 17 and 13 countries, respectively, did not have recent population expansions. These analyses also indicated that the population dynamics of *P. maculata* and *P. canaliculata* are quite stable. This is not a surprising result because *P. maculata* and *P. canaliculata* are typically r-selected species and widely distributed in many countries. In addition, the stable historical population size with a small recent expansion may be attributed to:

1) the glacial period has had an important influence on the spatial distribution pattern and genetic structure of species (Clark and Mccabe 2009). During the Quaternary glacial period there were many glacial processes in the high and middle latitudes of the world due to the decrease of global temperature, which might have contributed to the *Pomacea* populations experiencing a small recent expansion in South America (Per et al. 2017);
2) possibly a constant population size was the best fit for the model to the data set, suggesting that there was not much support for the recent expansion trend.

Conclusions

Biological invasions are of growing concern regarding the conservation of global biodiversity (Lv et al. 2013). In recent decades, there has been a dramatic increase in species introductions with the increasing travel and trade associated with rapid economic globalization (Blumental 2006). *Pomacea* is a successful invader which possesses many characteristics, such as high reproductive capability and dietary flexibility, rapid growth rate, and strong resistance to environmental conditions (Estebenet and Martín 2002; Cowie 2002; Boland et al. 2008). Currently, *Pomacea* show a tendency to expand in many countries (Rawlings et al. 2007; Hayes et al. 2008; Byers et al. 2013; Lv et al. 2013). In this study, *P. canaliculata* and *P. maculata* showed high genetic diversity, significant genetic differentiation, limited gene flow, and stable population dynamics among different countries.

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Supplementary material 1

List of all individual *Pomacea* spp. and outgroups used, and GenBank accession codes

Authors: Xiongjun Liu, Yu Zhou, Shan Ouyang, Xiaoping Wu

Data type: molecular data

Explanation note: List of all individual *Pomacea* spp. and outgroups used, and GenBank accession codes.

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Habitat preference and potential distribution of *Magnolia officinalis* subsp. *officinalis* and *M. o.* subsp. *biloba* in China

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Abstract

Magnolia officinalis subsp. *officinalis* and *M. officinalis* subsp. *biloba* are important medicinal plants in China. The bark of these two subspecies is commonly used in the production of a widely-used Chinese traditional medicine named ‘Houpu’. In recent years, *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* have become increasingly threatened owing to the over-harvesting of their bark and the fragmentation of their habitats. In this study, we aimed to support the conservation and cultivation of these two subspecies in China by: (1) assessing the relationship between numerous environmental variables and the geographical distributions of the subspecies; (2) analysing the environmental characteristics of suitable habitats for both subspecies and predicting the spatial distribution of these habitats in China; and (3) identifying conservation areas of both subspecies in China via overlay analysis. We also assessed the degree of human disturbance within suitable habitats. We found that temperature was a major determinant for the distribution of *M. o.* subsp. *officinalis*. Conversely, the distribution of *M. o.* subsp. *biloba* was primarily dependent on precipitation rather than temperature. Distinct habitat preferences were observed between *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*. Suitable habitats of *M. o.* subsp. *officinalis* were primarily distributed in the northern subtropical areas of China, with greater fluctuations in ambient temperature, lower extreme temperatures, less precipitation and greater fluctuations in precipitation. Habitats suitable for *M. o.* subsp. *biloba* were highly fragmented and were distributed in the central subtropical areas of China. We found that a large proportion of suitable habitats were not in the protected areas and that they were significantly disturbed by human activity. This analysis could provide useful information for the conservation of both *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* and could aid in the selection of cultivation sites.

Keywords
Habitat suitability, Maxent, receiver operating curve, nature reserves, human disturbance, Chinese medicine

Introduction

Magnolia officinalis subsp. *officinalis* and *M. officinalis* subsp. *biloba* are two important medicinal plants in the Magnoliaceae. The bark of these subspecies is used to make a famous traditional Chinese medicine named 'Houpu'. Houpu has been widely used in traditional Chinese medicinal practices for around 2000 years. Houpu has traditionally been thought to promote the flow of "qi" and blood and to reduce negative energy (Chinese Pharmacopoeia Commission 2015). The active ingredients of Houpu are magnolol and honokiol, both of which are known to possess broad pharmacological qualities, including anti-microbial, anti-inflammatory, anti-tumour and anti-aging effects and are also thought to aid muscle relaxation and cholesterol removal (Wang et al. 2005).

The demand for Houpu has increased dramatically over the last few decades. However, *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* grow at a relatively slow rate and bark can only be harvested from trees that are more than 15 years old. This has led to the over-harvesting of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* bark. In addition, the two subspecies have poor natural reproductive capacities (Liu et al. 1997) and their habitats have been greatly disturbed by human activity. As a result, wild *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* populations are greatly threatened and are at risk of extinction (Xiong et al. 2009). *Magnolia o.* subsp. *officinalis* and *M. o.* subsp. *biloba* are ranked as category II protected plant species (http://www.gov.cn/gongbao/content/2000/content_60072.htm) and have been identified as national key protected wild medicinal materials (http://www.forestry.gov.cn/portal/ynb/s/4769/content-802380.html). More recently, significant efforts have been made to conserve wild *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* populations. However, these wild populations are still at risk in China. Therefore, further efforts are required to conserve wild *M. officinalis* subsp. *officinalis* and *M. o.* subsp. *biloba* populations.

Habitat conservation is an efficient way to protect threatened species (Maxted et al. 2008). To improve the practicality and effectiveness of habitat conservation, it is essential to first understand the habitat preferences and distributions of at-risk species. This is imperative for the conservation of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* populations. Although *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* have a close phylogenetic relationship, they have different geographical distribution areas (Liu 1996). However, few studies have quantitatively assessed their habitat characteristics and evaluated habitat suitability for these two subspecies. Recently in China, during the conversion of farmland to forest, local governments have made significant efforts to promote the cultivation of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*. The medicinal efficacy of Houpu is closely related to the environmental conditions of the plants' habitat (Zhang et al. 2017). Inappropriate selection of cultivation sites not only results in the wasting of land and economic losses on the part of the farmers, but also reduced the medicinal quality of the Houpu. Therefore, proper evaluation of habitat suitability is essential for the cultivation and conservation of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*.

Thus, the objectives of this study were to: (1) analyse the relationship between environmental conditions and the distribution of *M. o.* subsp. *officinalis* and *M. officinalis* subsp. *biloba* and to improve our understanding of their habitat preferences; (2) estimate the spatial distribution of suitable habitats in China; and (3) identify the conservation areas and the extent of human disturbance in the suitable habitats. We hope this research will provide technical support for the conservation and cultivation of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* in China.

Methods

Spatial distribution of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*

According to the Flora of China (Volume 30(1)) (Liu 1996), *M. officinalis* subsp. *officinalis* is mainly distributed in the northern and central subtropical regions, which includes the southern area of Shaanxi province, the south-eastern areas of Gansu province and Henan province, the western area of Hubei province, the south-western area of Hunan province, the central and eastern areas of Sichuan province and the north-eastern area of Guizhou province (Fig. 1). *Magnolia o.* subsp. *biloba* is mainly distributed in the central subtropical regions, which includes the western areas of Anhui province and Zhejiang province, the southern areas of Jiangxi province, Fujian province and Hunan province, the northern area of Guangdong province and the northern and north-eastern areas of Guangxi province (Fig. 1).

A total of 241 specimens were reviewed from the Chinese Herbarium (http://www.cvh.ac.cn/) to retrieve the distribution information of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*. As most specimens were identified as *M. o.* in the original background information of these specimens, we invited plant taxonomists to re-identify these specimens to the level of subspecies.

We also collected geographical distribution information for both subspecies from the published literature (Zhang et al. 2009; Yu et al. 2010; Wang et al. 2016; Yan et al. 2016). Specifically, specimen records of cultivated trees were excluded from the dataset. Finally, 163 records of *M. o.* subsp. *officinalis* and 106 records of *M. o.* subsp. *biloba* were used to estimate the habitat suitability, respectively (Fig. 1).

Environmental variables

Information on nineteen bioclimatic variables was retrieved from WorldClim (http://www.worldclim.org/) (Table 2). We used the averages for each of the years from 1970 to 2000 and were of spatial resolution 2.5' (Hijmans et al. 2005).

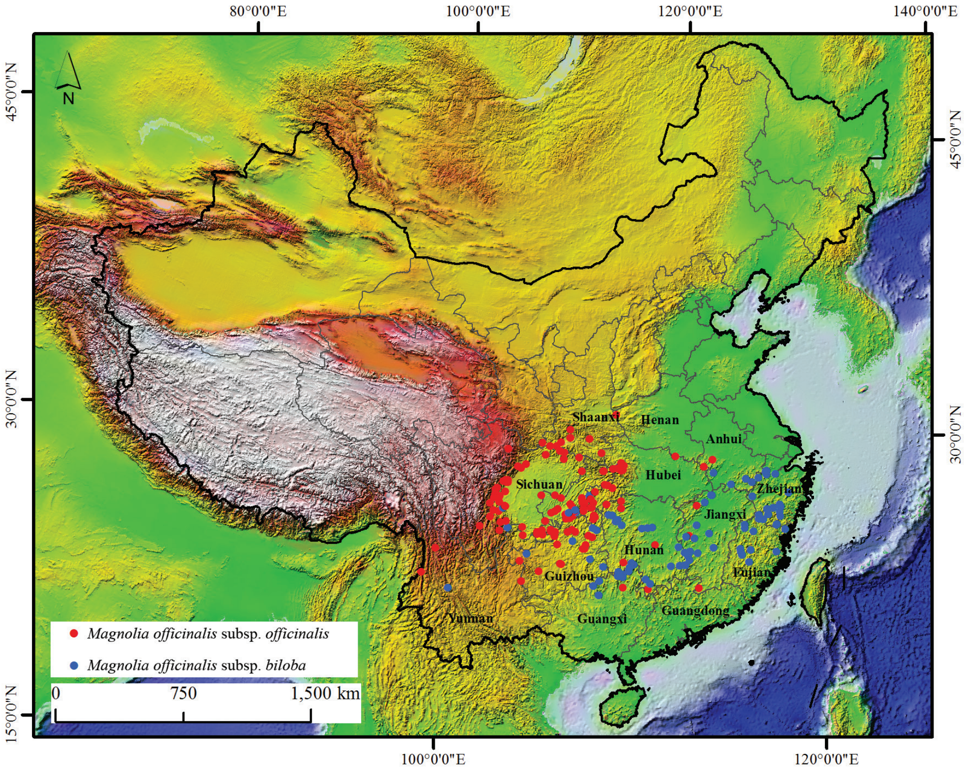

Figure 1. The distributions of *Magnolia officinalis* subsp. *officinalis* and *M. officinalis* subsp. *biloba* in China.

Table 1. Permutation importance of each environmental variable in determining *Magnolia officinalis* subsp. *officinalis* and *M. officinalis* subsp. *biloba* distributions

Variable	***M. o.* subsp. *officinalis***	***M. o.* subsp. *biloba***
altitude [m]	8.8	0.3
aspect [°]	0.1	0.1
slope [°]	0	0.1
temperature seasonality	11.1	18.5
min. temperature of coldest month [°C]	74.4	1.5
mean temperature of wettest quarter [°C]	0	1.4
mean diurnal range [°C]	1.5	4.2
annual precipitation [mm]	2.3	72.4
precipitation of warmest quarter [mm]	0.7	0.5
precipitation of driest month [mm]	1.1	1

The Advanced Spaceborne Thermal Emission and Reflection Radiometer Global Digital Elevation Model (ASTER GDEM) was used to derive topographic variables in this research. The vertical accuracy of the ASTER GDEM is 20 m and its horizontal accuracy is 30 m with 95% confidence (ASTER GDEM Validation Team 2011). We

Table 2. Approximate ranges of environmental variables suitable for each subspecies based on response curves

	Environmental variable	**Environmental ranges for *M. o.* subsp. *officinalis***	**Environmental ranges for *M. o.* subsp. *biloba***
Topography	altitude [m]	845–1750	156–594
	aspect[°]	87–151	68–140
Temperature	annual mean temperature [°C]	11–15	16–19.5
	mean diurnal range [°C]	6.7–9.2	6.4–7.8
	isothermality	26–30	25–28.5
	temperature seasonality	663–775	687.5–787.5
	max. temperature of warmest month [°C]	24.5–28	29.4–32.5
	min. temperature of coldest month [°C]	-3.8–-0.63	1.9–5
	temperature annual range [°C]	27–29	25.6–28.8
	mean temperature of wettest quarter [°C]	18.5–22.8	19.4–22.5
	mean temperature of driest quarter [°C]	1.3–5	6.9–13.1
	mean temperature of warmest quarter [°C]	20–24	>24.4
	mean temperature of coldest quarter [°C]	1.3–4.7	5–10
Precipitation	annual precipitation [mm]	1187.5–1500	1625–2156
	precipitation of wettest month [mm]	194–200	265–413
	precipitation of driest month [mm]	16–34	43–95
	precipitation seasonality	56–65	47–60
	precipitation of wettest quarter [mm]	525–712.5	712.5–1000
	precipitation of driest quarter [mm]	50–106	169–337.5
	precipitation of warmest quarter [mm]	530–750	562.5–750
	precipitation of coldest quarter [mm]	62–112.5	184–337.5

re-interpolated the ASTER GDEM to the same resolution as the bioclimatic variables (2.5') using a spline function.

Topographic variables used in this research include altitude, slope and aspect. Slope and aspect were calculated using the Spatial Analyst Tools in ArcGIS (v9.3). Slope helps to identify the rate of maximum change in z-value from each cell and the range of slope values in degrees is 0 to 90 (ESRI 2008). Aspect identifies the downslope direction of the maximum rate of change in value from each cell to its neighbours and it can be thought of as the slope direction (ESRI 2008). The output raster value of aspect is the compass direction, in which aspect is expressed in positive degrees from 0 to 360, measured clockwise from north, east, south and again to the north. In this research, we defined the north-facing slope as 0° and the south-facing slope as 180°. As the aspect changes from north to south, it increases gradually from 0 to 180°. We used this technique to transform aspect values from a circular variable (0–360°) into a continuous variable (0–180°).

To reduce the deleterious effects of collinearity on model fit, the maximum coefficient allowed between pairs of variables was set to 0.7 (Dormann et al. 2013). Correlation coefficients amongst environmental variables are presented in Suppl. material 1: Table S1. We then selected the following ten environmental variables for use in fitting the model: altitude, slope, aspect, mean diurnal range, temperature seasonality, min. temperature of coldest month, mean temperature of wettest quarter, annual precipitation, precipitation seasonality and precipitation of warmest quarter.

Nature reserve and land cover data

We retrieved data on the spatial distribution of national nature reserves from the Nanjing Institute of Environmental Sciences, Ministry of Ecology and Environment of China (http://www.nies.org/). We also retrieved land cover data (2015) of 1-km spatial resolution from the Resources and Environment Data Center of the Chinese Academy of Sciences (http://www.resdc.cn/).

Model fitting

Many software algorithms can be used to calculate habitat suitability using only species presence data, such as Bioclim (Busby 1986), Domain (Carpenter et al. 1993), Garp (Stockwell 1999), NFA (Hirzel et al. 2002) and Maxent (Phillips et al. 2006). The Maxent is one of the machine learning algorithm models and it is one of the most widely used and well-predicted species distribution models (Phillips et al. 2006; Elith et al. 2006; Harte et al. 2008; Wisz et al. 2008; Phillips et al. 2009; Yackulic et al. 2013).

In this study, we used Maxent to evaluate habitat suitability in *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*. We randomly selected 25% of the data entries for each subspecies for use as test data, with the remaining 75% being used to train the model (training data). The Maxent model parameters were set as follows: ‘maximum iterations’ was 500, ‘maximum number of background points’ was 10000, ‘replicates’ was 1 and ‘replicated run type’ was ‘cross-validate’. The ‘convergence threshold’ was 0.00001 and the ‘regularization multiplier’ was 1.

The output ASCII grid produced by Maxent is continuous probability data ranging from 0 to 1, which represents the habitat suitability of a species in a specified region. Based on previous research (Liu et al. 2013; Li et al. 2014; Liu et al. 2016), we adopted 0.7 as the threshold to transform the probability data into binary data (0/1). Grids, with probability higher than 0.7, were defined as suitable habitats.

Percent contribution and permutation importance are approaches available in the Maxent software which evaluate the contribution of variables to model predictions. Percent contribution and permutation importance are estimated based on the model gain, which is closely related to the deviance and is used to measure the goodness of fit of the model (Phillips 2017). Percent contribution depends on the path that the model used to reach the optimal solution (Songer et al. 2012; Smarter et al. 2012), whereas permutation importance depends on the final iteration of the fitted model. It is expressed as the differences between the AUC (area under the receiver operating curve) values of presence and background points caused by variation in the predictor variables (Songer et al. 2012). It is worth noting that when pairs of variables are strongly correlated with one another, the percent contributions of each should be interpreted with great care (Phillips 2017). Therefore, in this study, we used permutation importance to assess the contribution of each environmental variable to the fit of the produced models.

We also fitted response curves to evaluate how habitat suitability responded to variation in the environmental variables. In order to reduce the effects of correlation between pairs of environmental variables on model fit, each response curve was fitted using only one environmental variable. The fitted response curves are shown in Suppl. material 2: Figs S1–S6. Based on these response curves, we identified the characteristics of suitable habitats based on their environmental variables (Table 1).

Model validation

In numerous studies, only presence data (such as herbarium data) was available when attempting to model species distributions and habitat suitability. Absence data (negative records) are rare, despite their usefulness in assessing model specificity. Thus, it was difficult for us to use ROC (receiver operating characteristic curves) to evaluate the performance of the fitted models. Phillips et al. (2006) suggested an alternative approach which could avoid this issue through the distinguishing of presence from random, rather than presence from absence. In this way, ROC could be used to evaluate the performance of the fitted model.

In ROC, the ordinate axis represents the sensitivity, while the abscissa axis represents the false-positive fraction (1-specificity). The AUC (area under curve) is then used to measure the prediction success of the fitted model. AUC values range from 0 to 1; if AUC values are higher than 0.5, the imitative effect of the fitted model is deemed different from random (Fielding and Bell 1997). AUC values were then used to categorise model fits as follows (Swets 1988): poor (0.5–0.6), fair (0.6–0.7), good (0.7–0.8), very good (0.8–0.9) and excellent (0.9–1.0). In this study, the AUC calculated, based on the training data and test data, were both higher than 0.9 (Suppl. material 2: Fig. S7). This indicated that the fitted models performed excellently.

Assessment of conservation areas and the degree of human disturbance

We used overlay analysis and maps of national nature reserves and the identified suitable habitats to identify conservation areas where *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* populations were likely to occur. We also used overlay analysis of land cover and the identified suitable habitats to calculate the percentage for each land cover type in the suitable habitats and evaluated the extent to which these habitats have been disturbed by human activity.

Results

Contribution of environmental variables

Amongst the ten selected variables, the environmental variables which most affected the distribution of *M. o.* subsp. *officinalis* were min. temperature of coldest month, followed by temperature seasonality and altitude (Table 1). Given that many of the environmental variables were highly correlated with one another (Suppl. material 1: Table S1), we can reasonably conclude that extremely low temperatures (min. temperature of coldest month, mean temperature of driest quarter, mean temperature of coldest quarter) and temperature fluctuation (temperature seasonality and temperature annual range) are the most important variables which influence the distribution of *M. o.* subsp. *officinalis*. Both altitude and high temperature (max. temperature of warmest month, mean temperature of warmest quarter) also significantly influence the distribution of *M. o.* subsp. *officinalis* (Table 1), but to a lesser extent than extreme low temperatures and temperature fluctuations.

M. o. subsp. *biloba* seems to be more influenced by annual precipitation and extreme precipitation (precipitation of wettest month, precipitation of driest month, precipitation of wettest quarter, precipitation of driest quarter, precipitation of coldest quarter). Temperature fluctuation (temperature seasonality, temperature annual range, isothermality and mean diurnal range) also had notable influences on the distribution of *M. o.* subsp. *biloba*.

Habitat preference and potential distribution of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*

Habitats suitable for *M. officinalis* subsp. *biloba* are mainly located in central subtropical regions (Fig. 2). Small areas of suitable habitats could also be found in the northern areas of Taiwan. *Magnolia o.* subsp. *officinalis* habitats extend to locations in areas further north (Fig. 2), i.e. north subtropical areas. Habitats suitable for *M. o.* subsp. *officinalis* were also found in the south-eastern area of Tibet.

Compared to *M. officinalis* subsp. *biloba*, *M. officinalis* subsp. *officinalis* tends to grow at higher altitudes (Fig. 2) and is able to survive in areas experiencing greater fluctuations in ambient temperature, lower extreme temperatures, less precipitation and greater fluctuations in precipitation (Table 2).

Conservation areas and human disturbance

Overlay analysis of the identified suitable habitats and national nature reserves found that only 8.4% of the habitats suitable for *M. officinalis* subsp. *officinalis* were located in national nature reserves. Similarly, for *M. o.* subsp. *biloba*, only 3.4% of suitable habitats were located in national nature reserves. Thus, large areas of habitats, suitable for both subspecies, are not protected and are thus at risk (Fig. 2).

Overlay analysis of the identified suitable habitats and land cover found that the majority of land in habitats, suitable for both *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*, is woodland and forest (based on area) (Figs 3, 4). Suitable habitat areas were also constituted largely by agricultural land, residential land and mining and industry land (Figs 3, 4). In addition, suitable habitats were found to contain significant areas of grassland.

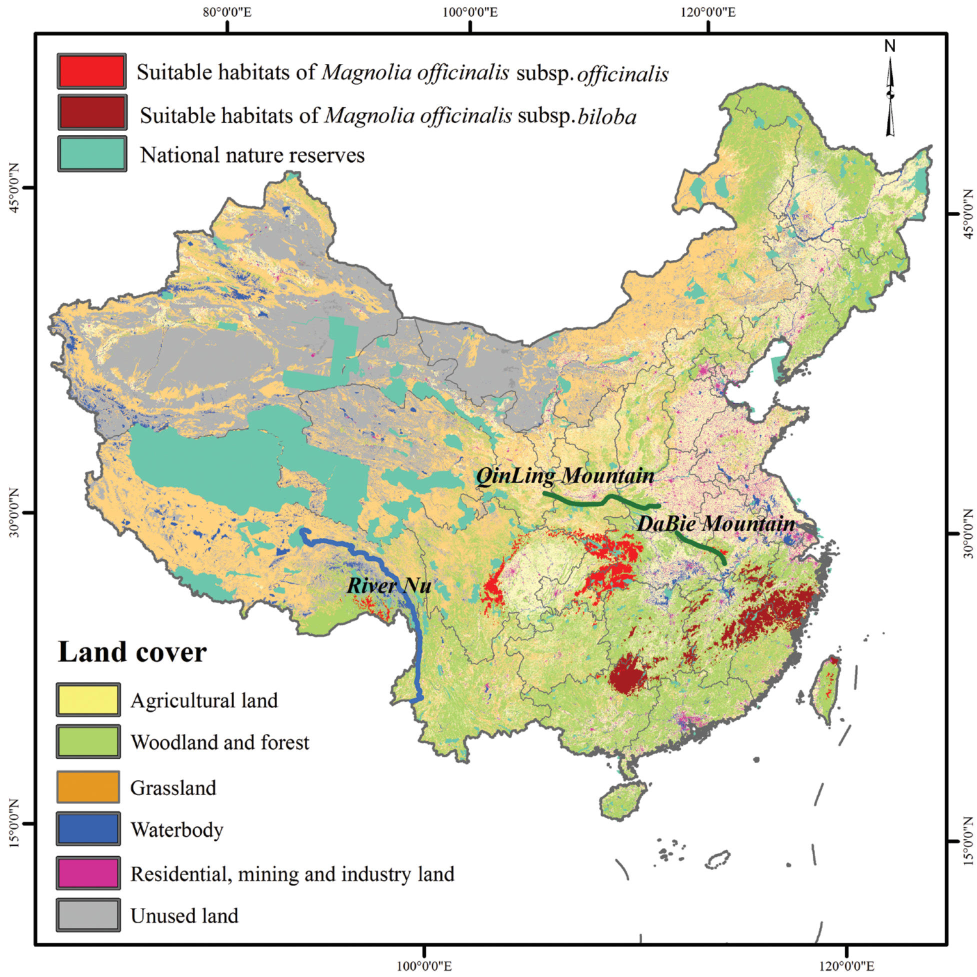

Figure 2. The spatial distributions of habitats suitable for *Magnolia officinalis* subsp. *officinalis* and *M. officinalis* subsp. *biloba*.

Discussion

Relationship between environments and the distribution of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*

The large-scale geographical distribution of vegetation is heavily influenced by the climate (Manthey and Box 2007; Punyasena et al. 2008). Amongst the myriad climatic variables, rainfall and temperature are the major factors which influence the distribution of vegetation; in particular, maximum and minimum rainfall and average temperature greatly limit the distribution and dispersal of vegetation (Thuiller et al. 2003; Crawford 2008). In our study, we found that extreme temperatures and precipitation

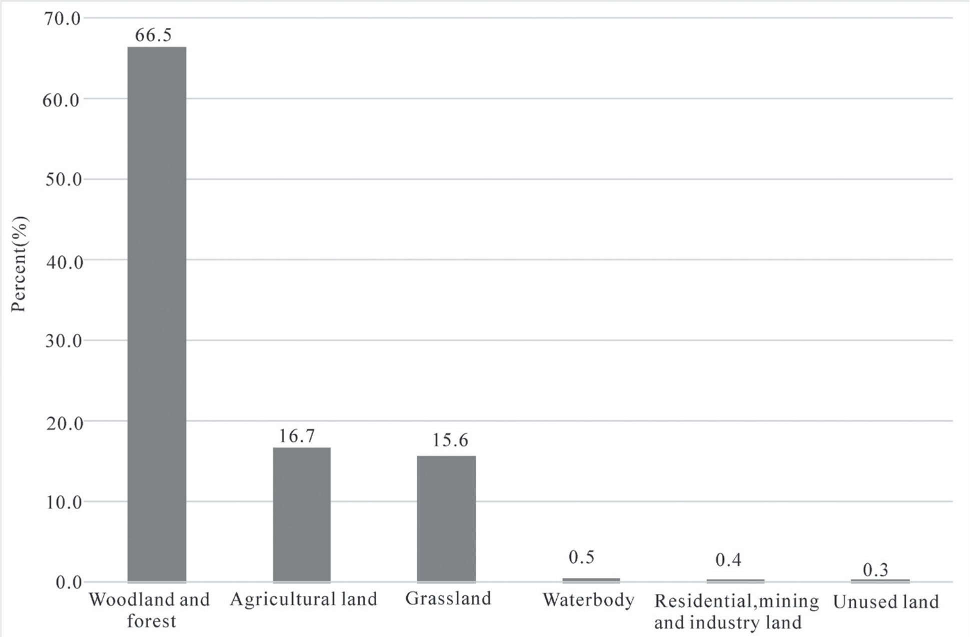

Figure 3. Land cover status (2015) within the suitable habitats of *Magnolia officinalis* subsp. *officinalis*.

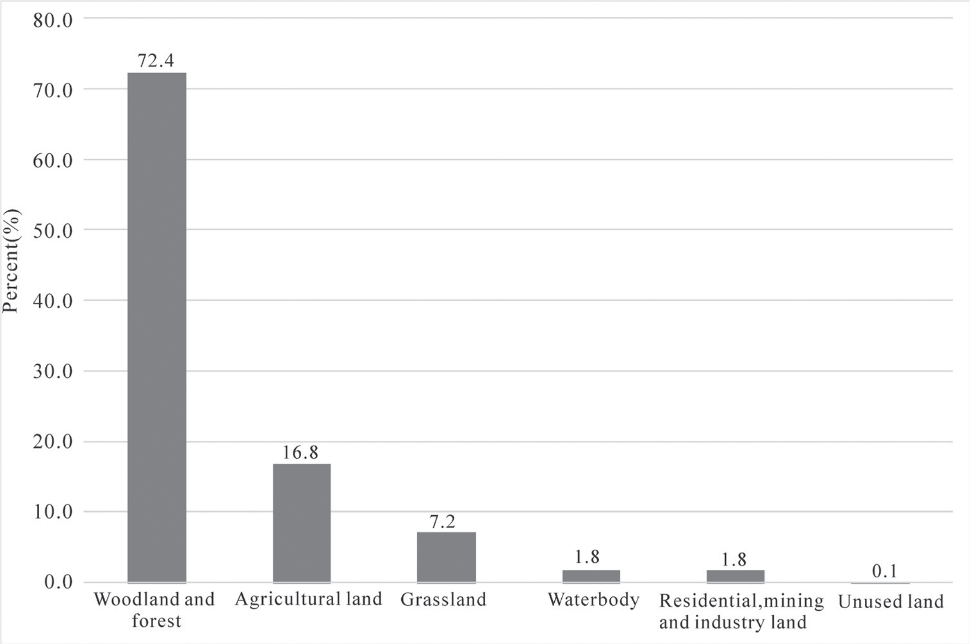

Figure 4. Land cover status (2015) within the suitable habitats of *Magnolia officinalis* subsp. *biloba*.

play important roles in determining the distributions of *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* (Table 1). However, the most influential variables varied between the two subspecies. For *M. o.* subsp. *officinalis*, temperature (min. temperature of coldest month, mean temperature of driest quarter, mean temperature of coldest quarter) was the most significant determinant of distribution. Conversely, in *M. o.* subsp. *biloba*, precipitation (annual precipitation, precipitation of wettest month, precipitation of driest month, precipitation of wettest quarter, precipitation of driest quarter, precipitation of coldest quarter) had a greater influence on distribution than temperature.

The reasons for this difference may be due to the different habitat preferences of the two subspecies. Habitats suitable for *M. o.* subsp. *officinalis* are mostly located in northern and central subtropical regions at high altitudes (Fig. 2). These areas are vulnerable to the Siberian cold current, resulting in extremely low temperatures in winter (Table 2). Therefore, extremely low temperature is the most important factor which limits the survival and spread of *M. o.* subsp. *officinalis.* Conversely, *M. o.* subsp. *biloba* prefers warmer, more southern regions (central subtropical areas) at low altitudes (Table 2). In southern areas, where low temperatures become less limiting, precipitation becomes the most important factor which prevents *M. o.* subsp. *biloba* from spreading to more northerly regions (Fig. 2). We also found that temperature fluctuations greatly influence the distributions of both *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* (Table 1). This finding was consistent with the hypothesis that temperature stability, in combination with sufficient rainfall, can promote colonisation by plant species (Slik et al. 2009; Zhang et al. 2013).

Threshold adopted to identify the suitable habitats

The output ASCII grid produced by Maxent is continuous probability data ranging from 0 to 1. A threshold is needed to transform the probability data into binary data (0/1) and to acquire the information about the spatial distribution of suitable habitats. In previous studies, 0.5 (Waltari and Guralnick 2009) and 0.8 (Ramírez-Barahona et al. 2009) were often used as arbitrary thresholds to transform the probability into binary data (0/1). Other thresholds, such as the minimum predicted value (Phillips et al. 2006), the 10th percentile training presence threshold (Brito et al. 2009), the 20th percentile training presence threshold (Donegan and Avendaño 2010), thresholds which result in a sensitivity of 95% (Newbold et al. 2009) and the maximisation of the sum of sensitivity and specificity (maxSSS) (Liu et al. 2013; Liu et al. 2016), have been utilised.

Maxent uses a variety of methods to determine thresholds, including the minimum training presence, 10th percentile training presence, equal training sensitivity and specificity, maximum training sensitivity plus specificity and so on (Suppl. material 1: Table S2). In this study, all thresholds estimated by Maxent were lower than 0.5 (Suppl. material 1: Table S2 and Table S3). Li et al. (2014) deemed that grids assigned probabilities higher than 0.6 could be reasonably defined as suitable habitats. Additionally, Liu et al. (2013, 2016) investigated which thresholds could be used confidently to transform probability data into binary data (0/1), reporting that the thresholds calculated by most algorithms are lower than 0.8.

Wilson et al. (2005) contend that the appropriate threshold should be selected according to the purpose for developing the species distribution model. Our primary purpose in this research was to find the most suitable habitats for *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* to aid in the conservation and plantation of both subspecies. Therefore, we used a larger threshold (0.7) to transform the continuous probability data into binary in order to ensure that only the most suitable habitats were selected, based on the assumption that areas exceeding the larger thresholds would be better able to support the conservation and plantation.

Conservation areas

Nature reserves provide effective refugia for wild plants. In this study, only a small number of habitats, suitable for either *M. officinalis* subsp. *officinalis* or *M. officinalis* subsp. *biloba*, were located in national nature reserves (Fig. 2). However, due to a lack of data, provincial, municipal and county-level nature reserves, forest parks, scenic spots and geological parks were unable to be included in our conservation area analysis. Therefore, the percentage of suitable habitats which are protected may in fact be higher than 8.4% (*M. o.* subsp. *biloba*) and 3.4% (*M. o.* subsp. *officinalis*).

Overlay analysis of land cover data and the locations of suitable habitats found that the majority of land in suitable habitats was woodland and forest (Figs 3, 4). However, plantations, including mulberry gardens, orchards and tea gardens were categorised within the "woodland and forest" category in the used datasets. It is thus difficult to know the exact proportion of plantation forests in the woodland and forest category. A considerable amount of land in suitable habitats was also used for agricultural land, residential land and mining and industry land (Figs 3, 4). Similarly, a large amount of land in suitable habitats was grassland (Figs 3, 4). In southern China, most grasslands are the result of secondary vegetation growth following deforestation. Therefore, we can conclude that human activities likely cause significant disturbances in habitats suitable for *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba*.

Limitations of this research

The geographical distributions of plants are heavily influenced by numerous environmental factors including climate, hydrology, soil, human activity and other factors (Gaston 2003). In our study, only climatic and terrain variables were used to assess habitat suitability. This limitation may have introduced some uncertainties into our assessments of habitat suitability. In addition, the latitudes and longitudes of some specimen collection sites were not directly recorded by GPS, being instead inferred from the names of the villages and towns near the collection sites which were recorded as background information for each specimen. Towns and villages are usually distributed in flat areas with low altitudes, which may have resulted in a tendency to assign lower altitudes than the actual altitudes of the sites from which the specimens were collected.

The habitat suitability assessment, conducted in this study, provides a scientific basis for the selection of priority protected areas in the conservation of *M. o.* subsp. *biloba* and *M. o.* subsp. *officinalis*. In addition to habitat suitability, the genetic diversity of endangered species needs to be considered when selecting priority areas for conservation (Cires et al. 2013). Genetic diversity plays a pivotal role in the maintenance of species populations (Beardmore 1983, Antonovics 1984, Yu et al. 2011). As we develop strategies for the conservation of threatened species, it is necessary to gain further insight into the genetic diversity in individual populations and that existing between populations (Hogbin and Peakall 1999; Yu et al. 2011). Previous studies have found that genetic diversity and population size are not always significantly correlated and the genetic diversity of populations of different sizes should be fully evaluated (Zhang et al. 2010). In conservation, proper attention should be paid to populations occupying smaller areas which have high genetic diversity, as they could be used to effectively reduce the loss of genetic resources (Zhang et al. 2010; Yu et al. 2011).

Prospects

Magnolia officinalis subsp. *officinalis* and *M. officinalis* subsp. *biloba* are closely related subspecies. However, there are marked differences in the geographical distributions of these two subspecies (Liu 1996). Few studies have investigated the characteristics and distributions of habitats suitable for these two subspecies. We mapped suitable habitats for both subspecies in China in this research (Fig. 2). Based on this information, we conclude that these identified suitable habitats should be selected as conservation priority areas in China to conserve *Magnolia* species or more natural reserves should be established to conserve these suitable habitats. This is very important for the *in situ* conservation of these subspecies. Moreover, the findings of our study can assist in the selection of suitable areas in which to cultivate these subspecies.

China is the ancestral home of the Magnoliaceae, with more than 40% of Magnoliaceae species having originated in southwest China (Wang and Jiang 2001; Cicuzza et al. 2007). In the past few decades, the survival of numerous Magnoliaceae species has been severely reduced by the activity of humans and many are greatly threatened by over-utilisation (for timber harvesting, bark, flower bud collection etc.) and habitat fragmentation (Wang and Jiang 2001; Cicuzza et al. 2007; Cires et al. 2013). According to the “China Biodiversity Red List,” 71.7% of Magnoliaceae species are defined as “Threatened” (Ministry of Ecology and Environment of China and Chinese Academy of Sciences 2013). Therefore, it is necessary to properly evaluate habitat suitability and genetic diversity for species in this family and to provide scientific support for their conservation.

Conclusion

In this study, we found that the environmental variables, which influence species distributions, are different for each subspecies. The distribution of *M. officinalis* subsp. *officinalis* was primarily determined by variation in minimum temperatures, while the distribution of *M. officinalis* subsp. *biloba* was primarily determined by variation in precipitation.

We identified the habitats suitable for both subspecies and found that the two subspecies have distinct habitat preferences. Compared to *M. o.* subsp. *biloba*, *M. o.* subsp. *officinalis* is found in more northerly areas, grows at higher altitudes and is able to survive in areas experiencing greater fluctuations in ambient temperature, lower extreme temperatures, less precipitation and greater fluctuations in precipitation.

The results of this analysis could provide useful information to support the *in situ* conservation of both *M. o.* subsp. *officinalis* and *M. o.* subsp. *biloba* and could aid in the selection of cultivation sites.

In the future, field survey data on the distribution of *Magnolia* species should be included in the assessment of habitat suitability to offset the deficiencies with regard to the specimen data. Genetic diversity assessment should be performed, together with habitat suitability assessment to provide stronger scientific support for the conservation of *Magnolia* species.

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Supplementary material 1

Tables S1–S3

Authors: Chuangye Song, Huiming Liu, Jixi Gao

Data type: statistical data

Explanation note: **Table S1.** Correlation coefficients between pairs of environmental variables. **Table S2.** Thresholds estimated by Maxent for the fitted model of *Magnolia officinalis* subsp. *officinalis*. **Table S3.** Thresholds estimated by Maxent for the fitted model of *Magnolia officinalis* subsp. *biloba*.

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Supplementary material 2

Figures S1–S7

Authors: Chuangye Song, Huiming Liu, Jixi Gao

Data type: statistical data

Explanation note: **Figure S1.** Response curves of habitat suitability to variables of topography of *Magnolia officinalis* subsp. *officinalis*. **Figure S2.** Response curves of habitat suitability to variables of temperature for *Magnolia officinalis* subsp. *officinalis*. **Figure S3.** Response curves of habitat suitability to variables of precipitation for *Magnolia officinalis* subsp. *officinalis*. **Figure S4.** Response curves of habitat suitability to variables of topography for *Magnolia officinalis* subsp. *biloba*. **Figure S5.** Response curves of habitat suitability to variables of temperature for *Magnolia officinalis* subsp. *biloba*. **Figure S6.** Response curves of habitat suitability to variables of precipitation for *Magnolia officinalis* subsp. *biloba*. **Figure S7.** Receiver operating characteristic curves of the fitted models (Fractional predicted area: the fraction of the total study area predicted present; Omission rate: the proportion of the localities falling outside the prediction.).

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A proposal for practical and effective biological corridors to connect protected areas in northwest Costa Rica

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Abstract

Habitat loss and increases in habitat isolation are causing animal population reductions and extirpations in forested areas of the world. This problem extends to protected areas, which, while often well-conserved, can be too small and isolated to maintain species that exist at low densities and require large contiguous areas of habitat (e.g. some large mammals). Costa Rica has been at the forefront of tropical forest conservation and a large proportion of the country's land area is currently under some form of protection. One such area is the northwest portion of Costa Rica, which is an extremely biodiverse region with several noteworthy national and privately-owned protected areas. However, each protected area is an isolated island in a sea of deforestation. Within Costa Rica's existing framework of biological corridors, we propose four sub-corridors as targets for restoration and full protection. These sub-corridors would link five major protected areas in northwest Costa Rica, with all of them linking to larger protected areas in the central portion of the country, while impacting a small number of people who reside within the corridors. After natural or active reforestation of the corridors, the result would be a contiguous protected area of 348,000 ha. The proposed sub-corridors would represent a 3.7% increase in protected area size in the region and only 0.2% of Costa Rica's total land area. Using the jaguar (*Panthera onca*) as a model umbrella species, we estimated that each current isolated protected area could support between 8–104 individuals. Assuming lack of dispersal between protected areas (distance between each ranges from 8.1 to 24.9 km), these population sizes are unlikely to be viable in the long term. However, the combined protected areas, connected by biological sub-corridors, could support about 250 jaguars, a population size with a higher probability of surviving. Our study shows that focusing conservation efforts on a relatively small area of Costa Rica could create a large protected area derived from numerous small isolated preserves.

Keywords
biological corridors, conservation, Costa Rica, *Panthera onca*, protected areas

Introduction

Biological corridors are important conservation tools for maintaining species diversity in places where habitat loss results in small isolated areas of natural landscapes (Taylor et al. 1993, Ewers and Didham 2006, Hilty 2012, Hilty et al. 2012) and may be vitally important for long-term survival of species under the ongoing human-induced climate change (Heller and Zavaleta 2009, Fung et al. 2017). Corridors can help facilitate dispersal (defined as movement of individuals with potential consequences for gene flow across space, Ronce 2007) and subsequently reduce extinction probabilities of some species, especially those that are unlikely to travel across large open spaces (e.g. primates, large terrestrial mammals, Weber and Rabinowitz 1996, Hilty et al. 2006, Crooks and Sanjayan 2006). While not a perfect replacement for large blocks of natural habitat, corridors often allow a number of small populations to effectively function as one large, viable population. Examples of effective corridors include those connecting a network of parks in Bhutan (Wangchuk 2007), the Terai Arc Landscape connecting 14 different protected areas in India and Nepal (Wikramanayake et al. 2004, Harihar and Pandav 2012), the Lower Rio Grande Valley National Wildlife Refuge in south-western United States (US Fish and Wildlife Service 2001) and the European Green Belt, which runs from the Finnish-Russian border all the way through the Balkans (Terry et al. 2006). Biological corridors appear to increase connectivity for a range of taxa, including vertebrates, arthropods and plants (Rosenberg et al. 1997, Bennett 1999, Haddad et al. 2003, Chetkiewicz et al. 2006).

Tropical rainforests continue to undergo rapid deforestation and fragmentation in many parts of the world (Hansen et al. 2010, Achard et al. 2014). However, some countries have slowed deforestation and have even begun to show net positive rates of forest cover (Meyfroidt and Lambin 2011, Keenan et al. 2015). Where deforestation has been largely halted and conservation efforts are strong, the opportunity exists to connect remaining blocks of isolated forest. For example, Costa Rica suffered extremely high rates of deforestation in the latter half of the 20th century, but by the year 2000, reforestation rates were higher than deforestation rates (Keenan et al. 2015). Although the exact amount of Costa Rica's forest cover is debatable, the country has been slowly gaining forest cover (to about 50%, Chacón-Cascante et al. 2012) since the turn of the century (Algeet-Abarquero et al. 2015), indicating that Costa Rica is going through the "forest transition," similar to what has been seen in some countries with highly developed economies (Mather and Needle 1998, Spray and Moran 2006, Jadin et al. 2016). Considering that 27% of Costa Rica is formally protected, much of the country's forest cover exists outside protected areas, forests that are likely necessary to conserve wide-ranging animals. Despite a strong and largely successful track record of forest conservation, many of Costa Rica's protected areas remain small islands of habitat, surrounded by deforested or highly fragmented lands (Sánchez-Azofeifa et al. 2003). These protected areas may lack sufficient area or connectivity to support some species, particularly those with low population densities and/or large home range requirements.

The Mesoamerican Biological Corridor proposal would connect a series of large existing protected areas via biological corridors, allowing connectivity from southern Mexico to Panama (Miller et al. 2001, Independent Evaluation Group 2011, Holland 2012). In part to meet the goals of the Mesoamerican Biological Corridor, Costa Rica's national system of conservation areas (Sistema Nacional de Areas de Conservación, SINAC 2018a) has established a system of biological corridors designed to connect protected areas throughout the country (National Program of Biological Corridors of Costa Rica, SINAC 2006, Fig. 1). However, the corridors established by SINAC are typically quite large (Chacón Cascante et al. 2012) and often include areas with large human populations (e.g. portions of the Paso de las Nubes have > 250 people/km^2, CIESIN 2016), extensive deforestation (greater than 50% in some corridors, Sánchez-Azofeifa et al. 2003, Morse et al. 2009, Fagan et al. 2016) and extensive economic activity, in particular agriculture (Fagan et al. 2013). These factors make true functional connectivity difficult or unlikely (i.e. connectivity that allows the successful dispersal of organisms between protected areas that increases the probability of species persistence, Rosenberg et al. 1997, Hess and Fischer 2001). Indeed, the Mesoamerican Corridor project has faced numerous political, social and ecological challenges since its inception (Vega 1994, Kaiser 2001, Miller et al. 2001, Grandia 2007, Mendoza et al. 2013). While Costa Rican efforts, directed at encouraging conservation in the large corridors, such as voluntary reforestation and ecosystem services payments, are likely to have environmental benefits, without coordinated efforts to reduce isolation of forest remnants, these activities are unlikely to produce truly contiguous areas of habitat outside of current protected areas. There have also been efforts (both ongoing projects and proposals) to create smaller biological corridors in Costa Rica that will likely provide direct connectivity between currently protected areas. Examples of these smaller corridors include: Maquenque National Park, which, when fully protected and reforested, would connect Costa Rican and Nicaraguan protected areas (Chassot and Monge-Arias 2012); the Barbilla-Destierro Corridor connecting the Central Volcanic Mountain Range to large protected areas in southern Costa Rica (Gamboa and Salom 2015); and the "Path of the Tapir" connecting protected areas in the south-western part of the country (Newcomer 2002). These corridors would be a fraction of the width of SINAC biological corridors, but would be a contiguous habitat between existing protected areas.

The north-western part of Costa Rica includes several noteworthy state- and privately-owned biological preserves such as Santa Rosa NP, Guanacaste NP, Rincón de la Vieja NP, Arenal NP, Monteverde Cloud Forest Reserve and the Children's Eternal Rainforest. The area is characterised by low elevation terrain punctuated by a series of young volcanic ranges. Habitat diversity is extremely high and includes tropical dry forests, pre-montane forests, cloud forests and tropical wet forests. Currently, pro-

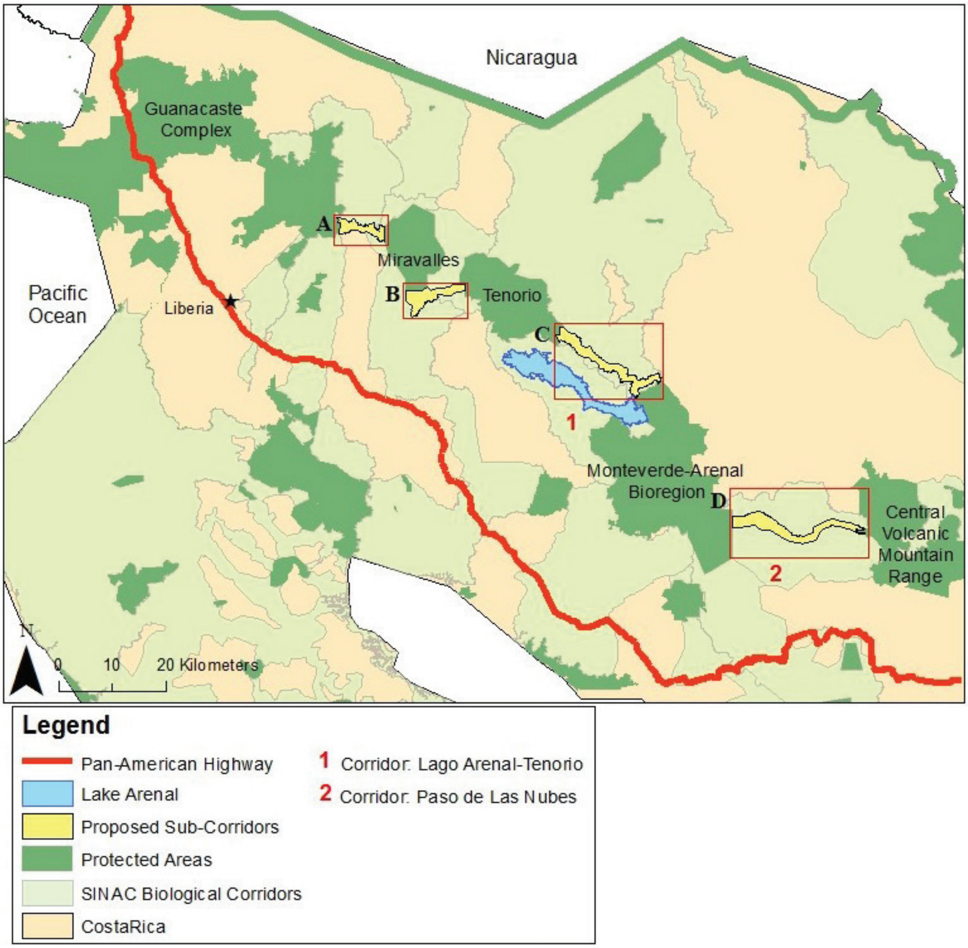


Figure 1. Proposed biological sub-corridors within the protected land and Costa Rican government designated biological corridors in north-western Costa Rica. **A** Guanacaste – Miravalles **B** Miravalles – Tenorio **C** Tenorio – Monteverde-Arenal Bioregion **D** Monteverde-Arenal Bioregion – Central Volcanic Mountain Range, 1 = Arenal – Tenorio Biological Corridor (SINAC-designated), 2 = Paso Las Nubes Biological Corridor (SINAC-designated).

tected areas are concentrated in the major volcanic features of the Guanacaste and Tilarán mountain ranges, plus a large area of tropical dry forest (Santa Rosa and Guanacaste National Parks) that stretches to the Pacific Ocean. With the exception of connectivity in the Guanacaste region, where three large national parks are connected either directly (Santa Rosa and Guanacaste) or by a protected corridor (Guanacaste and Rincón de la Vieja), protected areas are relatively small and isolated. For example, the Monteverde-Arenal Bioregion (MAB) is described as connected to its nearest neighbours, Tenorio National Park to the west and Juan Castro Blanco National park to the east, via SINAC's Arenal-Tenorio and Paso Las Nubes Biological Corridors, respectively. On the ground, however, functional connectivity (in terms of ability of forest-dependent organisms to move through these corridors) is less than ideal since the two biological corridors in question are composed mostly of fragments (see results) of forest with little or no formal protection. Recent monitoring of fragmented lands surrounding the MAB indicates that many large mammals found in the parks and preserves are not utilising their habitat outside the protected areas (Zamzow et al. 2018). Therefore, the MAB – which includes Arenal Volcano National Park, the Children's Eternal Rainforest, Monteverde Cloud Forest Biological Reserve, Santa Elena Cloud Forest Reserve and Alberto Manuel Brenes Biological Reserve – remains relatively isolated. This area has extremely high species diversity and endemism and supports a number of endangered species, including jaguar (*Panthera onca*), spider monkey (*Ateles geoffroyi*) and Baird's tapir (*Tapirus bairdii*). Two of the local communities surrounding the MAB, La Fortuna and Monteverde, are major ecotourism destinations (Koens et al. 2009) that generate millions of dollars of tourism revenue and support numerous ecotourism and related jobs (Aylward et al. 1996, Langholz et al. 2000, Stuckey et al. 2014).

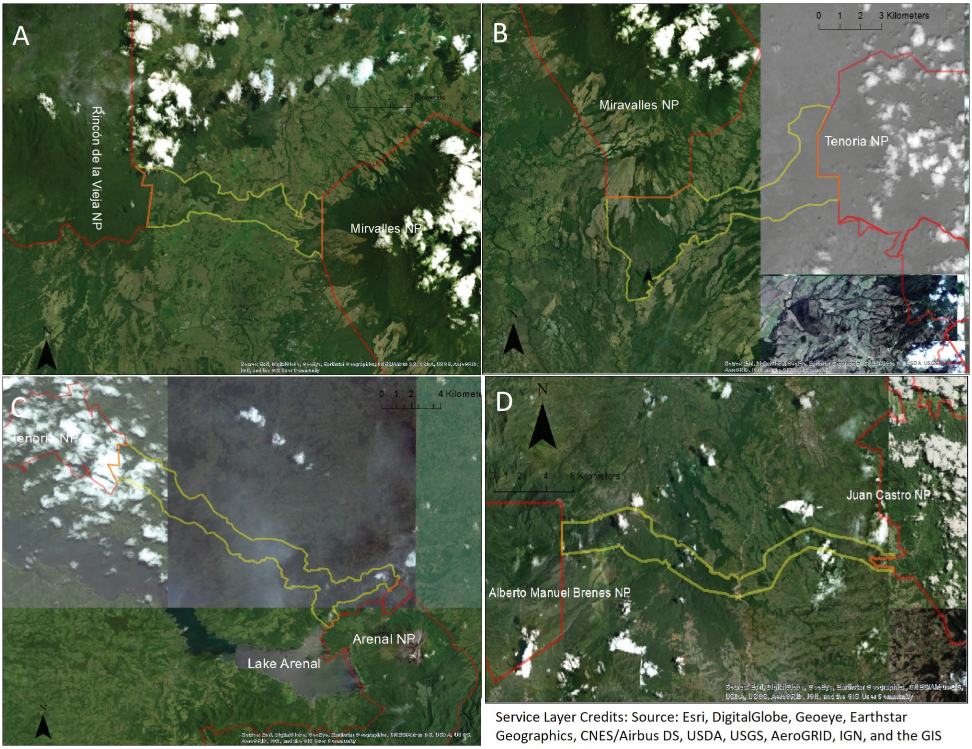
The distance between the isolated protected areas in the north-western part of Costa Rica is relatively small (minimum: 8km, maximum: 25 km) and there are areas of natural forest cover (variety of primary and secondary growth, M. Moran and L. Stallcup, personal observation) between these protected areas. Human population density is also relatively low, making for a high potential for creating biological corridors with low impact on human communities.

The goal of this study was to determine if we could identify potential sub-corridors within the larger SINCAC biological corridors that would have parameters that allow for the movement of forest-dependent species. These sub-corridors would meet the minimum size requirement (defined here as having a minimum width of 1000 m, Lees and Peres 2008) to achieve ecological connectivity for organisms unlikely to disperse across deforested or fragmented landscapes, while also having minimum impact on local human populations. We sought to find areas with high levels of current closed-canopy forest cover, as these areas likely retain a considerable amount of biodiversity and it would be relatively cost-effective to achieve complete forest cover through natural regeneration. We assumed that protection of currently forested lands would have less effect on economic activity in the region, since these lands are not currently being utilised for intensive agriculture. By proposing the smallest effective biological sub-corridors and minimising negative impact on current human populations and economic activity, we wanted to increase functional biological connectivity, conservation of natural resources, ecosystem services and benefits to the Costa Rican people. While interested in achieving connectivity of protected areas for the benefit of biodiversity in general, we modelled the current and potential population sizes of the jaguar as an umbrella species following Thornton et al. (2016) to determine if our proposed sub-corridors would be potentially effective in supporting a viable population of this sensitive species. The jaguar is a focal conservation species in Costa Rica (SINAC 2018b) and is declining in parts of the country (Salom-Pérez et al. 2007). It is often considered a valuable proxy for conserving biodiversity (Thornton et al. 2016), in that habitats capable of supporting this top predator species are likely to be able to support many other less sensitive species. In Costa Rica, the jaguar currently exists in several important "jaguar conservation units" that are poorly connected to each other with subsequent limited dispersal opportunities (jaguar conservation unit 154 in our study region, Sanderson et al. 2002).

Methods

We downloaded boundaries of legally protected terrestrial lands (UNEP-WCMC 2018) and the biological corridor boundaries regulated by the SINAC (Oficina Nacional Forestal 2006). We used the Global Forest Watch database (Hansen et al. 2013, parameters: 30 m × 30 m resolution, images from Landsat 7, GIS layer established in 2000, most recently updated in 2017) to locate forested areas between protected areas in north-western Costa Rica that could provide cover as feasible biological sub-corridors (See Appendix 1 for data sources). Our goals in defining a prospective sub-corridor were to minimise proposed size, minimise overall linear distance (km) between established protected areas, maximise current proportion of forest cover and minimise human population density. We first focused on finding land connecting existing protected areas that was relatively short in distance and maintained at least some forest cover. In all cases, we avoided any dense human population areas (i.e. cities and towns) using visual inspection of satellite data (explained below). When deciding on which route to choose, we ultimately had to make a judgement that considered distance of corridor, amount of forest cover and avoidance of densely populated areas. A limitation to the dataset is that the forest cover maps did not differentiate between native or non-native forest, nor forest quality (except for tree cover proportion).

We then obtained recent satellite views for each of the proposed sub-corridors from Google Earth Pro™. All corridor locations had satellite imagery from the year 2017. We established that the minimum width of any point along the corridor must be at least 1000 m, since this width appears to allow movement of practically all non-flying mammals in the Neotropics (Lees and Peres 2008). Each sub-corridor boundary was adjusted manually (using the Ruler tool in Google Earth) to minimise overlap with human structures and maximise areas of current forest cover (Fig. 2). Once sub-corridor boundaries were constructed, we measured (using visual determination and the Ruler tool) the amount of land that was forest, pasture, developed or other (i.e. barren land, either natural or due to human activity). Developed landscapes were classified as those covered by human-built structures (houses, roads etc.). We counted the number of human structures, other than roads, as a proxy for how many people live in the proposed sub-corridors. If there was some doubt about land cover classification, we used local ground observations (L. Stallcup, personal observations). Proportional land cover was then calculated by dividing the various land use categories by the total corridor area. We also measured the minimum length of each corridor – in other words, the minimal distance an animal would have to travel to move from one current protected area to another via the sub-corridor. Using Arc GIS™, we constructed maps of protected areas,



Service Layer Credits: Source: Esri, DigitalGlobe, Geoeye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

Figure 2. Satellite views of the four proposed biological sub-corridors (in yellow) with adjoining protected areas (in red). Dark green areas indicate the presence of forested habitat.

SINAC biological corridors and proposed sub-corridors in order to show the locations relative to other geographic features.

We then modelled the potential population size of jaguars, a commonly utilised "umbrella" species, whose presence may indicate a well-functioning ecosystem (Barua 2011, Thornton et al. 2016). The jaguar is a species that appears to have a viable population in some areas of our study (e.g. Guanacaste and Santa Rosa NPs, SINAC 2018b) but is very rare or absent in other protected areas (e.g. MAB, Zamzow et al. 2018). Therefore, if sub-corridor protection were to be initiated, this species could also be monitored in areas where it is currently absent or rare as a measure of whether our proposal was having some conservation success. Based on density estimates of jaguar from literature of published studies of the region (Appendix 2), we calculated the mean population density per 100 km^2 (± 2 standard errors of the mean). These values were used as the estimate of the potential carrying capacity of the forested habitat, based on the size of each current contiguous protected area. We then estimated population sizes of jaguars in all current protected areas as if these areas were already connected by the proposed sub-corridors (using area of currently protected areas, plus our proposed sub-corridor areas). In our estimates, we assumed no jaguar mortality other than natural density-dependent population regulation due to carrying capacity of the habitat.

Limitations and future improvements

Our analysis has limitations worth considering. Except for forest cover proportion, we did not utilise computer software (e.g. Marxan) to choose the best path for our sub-corridors. Instead, once we had located a pathway with the most forest cover that did not cross towns and other settlements, we made individual decisions on corridor shape and path based on local land use, shape of forest blocks, human structures, distance between existing protected areas and existing political frameworks (e.g. existence of current SINAC corridor designations). Our analysis, therefore, does not take into account costs, biodiversity protection or boundary length, as would be incorporated into some programmes (Ball et al. 2009). An important next step would be to determine land ownership patterns and potential interest in conservation amongst land owners within the sub-corridors. Biological monitoring (e.g. camera trap studies) would also be recommended if sub-corridor establishment is commenced to determine before and after use by target animals.

Results

The four proposed biological sub-corridors in northwest Costa Rica (Fig. 1) represent relatively small areas of the country (about 0.2% of total country area) and are relatively small as compared to the protected areas they will connect (3.7%, Table 1). Each proposed sub-corridor has high percentage forest cover (much of it native, M. Moran and L. Stallcup, personal observations) and low levels of development compared to surrounding landscape (Table 2, Fig. 3). Practically all lands not forested are in pasture, presumably utilised to raise cattle (M. Moran, personal observation).

The sub-corridor we propose to connect the Guanacaste complex (specifically Rincón de la Vieja National Park) to Miravalles National Park, Sub-corridor A, has the most deforested lands and contains the greatest number of human-built structures (Fig. 3A). It should be noted that areas north of proposed Sub-corridor A have a slightly higher proportion of forest cover, but also contain many more human structures (resulting in a judgement call minimising human impact for this recommended route). The corridor proposed to connect Miravalles and Tenorio National Parks, Sub-corridor B, is more than two-thirds forested and contains no visible human structures (Fig. 3B). The area north of Sub-corridor B represents a much shorter distance between protected areas and would be smaller in area, but would also pass directly through the town of Rio Naranjo (population = 1000+, INEC 2015), making it a less desirable route. The corridor connecting Tenorio and the MAB, Sub-corridor C, is also roughly two-thirds forested and contains a minimal number of human structures (Fig. 3C); however, because it connects two more distant protected areas in our study, its area and minimum distance is greater than proposed sub-corridors A and B (Table 2). The other potential locations for sub-corridors in this region between the MAB and Tenorio all have less forest cover, longer distance between protected areas and a greater number of

Table 1. Current physical characteristics of proposed biological sub-corridors in northwest Costa Rica that would produce a contiguous protected area from the Guanacaste Region (Santa Rosa NP) to the Central Volcanic Mountain Range. *indicates area covered in recent volcanic activity with sparse, but natural vegetation. †indicates barren land caused by ongoing road construction.

Corridor	Area (Ha)	Proportion forested	Proportion pasture	Proportion developed	Proportion Other	Length (km)	Human structures
Guanacaste–Miravalles	1,594	0.481	0.500	0.004	0.015*	8.2	28
Miravalles–Tenorio	2,532	0.715	0.227	<0.001	0.058*	9.7	0
Tenorio–MAB	3,946	0.674	0.326	<0.001	0.000	18.6	12
MAB–CVMT	4,277	0.765	0.224	0.004	0.007†	24.9	19
Total Corridor Area	**12,349**						
Current Protected Area	**348,000**						

Table 2. Area covered by all proposed corridors, currently protected lands and proportional contributions of these lands relative to current protected areas and Costa Rica as a whole.

Total corridor area (Ha)	Proportion addition to protected areas (region)	Proportion of Costa Rica
12,349	0.037	0.002

human structures. The fourth proposed corridor, Sub-corridor D, would connect the MAB to Juan Castro Blanco National Park, thereby connecting it to the greater Central Volcanic Mountain Range (Fig. 3D). This is the largest and longest sub-corridor. Sub-corridor D falls within the existing SINAC-designated Paso Las Nubes Biological Corridor (Fig. 1), which has a high proportion of forested land in the western portion. However, the eastern section is mostly deforested and passes close to several moderate-sized cities (most notably Ciudad Quesada). However, we were able to locate a route for Sub-corridor D with fewer human structures (Table 2). Regardless of the exact route for this sub-corridor, it is traversed in a north-south direction by the construction of a new highway connecting the cities of Ciudad Quesada and San Ramón, which means that wildlife passageways would need to be included to mitigate road impacts and enable Sub-corridor D to be ecologically functional (Fig. 3D).

The 23 studies that sampled jaguar density in tropical forests of Central America found an average of 7.09 jaguars/100 km^2 ± 1.16 (2SE, Appendix 2). Based on this mean value, each current isolated protected area in northwest Costa Rica can support less than 100 individuals (Table 3). The Central Volcanic Mountain Range protected area can support a population of just over 100 jaguars. None of these populations would be considered viable in the long-term (assuming N = 500 as minimum, Thomas 1990) unless there is some dispersal between populations (Rabinowitz and Zeller 2010). By connecting the protected areas in north-western Costa Rica to the Central Volcanic Mountain Range using our biological sub-corridors, the total estimated jaguar population would be more than 250. Assuming a conservative jaguar population estimate, all areas combined could support almost 200 individuals; at the higher range of the estimate, the area could potentially support more than 300 individuals (Table 3).

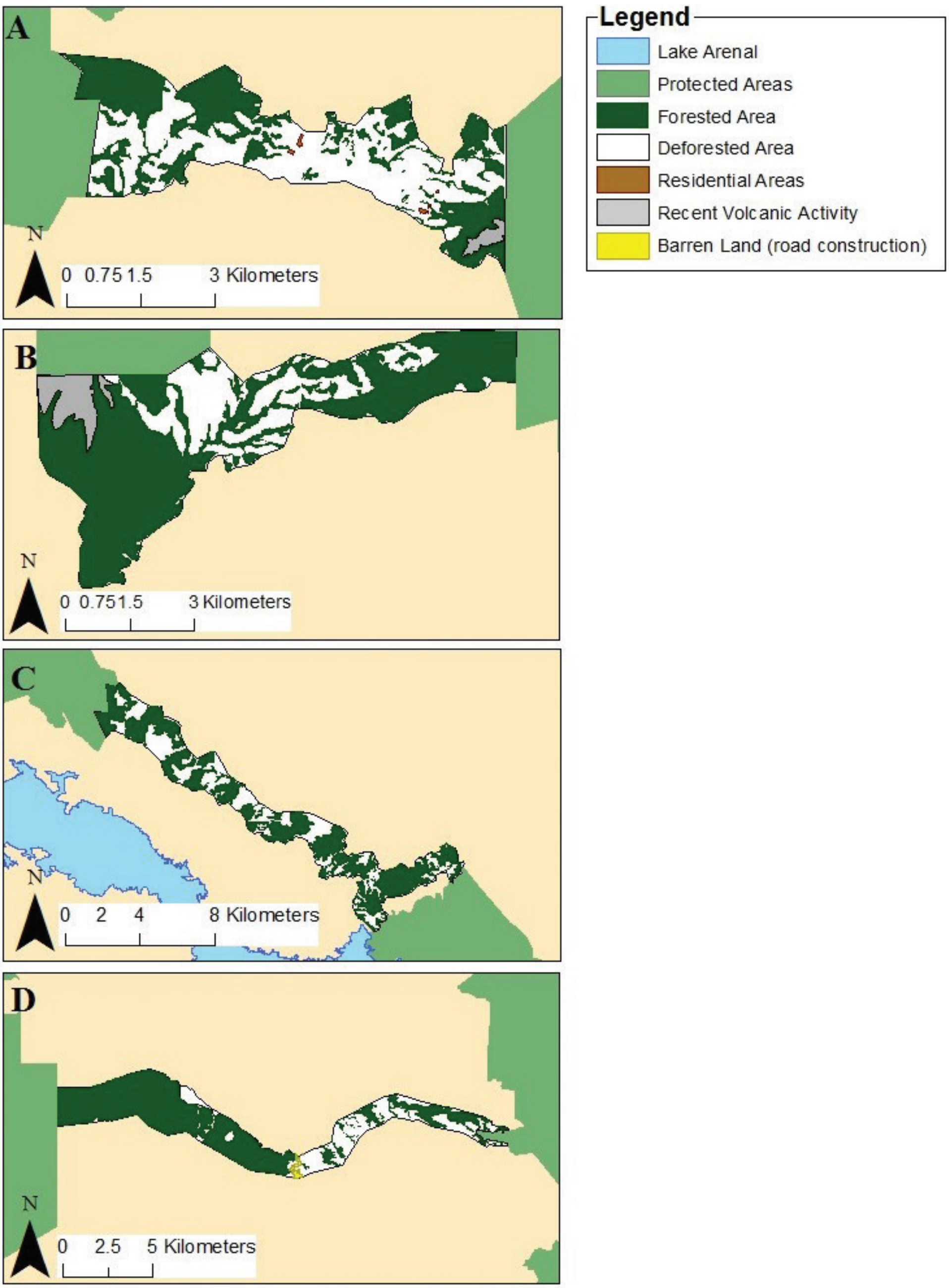


Figure 3. Details of land use for each proposed sub-corridor. **A** Guanacaste – Miravalles **B** Miravalles – Tenorio **C** Tenorio – Monteverde-Arenal Bioregion **D** Monteverde-Arenal Bioregion – Central Volcanic Mountain Range.

Table 3. Estimated population sizes of jaguar (*Panthera onca*) that could be supported in [1]current contiguous protected areas, [2]proposed contiguous protected areas connected by biological sub-corridors and habitat areas for each location. GC = Guanacaste Complex, M = Miravalles, T = Tenorio, MAB = Monteverde-Arenal Bioregion, CVMR = Central Volcanic Mountain Range, NWCR = Northwest Costa Rica including GC, M, T, MAB and three proposed corridors (Table 1), NWCR & CVMR = All protected areas and proposed corridors from Northwest Costa Rica + Central Volcanic Mountain Range.

Estimate	GC[1]	M[1]	T[1]	MAB[1]	CVMR[1]	NWCR[2]	NWCR & CVMR[2]
Mean	78	8	13	34	104	145	252
Low	60	6	10	27	80	112	195
High	96	10	16	42	128	179	310
Habitat Area (Ha)	110,241	11,670	18,492	48,500	146,794	196,975	348,049

Discussion

Overview of four corridors

The four functional sub-corridors proposed in this paper (Fig. 2) represent a preliminary, but potentially feasible, approach to protecting lands that could be useful in connecting populations of terrestrial mammals that are unlikely to travel between the current protected areas. Admittedly, the sub-corridors are relatively small, but human settlement patterns and large agricultural areas make the establishment of larger (in particular wider) contiguous corridors unlikely. However, by facilitating increased connectivity between currently protected areas, including some areas that do have existing jaguar populations (Sanderson et al. 2002, Rabinowitz and Zeller 2010), we create areas of contiguous habitat. This habitat could potentially support species such as the jaguar, which have naturally low population densities and require large areas with limited human activity to sustain viable populations. Some large mammals are missing or rare in isolated protected areas within our study region (Zamzow et al. 2018); often these mammals are species considered important for ecosystem functions, such as prey population regulation (e.g. top predators, Estes et al. 2011) and seed dispersal (e.g. Baird's tapir, Fragoso et al. 2003). Additional benefits of the current proposal include enhanced ecosystem services such as carbon sequestration, water resource protection and increased ecotourism, as has been seen in other parts of the Mesoamerican Biological Corridor (Harvey et al. 2008). These sub-corridors may also allow organisms to disperse more freely in response to climate change, a process that will likely become vitally important in the near future in many regions (Heller and Zavaleta 2009, Fung et al. 2017), including Mesoamerica (Imbach et al. 2013). However, each sub-corridor also has implementation challenges that will require creative solutions, as well as on-the-ground assessment (see Lees and Peres 2008, as an example). Below, we highlight details and challenges of each sub-corridor.

Sub-Corridor A

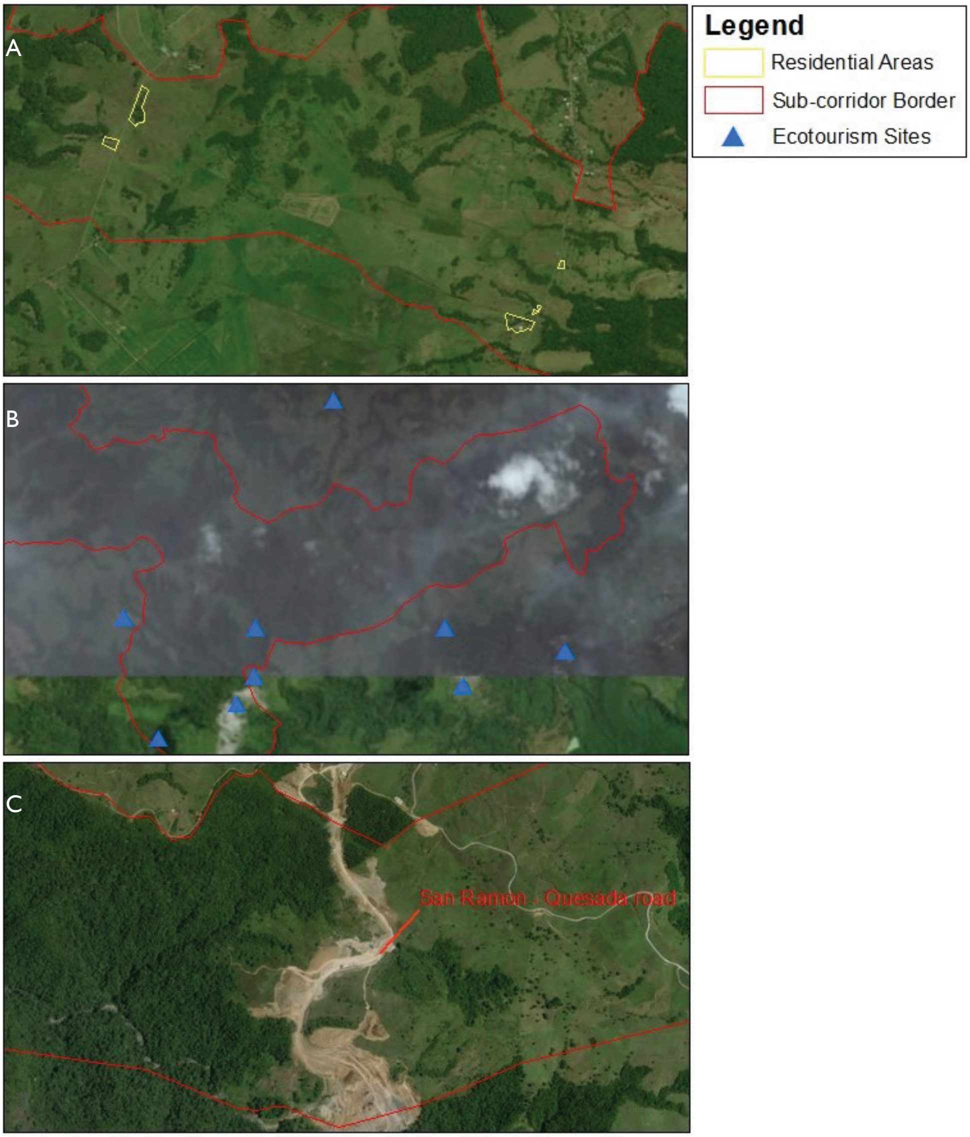
This proposed sub-corridor is the smallest and shortest, connecting the relatively large, biodiverse and contiguous reserves of the Guanacaste region (Santa Rosa, Guanacaste and Rincon de la Vieja National Parks) to Miravalles National Park. Presumably, this corridor would increase species richness in the latter since it would connect it to a much larger complex of conserved areas. However, Sub-corridor A also has the lowest level of forest cover (slightly less than 50%) and the greatest number of human structures, including some small developed areas, presumably containing a considerable number of people (Fig. 4A). Areas north and south of the proposed corridor have even larger settlements and less forest cover, so the proposed area seems the only practical option without additional active restoration or rezoning.

Sub-corridor B

This proposed sub-corridor, which connects Miravalles and Tenorio National Parks, is moderate in size and length, but it is probably the most practical under current land-use conditions. It is more than 75% forested and contains no human dwellings. The only development is a road (Route 6) that passes through the area. While this sub-corridor would be relatively easy to establish (if landowners were willing), it would only connect two relatively small protected areas, Miravalles and Tenorio National Parks. If neither of these conservation areas were connected via biological corridors to other regions, the conservation value, in terms of connecting populations of species that require large habitat areas, would presumably be modest.

Sub-corridor C

This proposed sub-corridor connects Tenorio with the Monteverde-Arenal Bioregion (MAB). It is relatively large, traverses a long distance, is about two-thirds forested and has relatively few human structures, most of which are located along Route 142 between Lake Arenal and the town of La Fortuna. Many of these developments are associated with ecotourism (Fig. 4B), so it is possible that conservation-minded business owners would be interested in conservation agreements (as has been effectively achieved in other parts of Costa Rica; Pagiola 2002) or other public-private conservation partnerships that could make this sub-corridor more likely. Portions of the land between Tenorio and Arenal Volcano National Parks include part of the watershed that drains to Lake Arenal, a major hydroelectric generating facility (about 15% of total) for Costa Rica, so forest protection and future reforestation in this sub-corridor could improve water quality and reduce sedimentation in Lake Arenal (sensu ecosystem services).



Service Layer Credits: Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

Figure 4. Highlighted areas of three biological corridors that have particularly problematic development and infrastructure patterns that would make corridor establishment logistically difficult. **A** Guanacaste – Miravalles **B** Tenorio – Monteverde-Arenal Bioregion **C** Monteverde-Arenal Bioregion – CVMR.

Sub-corridor D

This proposed sub-corridor connecting the MAB to the Central Volcanic Mountain Range has perhaps the highest conservation potential, but is challenging biologically (because of length, Rabinowitz and Zeller 2010), socially and politically. It would be the longest and largest corridor, passing near several large towns, most notably the city of Quesada (population = 44,000). There is also the road construction project (San Carlos-San Ramon Highway) that will bisect any conservation corridor established in this region (Fig. 4C). The western half of Sub-corridor D has a high level of forest cover (almost 100%). Additionally, hydroelectric facilities within the corridor would benefit from increased forest protection. Most important in conservation terms, this corridor would connect two large protected and biodiverse land complexes: the Monteverde-Arenal Bioregion and the Central Volcanic Mountain Range. However, the growing population of the region (Ciudad Quesada has grown 21% since 2000; INEC 2015), along with increasing development that will likely follow the construction of the San Carlos-San Ramon highway, will likely make corridor establishment challenging without mitigation. Large highways are particularly problematic for wildlife, causing direct mortality (from vehicle strikes), dispersal blockage and loss or damage to natural resources (Forman et al. 2003, Clevenger and Wierzchowski 2006, Beckmann et al. 2010). The current land use trajectory suggests maintaining connectivity between protected areas of northwest Costa Rica and the Central Volcanic Mountain Range requires proactive highway planning and mitigation (e.g. wildlife underpasses). Sub-corridor D should be a high priority for research and conservation focus. The implementation of planned mitigation, related to the construction of the San Carlos-San Ramon highway, is currently uncertain (Villalobos Sánchez 2018); however, the land under the long bridge being constructed over the Espino River (of which about 0.25 km will be elevated above ground) within our proposed corridor could act as an effective dispersal pathway. Once construction of the highway is completed, reclamation and restoration of the land disturbed by the bridge construction should be a priority.

Feasibility

The areas proposed for conservation in this study are very small relative to the size of Costa Rica and less than 5% the size of the current protected areas in the region (stretching from northwest Costa Rica to the Central Volcanic Mountain Range). In addition, the majority of the areas where sub-corridors are proposed already have relatively high proportions of forest cover and would therefore require a small amount of restoration work, such as active native tree planting. Other small corridors in the process of being established in Costa Rica also share these characteristics (Fagan et al. 2016). As has been seen across the country, simply allowing natural regeneration to occur would probably be sufficient to create a forested corridor within a relatively short period of time (e.g. secondary forest within 15 years, Shono et al. 2007). We suggest the proposed sub-corridors could be established with minimal disruption to local human populations and be ecologically effective for a relatively small initial expenditure of resources, once the land is purchased. However, long-term monitoring and management responsibilities would need to be determined prior to any land purchases. Once established, the proposed biological sub-corridors would still require active monitoring and protection, including law enforcement. Unauthorised encroachment, poaching, grazing and resource extraction remain a problem in currently protected areas in Costa Rica, even in places with high levels of enforcement (Hilborn et al. 2006). Protecting the sub-corridors, which in some cases would be located near areas of high human activity, would certainly be a challenge and should be considered as part of a regional protected areas strategy. Additionally, biological monitoring will need to be implemented to determine if the sub-corridors are functioning as intended. In addition to width, corridor qualities such canopy cover, tree height and anthropogenic intrusions affect use by target animals (Rosenberg et al. 1997, Lees and Peres 2008). With the advent of wildlife cameras, monitoring mammals, including secretive ones, is relatively straightforward and cost effective (O'Connell et al. 2010). Monitoring programmes beginning now, while the corridors are still fragmented and extending into the future when corridors become more contiguous, would provide valuable information on effectiveness (perhaps as a model for other regions as well).

One strategy could be the incorporation of these sub-corridors into existing protected areas. All proposed biological corridors would connect directly to existing parks and preserves owned by the Costa Rican government. While requiring additional public resources for purchase and protection, this method would work within the existing framework on land protection. In contrast to direct purchase of land for inclusion into the national system of protected areas, one could consider more creative land conservation methods. For example, targeted ecosystem service payments and/or conservation easements for high priority lands could be employed to promote connectivity in and around the proposed sub-corridors. Costa Rica's current system of environmental service payments, which include carbon emissions offsets, watershed protection, biodiversity maintenance and scenic and recreational enhancement (SINAC 2006), pays landowners to maintain forested lands on private property (Robalino et al. 2011). However, as of this writing, there is a limit of 600 hectares per private property owner (Executive Decree 2017). One alternative would be to lift this size restriction for targeted lands with the highest conservation value or other ecosystem services described above, although this could be controversial as it could benefit those with larger landholdings and presumably those who are wealthier. Discussion with all stakeholders (land owners, industries, conservation groups, government and local communities) will therefore be necessary. In addition to the proposed sub-corridors, there could be targeted reforestation in the vicinity of each sub-corridor, a process that could improve the habitat in the larger region (Mateo-Sánchez et al. 2015). In part, this priority already exists, since Costa Rica is moving in the direction of prioritising corridors and properties within the larger SINAC-designated biological corridors already receive preference for ecosystem service payments (Malavasi and Kellenberg 2002). Finally, the establishment of conservation easements, by which landowners are provided a payment (by a government or non-government conservation group) for maintaining in perpetuity land in its natural state and agreeing to certain management practices, has been effective for protecting lands in many other locations across the globe (Rissman et al. 2007). If combined with good vigilance and enforcement, conservation easements could also be considered for these corridor parcels in Costa Rica, which would offer financial incentives to landowners.

Conclusions

The current protected areas of northwest Costa Rica are each too small to provide long-term survival to some species, such as the jaguar. While jaguars are present in the region (SINAC 2018b), very few isolated populations are likely to survive long-term (Quigley and Crawshaw 1992). In fact, viable jaguar populations may only occur in only two of the areas of this study: the reserves of the Guanacaste Conservation Area and the Central Volcanic Mountain Range (Sanderson et al. 2002, SINAC 2018b), although our analysis indicates these are also too small to survive in the long-term (Thomas 1990). A recent camera study of the MAB found that jaguars were very rare (and probably non-viable) in that isolated (although relatively large, about 50,000 ha) conservation area (Zamzow et al. 2018). However, if the sub-corridors proposed here were established and protected, they would increase connectivity, presumably increasing the combined population size in all protected areas from the Guanacaste region to the Central Volcanic Mountain Range (jaguar conservation unit 154 in Sanderson et al. 2002). This process would thereby improve long-term viability of jaguar populations (although still with some risk of extinction) and provide additional conservation value to other species that also require large contiguous areas of habitat. Even if these small corridors facilitate limited jaguar use, evidence suggests that even an occasional individual successfully dispersing would improve survivability of the existing isolated populations (Rabinowitz and Zeller 2010). Furthermore, efforts are underway to establish biological corridors across international boundaries; one proposed corridor would connect protected areas near La Selva Biological Station in north-eastern Costa Rica to the Indio Maíz Biological Reserve in south-eastern Nicaragua (Morse et al. 2009), while another would connect the Turrialba region of Costa Rica with the large complex of protected areas in the Talamanca Mountains of Costa Rica and Panama (Cusack and Dixon 2006). If all of these corridors, including our proposed examples, were to be established and subsequently protected, species that occur at low densities, require large territories and which need abundant prey could conceivably roam across almost all of Costa Rica's protected areas (Redondo-Brenes et al. 2010). The proposed corridors would directly connect multiple Central American jaguar conservation units that currently have poor dispersal frequency (Sanderson et al. 2002, Rabinowitz and Zeller 2010). Therefore, species like the jaguar would have a much better chance of survival within Costa Rica, simultaneously ensuring the country's critical role within the Mesoamerican Biological Corridor initiative. Regardless, the chance of survival for sensitive species could be vastly improved in the three-country region (Costa Rica, Panama and Nicaragua) with the addition of the proposed functional biological sub-corridors, supporting the conservation of other key species, as well as ecosystem services in the process.

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Appendix 1

Sources for GIS data utilised for corridor construction.

Data Description	Source	Link	Year
Protected areas of Costa Rica	Protected Planet	https://www.protectedplanet.net/country/CR	2017
Biological Corridors of Costa Rica	Sistema Nacional de Áreas de Conservación de Costa Rica (SINAC)	http://www.onfcr.org/psa/capas-de-prioridades	2016
Forest Cover	Global Forest Watch	https://beta-gfw.opendata.arcgis.com/items/7876b225f8034a0ebba79fad4afb80ad	2017
Human Population Estimates	Center for International Earth Science Information Network	http://beta.sedac.ciesin.columbia.edu/data/set/gpw-v4-population-density/data-download	2015
Administrative Roads of Costa Rica	MapCruzin	https://mapcruzin.com/free-costa-rica-country-city-place-gis-shapefiles.htm	2018
Costa Rica and Nicaragua Boundaries	Diva-GIS	http://www.diva-gis.org/datadown	2001

Appendix 2

Data sources for the estimation of jaguar (*Panthera onca*) densities in Central American habitats.

Density (N/100 km^2)	Habitat	Location	Source
8.80	Moist Tropical Rainforest	Cockscomb, Belize	Silver 2004
7.48	Moist Tropical Rainforest	Chiquibul, Belize	Silver 2004
5.40	Tropical Dry Forest	Jalisco, Mexico	Núñez-Pérez 2011
8.80	Moist Tropical Rainforest	Cockscomb, Belize	Harmsen 2006
4.82	Moist Tropical Rainforest	Cockscomb, Belize	Harmsen 2006
18.29	Moist Tropical Rainforest	Cockscomb, Belize	Harmsen 2006
11.45	Moist Tropical Rainforest	Cockscomb, Belize	Harmsen 2006
5.30	Tropical Dry Forest	Fireburn, Belize	Miller 2005
11.28	Moist Tropical Rainforest	Gallon Jug, Belize	Miller 2005
8.82	Moist Tropical Rainforest	Gallon Jug, Belize	Miller 2006
6.98	Moist Tropical Rainforest	Corcovado, Costa Rica	Salom-Pérez et al. 2007
2.00	Moist Tropical Rainforest	Golfo Dulce, Costa Rica	Bustamante 2008
6.70	Moist Tropical Rainforest	Guanacaste, Costa Rica	Rojas 2006
1.34	Moist Tropical Rainforest	Talamanca, Costa Rica	Gutierrez and Porras 2008
5.42	Moist Tropical Rainforest	Talamanca, Costa Rica	González-Maya 2007
11.28	Moist Tropical Rainforest	Carmelita, Guatemala	Moreira et al. 2008a
1.54	Moist Tropical Rainforest	La Gloria, Guatemala	Moreira et al. 2007
1.99	Moist Tropical Rainforest	Mirador, Guatemala	Moreira et al. 2005
11.14	Moist Tropical Rainforest	Dos Lagunas, Guatemala	Moreira et al. 2008b
6.63	Moist Tropical Rainforest	Tikal, Guatemala	García et al. 2006
6.04	Moist Tropical Rainforest	Mechor de Mecos, Guatemala	Moreira et al. 2010
6.32	Moist Tropical Rainforest	Laguna del Tigre, Guatemala	Moreira et al. 2009
5.20	Moist Tropical Rainforest	La Mosquitia, Honduras	Portillo-Reyes and Hernández 2011
Mean (per 100 km^2)	7.09		
Standard Error	0.81		

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