Nature Conservation 17: 1–17 (2017)
doi: 10.3897/natureconservation.17.9714
http://natureconservation.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Nature Conservation
Launched to accelerate biodiversity conservation

Conservation genetics of American crocodile, *Crocodylus acutus*, populations in Pacific Costa Rica

Laurie A. Mauger[1], Elizabeth Velez[2], Michael S. Cherkiss[3], Matthew L. Brien[4], Frank J. Mazzotti[5], James R. Spotila[6]

1 *Department of Biology, Southern Utah University, 351 West University Boulevard, Cedar City, UT 84720, USA* **2** *Kelonia Conservation Society, P.O. Box 473-3000, Heredia, Costa Rica* **3** *Wetland and Aquatic Research Center, 3321 College Avenue, Davie, FL 33314, USA* **4** *Wildlife Management International Pty. Limited, P.O. Box 350, Karama, NT 0813, Australia* **5** *University of Florida Fort Lauderdale Research and Education Center, 3205 College Avenue, Davie, FL 33314, USA* **6** *Department of Biodiversity, Earth and Environmental Science, Drexel University, 3245 Chestnut Street, Philadelphia, PA 19104, USA*

Corresponding author: *Laurie A. Mauger* (lauriemauger@suu.edu)

Academic editor: *W. Magnusson* | Received 29 June 2016 | Accepted 6 February 2017 | Published 8 March 2017

http://zoobank.org/8D9D547C-AC5B-45C4-8208-37AC4A38B6C5

Citation: Mauger LA, Velez E, Cherkiss MS, Brien ML, Mazzotti FJ, Spotila JR (2017) Conservation genetics of American crocodile, *Crocodylus acutus*, populations in Pacific Costa Rica. Nature Conservation 17: 1–17. https://doi.org/10.3897/natureconservation.17.9714

Abstract

Maintaining genetic diversity is crucial for the survival and management of threatened and endangered species. In this study, we analyzed genetic diversity and population genetic structure at neutral loci in American crocodiles, *Crocodylus acutus*, from several areas (Parque Nacional Marino Las Baulas, Parque Nacional Santa Rosa, Parque Nacional Palo Verde, Rio Tarcoles, and Osa Conservation Area) in Pacific Costa Rica. We genotyped 184 individuals at nine microsatellite loci to describe the genetic diversity and conservation genetics between and among populations. No population was at Hardy-Weinberg Equilibrium (HWE) over all loci tested and a small to moderate amount of inbreeding was present. Populations along the Pacific coast had an average heterozygosity of 0.572 across all loci. All populations were significantly differentiated from each other with both F_{ST} and R_{ST} measures of population differentiation with a greater degree of molecular variance (81%) found within populations. Our results suggest *C. acutus* populations in Pacific Costa Rica were not panmictic with moderate levels of genetic diversity. An effective management plan that maintains the connectivity between clusters is critical to the success of *C. acutus* in Pacific Costa Rica.

Keywords

American crocodile, heterozygosity, microsatellites, population genetics, genetic structure

Introduction

Threatened and endangered species face many challenges including habitat fragmentation and destruction, human population growth, and loss of genetic variability. Maintenance of genetic diversity is of increasing importance in the preservation of threatened and endangered species (Lacy 1997; Haig 1998; Reed and Frankham 2003; Reed et al. 2007). Lack of genetic diversity can lead to inbreeding depression (Frankham 1995), decreased immunity (O'Brien et al. 1985), decreased reproductive performance (O'Brien et al. 1985; Parker et al. 1991) and eventual extinction (Frankham 2005). Effective management strategies for threatened and endangered species require integration of all aspects of the species' biology, including both demography and genetics (Lande 1988)

The American crocodile (*Crocodylus acutus*) is mainly a coastal species ranging from the extreme southern tip of Florida, through the Caribbean, and Central and northern South America (Mazzotti 1999; Thorbjarnarson 2010). Populations range-wide are threatened by habitat destruction and fragmentation, poaching, and past overexploitation (Ross 1998; Thorbjarnarson et al. 2006; Mazzotti et al. 2007). Current threats to *C. acutus* in Costa Rica include habitat loss (particularly nesting habitat), deliberate killing (Machkour-M'Rabet et al. 2009), and pollution (Rainwater et al. 2007; Rainwater et al. 2011). *Crocodylus acutus* is a wide ranging and ecologically plastic species (Thorbjarnarson 2010) with substantial genetic differentiation among populations (Menzies and Kushlan 1991; Rodriguez 2007; Porras et al. 2008; Thorbjarnarson 2010). Determining the status and ecology of *C. acutus* in Costa Rica is a priority project of the IUCN Crocodile Specialist Group Action Plan (Ross 1998). Genetic evaluations of *C. acutus* range-wide were further named as a moderate priority project in the 2010 Action Plan (Thorbjarnarson 2010). Therefore, it is important to describe genetic diversity and differentiation of this species throughout its range, including Costa Rica.

In the present study, we investigated the genetic structure of *C. acutus* populations in several areas of Pacific Costa Rica (Fig. 1) and the degree of effective migration occurring between populations. A series of estuaries provide pockets of suitable crocodile habitat along the Pacific coast which made it an optimal area for studying gene flow between potential metapopulations. Crocodiles are known to migrate long distances (Kay 2004; Campos et al. 2006; Read et al. 2007; Campbell et al. 2013). There have been accounts of *C. acutus* migrating over 35 km for nesting (Cherkiss et al. 2006) and movements over 388 km (Cherkiss et al. 2014) in southern Florida. The ability of crocodilians to migrate and disperse long distances increases the amount of potential gene flow between neighboring or distant populations. It is possible that crocodiles are dispersing between habitat patches in Pacific Costa Rica; thus, facilitating gene flow along the coast. We used microsatellites to test the hypothesis that *C. acutus* populations do not exist as a continuous, panmictic population in Pacific Costa Rica.

Methods

Study area

We sampled 5 localities in Pacific Costa Rica for *C. acutus* (Fig. 1). Site LB (Parque Nacional Marino Las Baulas) was in the Tempisque Conservation Area (ACT); site PV (Parque Nacional Palo Verde) was in the Arenal-Tempisque Conservation Area (ACA-T); site SR (Parque Nacional Santa Rosa) was in the Guanacaste Conservation Area (ACG); site RT (Rio Tarcoles) was in Central Pacific Conservation Area (ACOPAC); sites RS (Rio Sierpe), T (Terraba Delta), PL (Pejeperro Lagoon), PTL (Pejeperrito Lagoon), RE (Rio Esquinas), RC (Rio Coto) and PB (Parrot Bay Lodge, Puerto Jimenez) were in the Osa Conservation Area (ACOSA). Crocodiles were sampled from seven areas in ACOSA; however, they have been combined as one population due to low sample numbers. Localities ranged from large river systems (PV, RT, and ACOSA), to estuaries (LB and SR) and coastal lagoons (SR and ACOSA). (See Mauger et al. 2012 for additional study location information.)

Sample collection

We collected blood and tissue samples at the beginning of the rainy season in SR (2007) and PV (2005, 2008 and 2009), throughout the year in LB (2007 – 2009), during the rainy season in RT (2005 – 2006) and during the end of the dry season in ACOSA (2006, 2008 and 2009). We captured crocodiles mainly during spotlight surveys using the break-away snare method (Hutton et al. 1987; Hutton and Woodhouse 1989), snake tongs or by hand (see Mauger et al. 2012 for additional information on sample collection). Blood and/or tissue was collected from 184 individuals (see Table 1 for size class distribution of samples). In sites where a large number of hatchlings were captured, a random number selector was used to randomly select four to six hatchlings for genetic analysis. Tissue was collected from the caudal scutes during marking and blood was collected from the caudal vein or the dorsal sinus. Tissue samples were preserved in 95–100% ethanol. Blood was preserved on Whatman FTA Cards for DNA Preservation® (GE Life Sciences).

DNA isolation and microsatellite amplification

We isolated DNA from tissue samples using the DNeasy Blood and Tissue Kit™ (Qiagen) and purified from blood cards with two five-minute washes with FTA Purification Reagent (Whatman) and two five-minute washes with Tris-EDTA (TE; 10 mM Tris-Cl, pH 7.5, 1 mM EDTA) buffer. Each wash consisted of 50 µl of solution.

We amplified nine microsatellite DNA loci using previously characterized primers (Dever and Densmore 2001; Fitzsimmons et al. 2001) C391, Cj16, Cj18, Cj20,

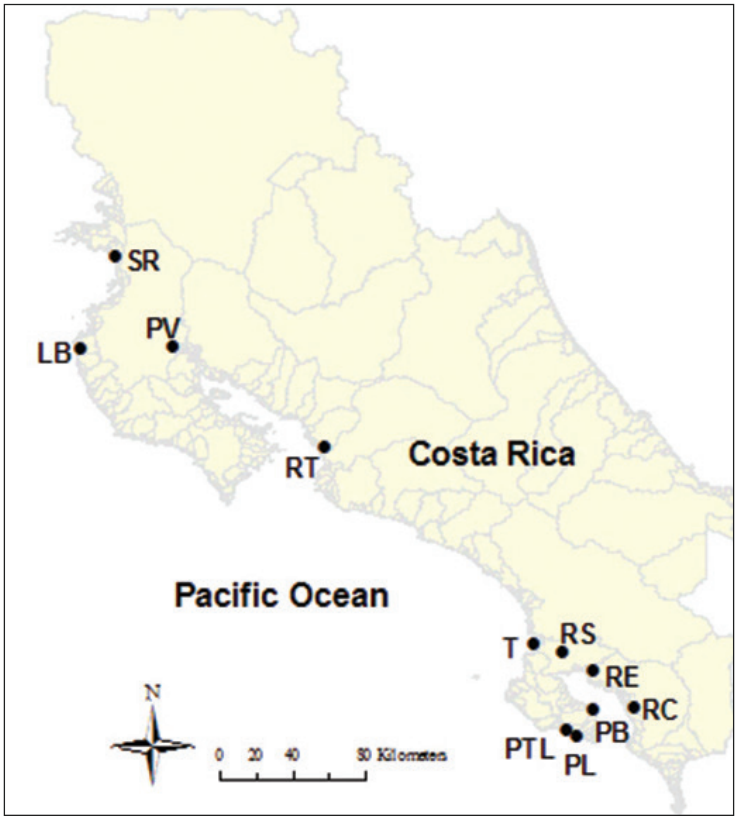

Figure 1. Map of collection sites in Costa Rica of the American crocodile, *Crocodylus acutus*. Parque Nacional Marino Las Baulas (LB), Parque Nacional Palo Verde (PV), Parque Nacional Santa Rosa (SR), Rio Tarcoles (RT), Rio Sierpe (RS), Terraba Delta (T), Pejeperitto Lagoon (PTL), Pejeperro Lagoon (PL), Rio Esquinas (RE), Rio Coto (RC) and Parrot Bay Lodge (PB). Sites RS, T, PTL, PL, RE, RC, and PB were grouped together as Osa Conservation Area (ACOSA). Localities ranged from large river systems (PV, RT, and ACOSA) to estuaries (LB, SR) and coastal lagoons (SR and ACOSA).

Table 1. Size class distribution of genotyped crocodiles for each site.

Site	Hatchling	Juvenile	Subadult	Adult	Total
LB	12	17	13	4	46
SR	4	8	5	0	17
ACOSA	0	44	3	1	48
PV	9	32	2	9	53
RT	0	13	0	3	19
Total	25	114	23	17	184

Cj109, Cj131, CU5-123, CUD68, and CUJ131 via polymerase chain reaction (PCR). The forward primer of each pair was labeled with a fluorescent dye (6-FAM, HEX or NED; Applied Biosystems) to allow for the detection and sizing of DNA fragments.

The DNA was amplified in 25 µl reactions containing 1.25 units of EconoTaq DNA Polymerase (Lucigen), 2.5 µl 10X buffer (100 mM Tris-HCl (pH 9.0), 500 mM KCl, 1% Triton X-100, 15 mM $MgCl_2$), 1.0 µl 25 mM $MgCl_2$ (Cj16 and Cj20) or 0.5 µl 25 mM $MgCl_2$ (all other primers), 1.0 µl of 10 mM dNTP's (Qiagen), 1.0 µl each of the forward and reverse primer, approximately 100 ng template DNA and purified water to the final volume. Microsatellites were amplified according to the following parameters: initial denaturation at 94°C for 2 minutes, 33 cycles of 94°C for 1 minute, 58°C (C391, Cj18, Cj131, CU5-123, CUD68, CUF131), 59°C (Cj16, Cj20) or 62°C (Cj109) for 1 minute, and 72°C for l minute, and a final extension at 72°C for 10 minutes. Amplified loci were separated on an Applied Biosystems (ABI) 3730xl Genetic Analyzer and sized with LIZ-500 size standard by Genewiz, Inc (www.genewiz.com). Genotypes were assigned using PeakScanner 1.0 (Applied Biosystems).

Genetic diversity

Data files were converted to formats supported by various genetic programs in CREATE 1.0 (Coombs et al. 2007). Probability of Identity (PI) was estimated in GENALEX 6 (Peakall and Smouse 2006) to determine the minimum number of microsatellites needed to identify individuals. Allelic richness (A_R) and the number of private alleles (A_{Priv}) were estimated in HP-RARE (Kalinowski 2005). We estimated numbers of alleles, allele frequencies and gene diversities in FSTAT 2.9.3.2 (Goudet 1995).

Observed versus expected number of heterozygotes were estimated in Genepop on the Web (Raymond and Rousset 1995; Rousset 2008). Departure from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) were estimated in Genepop on the Web (Raymond and Rousset 1995; Rousset 2008). Departure from HWE was tested using an exact test (Guo and Thompson 1992) and a chi-square goodness of fit test with a dememorization number of 10,000, and 1,000 batches of 10,000 iterations each. Linkage disequilibrium was tested to determine if small effective population sizes within the different localities caused nonrandom association of alleles at different loci. Linkage disequilibrium was tested for all pairs of loci used by the log likelihood ratio statistic under the same parameters as HWE. All p-values were adjusted to allow for multiple comparisons. Weir and Cockerham's (1984) inbreeding coefficient, F_{IS}, was estimated for each population in FSTAT 2.9.3.2 (Goudet 1995) with and without randomly selected hatchlings. Homozygote excess at each locus in each locality was estimated by MICROCHECKER (Van Oosterhout et al. 2004). MICROCHECKER was also used to identify if null alleles were present at each locus in each locality. Null alleles were suggested for loci with a general excess of homozygotes for most allele size classes.

We identified whether rare alleles had been lost due to previous genetic bottlenecks under the infinite alleles (IAM) and stepwise mutation (SMM) models in BOTTLENECK version 1.2.02 (Piry et al 1999). Both the standardized differences and Wilcoxon tests were run.

Population genetic structure

We used an analysis of molecular variance (AMOVA) to estimate the percentage of variance within and among populations with GENALEX 6 (Peakall and Smouse 2006). Population differentiation was estimated for all population pairs using several methods. We estimated F_{ST} and R_{ST} for each population pair in FSTAT 2.9.3.2 (Goudet 1995) and Arlequin ver. 3.11 (Excoffier et al. 2005) respectively. All p-values were adjusted to allow for multiple comparisons.

We used Mantel's test to determine the relationship between geographic and genetic distance. Isolation by Distance Web Service (IBDWS; Jensen et al. 2005) was used to test for the presence of isolation by distance (IBD) between population pairs. Sites RT and T were excluded from the analysis because GPS coordinates of crocodile captures were not available. Each Mantel test was performed with 30,000 randomizations. Distances between populations were estimated using an oceanic/coastline route. Rousset's genetic distance (F/(1-F)) was calculated using genetic differentiation (F_{ST}).

Results

Genetic diversity

The nine microsatellites chosen for this study had an average probability of identity (PI) of 4.96^{-6} across all five Costa Rican populations (SR=5.4^{-6}; LB=7.9^{-6}; ACOSA=8.7^{-8}; PV=1.5^{-7}; RT=1.1^{-5}). This indicated that there was a low probability that two individuals chosen at random would have the same genotype. These microsatellites were sufficient for this study.

We identified 88 alleles in five *C. acutus* populations sampled in Pacific Costa Rica across all nine microsatellite loci. Average A_R and A_{Priv} over all loci were estimated using a corrected sample size of 34 alleles. The A_R ranged between 4.22 and 5.64 and A_{Priv} ranged between 0.27 and 1.36 in the sampled locations (Table 2). Allele frequencies for each microsatellite locus ranged from 0 (in localities where the allele was not genotyped) to 0.882 (Appendix 1: Allele Frequencies). Allele frequencies were also calculated with the hatchlings removed. There were no substantial differences in allele frequencies when hatchlings were removed. We tested for genetic bottlenecks under the IAM and SMM models for all samples combined and each site separately. Bottlenecks were not detected under IAM ($p>0.05$), but were detected in all populations under SMM ($p<0.009$) with the standardized differences test.

No population was in Hardy-Weinberg Equilibrium (HWE) over all nine microsatellite loci tested (Table 3). Site LB was not in HWE at loci Cj16 ($p<0.001$), Cj109 ($p=0.03$) and Cj131 ($p=0.01$); site SR was not in HWE equilibrium at locus C391 ($p=0.005$); site ACOSA was not in HWE at loci C391 ($p=0.03$), Cj18 ($p=0.02$), Cj20 ($p=0.04$), Cj109 ($p=0.002$), CU5-123 ($p<0.001$) and CUD68 ($p<0.001$); site PV was not in HWE at loci Cj18 ($p=0.001$), Cj109 ($p=0.03$), Cj131 ($p=0.01$), CU5-123

Table 2. Genetic variability estimates for *Crocodylus acutus* populations in Pacific Costa Rica.

Sample Site	Code	N	A_R	A_{Priv}
Area of Conservation Tempisque	ACT			
Las Baulas National Park	LB	46	4.31	0.37
Palo Verde National Park	PV	54	5.19	0.58
Area of Conservation Guanacaste	ACG			
Santa Rosa National Park	SR	17	4.22	0.3
Central Pacific Conservation Area	ACOPAC			
Rio Tarcoles	RT	17	4.22	0.27
Osa Conservation Area	ACOSA	49	5.64	1.36

N = sample size.

A_R = allelic richness.

A_{Priv} = number of private alleles.

Table 3. Expected (H_E) and observed heterozygosities (H_O) for microsatellite loci in *Crocodylus acutus* populations.

	LB (N = 46)		SR (N = 17)		PV (N = 54)		RT (N = 17)		ACOSA (N = 49)	
Locus	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O
C391	0.46	0.52	0.65$^{\#}$	0.35^{*+}	0.59	0.57	0.36	0.35	0.57$^{\#}$	0.53*
Cj16	0.48	0.28$^{*+\#}$	0.51	0.53	0.62	0.59	0.39	0.47	0.64	0.65
Cj20	0.41	0.61	0.56	0.35	0.67	0.59	0.75$^{\#}$	0.41*	0.66$^{\#}$	0.63^{+}
Cj131	0.49	0.48	0.22	0.24	0.37$^{\#}$	0.43	0.39	0.35	0.45	0.47*
Cj18	0.45	0.41	0.47	0.53	0.76$^{\#}$	0.70*	0.73$^{\#}$	0.41^{*+}	0.89$^{\#}$	0.80^{*+}
Cj109	0.64	0.59$^{*\#}$	0.78	0.65	0.78$^{\#}$	0.67*	0.62	0.76^{+}	0.61$^{\#}$	0.39*
CU5-123	0.40	0.37*	0.06	0.06	0.62$^{\#}$	0.33^{*+}	0.49	0.53^{+}	0.45$^{\#}$	0.29*
CUD68	0.70	0.54^{*+}	0.68	0.65	0.73$^{\#}$	0.46^{+}	0.65$^{\#}$	0.35^{*+}	0.76$^{\#}$	0.39^{*+}
CUJ131	0.68	0.67$^{\#}$	0.63	0.41*	0.57$^{\#}$	0.61	0.56	0.76	0.54	0.51

*heterozygote deficiency at these loci (Wilcoxon test; p=0.05).

$^{+}$homozygote excess at these loci suggested that null alleles may be present (p=0.05).

$^{\#}$loci removed from Hardy-Weinberg Equilibrium.

(p<0.001), CUD68 (p<0.001) and CUJ131 (p=0.02); and site RT was not in HWE at loci Cj18 (p=0.03), Cj20 (p=0.02) and CUD68 (p=0.0026). The Wilcoxon test identified heterozygote deficiencies in all localities for at least one microsatellite loci (p=0.002-0.02; Table 3). Null alleles were suggested for at least one locus in each locality by general excess of homozygote for most allele size classes (Table 3). Average F_{IS} values ranged from 0.096 to 0.179 for each site. Inbreeding levels were 0.179 and 0.103 in ACOSA and RT, respectively. Inbreeding coefficients were calculated with hatchlings included and removed in the remaining sites. Site PV had a lower F_{IS} when hatchlings were included (0.127 and 0.142 with and without hatchlings, respectively). Site SR had higher F_{IS} when hatchlings were included (0.179 and 0.166 with and without hatchlings, respectively). There was no difference in F_{IS} (0.096) in site LB.

Linkage disequilibrium (LD) tests were performed to investigate the distribution of the nine microsatellite loci for *C. acutus* populations on the Pacific coast of Costa Rica. Pairwise comparisons were performed for each population. LD did not play a strong role in the nine microsatellites tested (p=0.001-0.99). All p values were adjusted for multiple tests.

Population genetic structure

An analysis of molecular variance estimated that 19% of the variation occurred between populations, while 81% of molecular variance occurred within individual populations. This suggested that individual populations were genetically diverse. Population differentiation was measured between all population pairs using F_{ST} and R_{ST}. All population pairs were significantly differentiated (p=0.05) using both measures of population differentiation (Table 4). We observed the least amount of differentiation between ACOSA with LB and SR and the highest level of differentiation between LB with SR and RT.

Isolation by distance (IBD) was estimated between each sampled crocodile in all localities in Pacific Costa Rica. No IBD was observed (p=0.92; Fig. 2). Isolation between populations did not restrict gene flow.

Discussion

The nine microsatellites chosen in this study provided data on the genetic structure of *C. acutus* populations along the Pacific coast of Costa Rica. Average heterozygosity of crocodiles along the Pacific coast of Costa Rica was slightly higher than or comparable to that in other crocodilian populations (Glenn et al. 1998; Davis et al. 2001; Dever et al. 2002; Ryberg et al. 2002; Verdade et al 2002; de Thoisy et al. 2006; Rodriguez et al. 2008). However, in this study, several individual loci did have lower heterozygosity values, possibly due to inbreeding observed in all populations. Crocodiles within site SR had higher F_{IS} values than other sites. This may be because we observed and captured few individuals that had exceeded minimum breeding size. This site also represented the smallest crocodile habitat with lower encounter rates (Mauger et al. 2012). Fewer breeding crocodiles and lower encounter rates could explain the higher inbreeding levels since presumably the gene pool is limited to fewer individuals. In all surveyed localities, size class distributions estimated during the study showed a higher percentage of juveniles (0.5 – 1.25 m) than adults (>2.25 m) (Mauger et al. 2012). The higher percentage of juveniles in these localities could indicate population recovering from past bottlenecks (Ouboter and Nanhoe 1989) and explain the higher inbreeding levels observed in this study.

We identified previous genetic bottlenecks in all sampled localities. Past bottlenecks were confirmed under the SMM model by BOTTLENECK (Piry et al. 1999).

Table 4. Population differentiation between all *Crocodylus acutus* population pairs using R_{ST} (above 0 line) and F_{ST} (below 0 line).

Site	LB	SR	ACOSA	PV	RT
LB	–	0.66⁺	0.04⁺	0.23⁺	0.65⁺
SR	0.19⁺	–	0.04*	0.18⁺	0.11*
ACOSA	0.15⁺	0.1⁺	–	0.10⁺	0.06*
PV	0.1⁺	0.08⁺	0.07⁺	–	0.24⁺
RT	0.24⁺	0.14⁺	0.10⁺	0.10⁺	–

*significant at p=0.05

⁺significant at p=0.001

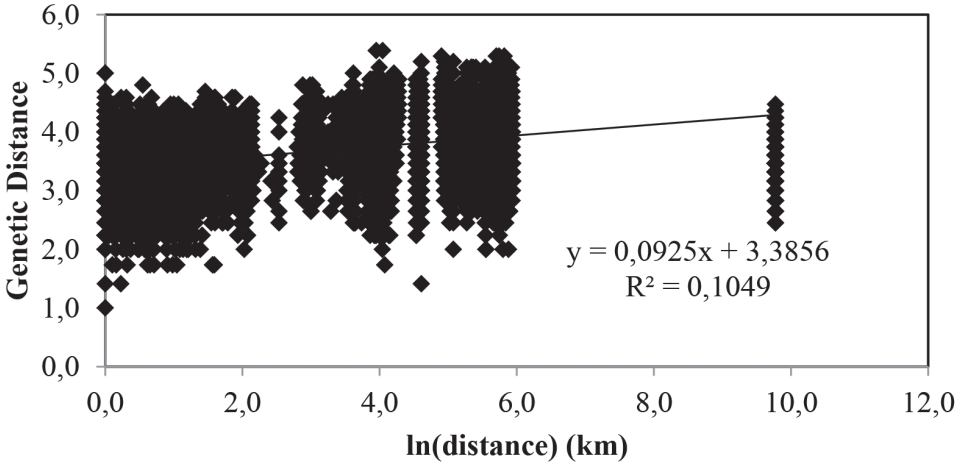

Figure 2. Mantel test for isolation by distance between individual crocodiles in all localities. No isolation by distance was observed (p=0.92). Rousset's distance (F/(1-F)) was used for genetic distance. Geographic distance was the log of a straight line distance (km) between populations.

Based on these analyses, we concluded that all populations underwent a previous reduction in population size. Population bottlenecks lead to the rapid loss of rare alleles and results in the loss of the total number of alleles at a faster rate than a loss in overall heterozygosity (Ortego et al. 2010). The low number of private alleles observed in this study (Table 2) could be an artifact of past genetic bottlenecks. Null alleles were also detected for at least one locus in each locality (Table 3). The absence of these alleles in the analysis could help explain the loss of genetic variation observed in these localities. *Crocodylus acutus* populations declined range-wide through the mid-20th century as a result of hunting and illegal poaching (Ross 1998; Thorbjarnarson et al. 2006; Thorbjarnarson 2010). These human activities have not been documented in Costa Rica; however, it is possible that populations here experienced similar pressures and population declines. We observed some poaching and killing of large individuals in LB during the study and have received reports of similar activities at other sites in Pacific

Costa Rica (personal observation and communication with local people). Anecdotal data suggests that crocodile numbers are also increasing in LB (F. Paladino, personal communication) and PV (Bolaños-Montero 2012). Recent introduction of tilapia into the Tempisque River Basin (site PV) has provided a continuous food source for crocodiles (Sandlund et al. 2010), potentially contributing to the recent population growth. As a result, crocodile numbers in this region have increased precipitously in recent years, causing adverse interactions between human and crocodile populations (Bolaños-Montero 2012). Additional factors are most likely at play, which are contributing to population growth.

No population was in Hardy-Weinberg Equilibrium for all microsatellite loci. This could be due to the heterozygote deficiency observed at loci that were not in HWE, to inbreeding documented in several populations, or to effective migration. Our results suggest that crocodile populations in Pacific Costa Rica were differentiated from each other, as supported by studies on *C. acutus* and other crocodilian species (Farias et al. 2004; Porras et al. 2008; Machkour-M'Rabet et al. 2009; Thorbjarnarson 2010). All population pairs exhibited significant genetic differentiation, with all but two population pairs (LB and ACOSA, and SR and ACOSA) showing moderate differentiation from each other (R_{ST}<0.05; Table 4). These values supported the hypothesis that migration occurred between populations; however, some population pairs were more differentiated from each other and thus had lower historical migration rates between patchily distributed habitats in Costa Rica. The level of subdivision observed suggested that crocodiles in Pacific Costa Rica were not panmictic; however, genetic connections did exist. Additionally, the majority of molecular variance was observed within populations. This could explain why moderate differentiation was observed between most population pairs. However, the highly mobile nature of crocodiles (Kay 2004; Campos et al. 2006; Read et al. 2007; Campbell et al. 2013) could be facilitating gene flow between populations along the Pacific coast. A recent study on the spatial ecology of *C. acutus* in Panama, suggests that males have a larger home ranges, but females have larger average movement distances (Balaguera-Reina et al. 2016). Balaguera-Reina et al. (2016) also noted dispersal differences between age classes and dry and wet seasons. Dispersal differences between age classes, i.e. subadults dispersing to find mating territories, could also explain the departure from Hardy-Weinberg Equilibrium and moderate differentiation levels. GPS-based tracking studies of Costa Rican *C. acutus* would contribute important information on contemporary crocodile dispersal abilities and maximum home ranges in patchily distributed habitats.

Conclusions

The data presented here supported moderate differentiation and an absence of isolation by distance in Pacific Costa Rica. Our results suggested the loss of genetic variation through a lack of connectivity between some localities and previous population bottlenecks. The moderate heterozygosity values and genetic differentiation described

here emphasized the need to protect all potential crocodile habitat, to write management plans across conservation areas and national parks in Costa Rica, and the need for conservation and management units to extend over the entire span of a species' range.

Acknowledgements

We would like to thank everyone that helped us in the field, especially Bernal Cortes, Luis Fernando Lopez Lara, Juan Jose Victor Villalobos, Guillermo Briceo, Ademar Rosales, Fabricia Alvarez, Issac Ehresman, Gareth Blakemore, Mike Boston and Jim Tamarack. We would also like to thank the Ministerio del Ambiente, Energa y Telecomunications (MINAET) and the Sistema Nacional de Áreas de Conservación (SINAC), especially Rotney Piedra, Jose Quiroz and Roger Blanco for research permits. Luz Barrantes Bahder and Juan Rafael Boloños Montero provided samples from Rio Tarcoles and Tempísque. This work was largely supported by the Betz Chair of Environmental Sciences at Drexel University, and the Sophie Danforth Conservation Biology Fund from Roger Williams Park Zoo in Rhode Island. Research in the Osa Peninsula was also partially funded by the El Tigre Fund. The field research in Las Baulas National Park was partially supported by the Leatherback Trust. The Leatherback Trust provided vehicles in Guanacaste, lodging at the Goldring Gund Marine Biology Station at Playa Grande, and a boat for many of the crocodile surveys conducted to collect genetic material. Any use of trade, product or firm names is for descriptive purposed only and does not imply endorsement by the U.S. Government.

References

Balaguera-Reina S, Venegas-Anaya M, Sánchez A, Arbelaez I, Lessios HA, Densmore LD (2016) Spatial ecology of the American crocodile in a tropical Pacific island in Central America. PLoS ONE 11. https://doi.org/10.1371/journal.pone.0157152

Bolaños-Montero JR (2012) Survey of American crocodiles in Tempisque Great Wetlands, Costa Rica. Crocodile Specialist Group Newsletter 31: 5–7.

Campbell HA, Dwyer RG, Irwin TR, Franklin CE (2013) Home range utilization and long-range movement of estuarine crocodiles during the breeding and nesting season. PLoS ONE 8: https://doi.org/10.1371/journal.pone.0062127

Campos Z, Coutinho M, Mourão G, Bayliss P, Magnusson WE (2006) Long distance movements by *Caiman crocodilus yacare*: implications for management of the species in the Brazilian Pantanal. Journal of Herpetology 16: 123–132.

Cherkiss MS, Parry M, Mazzotti FJ (2006) *Crocodylus acutus* (American crocodile) migration. Herpetological Review 38: 72–73.

Cherkiss MS, Mazzotti FJ, Hord L, Aldecoa M (2014) Remarkable movements of an American crocodile (*Crocodylus acutus*) in Florida. Southeastern Naturalist 13: N52–N56. https://doi.org/10.1656/058.013.0407

Coombs JA, Letcher BH, Nislow KH (2007) CREATE: a software to create input files from diploid genotypic data for 52 genetic software programs. Molecular Ecology Notes 8: 578–580. https://doi.org/10.1111/j.1471-8286.2007.02036.x

Davis, LM, Glenn TC, Elsey RM, Dessauer HC, and Sawyer RH (2001) Multiple paternity and mating patterns in the American alligator, *Alligator mississippiensis*. Molecular Ecology 10: 1011–1024. https://doi.org/10.1046/j.1365-294X.2001.01241.x

de Thoisy BT, Hrbek T, Farias IP, Vasconcelos WR, Lavergne A (2006) Genetic structure, population dynamics, and conservation of Black caiman (*Melanocuchus niger*). Biological Conservation 133: 474–482. https://doi.org/10.1016/j.biocon.2006.07.009

Dever JA, Densmore LD (2001) Microsatellite's in Morelet's crocodile (*Crocodylus moreletti*) and their utility in addressing crocodilian population genetics questions. Journal of Herpetology 35: 541–544. https://doi.org/10.2307/1565981

Dever JA, Strauss RE, Rainwater TR, McMurray ST, Densmore LD (2002) Genetic diversity, population subdivision and gene flow in Morelet's crocodile (*Crocodylus moreletti*) from Belize, Central America. Copeia 4: 1078–1091. https://doi.org/10.1643/0045-8511(2002)002[1078:GDPSAG]2.0.CO;2

Excoffier L, Laval G, Schneider S (2005) Arlequin ver 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1: 47–50.

Farias IP, da Silveira R, de Thoisy B, Monjelo LA, Thorbjarnarson J, Hrbek T (2004) Genetic diversity and population structure of Amazonian crocodilians. Animal Conservation 7: 265–272. https://doi.org/10.1017/S136794300400143X

Fitzsimmons NN, Tanksley S, Forstner MRJ, Louis EE, Daglish R, Gratten J, Davis S (2001) Microsatellite markers for *Crocodylus*: new genetic tools for population genetics, mating system studies and forensics. In: Grigg GC, Seebacher F, Franklin CE (Eds) Crocodilian Biology and Evolution. Surrey Beaty & Sons (Australia), 51–57.

Frankham R (1995) Inbreeding and extinction: A threshold effect. Conservation Biology 9: 792–799. https://doi.org/10.1046/j.1523-1739.1995.09040792.x

Frankham R (2005) Genetics and extinction. Biological Conservation 126: 131–140. https://doi.org/10.1016/j.biocon.2005.05.002

Glenn TC, Dessauer HC, Braun MJ (1998) Characterization of microsatellite DNA loci in American alligators. Copeia 1998: 591–601. https://doi.org/10.2307/1447789

Goudet J (1995) FSTAT (version 1.2): A computer program to calculate F-statistics. Journal of Heredity 86: 485–486.

Guo S, Thompson E (1992) A monte-carlo method for combined segregation and linkage analysis. American Journal of Human Genetics 51: 1111–1126.

Haig SM (1998) Molecular contributions to conservation biology. Ecology 79: 413–425. https://doi.org/10.1890/0012-9658(1998)079[0413:MCTC]2.0.CO;2

Hutton JM, Loveride JP, Blake DK (1987) Capture methods for the Nile crocodile in Zimbabwe. In: Webb GJW, Manolis SC, Whitehead PJ (Eds) Wildlife management: Crocodiles and alligators. Surrey Beatty & Sons (Australia), 243–247.

Hutton JM, Woodhouse ME (1989) Mark-recapture to assess factors affecting the proportion of a Nile crocodile population seen during spotlight counts at Ngezi, Zimbabwe, and the

use of spotlight counts to monitor crocodile abundance. Journal of Applied Ecology 26: 381–395. https://doi.org/10.2307/2404068

Jensen JL, Bohonak AJ, Kelley ST (2005) Isolation by distance, web service. BMC Genetics 6:13. https://doi.org/10.1186/1471-2156-6-13

Kalinowski S (2005) HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Notes 5: 187–189. https://doi.org/10.1111/j.1471-8286.2004.00845.x

Kay WR (2004) Movements and home ranges of radio-tracked *Crocodylus porosus* in the Cambridge Gulf region of Western Australia. Wildlife Research 31: 495–508. https://doi.org/10.1071/WR04037

Lacy RC (1997) Importance of genetic variation to the viability of mammalian populations. Journal of Mammalogy 78: 320–335. https://doi.org/10.2307/1382885

Lande R (1988) Genetics and demography in biological conservation. Science 241: 1455–1460. https://doi.org/10.1126/science.3420403

Machkour-M'Rabet S, Hénaut Y, Charruau P, Gevrey M, Winterton P, Legal L (2009) Between introgression events and fragmentation, islands are the last refuge for the American crocodile in Caribbean Mexico. Marine Biology 156: 1321–1333. https://doi.org/10.1007/s00227-009-1174-5

Mauger LA, Velez E, Cherkiss MS, Brien ML, Boston M, Mazzotti FJ, Spotila JR (2012) Population assessment of the American crocodile, *Crocodylus acutus* (Crocodilia: Crocodylidae) on the Pacific coast of Costa Rica. Revista de Biología Tropical Trop 60: 1889–1901. https://doi.org/10.15517/rbt.v60i4.2188

Mazzotti FJ (1999) The American Crocodile in Florida Bay. Estuaries 22: 552–561. https://doi.org/10.2307/1353217

Mazzotti FJ, Brandt LA, Moler P, Cherkiss MS (2007) American crocodile (*Crocodylus acutus*) in Florida: Recommendations for endangered species recovery and ecosystem restoration. Journal of Herpetology 41: 122–132. https://doi.org/10.1670/0022-1511(2007)41[122:ACCAIF]2.0.CO;2

Menzies RA, Kushlan JA (1991) Genetic variation in populations of the American Crocodile. Journal of Herpetology 25: 357–361. https://doi.org/10.2307/1564598

O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M, Wildt DE (1985) Genetic basis for species vulnerability in the cheetah. Science 227: 1428–1434. https://doi.org/10.1126/science.2983425

Ortego J, Aguirre MP, Cordero PJ (2010) Population genetics of *Mioscirtus wagneri*, a grasshopper showing a highly fragmented distribution. Molecular Ecology 19: 472–483. https://doi.org/10.1111/j.1365-294X.2009.04512.x

Ouboter, PE, Nanhoe MR (1989) Notes on the dynamics of a population of *Caiman crocodilus* in Northern Suriname and its implications for management. Biological Conservation 48: 243–264. https://doi.org/10.1016/0006-3207(89)90101-8

Parker C, Pusey AE, Rowley H, Gilbert DA, Martenson J, O'Brien SJ (1991) Case study of a population bottleneck: Lions of Ngorongoro Crater. Conservation Biology 5: 219–230. https://doi.org/10.1111/j.1523-1739.1991.tb00127.x

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288–295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity 90: 502–503. https://doi.org/10.1093/jhered/90.4.502

Porras LPM, Boloños Montero JR, Barr BR (2008) Genetic variation and gene flow among populations of *Crocodylus acutus* (Crocodylia: Crocodylidae) in three rivers of Central Pacific, Costa Rica. Revista de Biología Tropical 56: 1471–1480.

Rainwater TR, Millichamp NJ, Barrentes LDB, Barr BR, Montero JRB, Platt SG, Abel MT, Cobb GP, Anderson TA (2011) Occular disease in American crocodiles (*Crocodylus acutus*) in Costa Rica. Journal of Wildlife Diseases 47: 415–426. https://doi.org/10.7589/0090-3558-47.2.415

Rainwater TR, Wu TH, Finger AG, Cañas JE, Yu L, Reynolds KD, Coimbatore G, Barr B, Platt SG, Cobb GP, Anderson TA, McMurry ST (2007) Metals and organochlorine pesticides in caudal scutes of crocodiles from Belize and Costa Rica. Science of the Total Environment 373: 146–156. https://doi.org/10.1016/j.scitotenv.2006.11.010

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and exumenicism. Journal of Heredity 86: 248–249.

Read MA, Grigg GC, Irwin SR, Shanahan D, Franklin CE (2007) Satellite tracking reveals long distance coastal travel and homing by translocated estuarine crocodiles, *Crocodylus porosus*. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0000949

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. Conservation Biology 17: 230–237. https://doi.org/10.1046/j.1523-1739.2003.01236.x

Reed DH, Nicholas AC, Stratton GE (2007) Genetic quality of individuals impacts population dynamics. Animal Conservation 10: 275–283. https://doi.org/10.1111/j.1469-1795.2007.00120.x

Rodriguez D (2007) Crocodilian evolution, systematics and population genetics: recovery and ecological interactions of the American crocodile (*Crocodylus acutus*). PHD Thesis. Texas Tech University.

Rodriguez D, Cedeño-Vazquez JR, Forstner MR, Densmore LD (2008) Hybridization between *Crocodylus acutus* and *Crocodylus moreletti* in the Yucatan Peninsula: II Evidence from microsatellites. Journal of Experimental Zoology Part B 309A: 674–686. https://doi.org/10.1002/jez.499

Ross JP (1998) Crocodiles: an action plan for their conservation, IUCN/SSG Crocodile Specialist Group Publication. Oxford Press (Oxford).

Rousset F (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular Ecology Resources 8: 103–106. https://doi.org/10.1111/j.1471-8286.2007.01931.x

Ryberg WA, Fitzgerald LA, Honeycutt RL, Cathey JC (2002) Genetic relationships of American alligator populations distributed across different ecological and geographic scales. Journal of Experimental Zoology Part B 294: 325–333. https://doi.org/10.1002/jez.10207

Sandlund OT, Daverdin RH, Choudhury A, Brooks DR, Diserud OH (2010) A survey of freshwater fishes and their macroparasites in the Guanacaste Conservation Area (ACG), Costa Rica. NINA Report 635, 45 pp.

Thorbjarnarson JB, Mazzotti FJ, Sanderson E, Buitrago F, Lazcano M, Minkowski K, Muniz M, Ponce P, Sigler L, Soberon R, Trelancia AM, Velasco A (2006) Regional habitat conservation priorities for the American crocodile. Biological Conservation 128: 25–36. https://doi.org/10.1016/j.biocon.2005.09.013

Thorbjarnarson JB (2010) American Crocodile (*Crocodylus acutus*). In: Manolis SC, Stevenson C (Eds) Crocodiles. Status Survey and Conservation Action Plan, 3rd ed. Crocodile Specialist Group (Darwin): 46–53.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Resources 4: 535–538. https://doi.org/10.1111/j.1471-8286.2004.00684.x

Verdade LM, Zucoloto RB, Coutinho LL (2002) Microgeographic variation in *Caiman latirostris*. Journal of Experimental Zoology Part B 294: 387–396. https://doi.org/10.1002/jez.10200

Weir B, Cockerham C (1984) Estimating F-statistics for the analysis of population structure. Evolution 38: 1358–1370. https://doi.org/10.2307/2408641

Appendix 1

Allele Frequencies. Allele frequencies for each locus in each *Crocodylus acutus* population studied in Pacific Costa Rica. The number in parenthesis indicates the sample size in that location.

Locus	Allele	LB (N=46)	SR (N=17)	PV (N=54)	RT (N=17)	ACOSA (N=49)
C391	139	0	0	0.009	0	0
	141	0.022	0	0.093	0.088	0.02
	143	0.011	0	0	0	0
	147	0.022	0.029	0	0	0
	149	0.065	0.059	0.028	0	0.041
	151	0.033	0.206	0.037	0	0.02
	153	0.728	0.559	0.611	0.794	0.602
	155	0.022	0.029	0	0	0.031
	157	0	0.029	0.167	0.088	0.265
	159	0	0	0.009	0	0
	161	0	0.088	0.046	0.029	0.01
	163	0.098	0	0	0	0.01
Cj16	151	0	0	0	0	0.031
	153	0	0.676	0.287	0.765	0.551
	155	0	0	0.009	0	0.041
	157	0	0	0	0	0.01
	173	0	0	0.009	0.029	0
	175	0.13	0.029	0.093	0.176	0.02
	183	0	0	0.019	0	0.071

Locus	Allele	LB (N=46)	SR (N=17)	PV (N=54)	RT (N=17)	ACOSA (N=49)
Cj16	185	0.696	0.147	0.537	0	0.224
	187	0.163	0.147	0.046	0.029	0.051
	189	0.011	0	0	0	0
Cj18	195	0	0	0	0	0.02
	197	0.011	0	0	0	0
	199	0.054	0	0.157	0.088	0.071
	201	0.739	0.029	0.231	0.147	0.133
	203	0	0	0	0.176	0.041
	205	0	0	0	0	0.01
	215	0.043	0	0	0	0.163
	217	0.054	0.059	0.046	0.029	0.112
	219	0.011	0.206	0.111	0.088	0.01
	221	0.054	0	0.019	0	0.041
	223	0.033	0.706	0.389	0.471	0.204
	225	0	0	0.037	0	0.031
	227	0	0	0	0	0.092
	229	0	0	0.009	0	0.071
Cj20	168	0.011	0	0	0	0.031
	170	0.022	0.059	0.019	0.029	0.02
	172	0.054	0.265	0.296	0.206	0.51
	174	0.75	0.618	0.491	0.206	0.143
	176	0	0.029	0.037	0.118	0
	178	0.163	0.029	0.074	0.412	0.245
	186	0	0	0.046	0	0
	196	0	0	0	0	0.051
	200	0	0	0	0.029	0
	206	0	0	0.019	0	0
	212	0	0	0.019	0	0
Cj109	364	0	0	0.065	0.029	0
	366	0.054	0.176	0.343	0.412	0.214
	368	0.511	0.324	0.194	0.059	0.551
	370	0.141	0.235	0.231	0.471	0.204
	372	0	0.029	0.037	0.029	0.01
	374	0.293	0.235	0.12	0	0.01
	376	0	0	0.009	0	0
	378	0	0	0	0	0.01
Cj131	209	0	0.029	0	0	0
	211	0.087	0	0.019	0.059	0.061
	213	0.141	0.059	0.194	0.176	0.143
	215	0.696	0.882	0.769	0.765	0.724
	217	0.065	0.029	0	0	0.051
	219	0.011	0	0.019	0	0
	231	0	0	0	0	0.02

Locus	Allele	LB (N=46)	SR (N=17)	PV (N=54)	RT (N=17)	ACOSA (N=49)
CU5-123	218	0	0	0.01	0	0.013
	220	0	0	0.029	0	0
	222	0.011	0	0	0	0
	224	0.189	0	0.279	0	0.079
	226	0.744	0.971	0.519	0.618	0.605
	228	0.033	0.029	0.106	0	0.026
	230	0.022	0	0.01	0	0
	232	0	0	0	0	0.066
	234	0	0	0.048	0.382	0.211
	220	0	0	0.029	0	0
	222	0.011	0	0	0	0
	224	0.189	0	0.279	0	0.079
	226	0.744	0.971	0.519	0.618	0.605
	228	0.033	0.029	0.106	0	0.026
	230	0.022	0	0.01	0	0
	232	0	0	0	0	0.066
	234	0	0	0.048	0.382	0.211
CUD68	105	0	0	0	0	0.031
	121	0	0	0	0	0.02
	123	0.054	0	0.009	0	0.051
	125	0.261	0.294	0.389	0.029	0.173
	127	0.348	0	0.139	0.147	0.367
	129	0.337	0.412	0.231	0.353	0.265
	131	0	0	0.231	0.471	0.092
	133	0	0.294	0	0	0
CUJ131	141	0.011	0	0	0	0
	155	0	0	0.019	0.059	0
	171	0.272	0.088	0.009	0.265	0
	175	0	0	0.009	0	0.01
	179	0.011	0.059	0.009	0	0.02
	181	0.337	0.5	0.389	0.059	0.582
	183	0	0	0.028	0	0.031
	185	0.37	0.353	0.528	0.618	0.357
	187	0	0	0.009	0	0

Nature Conservation 17: 19–33 (2017)
doi: 10.3897/natureconservation.17.10939
http://natureconservation.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Nature Conservation
Launched to accelerate biodiversity conservation

Natural habitats uncovered? – Genetic structure of known and newly found localities of the endangered bitterling *Pseudorhodeus tanago* (Cyprinidae)

Kenji Saitoh[1], Noriyasu Suzuki[2], Masumi Ozaki[3], Kazuhiro Ishii[4], Tetsuya Sado[5], Takahiro Morosawa[6], Takatoshi Tsunagawa[7], Masaru Tsuchiya[8]

1 *National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency, Fukuura 2-12-4, Kanazawa, Yokohama 236-8648, Japan* **2** *Chiba Biodiversity Center, Natural History Museum & Institute, Chiba, Aoba 955-2, Chuo, Chiba 260-8682, Japan* **3** *Chiba Prefectural Fisheries Research Center, Usuidai 1390, Sakura, Chiba 285-0866, Japan* **4** *Ibaraki Prefectural Government, Kasahara 978-6, Mito, Ibaraki 310-8555, Japan* **5** *Natural History Museum & Institute, Chiba, Aoba 955-2, Chuo, Chiba 260-8682, Japan* **6** *Japan Wildlife Research Center, Kotobashi 3-3-7, Sumida, Tokyo 130-8606, Japan* **7** *Tochigi Prefectural Fisheries Experimental Station, Sarado 2599, Ohtawara, Tochigi 324-0404, Japan* **8** *Ibaraki Nature Museum, Osaki 700, Bando, Ibaraki 306-0622, Japan*

Corresponding author: *Kenji Saitoh* (ksaitoh@affrc.go.jp)

Academic editor: *S. Lengyel* | Received 31 October 2016 | Accepted 3 March 2017 | Published 9 March 2017

http://zoobank.org/4AEE22C2-58A5-4362-A53C-23C217AD5109

Citation: Saitoh K, Suzuki N, Ozaki M, Ishii K, Sado T, Morosawa T, Tsunagawa T, Tsuchiya M (2017) Natural habitats uncovered? – Genetic structure of known and newly found localities of the endangered bitterling *Pseudorhodeus tanago* (Cyprinidae). Nature Conservation 17: 19–33. https://doi.org/10.3897/natureconservation.17.10939

Abstract

Overuse of natural resources by humans is a major threat to biodiversity. Overuse often involves species of economic or esthetic value, and fish are a typical example for a group that is exploited both for economic reasons (for human consumption) and for esthetic reasons (e.g. by aquarists). *Pseudorhodeus tanago* (Tanaka, 1909) (formerly known as *Tanakia tanago*) is a small colorful but legally protected (fishing, keeping and transfer are banned) bitterling fish distributed around Tokyo, Japan. Whereas it is critically endangered and more and more habitat loss has occurred, at least four stocks have been newly found during the last decade. To explore whether emergence of these newly found habitats is a consequence of incomplete survey, we genotyped mitochondrial cytochrome b sequence of *P. tanago* from 17 localities and an illegal home aquarium. Populations known by the past extensive survey (13 localities) showed geographically structured population genetic characteristics. Population-specific haplotypes were common indicating past divergence and bottleneck events. Four (north, {center + west}, south_1, south_2) or five

(north, center, west, south_1, south_2) geographic groups were detectable as for these known localities. On the other hand, newly found stocks were polymorphic and showed identical haplotypes from distant known localities. If we assume historical basis of distribution and genetic characteristics of these newly found stocks, it must be a series of unlikely geological events and haplotype sorting. We discuss potential issues posed by these questionable stocks.

Keywords
Bottleneck, bucket biology, conservation genetics, fish dumping, home aquarium, phylogeography, poaching, satoyama, *Tanakia tanago*

Introduction

Overuse of organisms by hunting or fishing for trade or esthetic purposes is one of the biggest threats to biodiversity. Controlling these activities is fundamental for ensuring the persistence of endangered organisms, particularly those that have traits attractive to humans. Alerts to poaching are thus necessary on those organisms legally protected. Not only tusks of elephants and rhinos but beautifully colored bodies of fish as well have attracted violators. The red list of Japanese brackish and freshwater fish (Ministry of the Environment, Japan 2015) acknowledges 39 species out of 168 of endangered and vulnerable categories have threats of overfishing in connection with home aquarium. Inversely, unauthorized fish release also threatens the integrity of natural fish population structures. A big source of invasion of alien freshwater fish is fish dumping from home aquaria (Lintermans 2004, Gertzen et al. 2008, Fuller et al. 2013, Ishikawa and Tachihara 2014). Poaching and dumping of endangered and protected fish, if any, disturb conservation programs and policy making and ultimately threat that species.

Pseudorhodeus tanago (Tanaka, 1909) (formerly known as *Tanakia tanago* transferred to the new genus, Chang et al. 2014) is a colorful small bitterling fish endemic to Japan with a limited geographic range around Tokyo. Habitats of *P. tanago* are in small water bodies such as ditches or ponds with spring water in hills (Nakamura 1969, Mochizuki 1997, Maita 2002, Ishinabe 2014). These habitats link with traditional agricultural landscape known as 'satoyama'. Traditional farming activities have maintained ditches, ponds and vegetation around waters in farmlands in hills (Kobori and Primack 2003), and thus maintained habitats of *P. tanago*. However, because of heavily populated and highly developed areas around its habitats, *P. tanago* is critically endangered. Urbanization accompanying change of farming style gives rise to habitat loss (Mochizuki 1997, Ishinabe 2014). Agency for Cultural Affairs, and Ministry of the Environment, Japan have endorsed this bitterling as a legally protected species (Ministry of the Environment, Japan 2015). Fishing, keeping and transfer of *P. tanago* are banned. Since around 1970, conservation measures of *P. tanago* have been taken under local bases including extensive search for habitats, development of ex situ breeding techniques, protected area enclosure, mitigation of civil engineering activities (Tochigi Prefectural Fisheries Experimental Station 1973, Akiyama et al. 1994, Mochizuki 1997, Kubota et al. 2010, Ishinabe 2014). A national breeding program for the

protection of the species began in 1995, which included ex situ breeding and habitat restoration (Agency of Environment, Japan et al. 1995).

Records of 45 localities of *P. tanago* habitats were established as part of an extensive survey (Tanaka 1909, Nakamura 1969, Maita 2002, Ishinabe 2014, present report) (Fig. 1a). Some of the localities have been kept secret by authorities because of risks of poaching. By around 2000, most known habitats were lost, and further local extinction has occurred in these days in spite of conservation measures (Fig. 1b). Many populations have undergone habitat degradation to decline to ca. 1/100 in a few years, and in an extreme case the population is vanishing in the final remnant habitat of a short (< 100 m) stretch of a small (ca. 60 cm wide) stream (Mochizuki 1997).

From the habitat characteristics of *P. tanago*, confined and scattered among headwaters of fine branching dales in agricultural landscape on hill terrains, we assume geographically structured population genetic characteristics of this species. Kubota et al. (2010) outlined geographic population structure of this species, but lack of localities from southern part of its geographic range and usage of questionable specimens obscured clear-cut geographic structure. Analysis of geographic population structure of this critically endangered fish with habitat loss based on specimens from reliable sources is necessary for setting up conservation programs (Waples and Gaggiotti 2006).

Whereas more and more local populations have been lost recently, *P. tanago* were newly found in a few localities in the last decade ('newly found' locality or stock hereafter) (Fig. 1b, hatched areas). Are these newly found stocks are simply because of incomplete survey of distribution? The aims of this study were to delineate geographical genetic structure of *P. tanago* and to identify symptom of disturbance, if any, in this structure by an analysis of genotypes of 80 individuals from 18 populations or stocks. Four of them are from newly found localities, one from an illegal home aquarium, and the others are from localities previously known by the past extensive survey ('known' localities or populations hereafter).

Materials and methods

Pseudorhodeus tanago from 13 known localities collected from 1993 through 2013 (55 individuals), four newly found stocks collected from 2010 through 2014 (22 individuals), and three individuals seized from an illegal home aquarium were materials of this research (Table 1, Fig. 1b). Fishes from two out of the 13 known localities were of ex situ preserved stock extinct in the wild in early 1990s (#8) and late 1970s (#10). Habitat characteristics of the collecting localities were identical to descriptions in literatures (Nakamura 1969, Mochizuki 1997, Maita 2002) except for two newly found localities (#14, 17) where civil engineering activities including straight cut of channels with concrete enforcement have suffered their habitats.

Extraction of DNA was done from fin clips with QuickGene DNA Tissue kit on QuickGene-810 (Kurabo, Neyagawa, Japan). PCR primers were L14695 on the L-strand (AATTYTTGCTCRGACTCTAACC) and H15910 on the H-strand

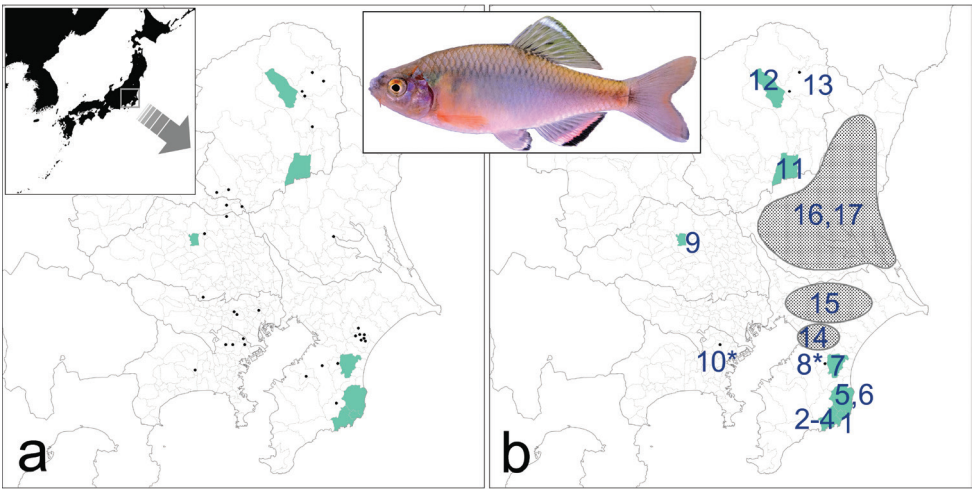

Figure 1. Past (**a**) and present (**b**) geographic distribution of *Pseudorhodeus tanago*. Dots indicate records of presence of the bitterling. Light blue painted areas contain one to three localities from which specimens of the present report came. Exact places of these localities are kept secret by authorities to protect from poaching. Numbers on map b stand for localities of samples listed in Table 1. Asterisks indicate extinction in the wild and specimens came from ex situ preserved stocks. Habitats #14–17 in hatched areas (exact places are kept secret by authorities) are newly found. Hatched areas thus do not represent exact geographic ranges.

Table 1. Localities and specimens used in this study.

#	Locality	Collecting date, year	Number of individuals
1	Onjuku, Chiba	Dec. 19, 2012	4
2	Katsuura 1, Chiba	Jun. 14, 2012	8
3	Katsuura 2, Chiba	Jun. 14, 2012	4
4	Katsuura 3, Chiba	Aug. 22, 2012	4
5	Isumi A, Chiba	Jun. 21, 2012	4
6	Isumi B, Chiba	Jun. 26, 2012	8
7	Mobara, Chiba	Oct. 26, 2012	4
8	Nagara, Chiba*	Jan. 28, 2013	4
9	Namegawa, Saitama	2003	5
10	Small pond near Yokohama*	2003 year class	3
11	South-east Tochigi	2006	3
12	North Tochigi	1993-1994	1
13	Handa natural habitat conservation area, Tochigi	1993	3
14	Central Chiba**	May 16, 2010	3
15	North Chiba**	Jun. 3, 2010, Jul. 25, 2011	2
16	Ibaraki A**	Nov. 6, 2014	16
17	Ibaraki B**	2014	1
18	Seized from a home aquarium	2014	3

* Preserved stock ex situ (extinct in the wild).

** Newly found stocks.

(GATCTTCGGATTACAAGACCGAT) which worked for amplifying a 1219 bp mitochondrial DNA fragment encompassing the whole cytochrome b gene and flanking tRNA partial sequences. PCR reaction mixture of 12.5 μL contained 1μL of template DNA, 0.96 μL of dNTP mix (2.5 nmol each), 1.2 μL of 10x Ex*Taq* buffer, 0.06 μL (0.3 U) of Ex*Taq* (Takara, Shiga, Japan), 1 μL of primers (5 pmol each), 7.28 μL of Milli-Q grade water. PCR reaction was of touchdown profile (Don et al. 1991) in which annealing temperatures dropped from 59°C down to 53°C in the initial 7 cycles and was constant at 55°C in the remaining 28 cycles (35 cycles in total). The PCR reaction started with 3 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at the above touchdown annealing temperatures, 120 sec at 72°C with final extension at 72°C for 5 min. The PCR primers and two internal primers (L15438 [TTTTCCTACAAAGATCTATTAGG] and H15569 [CGTAAGATGGCGTAGGCAAATAA] on the L- and H-strand, respectively) worked for overlapping double-stranded sequencing with BigDye terminator v.3.1 kit run on an ABI3730 sequencer (ABI, Foster City CA, USA). The complete mitochondrial cytochrome b sequences used in this research have DDBJ/GenBank entries of LC17598–LC170677.

Indices of nucleotide divergence were calculated with Arlequin v.3.5 (Excoffier and Lischer 2010). Geographic population structure was assessed with SAMOVA v.2.0 (Dupanloup et al. 2002). The net nucleotide divergence was calculated by π_{xy}-(π_x+π_y)/2 where π_{xy} is average number of nucleotide differences between populations x and y, and π_x and π_y stand for this value between individuals within populations x and y. Parsimonious haplotype network was drawn with TCS v.1.2.1 (Clement et al. 2000).

Results

Haplotype grouping

Sequencing *Pseudorhodeus tanago* mitochondrial cytochrome b revealed 10 haplotypes (Table 2, Hap01–10). There were 19 variable sites, and 14 nucleotide substitutions observed between the most distant haplotypes (Hap01, 10) (Table 3). All the observed nucleotide substitutions were transitions, two of which were non-synonymous (positions 15100 and 15415). Most specimens from known localities (11/13) were monotypic, whereas newly found and seized stocks except for #17 with a single individual examined were polymorphic.

A haplotype network coincides with geographically structured population genetic characteristics of this species (Fig. 2). Regarding 13 known localities, haplotypes from each locality frequently were specific to that locality (9/13). Haplotypes placed on the upper-left side of the figure (Hap09, 10) were from northern part of the geographic range (Fig. 1b, #12, 13). Likewise, haplotypes arranged on the right side (Hap01–05) were all from the southern part (Fig. 1b, #1–6), and Hap08 on the lower-left side of the network was from the western part (Fig. 1b, #10) of the range. Placement of the other two haplotypes (Hap06, 07) was on the center of the network, and their geo-

Table 2. Haplotype composition of localities.

# \ Haplotype	Hap01	Hap02	Hap03	Hap04	Hap05	Hap06	Hap07	Hap08	Hap09	Hap10
1	4									
2					8					
3	4									
4		4								
5			2	2						
6	7					1				
7						4				
8*						4				
9							5			
10*								3		
11						3				
12									1	
13										3
14**			1			2				
15**	1	1								
16**	8	5		3						
17**				1						
18	1			2						

* Preserved stock ex situ (extinct in the wild).

** Newly found stocks.

graphic placement was roughly the center of the range (Fig. 1b, #7–9) except for a single exception from locality #6 in the southern part (Table 2). This haplotype grouping was delineated by gaps with three or more missing haplotypes, whereas one or no gap lay between haplotypes within these groups. Haplotypes of the south group, however, contained distant types (four nucleotide differences at most), and there might be subgrouping among them.

Geographic grouping

Geographic grouping of localities by AMOVA (SAMOVA) under assumptions of four or five geographic groups was similar to the haplotype grouping based on the haplotype network without geographic information (Table 4, Fig. 3). Values of sum of squares and coverage of variation among geographic groups were much higher than those values both among localities within geographic groups and within localities indicating clear geographic structures. The North haplotype group (Hap09, 10) appeared in a North geographic group that consisted of localities #12, 13. Regarding 13 known habitats, the SAMOVA grouping divided the South haplotype group into two geographic groups (south_1 of localities #1, 3, 6 and south_2 of #2, 4, 5). The former consisted predominantly of Hap01 which did not appear among specimens from the latter. The Center (#7–9, 11) and the West (#10) geographic groups under

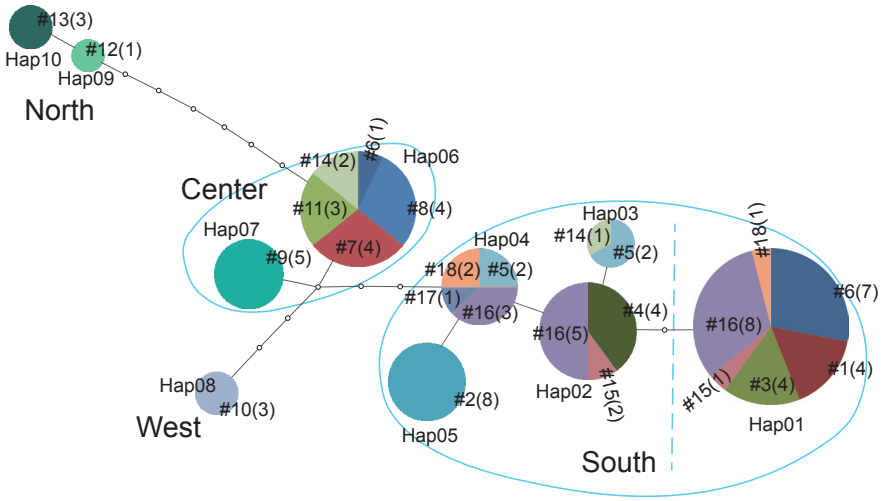

Figure 2. Parsimonious haplotype network of mitochondrial DNA sequences of *Pseudorhodeus tanago* (Hap01–10). Each line connecting haplotypes indicates one nucleotide difference. Small open circles stand for missing haplotypes. Locality number (#) and number of individuals observed (parentheses) indicate haplotype sharing. Blue solid lines encircle haplotype groups (north, center, west and south). Blue broken line indicates sub-grouping within the south haplotype group exhibited by SAMOVA analysis (right, south_1; left, south_2).

Table 3. Haplotypes and variable sites of *Pseudorhodeus tanago* mitochondrial cytochrome b.

Nucleotide site *	1 4 4 1 2	1 4 4 7 2	1 4 5 2 0	1 4 5 2 6	1 4 5 2 9	1 4 5 8 0	1 4 8 0 7	1 4 9 3 7	1 4 9 5 5	1 4 9 6 7	1 4 9 9 4	1 5 0 3 0	1 5 1 0 0 **	1 5 2 2 8	1 5 3 0 1	1 5 4 1 5 **	1 5 4 3 2	1 5 4 4 7	1 5 5 1 3
Haplotype																			
Hap01	T	G	A	C	G	G	G	C	G	G	G	T	A	A	T	G	C	A	G
Hap02	T	G	A	C	G	G	A	C	G	G	G	C	A	A	T	G	C	A	G
Hap03	T	G	A	C	G	G	A	C	G	G	G	C	A	G	T	G	C	A	G
Hap04	T	G	A	C	G	G	A	C	G	G	G	C	A	A	T	G	T	A	G
Hap05	T	G	A	C	G	G	A	C	G	G	G	C	A	A	T	A	T	A	G
Hap06	T	A	A	T	G	G	A	C	G	C	A	C	A	A	T	G	T	A	G
Hap07	T	A	A	T	G	G	A	C	G	G	A	C	A	A	C	G	T	A	G
Hap08	T	A	A	T	G	A	A	T	G	G	A	C	A	A	T	G	T	G	G
Hap09	C	A	G	T	A	G	A	C	A	A	A	C	G	A	T	G	T	A	A
Hap10	C	A	G	T	A	G	A	C	A	A	A	C	G	A	T	G	T	G	A

* Positions corresponding with those of AP012526 (Miya et al. 2015).

** Nucleotide sites with non-synonymous substitutions.

an assumption of five groups coincided with those of haplotype groups, but they were combined under a four groups assumption. Small but clear-cut nucleotide differences between localities within the south haplotype groups in spite of geographic proximity

Table 4. Contribution to variation in different patterns of population/stock grouping.

Grouping	Number of groups	Among groups				Among localities within groups				Within localities				Variance among groups
		Sum of squares	% of variation	Fct	P(Fct)	Sum of squares	% of variation	Fst	P(Fst)	Sum of squares	% of variation	Fsc	P(Fsc)	
Known localities only														
North + center + west + south_1 (#1 + #3 + #6) + south_2 (#2 + #4 + #5)	5	110,494	80,50	0,805	low*	15,562	13,35	0,939	low	8,125	6,15	0,685	low	2,532
North + {center + west} + south_1 (#1 + #3 + #6) + south_2 (#2 + #4 + #5)	4	101,475	74,43	0,744	low	24,582	19,52	0,940	low	8,125	6,04	0,764	low	2,382
All localities (known + newly found)														
North + {center + #14} + west + south_3 (#2) + south4 (others)	5	121,061	76,18	0,762	low	22,598	11,29	0,875	low	23,562	12,52	0,474	low	2,389
North + {center + #14} + west + south	4	105,356	72,76	0,728	low	38,302	16,08	0,888	low	23,562	11,16	0,590	low	2,560

* P-value lower than 0.00001.

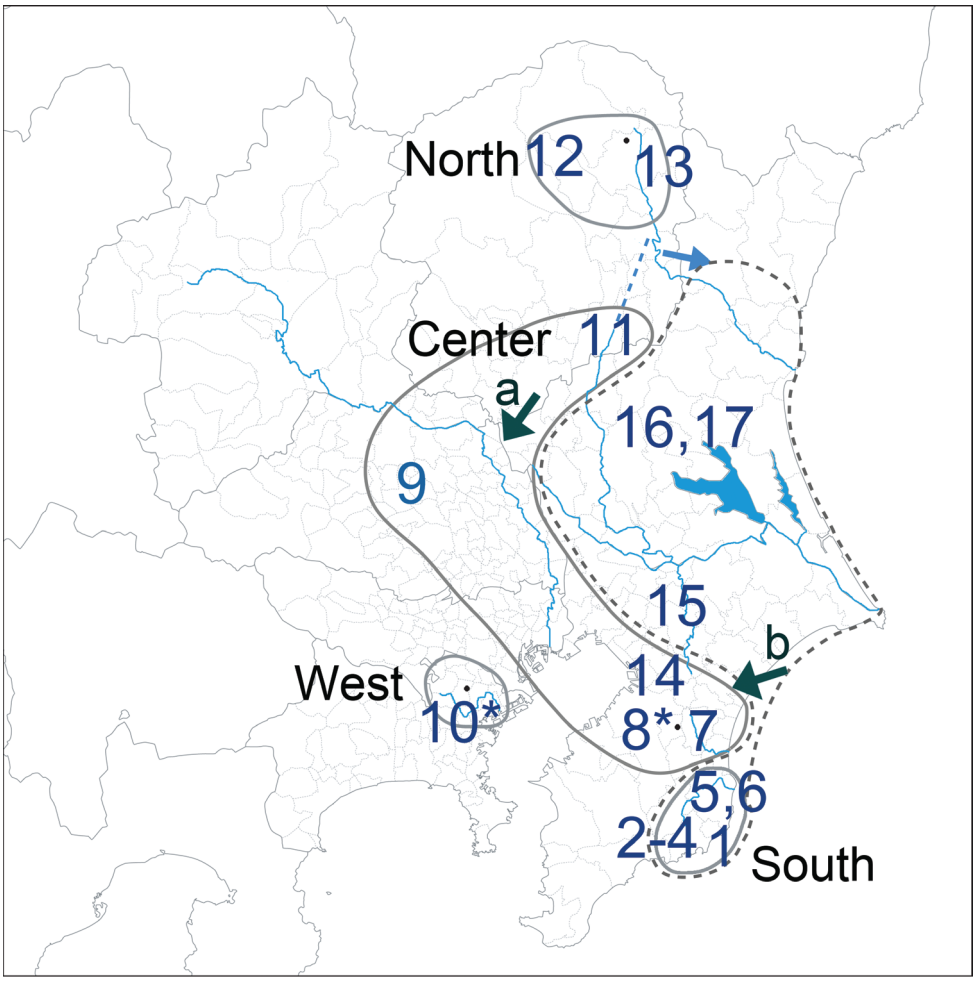

Figure 3. Geographic grouping of localities (encircled by gray solid lines) and river connectivity in 16th century (blue solid lines). Gray broken line encircles localities with the South geographic group including newly found localities (#15–17). Another newly found locality (#14) could be a member of the Center geographic group, but its haplotype composition is not typical among localities of the group. Arrows a and b denote historical overflow or stream capture terrains between river basins. Blue broken line indicates ancient river flow, and a blue arrow denotes change of the river flow at 500 ka.

(ca. 1–16 km, Table 5) divided the southern localities into two geographic groups, whereas moderate differences (0 to four base changes) among localities of the center and west geographic groups distant from each other (ca. 60–100 km except between #7 and #8) could be pooled.

Geographic grouping of known localities was largely coincident with river connectivity (Fig. 3). The North and the West geographic groups appeared each in a single river basin respectively. The South geographic group covered two river basins close to each other, one of which is kept secret by the authority (not shown on the map).

Table 5. Genetic (π_{xy}, below diagonal) and geographic (km, above diagonal) distance among collecting localities.

#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		3.45	4.41	5.10	15.18	6.54	32.34	30.18	131.78	77.30	137.40	186.42	192.10	44.87	61.76	100.22	97.24
2	4.000		1.00	2.41	15.54	9.22	32.37	29.88	129.87	74.61	137.21	186.22	192.17	44.35	61.48	101.46	96.42
3	0.000	4.000		1.79	16.07	10.19	32.75	30.17	129.62	74.09	137.49	186.49	192.51	44.57	61.75	102.10	96.52
4	2.000	2.000	2.000		17.85	11.39	34.54	31.94	130.99	75.05	139.25	188.25	194.29	46.31	63.51	103.84	98.20
5	2.333	1.333	2.333	0.333		11.27	17.19	15.30	119.45	68.91	122.25	171.28	176.92	30.06	46.69	86.17	82.61
6	0.000	3.250	0.000	1.500	1.708		28.08	26.52	130.38	78.05	132.94	181.94	187.25	41.26	57.63	94.19	93.82
7	7.000	5.000	7.000	5.000	4.333	5.250		3.81	105.55	61.36	105.06	154.09	159.82	13.64	29.55	71.49	65.94
8	7.000	5.000	7.000	5.000	4.333	5.250	0.000		105.40	59.23	107.34	156.35	162.36	14.76	31.62	75.08	67.31
9	7.000	5.000	7.000	5.000	4.333	5.500	2.000	2.000		62.86	70.58	98.52	112.62	92.31	82.41	109.34	51.58
10*	9.000	7.000	9.000	7.000	6.333	7.500	4.000	4.000	4.000		103.40	146.69	140.01	52.65	55.93	106.07	59.01
11	7.000	5.000	7.000	5.000	4.333	5.250	0.000	0.000	2.000	4.000		49.02	56.15	93.02	75.74	61.16	45.70
12	13.000	11.000	13.000	11.000	10.333	11.375	7.000	7.000	8.000	10.000	7.000		15.79	141.99	124.74	103.09	92.74
13	14.000	12.000	14.000	12.000	11.333	12.375	8.000	8.000	9.000	9.000	8.000	1.000		148.38	130.99	103.10	101.6
14**	3.667	2.333	3.667	1.667	1.000	2.333	0.000	0.000	1.333	3.333	0.000	6.667	7.667		17.40	65.84	52.56
15**	0.000	2.000	0.000	0.000	0.333	-0.250	5.000	5.000	5.000	7.000	5.000	11.000	12.000	1.667		52.55	37.24
16**	3.000	1.000	3.000	1.000	0.333	2.250	4.000	4.000	4.000	6.000	4.000	10.000	11.000	1.333	1.000		58.96
17**	0.492	2.117	0.492	0.492	0.638	0.195	5.117	5.117	5.117	7.117	5.117	11.117	12.117	1.908	-0.508	1.117	

* Preserved stock ex situ (extinct in the wild).

** Newly found stocks.

Potential connection between these two basins through deltas is possible. The Center geographic group spanned a wider range on the periphery of a lowland plain which roughly corresponds with the area encircled by the gray solid line of this group plus coastal area on the east (Fig. 3). Localities of this geographic group presented in three river basins potentially connected with each other through flat low overflow terrain of 12 m high (Fig. 3, arrow a) or overflow plus switching dales (Kagose 1979) (flat hill top at 90m high and opposing headwaters with imbalanced slopes at 60 m high, arrow b).

On the other hand, placement of specimens from newly found localities onto the haplotype network obscured the geographic population genetic structure, though SAMOVA analysis apportioned these localities to either of the South or Center geographic groups according to their haplotype composition. Coverage of variation among geographic groups reduced from 74.43–80.5% to 72.76–76.18% (Table 4). Newly found localities except for #14 contained haplotypes of only the South group in spite of potential river connectivity with localities with the Center or the North geographic groups (Fig. 3). Locality #14 showed a mixed composition of both the Center and the South haplotype groups.

Discussion

Genetic architecture of known populations

The low sequence diversity of *Pseudorhodeus tanago* represented as monotypy in most known localities (11/13) conformed to their habitat characteristics (Table 2). Specific haplotypes from many of the known localities (9/13) indicated that populations of *P. tanago* rarely exchange with each other. Confinement in small isolated water bodies and subsequent bottleneck events might have brought about the present genetic structure of populations.

Isolation on a wider and geological scale is also responsible for divergence among geographic groups. The North geographic group contained haplotypes distant from others (Hap01 and Hap10), and ancestors of the North and other geographic groups thus diverged first. The central part of the entire geographic range of *P. tanago* encircled the flat plain that underwent repeated marine transgressions (Fig. 3). Marine transgression at > 400 ka (Sugai et al. 2013) and/or change of river flow at 500 ka (Fig. 3, blue arrow) (Koike et al. 1985, Kubota et al. 2010) might be responsible for the isolation of these geographic groups. Repeated marine transgressions afterward have facilitated further isolation among geographic groups.

Implication of genetic characteristics of newly found stocks

Polymorphism and haplotype composition among newly found stocks, on the other hand, posed a question about the characteristics of genetic and geographic population

structure of *P. tanago*. If we assume historical basis of distribution and genetic characteristics of these newly found stocks, it must be a series of unlikely geological events and haplotype sorting.

Including these newly found stocks, *P. tanago* as a whole showed a bipartite population structure in which some localities show genetic variation while others rarely do so (Table 2). We should assume population admixture and rampant migration among these newly found stocks or one way migration from northwest part of the map including known populations (Fig. 1a), whereas known populations underwent isolation and bottleneck events. Marine transductions were, however, more frequent near the east coast since geological ancient until historic medieval times where newly found stocks inhabit (Kubo 2007, Sugai et al. 2013), and thus habitats of newly found stocks might be ephemeral prone to bottleneck. River connectivity implies that the hypothetical migration was from central or north geographic group (Fig. 3), but newly found localities (#15–17) consisted solely of south haplotype group. Admixture between populations around south group of known and newly found localities upon ace age marine regression is unlikely because of absence of continental shelf at the south-east coast (Sugai et al. 2013). Even if it was possible, river connection should involve localities of center geographic group (#7, 8) (Fig. 3), the main haplotype of which is absent from the newly found localities. Among newly found stocks, #14 consisted of haplotype of center group and was close to populations of center geographic group (#7, 8), but its natural distribution is questionable because of disturbance of the habitat by civil engineering. Straight cut channelization with concrete enforcement both on the banks and bottom has eliminated mud-sandy substrates for bitterlings' spawning host mussels.

There is no information such as confession or witness of violators about unauthorized releasing of *P. tanago* into the newly found localities. Genetic polymorphism of newly found localities may arrange stocks of these localities as conservation target of higher priority, if habitats are under risks of extinction. Recent decline of habitats indicates mitigation activities have not worked well (Mochizuki 1997, Ishinabe 2014) or just under trials (Tsunagawa et al. 2016). Then, ex situ breeding which works currently (Kubota et al. 2010, Ishinabe 2014) would be an interim choice. Under the assumption of natural distribution, stocks from newly found localities would be of higher priority for ex situ preservation.

Natural distribution in these newly found localities is, however, questionable because of unlikely association between haplotype/population relationships and river connectivity. Because bitterling fishes have attractive coloration to humans, there have been reports on unauthorized intentional releasing activities possibly implicated in home aquaria (Miyake et al. 2011, Kitazima et al. 2015, Saitoh et al. 2016). Under an assumption of unauthorized releasing or dumping of *P. tanago*, we confront difficult issues. Genetic polymorphism of the newly found and seized stocks (Table 2) implied a number of poaching activities. Because violators do not assess stock size of their target fish in the field, poaching would sometimes be devastating. Unauthorized releasing or dumping, if any, also poses problems. Unauthorized releasing of *P. tanago* is illegal, because unauthorized transfer of this legally protected fish is prohibited. Released *P.*

tanago individuals themselves are, however, legal target of conservation (Ministry of the Environment, Japan 2015). Presence of questionable stocks thus disturbs prioritization of conservation targets under limited funds and human resources. Releasing or dumping onto habitats of natural populations is a direct threat to the integrity of natural properties of *P. tanago*. Our report has cautionary implication to potential violators.

Acknowledgments

We dedicate this paper to Toshihiro Ishinabe (deceased) (Kannonzaki Nature Museum) and his contributions to conservation of *Pseudorhodeus tanago* over two decades carrying out habitat monitoring and ex situ breeding. KS, NS and KI conceived this research. KS analyzed data and drafted the manuscript. NS, MO, KI, TS, TM, TT and MT conducted field survey. All authors read, improved and approved the manuscript. This research was financially supported by a Protective Breeding Program of Ministry of the Environment, Japan. Permission for research use of *P. tanago* was provided by Agency for Cultural Affairs and Ministry of the Environment, Japan to NS and KI. We have no financial conflict of interests about contents of this publication. Specimens were collected under a condition in which the authorities expect non-disclosure of exact places of habitats except for a protected and publicly opened locality (#13). NS and KI are employees of two of these authorities that do not disclose some localities. Toshihiro Ishinabe, Hitoshi Kubota (Tochigi Prefectural Government), Kouki Kano (Ibaraki University), Masaki Miya (Natural History Museum and Institute, Chiba), Naoyuki Suguro (Kanagawa Prefectural Fisheries Technology Center) and Kazuhiro Umezawa (Saitama Prefectural Government) helped us collecting specimens. Maya Suzuki (Kanto Regional Environment Office, Ministry of the Environment) helped and coordinated field survey and sample collection. Takahiro Ohtsuka (National Research Institute of Fisheries Science) helped us for laboratory works.

References

Agency of Environment Japan, Ministry of Education Science and Culture Japan, Ministry of Agriculture Forestry and Fisheries Japan, Ministry of Construction Japan (1995) Protective Breeding Program of Tokyo Bitterling, *Tanakia tanago*. Agency of Environment, Japan, 3 pp. [In Japanese]

Akiyama N, Imai H, Ogasawara Y (1994) Effectiveness of *Margaritifera laevis* as spawning bed for *Tanakia tanago*. Aquaculture Science (Suisanzoshoku) 42: 231–238. [In Japanese with English abstract]

Chang C-H, Li F, Shao K-T, Lin Y-S, Morosawa T, Kim S, Koo H, Kim W, Lee J-S, He S, Smith C, Reichard M, Miya M, Sado T, Uehara K, Lavoué S, Chen W-J, Mayden RL (2014) Phylogenetic relationships of Acheilognathidae (Cypriniformes: Cyprinoidea) as revealed from evidence of both nuclear and mitochondrial gene sequence variation:

Evidence for necessary taxonomic revision in the family and the identification of cryptic species. Molecular Phylogenetics and Evolution 81: 182–194. https://doi.org/10.1016/j.ympev.2014.08.026

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657–1660. https://doi.org/10.1046/j.1365-294x.2000.01020.x

Don R, Cox P, Wainwright B, Baker K, Mattick J (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research 19: 4008. https://doi.org/10.1093/nar/19.14.4008

Dupanloup I, Schneider S, Excoffier L. (2002) A simulated annealing approach to define the genetic structure of populations. Molecular Ecology 11: 2571–2581. https://doi.org/10.1046/j.1365-294X.2002.01650.x

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Fuller P, Neilson M, Huge DH (2013) The NAS alert system: A look at the first eight years. Fisheries 38: 128–138. https://doi.org/10.1080/03632415.2013.767241

Gertzen E, Familiar O, Leung B (2008) Quantifying invasion pathways: fish introductions from the aquarium trade. Canadian Journal of Fisheries and Aquatic Sciences 65: 1265–1273. https://doi.org/10.1139/F08-056

Ishikawa T, Tachihara K (2014) Introduction history of non-native freshwater fish in Okinawa-jima Island: ornamental aquarium fish pose the greatest risk for future invasions. Ichthyological Research 61: 17–26. https://doi.org/10.1007/s10228-013-0367-6

Ishinabe T (2014) Conservation practices of the Tokyo bitterling, *Tanakia tanago*, in Chiba Prefecture. In: Nagata Y (Ed.) An Introduction to Freshwater Fish Research: Snoop into Fish Life under the Water. Tokai University Press, Hadano, 103–112.

Kagose Y (1979) Stream capture by Konakaike catchment at the edge of Shimousa upland. Chiri (Geography) 24(11): 78–81. [In Japanese]

Kitazima J, Matsuda M, Mori S, Kokita T, Watanabe K (2015) Population structure and cryptic replacement of local populations in the endangered bitterling *Acheilognathus cyanostigma*. Ichthyological Research 62: 122–130. https://doi.org/10.1007/s10228-014-0412-0

Kobori H, Primack RB (2003) Participatory conservation approaches for satoyama, the traditional forest and agricultural landscape of Japan. AMBIO: A Journal of the Human Environment 32: 307–311. https://doi.org/10.1579/0044-7447-32.4.307

Koike K, Iwasaki T, Danbara T, Momose M (1985) Fission-track ages and their geological meanings of quaternary volcanic ashes (Shimotsuke-loam) covering the Kitsuregawa Hills, Tochigi Prefecture, central Japan. Science Reports of Geographical Institute, Komazawa University 21: 39–67. [In Japanese]

Kubo S (2007) Joso Inland Sea: Topography and environmental changes of Katori Plain in historic times. In: Ichimura T, Ibaraki Prefectural Museum of History (Eds) Outlook of Inland Seas in Medieval East Japan. Koshi Shoin, Tokyo, 39–64. [In Japanese]

Kubota H, Watanabe K, Suguro N, Tabe M, Umezawa K, Watanabe S (2010) Genetic population structure and management units of the endangered Tokyo bitterling, *Tanakia tanago* (Cyprinidae). Conservation Genetics 11: 2343–2355. https://doi.org/10.1007/s10592-010-0120-x

Lintermans M (2004) Human-assisted dispersal of alien freshwater fish in Australia. New Zealand Journal of Marine and Freshwater Research 38: 481–501. https://doi.org/10.1080/00288330.2004.9517255

Maita A (2002) The study of social management systems for conservation of miyakotanago habitats in satochi. Bulletin of the Tokyo University Forests 107: 127–223. [In Japanese with English summary]

Ministry of the Environment, Japan (Ed.) (2015) Red Data Book 2014 – Threatened Wildlife of Japan – Vol.4 Pisces – Brackish and Fresh Water Fishes. Gyosei, Tokyo, 414 pp. [In Japanese]

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W (2015) MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. Royal Society Open Science 2: 150088. https://doi.org/10.1098/rsos.150088

Miyake T, Nakajima J, Onikura N, Ikemoto S, Iguchi K, Komaru A, Kawamura K (2011) The genetic status of two subspecies of *Rhodeus atremius*, an endangered bitterling in Japan. Conservation Genetics 12: 383–400. https://doi.org/10.1007/s10592-010-0146-0

Mochizuki K (1997) *Tanakia tanago* (Tanaka). In: Nagata Y, Hosoya K (Eds) Circumstances in Endangered Japanese Freshwater Fishes and Their Protection. Midori Shobo, Tokyo, 64–75. [In Japanese]

Nakamura M (1969) Cyprinid Fishes of Japan: Studies on the Life History of Cyprinid Fishes of Japan. Special Publication of the Research Institute for Natural Resources 4, Tokyo, 455 pp. [In Japanese with English summary]

Saitoh K, Shindo K, Fujimoto Y, Takahashi K, Shimada T (2016) Mitochondrial genotyping of an endangered bitterling *Acheilognathus typus*. ZooKeys 623: 131–141. https://doi.org/10.3897/zookeys.623.8981

Sugai T, Matsushima [Ogami] H, Mizuno K (2013) Last 400 ka landform evolution of the Kanto Plain: Under the influence of concurrent glacio-eustatic sea level changes and tectonic activity. Journal of Geography (Chigaku Zasshi) 122: 921–948. [In Japanese with English abstract] https://doi.org/10.5026/jgeography.122.921

Tanaka S (1909) Descriptions of one new genus and ten new species of Japanese fishes. The Journal of the College of Science, Imperial University of Tokyo, Japan 27: 1–27

Tochigi Prefectural Fisheries Experimental Station (1973) Interim report of research on conservation measures of Tokyo Bitterling and stickleback. Annual Report of the Tochigi Prefectural Fisheries Experimental Station 15: 103. [In Japanese]

Tsunagawa T, Sakai T, Kubota H (2016) Activities for long-term persistence of a wild population of Tokyo Bitterling: An approach to supportive breeding and habitat restoration. In: Nature Conservation Committee of Ichthyological Society of Japan, Watanabe K, Mori S (Eds) The Challenges of Freshwater Fish Conservation: Concepts and Practice for Restoration of Aquatic Biodiversity. Tokai University Press, Hadano, 51–65. [In Japanese]

Waples RS, Gaggiotti O (2006) What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. Molecular Ecology 15: 1419–1439. https://doi.org/10.1111/j.1365-294X.2006.02890.x

Nature Conservation 17: 35–56 (2017)
doi: 10.3897/natureconservation.17.12251
http://natureconservation.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Nature Conservation
Launched to accelerate biodiversity conservation

Temporal changes in vegetation of a virgin beech woodland remnant: stand-scale stability with intensive fine-scale dynamics governed by stand dynamic events

Tibor Standovár[1], Soma Horváth[1], Réka Aszalós[2]

1 *Department of Plant Systematics, Ecology and Theoretical Biology, Institute of Biology, L. Eötvös University, Pázmány P. sétány 1/c, H-1117 Budapest, Hungary* **2** *MTA Centre for Ecological Research, Institute of Ecology and Botany, Alkotmány u. 2-4., H-2163 Vácrátót, Hungary*

Corresponding author: *Tibor Standovár* (standy@caesar.elte.hu)

Academic editor: *K. Henle* | Received 13 February 2017 | Accepted 1 March 2017 | Published 16 March 2017

http://zoobank.org/6F0EEA14-F1C4-40D6-A0B3-249B0A24AFF1

Citation: Standovár T, Horváth S, Aszalós R (2017) Temporal changes in vegetation of a virgin beech woodland remnant: stand-scale stability with intensive fine-scale dynamics governed by stand dynamic events. Nature Conservation 17: 35–56. https://doi.org/10.3897/natureconservation.17.12251

Abstract

The aim of this resurvey study is to check if herbaceous vegetation on the forest floor exhibits overall stability at the stand-scale in spite of intensive dynamics at the scale of individual plots and stand dynamic events (driven by natural fine scale canopy gap dynamics). In 1996, we sampled a 1.5 ha patch using 0.25 m² plots placed along a 5 m × 5 m grid in the best remnant of central European montane beech woods in Hungary. All species in the herbaceous layer and their cover estimates were recorded. Five patches representing different stand developmental situations (SDS) were selected for resurvey. In 2013, 306 plots were resurveyed by using blocks of four 0.25 m² plots to test the effects of imperfect relocation.

We found very intensive fine-scale dynamics in the herbaceous layer with high species turnover and sharp changes in ground layer cover at the local-scale (< 1 m²). A decrease in species richness and herbaceous layer cover, as well as high species turnover, characterized the closing gaps. Colonization events and increasing species richness and herbaceous layer cover prevailed in the two newly created gaps. A pronounced decrease in the total cover, but low species turnover and survival of the majority of the closed forest specialists was detected by the resurvey at the stand-scale. The test aiming at assessing the effect of relocation showed a higher time effect than the effect of imprecise relocation.

The very intensive fine-scale dynamics of the studied beech forest are profoundly determined by natural stand dynamics. Extinction and colonisation episodes even out at the stand-scale, implying an overall compositional stability of the herbaceous vegetation at the given spatial and temporal scale. We argue that fine-scale gap dynamics, driven by natural processes or applied as a management method, can warrant the survival of many closed forest specialist species in the long-run.

Nomenclature: Flora Europaea (Tutin et al. 2010) for vascular plants; Soó 1968–1980 for syntaxa

Keywords

Deciduous forest, temperate forest, resurvey, forest reserve, forest developmental stage, *Fagus sylvatica*, ancient forest herbs

Introduction

The significance of the herbaceous layer in forest biodiversity and ecosystem functioning has been widely appreciated (Whigham 2004, Gilliam 2014), generating an interest in its dynamics. During the last decade, remarkable changes in the herbaceous layer have been shown by several studies (e.g. reviewed in Verheyen et al. 2012). Resurvey studies investigated the understorey to detect potential long-term changes and to assess the role of different environmental variables as driving forces in deciduous forests. The observed changes were attributed to different background causes, such as combined effect of temperature increase and canopy opening (De Frenne et al. 2013, 2015), abandonment of former forest management practices (Hédl et al. 2010, Heinrichs et al. 2014), lack of disturbance (Brewer 1980, Taverna et al. 2005), deer herbivory (Rooney and Dress 1997, Taverna et al. 2005, Wiegmann and Waller 2006), increased soil acidity (Falkengren-Grerup and Tyler 1991, Durak 2010, Šebesta et al. 2011), nitrogen deposition (Verheyen et al. 2012) and often the intermingled influence of several factors (Heinrichs et al. 2014, Vanhellemont et al. 2014, Bernhardt-Römermann et al. 2015, Naaf and Kolk 2016). The majority of the observed changes in the herbaceous layer show an explicit trend, as a consequence of direct or indirect anthropogenic effects. A marked successional shift towards species-poorer herbaceous layer communities was detected as a result of abandonment of former management practices (Baeten et al. 2010, Hédl et al. 2010), lack of disturbance (Brewer 1980) and herbivory (Rooney and Dress 1997). A loss of rarer native plants (Hédl 2004) and the homogenization of the herbaceous layer was detected, and was attributed to high browsing pressure by deer herbivory (Wiegmann and Waller 2006), acidification (Durak 2010) and light deficit (Davison and Forman 1982, Heinrichs et al. 2014).

Fine-scale herbaceous layer dynamics is also often linked to stand-scale dynamics. Gap formation significantly increases solar radiation and soil moisture (Collins et al. 1985, Moore and Vankat 1986, Gálhidy et al. 2006) leading to intensive alteration of composition and cover of the herbaceous layer (Gálhidy et al. 2006,

Kelemen et al. 2012) in large (Degen et al. 2005) and small gaps (Mountford et al. 2006). Stand dynamic events were matched with certain vegetation changes in different Slovakian primeval forests (Ujházy et al. 2005, 2007, Martináková and Martinák 2012). The herbaceous layer was best developed at the beginning of the decaying stage, and its cover sharply decreased when the tree seedlings and saplings overgrew them at the end of the decaying stage and in the growing stage. Herbaceous layer vegetation change can have a cyclic pattern, especially in forest stands that do not experience the strong successional effect of abandonment or other marked environmental change. Ujházy et al. (2007) detected low species turnover and cyclic changes in a Slovakian primeval forest at the stand-scale. Changing abundance or dominance of herb species characterized the cyclic changes, which was interpreted as internal community dynamics (Van Der Maarel 1996, Ujházy et al. 2005). Carøe et al. (2000) could not detect a successional trend either, only changes in species abundance in a managed Danish beech forest. Neither could Martináková and Martinák (2012) in a Slovakian natural fir-beech forest; however, the time interval was short in both investigations.

These short- and long-term changes in the herbaceous layer are most often detected by resurveys of phytosociological relevés or smaller quadrats after a few years (Ujházy et al. 2007, Martináková and Martinák 2012), one or a few decades (Davison and Forman 1982, Taverna et al. 2005, Łysik 2008, 2009, Vanhellemont et al. 2014) or even after a half-century interval (Brewer 1980, Hédl 2004, Durak 2010, 2012, Hédl et al. 2010, Šebesta et al. 2011). In some cases the relocation can be precise, when the time span is short or permanent corner markers were used, but more often former plots are relocated only approximately. The error resulting from relocation uncertainty can be high, especially in the case of small plots (Kopecký and Macek 2015). However, if relocation is done carefully, the resurvey can be robust to localization uncertainty, and can provide evidence of long-term changes in the herbaceous layer (Chytrý et al. 2014, Kopecký and Macek 2015). Ross et al. (2010) also demonstrated that if temporal change in the vegetation is greater than recent spatial heterogeneity, results of the resurveys can be interpreted with some confidence.

In our study, we were especially interested in the dynamics of the herbaceous layer at multiple spatial scales in relation to simple stand dynamic events, such as the opening and closure of smaller and larger gaps of a primeval beech forest remnant in Hungary. The exact location of the original sampling plots surveyed in 1996 was unknown, but the sampling plots could be relocated with 1 m accuracy after 17 years.

We hypothesized that in this virgin woodland remnant: 1) fine-scale changes in the herbaceous layer are evened out at the stand-scale, where no significant changes can be detected in the species pool (low species turnover) and total cover of the herbaceous layer at this temporal and spatial scale; 2) fine-scale changes in the herbaceous layer are governed by dynamic events in the tree canopy, such as opening and closing of the canopy gaps; 3) small gap stand dynamics warrant the survival of closed forest specialist herbs at the stand-scale.

Methods

Study area

The study was carried out in Kékes Forest Reserve (63 ha), which is one of last and best remnant of central European montane beech woods in Hungary. Kékes is the highest point in Hungary (1014 m) and is situated in the Mátra Mountains, northern Hungary. The climate is relatively continental. Mean annual precipitation is around 840 mm, of which 480 mm fall during the growing season. Mean annual temperature is 5.7°C, with cold winter (-4.7°C in January) and mild summer temperatures (15.5°C in July). The bedrock is andesite and andesitic tuff. The topography is extremely steep; scree slopes are characteristic. The shallow brown forest soils are mainly covered by montane beech forest (*Aconito-Fagetum* Soó (1930) 1960). Mixed maple-ash-lime forest (*Phyllitidi-Aceretum* Moor, 1952 *subcarpaticum* (Dostál 1933) Soó 1957) occurs in the most humid and rocky patches of the reserve on ranker type soils (Kovács 1968, Soó 1980).

According to historical records (Czájlik 2009) the area has never been used for timber production. As a result, most parts of the reserve show the characteristic fine-scale mosaic of forest developmental stages – sensu Korpeľ (1995) – of central European montane beech forests. For the purpose of the original survey we selected a roughly 1.5 ha plot in the reserve that contained a closed old beech stand, a small gap in the beech stand, and a larger collapse on a rockier site.

Data collection

In 1996, vegetation of the herbaceous layer was systematically sampled in a 120 m × 120 m patch (approximately 1.5 ha), which was divided into 16 30 m × 30 m squares with wooden sticks as field marks in the four corners. On the 120 m × 120 m patch a grid with 5 m intervals was laid out. Altogether 576 plots with 0.25 m^2 were set out on the grid (see Suppl. material 1). All grasses, sedges, herbs as well as trees and shrubs lower than 0.5 m in height were included in the sample (Gilliam 2007). Total vegetation cover and cover for each vascular plant species were estimated in percentage.

To make future assessment of stand dynamics possible, in 1997 a tree stand position map (each tree individual recorded by X and Y coordinates) was created for the 1.5 ha plot (see Suppl. material 2). The map was also used to assist the approximate relocation of the 0.25 m^2 plots, as they were not permanently marked in 1996. Partial resurvey of the herbaceous vegetation was carried out in 2013. The aim was to compare 17-year vegetation change within and between the patches with different histories of stand dynamics. To select the appropriate patches, we used the stand position map and a series of archive aerial photographs to detect the location and time of certain events in stand development. Using the same methods, we resampled 306 plots representing five different stand developmental situations (see Suppl. materials 1 and 2). However, because of the lack of permanent marking of the original sampling locations, reloca-

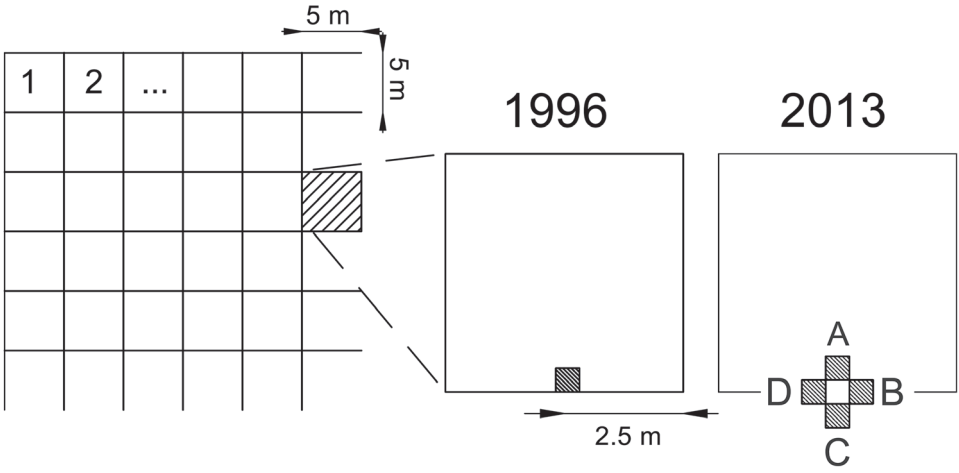

Figure 1. Design of the sampling methods. In each cell of a 5 m × 5 m grid a 0.25 m² plot was sampled in 1996. Blocks of four 0.25 m² plots were resampled in the same grid in 2013.

tion was only possible with a 1 m accuracy. To overcome this problem we used a cluster of four 0.25 m² plots at each of the 306 points to represent the original locations (Figure 1).

The five stand developmental situations (SDS) were identified as follows (Table 1, Figure 2, Suppl. material 2):

Control (C): This patch was selected because the tree canopy of the closed old beech stand was undisturbed during the study period.

One-year-old gap (1YG): This gap was formed in 2012 by the fall of two large beech trees.

Three-year-old gap (3YG): This gap was created by the fall of five trees in spring 2010.

Old beech gap (OBG): This gap was formed by the fall of a single large beech tree in the early 1990s.

Old collapse (OCO): It was opened in the early 1980s.

The 306 plots resurveyed in 2013 were selected to include both the centres and the peripheries of the five SDSs (see Suppl. material 1). The results are presented for three different spatial scales as follows (Table 2):

Stand-scale: Data from all the 306 plots are lumped together to characterise the 1 ha patch.

Stand developmental situation (SDS) scale: Each SDS is represented by the joined data of 25 plots located in the centre of an individual SDS. Thus, an approximately 400 m² area is sampled (which is comparable to the size of standard phytosociological relevé) for each SDS.

Table 1. Summary information on the five stand developmental situations (SDS) studied. For explanations of the stand developmental situations, see methods.

SDS	Soil depth	Canopy in 1996	Canopy in 2013	Herb layer in 1996	Herb layer in 2013
C	shallow	closed	closed	sparse	unchanged
1YG	shallow	closed	open	sparse	increased
3YG	shallow	closed	open	sparse	increased
OBG	deep	open	closed	dense	decreased
OCO	rocky	open	closed	dense	decreased

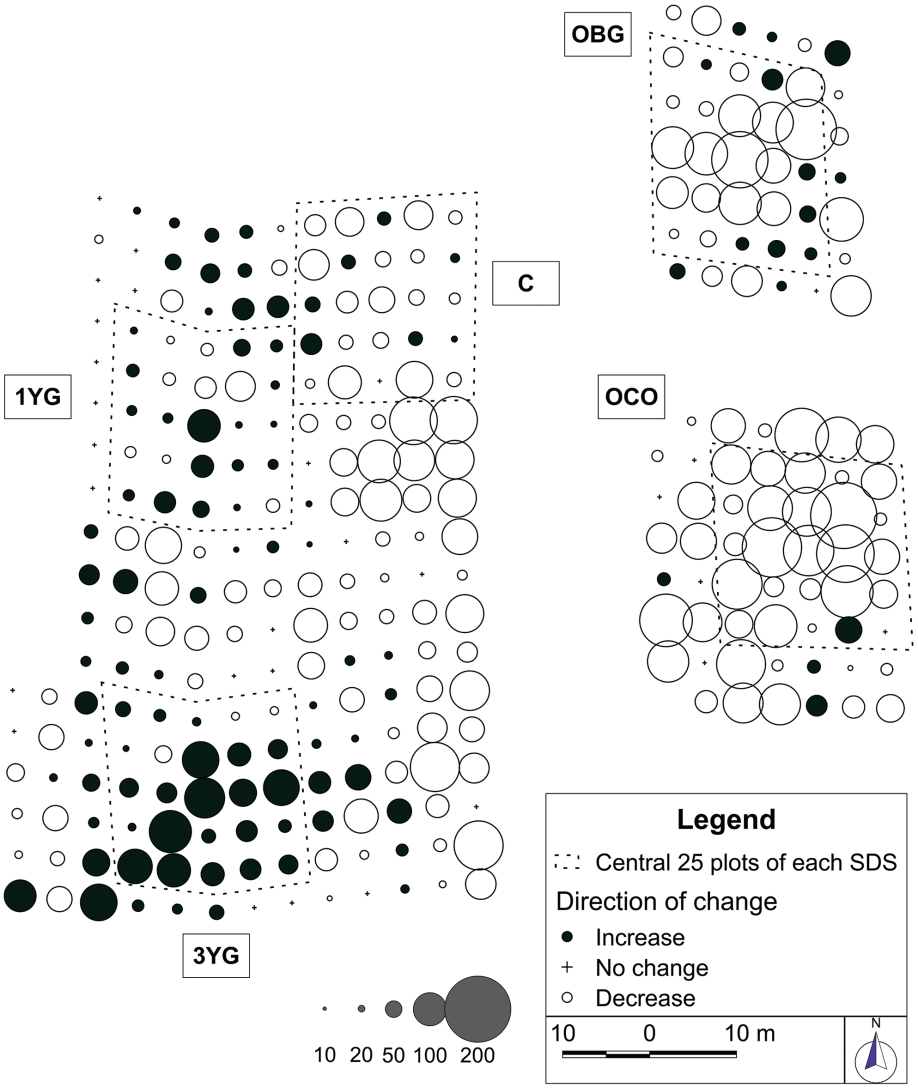

Figure 2. Changes in total herbaceous-layer vegetation cover (%) between 1996 and 2013. Dotted lines indicate the borders of the samples representing the five stand developmental situations (C, 1YG, 3YG, OBG, OCO).

Table 2. Summary information on spatial scales and number of sampling units used in this study (SDS = stand developmental situation).

Scale of study	Represented area	Number of plots	
		In 1996	In 2013
Stand	1 ha	306	306×4 = 1224
SDS	400 m²	5 SDS×25 = 125	5 SDS×25×4 = 500
Local	0.25 m²	306	306×4 = 1224

Fine-scale: All the 306 plots are treated separately so changes at the 0.25 m² scale can be described. Table 2 summarises the scales and numbers of sampling units in this study.

Data analyses

For our analyses we used the following variables:

Cover of the herbaceous layer: For each plot we used the sum of estimated cover values of individual species, so > 100% values can occur. Mean cover and maximal cover were calculated from the plot data for the whole stand and for the five SDSs. For the 2013 data the overall means were used: 1224 plots (306×4) for the stand-scale, 500 plots (4×125) for the SDS-scale. Changes in the abundance of herbaceous vegetation were calculated as change in total cover. To get a better understanding of the dynamics of individual species at the stand- and SDS-scales, both net and absolute cover change were calculated.

Species richness of the herbaceous layer: The number of species was calculated for all the three spatial scales and for the two sample years. The average species number per plot was also compared.

To quantify changes that occurred during the 17 years we used the mean values of the four subsamples of 2013 data (mean species richness, mean cover of the herbaceous layer of each plot). In this way we used an equal number of plots as in 1996 for the comparisons (306 plots for stand- and fine-scale, and 125 plots for SDS-scale analyses). The relationships between stand developmental situations (SDS) and magnitude of change in species richness and total herbaceous cover were studied by using Kruskal-Wallis test (H statistics), the non-parametric analogue of classical analysis of variance (Conover 1980). Wilcoxon matched pairs test was used to test the differences between the two surveys regarding temporal changes of species richness and total herbaceous cover at SDS- and fine-scale.

In order to quantify the changes in a species pool the following variables were calculated, where in the case of 2013 data the average values of the four subsamples were used for all calculations:

Number of colonisation events: Individual colonisation events were detected at the fine-scale (0.25 m²). The number of colonisation events (appearance of a species) was

expressed as sum (all new occurrences of all species) and mean (average number of new occurrences per plot) for each spatial scale studied.

Number of extinction events: Individual extinction events were detected at the fine-scale (0.25 m²). The number of extinction events (disappearance of a species) was expressed as sum (all disappearances of all species) and mean for each spatial scale studied.

Absolute species turnover in the herbaceous layer: Absolute turnover in species composition between successive sampling years was calculated as (E + C)/2, where E and C are the number of species extinctions and colonisations, respectively (Williamson 1978).

Relative species turnover in the herbaceous layer: Relative turnover was calculated as (E + C)/(S1 + S2) × 100%, where E and C are as above and S1 and S2 are the number of recorded species present in the two years (Diamond 1969).

Colonization and extinction events and the species turnover in the herbaceous layer were also assessed in a qualitative way. The behaviour of individual species was analysed at the stand- and SDS-scales.

When the effect of relocation was analysed, we compared the size of time effect (i.e. the difference between the 1996 value and the average value of the four 2013 subsamples) and the size of relocation effect (i.e. average of paired differences between the four subsamples) by Wilcoxon matched pair test. A one-sided alternative hypothesis was applied, i.e. higher time than relocation effect. Significant differences imply that the observed differences between two sampling times cannot be caused by the imprecise re-allocation of the subsamples only.

Results

Cover of the herbaceous layer

At the stand-scale we observed a general decrease in the abundance of herbaceous vegetation between 1996 and 2013 (Figure 2). The mean total cover/plot changed from 20.2% in 1996 to 7% in 2013.

At the SDS-scale we found profound differences in the magnitude and tendency of cover change from 1996 to 2013 (Table 3; Kruskal–Wallis test results, $H = 57.31336$, $p < 0.00001$). Wilcoxon matched pair test also showed significant differences between the two surveys in cover at the fine-scale (306 pairs of plots, Wilcoxon matched pair test results, $T = 10863.50$, $Z = 6.36$, $p < 0.00001$) and at SDS-scale for each SDS except for 1YG (25 pairs of plots, Wilcoxon matched pair test results, C – T = 53.00, $Z = 2.771429$, $P = 0.005581$; 1YG – T = 120.00, $Z = 1.143544$, $p = 0.252814$; 3YG – T = 17.5000, $Z = 3.901502$, $p < 0.0001$; OBG – T = 48.50, $Z = 3.067388$, $p = 0.002160$; OCO – T = 8.00, $Z = 4.057143$, $p < 0.0001$).

Vegetation cover decreased considerably in infilling old gaps (OCO: -50.3%; OBG: -30.3%), whereas it increased slightly (1YG: 1.6%) and more substantially (3YG: 16.7%) in recently created gaps.

Table 3. Total vegetation cover (%) in 1996 and 2013 in the five stand developmental situations. For explanations of the stand developmental situations, see methods.

	Mean cover in 1996 (%)	Mean cover in 2013 (%)	Max. cover in 1996 (%)	Max. cover in 2013 (%)	Max. cover change/ plot in 1996 (%)
C	14.1	5.1	56	29	-46.6
1YG	6.2	7.8	46	74	35.5
3YG	4.1	20.8	25	112	62.7
OBG	36.7	6.4	140	36	-137.4
OCO	55.0	4.7	176	75	-165.7

Table 4. Net and absolute cover change (%) of the ten most responsive species between 1996 and 2013.

Species name	Stand-scale	SDS-scale (5×25 plots)				
	306 plots	3YG	1YG	C	OBG	OCO
Net Change (%)						
Galium odoratum	-1270.80	45.00	-24.12	-123.25	-351.32	-70.00
Urtica dioica	-765.00	72.00	4.25	0.00	-25.00	-432.75
Dryopteris filix-mas	-418.55	25.00	40.75	-19.25	-147.00	-129.10
Athyrium filix-femina	-766.75	18.75	2.25	0.00	16.25	-429.25
Mercurialis perennis	-375.25	12.25	2.00	-9.87	-56.62	0.00
Fagus sylvatica	-115.95	40.50	0.90	5.00	-121.25	0.00
Geranium robertianum	-279.35	0.75	-24.50	-2.00	-5.10	-24.50
Cardamine bulbifera	34.50	0.00	11.75	-29.50	20.72	0.50
Solanum dulcamara	108.00	41.50	8.75	0.00	0.00	-15.00
Acer pseudoplatanus	105.87	80.12	0.37	1.75	3.42	-1.00
Absolute change (%)						
Galium odoratum	1646.35	90.00	72.37	131.75	353.37	70.00
Urtica dioica	948.50	72.00	4.25	0	25.00	432.75
Dryopteris filix-mas	899.35	25.00	49.75	22.75	173.00	142.10
Athyrium filix-femina	885.75	18.75	2.25	0.00	16.25	436.75
Mercurialis perennis	640.75	71.25	36.00	54.37	80.37	0.00
Fagus sylvatica	364.95	40.50	9.10	15.00	147.25	0.00
Geranium robertianum	310.85	6.75	25.50	6.00	5.10	73.00
Cardamine bulbifera	263.95	0.00	11.75	77.50	36.17	0.50
Solanum dulcamara	236.50	41.50	8.75	0.00	0.00	21.00
Acer pseudoplatanus	235.47	82.12	12.37	12.25	7.62	1.00

At the fine-scale, i.e. at the scale of individual plots, the largest decrease and increase in total cover was -165.7% and 62.7% respectively.

The observed changes in mean cover were obtained as the balance of positive and negative changes in individual species. Most changes were attributed to only a few species. As Table 4 shows, the sum of absolute cover changes was the highest for *Galium odoratum*, followed by *Urtica dioica* and the two fern species. A large difference between absolute and net change within an SDS indicates intensive fine-scale dynam-

ics (increase and decrease in cover occur simultaneously in individual plots belonging to an SDS). This behaviour is characteristic of forest specialist species such as *Galium odoratum* (3YG, 1YG), *Cardamine bulbifera* (C, OBG) and *Mercurialis perennis* (3YG, 1YG, C, OBG)

Species richness of the herbaceous layer

In 1996 and 2013 we recorded 42 and 48 species, respectively, though in the latter case a four times larger area was sampled because of the four subsamples used (306 plots×4). At the stand-scale our full sample (306 locations, two surveys) contained data from 54 species (45 herbs, 6 trees, 3 shrubs), of which 50 species occurred in the 125 plots representing the five SDSs.

In contrast to the relative stability in species richness at the stand-scale, a much more variable picture was obtained when the SDS-scale was studied. In 1996 species richness varied between 7 and 23 (Table 5) in the five SDSs (25 plots each), whereas the same number were in the range of 14–31 in 2013. As Table 5 and Figure 3 show, the largest change in SDS-scale species richness took place in the three-year-old gap (3YG), where it grew from 7 to 31. Species richness was the most stable in the control, where it changed from 15 to 14. When mean species richness/plot was considered, the largest change was found in OCO, where it decreased to almost one-tenth of the original 3.7 in 1996.

We found significant differences between the five SDSs in changes in species richness from 1996 to 2013 (Kruskal–Wallis test results, $H = 56.1961$, $p < 0.00001$). At the fine-scale (individual 0.25 m² plots) species richness was in the range of 0-10 and 0-8 in 1996 and in 2013, respectively, with high variation among the five SDSs (Table 5). Significant differences were also found between the two surveys for each SDS (25 pairs of plots, Wilcoxon matched pair test results, C – T = 47.00, $Z = 2.942857$, $p = 0.003252$; 1YG – T = 8.00, $Z = 3.84718$, $p = 0.00012$; 3YG – T = 21.00, $Z = 3.807328$, $p = 0.000141$; OBG – T = 17.00, $Z = 3.680209$, $p = 0.000233$; OCO – T = 0.00, $Z = 4.285714$, $p = 0.00018$), as well as at the fine-scale (306 pairs of plots, Wilcoxon matched pair test results, T = 9443.500, $Z = 7.023608$, $p < 0.00001$).

Table 5. Species richness in 1996 and 2013 in the five stand developmental situations (SDS). For explanations of the stand developmental situations, see methods. SpNo = species number.

SDS	Species richness in 1996	Species richness in 2013	Mean SpNo/plot in 1996	Mean SpNo/plot in 2013
C	15	14	2.44	1.27
1YG	12	24	1.12	1.48
3YG	7	31	0.6	2.21
OBG	23	18	4.12	1.53
OCO	19	15	3.68	0.4

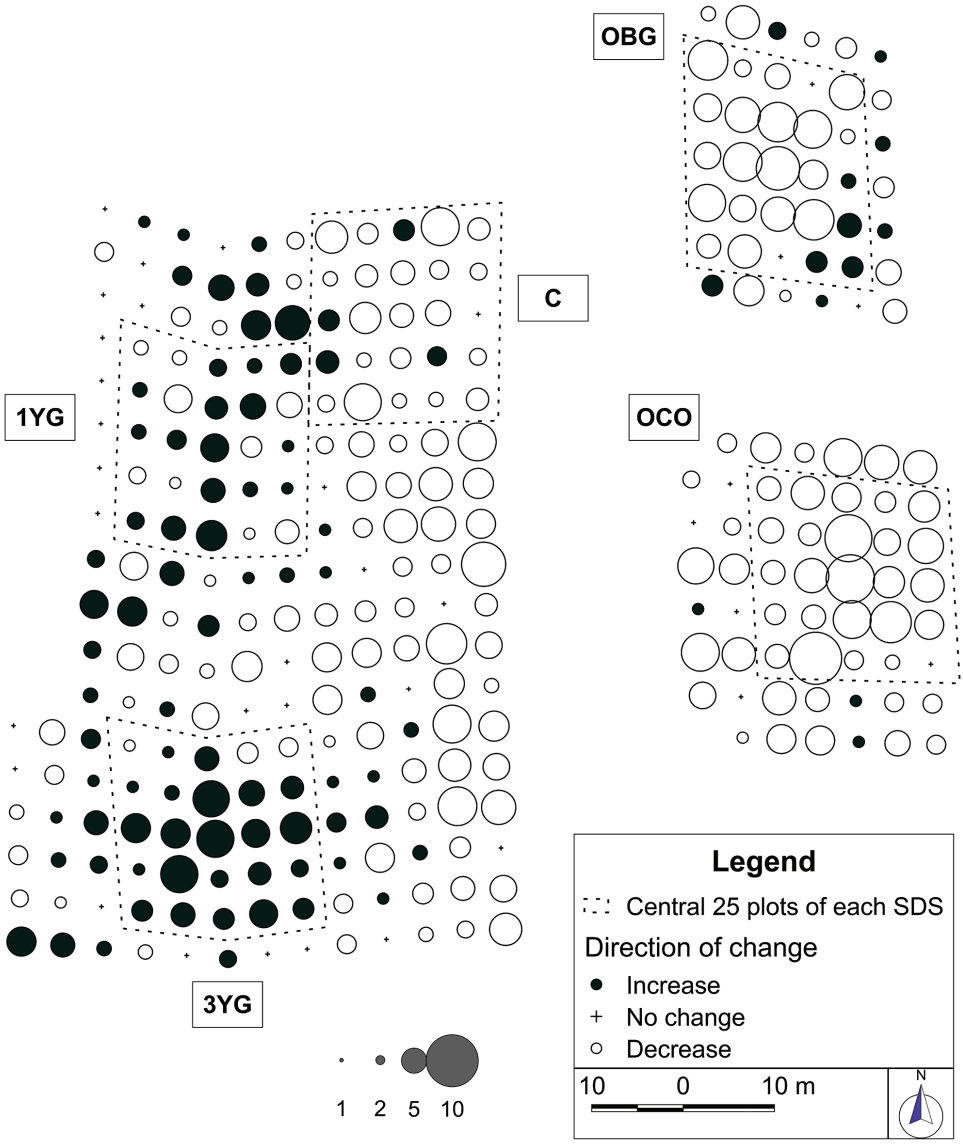

Figure 3. Changes in species richness between 1996 and 2013. Dotted lines indicate the borders of the samples representing the five stand dynamic situations (C, 1YG, 3YG, OBG, OCO).

Number of extinction and colonization events and species turnover in the herbaceous layer

Quantitative

Species extinctions and colonisation occurred at all spatial scales studied during the 17 years between the two samplings. At the stand-scale there were only six species that dis-

Table 6. Number of extinction and colonisation events (overall mean of the four subsamples at each plot), absolute and relative species turnover in the five stand developmental situations (SDS) based on resurvey data collected in 1996 and 2013. For explanations of the stand developmental situations, see methods.

SDS	Extinction	Colonisation	Mean Absolute Turnover/Plot	Mean Relative turnover (%)
C	77.25	20.5	1.95	117.11
1YG	43	30.25	1.46	121.07
3YG	19.75	48.25	1.36	105.26
OBG	109.75	16.25	2.52	99.53
OCO	127.5	5.75	2.66	137.25

appeared, but all of them were present at very low frequencies and abundances in 1996. Half of the recorded 12 newly occurring species were actually present in the 576 plots sampled in 1996, but were not included in the sample (306 plots) used for the resurvey.

At the SDS-scale there were considerable differences among the individual SDS. As Table 6 shows, the number of extinctions was the highest in OCO and OBG, whereas – as was expected – colonisation was the most intensive in 3YG. Mean absolute turnover was the highest in OCO and OBG, mostly because of the high number of extinctions. If we also consider the size of the species pool, relative turnover was the highest in OCO, followed by 1YG, where the initial species richness was very low. Fine-scale (0.25 m²) colonisation was in the range of 0 and 4.75 in the 306 plots, with a mean value of 0.717. The same values for extinctions were 0, 10 and 1.4, respectively.

Qualitative

Quantitative results indicate relative stability at the stand-scale and rather intensive dynamics at the SDS- and fine-scales. However, to get a better understanding of the processes, it is worth looking at what species become extinct or colonise in different situations. Special interest is devoted to closed forest specialist species (Schmidt et al. 2011), which are marked in bold in the text below.

At the stand-scale, the six species that were not included in the 2013 sample were all forest species (***Chrysosplenium alternifolium***, *Epilobium montanum*, ***Hordelymus europaeus***, ***Melica uniflora***, ***Monotropa hypopitys***, *Viola odorata*) that occurred at very low abundances in 1996. Half of the newly colonising 12 species were forest species (***Actaea spicata***, *Campanula rapunculoides*, ***Epipactis helleborine***, *Hieracium acuminatum*, *Poa nemoralis* and ***Polygonatum verticillatum***) that were recorded as new because of similar chance effects since, according to our field records, they were all present in 1996 within the studied 1.5 ha stand. Among the colonisers there were two ruderal species (*Tussilago farfara*, *Cirsium arvense*) and one newly appearing adventive species (*Impatiens parviflora*).

At the SDS-scale it is worth comparing the list of disappearing and newly appearing species. In the OBG, most extinctions were of closed forest species (e.g. ***Galium odoratum***,

Table 7. Closed forest specialist species that disappeared from individual SDS (based on data of 25 plots each) by 2013. Numbers in brackets indicate the original number of occurrence of the species in 1996. For explanations of the stand developmental situations, see methods.

	Closed forest specialist herbaceous species	
SDS	**Disappearing**	**Surviving**
C	*Melica uniflora* (1)	*Cardamine bulbifera, Dryopteris filix-mas, Euphorbia amygdaloides, Galium odoratum, Mercurialis perennis, Viola reichenbachiana*
1YG	none	*Dryopteris filix-mas, Galium odoratum, Galium odoratum, Viola reichenbachiana*
3YG	none	*Galium odoratum, Mercurialis perennis,*
OBG	*Circaea lutetiana* (1); *Hordelymus europaeus* (1); *Moehringia trinervia* (1); *Oxalis acetosella* (2); *Pulmonaria obscura* (1)	*Cardamine bulbifera, Dryopteris filix-mas, Euphorbia amygdaloides, Galium odoratum, Mercurialis perennis, Viola reichenbachiana*
OCO	*Chrysosplenium alternifolium* (1); *Galium odoratum* (2)	*Dryopteris filix-mas, Lamiastrum galeobdolon luteum, Impatiens noli-tangere, Oxalis acetosella, Polystichum braunii*

Mercurialis perennis, ***Viola reichenbachiana***, ***Cardamine bulbifera***) and there were only a few extinctions of gap or disturbance indicating species (*Chelidonium majus*, *Geranium robertianum*, *Rubus idaeus*, *Urtica dioica*). Colonisation events were almost exclusively due to closed forest specialist species. At the rocky OCO site the observed extinctions were mainly attributable to those species (e.g. ***Dryopteris filix-mas***, ***Impatiens nolitangere***, *Solanum dulcamara*, *Urtica, dioica*) that occurred with high frequencies in 1996. The low number of colonisation was due to similar species because site conditions are not favourable for most closed forest specialist herbs. On the contrary, in the control plot the species pool did not change much, despite the relatively intensive dynamics (77 extinctions, 20 colonisations). Extinctions and colonisations were both due to real closed forest specialist herbs (e.g. ***Cardamine bulbifera***, ***Euphorbia amygdaloides***, ***Galium odoratum***, ***Mercurialis perennis***). In 3YG and 1YG only a low number of extinctions were recorded, mostly due to the disappearance of forest species. The large number of colonisations in these gaps was attributable to both typical gap species and to those forest species that are able to react relatively fast to changing light conditions.

Table 7 shows how successfully closed forest species (Schmidt et al. 2011) survived in the individual SDS. Only a few species with low original frequencies disappeared, indicating the overall successful survival of this species group.

Effect of relocation error

Our results on the effects of relocation error showed that time effect was significantly higher than relocation effect both in the case of species richness (306 pairs of plots, Wilcoxon matched pair test results, $T = 8475$, $Z = 7.769055$, $p < 0.00001$) and in the case of total cover of the herbaceous layer (306 pairs of plots, Wilcoxon matched pair

test results, T = 9932, Z = 6.983762, p < 0.00001). Consequently, differences between data from 1996 and average data of the four subsamples of 2013 cannot be merely attributable to imprecise relocation of subsamples.

Discussion

After 17 years we found low species turnover and a general decrease in herbaceous cover at the stand-scale. Therefore, our first hypothesis is only partly supported. We found an overall stability of the species pool in the herbaceous layer at the stand-scale, whereas the abundance of vegetation (measured as total herb cover) changed considerably.

Several analyses pointed out that a decrease in herbaceous cover in a temperate forest is attributed to light deficit caused by a denser canopy. This can be a consequence of less intensive forest management or even abandonment, as well as the lack of natural disturbance including the activities of grazers (Brewer 1980, Davison and Forman 1982, Vera 2000, Verheyen et al. 2012, Kopecky et al. 2013). A drastic decrease in herbaceous species cover was found by Łysik (2008) in a primeval beech forest in Poland and by Ujházy et al. (2007) in a primeval fir-beech forest in Slovakia. Both studies connected this sharp decrease with the massive recruitment of woody regeneration. The observed decrease in our case can also be related to the partial closure of the canopy, and to the infilling of the two main former canopy gaps with young woody species (see below). The majority of the reduction in the herb cover was due to a few forest species (***Galium odoratum***, ***Mercurialis perennis***) and to species (*Urtica dioica*, ***Dryopteris filix-mas***, ***Athyrium filix-femina***) that were abundant in gaps in 1996. Hence, we argue that stability of herbaceous cover might be realized on a larger study site, where all stand dynamic situations are represented in natural proportions. We also assume that the extremely dry years of 2012 and 2013 as a potential climate factor could have contributed to the observed decrease.

The majority of the temperate forest resurvey studies document high species turnover, as a consequence of environmental change. Denser canopies effect not only the cover of the herbaceous layer, but also trigger trait-based reactions as the decrease in light-demanding species and increase in species with good abilities for living in shade (Brewer 1980, Hédl et al. 2010, Heinrichs et al. 2014). A direct trend in species trait shift can be observed in spite of the formerly assumed or postulated stability of a site (Brewer 1980, Taverna et al. 2005, Łysik 2008). Despite the expected stability, results of Taverna et al. (2005), for example, showed systematic changes in herbaceous species richness in a climax hardwood forest in North-Carolina, as a potential result of the elimination of ground fires and widespread grazing typical at the beginning of the twentieth century. Invasive species can significantly change the composition of the herbaceous layer even in primeval forests (Łysik 2008). One of the few examples, where the ground vegetation showed an overall stability, is reported from a Danish beech forest by Carøe et al. (2000), but the temporal scale was very short (5 years). Similar stability of species pool was found by Woods et al. (2012) in unmanaged northern

hardwood forests over a three-decade period. These results highlight the rarity of the observed low species turnover of Kékes Forest Reserve after almost two decades, where all extinct species were represented with very low abundances in 1996; similarly, all colonization happened with low abundance and many of the colonizing species were present in the species pool of the close surroundings.

Our results support the concept that the experienced intensive fine-scale dynamics is profoundly governed by the stand dynamic events of the studied forest stand. Significant differences were found between the individual SDSs and between years in herbaceous cover, species richness and species turnover at the SDS-scale. Highest species richness, mean species number/plot and highest herb cover characterized the two main gaps (OCO and OBG) in 1996. An expressed decrease in the herbaceous layer cover was observed after 17 years in these two, gradually closing gaps, where the saplings have overgrown and started to cast shadow on the herbaceous plants. Lateral canopy expansion of bordering trees has also contributed to gap closure. A very high number of extinctions with high absolute species turnover were detected in these situations. In the old collapse (OCO) the cover of ***Dryopteris filix-mas***, ***Athyrium filix-femina***, and *Urtica dioica* was drastically reduced. In the old beech gap (OBG) pronounced recession of ***Galium odoratum***, ***Dryopteris filix-mas***, ***Fagus sylvatica*** (young beech individuals have grown out of the herbaceous layer) and ***Mercurialis perennis*** was detected. Competition between the herbaceous plants and the saplings, especially with the very competitive beech saplings, and the resulting drop in herbaceous plant cover, was observed by several authors (Davis et al. 1998, Łysik 2008, Ujházy et al. 2007, 2013). On the other hand, the two younger gaps, created in 2010 and 2012 (3YG, 1YG) had low species richness, low mean species/plot and low herbaceous cover at the beginning, as the canopy was closed in 1996. The resurvey detected a sharp increase in both species richness and cover in the case of the three-year-old gap (3YG), where the massive colonization process was performed by gap specialists (*Urtica dioica*, *Solanum dulcamara*), by responsive forest species (***Dryopteris filix-mas***, ***Galium odoratum***) and by tree seedlings (***Fagus sylvatica***, *Acer pseudoplatanus*). All these changes were more moderate in the case of 1YG, due to the shorter time-span. Despite the high absolute species turnover, the cover of the herbaceous layer and species richness changed only slightly in the control SDS. These results support our second hypothesis that stand dynamic events govern the herb cover, and drive the herbaceous layer changes, at least during this nearly two decades in our study site.

The Slovakian fir-beech forest investigated by Ujházy et al. (2005, 2007) had a very similar species pool in the herbaceous layer as our studied site. They found relatively low species turnover – compared to commercial forests – in the three developmental stages; rather the abundance or dominance values differentiated the growing, optimum and decay stages (Ujházy et al. 2005). The investigated short-term (4 year) changes were attributable to stand dynamics; they recorded a rapid change in the herb layer during the four-year period established in young gaps (Ujházy et al. 2007). However, they did not analyse colonisation and extinction patterns for all species.

Although the four subsamples of each plot were relocated only with a 1 m accuracy in 2013, statistical tests proved that the time effect (change in vegetation) was significantly higher than the relocation effect. With this we managed to assure that the interpreted temporal changes were not artefacts resulting from imperfect relocation.

Conclusion

We found that intensive local-scale extinction and colonization episodes were balanced at the stand-scale, resulting in overall stability of the species pool in the herbaceous layer vegetation. These results have important implications for both forest management and conservation.

The observed stand dynamics driven changes of vegetation indicate that the use of small regeneration areas in forest management can prevent competitive ruderal species (e.g. *Calamagrostis epigejos*) from causing serious problems in regeneration (Gálhidy et al. 2006, Kelemen et al. 2012).

From a conservation viewpoint, one of the most important species-related implications is that the long-term survival of closed forest specialists, including ancient forest indicators (Peterken and Game 1984, Hermy et al. 1999), could be guaranteed by management techniques mimicking natural small gap dynamics. This is important for several of those species that are seriously dispersal limited, meaning that once they disappear it is extremely hard, or it takes decades to centuries, to recolonize secondary forests (Kelemen et al. 2014). We assume that the observed stability in the species pool was secured by the primeval character of Kékes Forest Reserve. By primeval we refer to both structurally rich old-growth character and long forest continuity. The former is illustrated by the presence of all important forest developmental phases produced by natural stand dynamics (natman report 2002) and the high amount and diversity (size and decay phase) of deadwood (Christensen et al. 2005) and also of forest specialist organisms (e.g. Ódor 2000, Ódor and Standovár 2001, Ódor et al. 2006, Standovár et al. 2006, Heilmann-Clausen et al. 2014). Long continuity is convincingly documented by Czájlik (2009). Similar relationships between the richness in ancient forest indicator plants and that of other organism groups were shown, for example, by Hofmeister et al. (2014) for macrofungi.

Several studies documented that habitat specialist species occur in the highest numbers in forested landscapes with long continuity of forest structures and habitats (for a review see e.g. Nordén et al. 2014). This emphasizes the specific conservation role of even small remnants of virgin or ancient forest, as they secure the long-term survival of habitat specialists, and they might also serve as a source for dispersal from these remnants into the surrounding landscape.

In areas, where nature conservation is not the sole purpose of management, managers have started to apply retention forestry as a means of integrating conservation concerns into forest management. A thorough review (Fedrowitz et al. 2014) showed that retention cuts supported greater abundance and higher species richness of forest species than clear-cuts. This positive effect increases with the proportion of retained

trees and time since harvest. However, they also showed that retention cuts cannot be a substitute for uncut forest because it had negative impacts on mostly closed forest specialist species.

All these suggest that for successful conservation of forest biodiversity we still need to preserve existing remnants of woodlands with high conservation value and also to apply a mix of management approaches in their immediate surrounding that support natural processes and the creation of important habitats.

Acknowledgements

The authors are grateful to all those friends and colleagues who helped in any phase of the fieldwork during the original or the resurvey, especially to Péter Ódor, Erzsébet Szurdoki and Ilona Paszterkó and László Gálhidy. We thank the constructive comments and suggestion of an anonymous reviewer, which helped to improve our paper. We also thank Zoltán Botta-Dukát for his statistical advice. This work was partially supported by a grant from Switzerland through the Swiss Contribution (SH-4/13).

References

Baeten L, Hermy M, Van Daele S, Verheyen K (2010) Unexpected understorey community development after 30 years in ancient and post-agricultural forests. Journal of Ecology 98: 1447–1453. https://doi.org/10.1111/j.1365-2745.2010.01711.x

Bernhardt-Römermann M, Baeten L, Craven D, De Frenne P, Hédl R, Lenoir J, Bert D, Brunet J, Chudomelová M, Decocq G, Dierschke H, Dirnböck T, Dörfler I, Heinken T, Hermy M, Hommel P, Jaroszewicz B, Keczyński A, Kelly DL, Kirby KJ, Kopecký M, Macek M, Máliš F, Mirtl M, Mitchell FJG, Naaf T, Newman M, Peterken G, Petřík P, Schmidt W, Standovár T, Tóth Z, Calster HV, Verstraeten G, Vladovič J, Vild O, Wulf M, Verheyen K (2015) Drivers of temporal changes in temperate forest plant diversity vary across spatial scales. Global Change Biology 21: 3726–3737. https://doi.org/10.1111/gcb.12993

Brewer R (1980) A Half-century of Changes In the Herb Layer of A Climax Deciduous Forest In Michigan. Journal of Ecology 68: 823–832. https://doi.org/10.2307/2259458

Carøe I, Barfod A, Lawesson J (2000) Temporal dynamics of the ground vegetation in a Danish beech forest. Nordic Journal of Botany 20: 585–597. https://doi.org/10.1111/j.1756-1051.2000.tb01609.x

Christensen M, Hahn K, Mountford EP, Ódor P, Standovár T, Rozenbergar D, Diaci J, Wijdeven S, Meyer P, Winter S, Vrska T (2005) Dead wood in European beech (Fagus sylvatica) forest reserves. Forest Ecology and Management 210: 267–282. https://doi.org/10.1016/j.foreco.2005.02.032

Chytrý M, Tichý L, Hennekens SM, Schaminée JHJ (2014) Assessing vegetation change using vegetation-plot databases: a risky business. Applied Vegetation Science 17: 32–41. https://doi.org/10.1111/avsc.12050

Collins BS, Dunne KP, Pickett STA (1985) Responses of forest herbs to canopy gaps. In: Pickett STA, White PS (Eds) The ecology of natural disturbance and patch dynamics. Academic Press, New York, 218–234.

Conover WJ (1980) Practical nonparametric statistics. Wiley, New York.

Czájlik P, Kenderes K, Standovár T, Tímár G (2003) Report on Site-based Permanent Plot, Second-phase and New Mapping Studies: Kékes Forest Reserve. Nat-Man Working Report 51., p. 28.

Czájlik P (2009) A Kékes-Észak erdőrezervátum és térségének története: egy őserdőfragmentum fennmaradása [History of the Kékes Forest Reserve and the surrounding region]. In Borhidi A, Horváth F (Eds), Az erdőrezervátum-kutatás eredményei, 3: 7–86. Ecological and Botanical Institute – Hungarian Academy of Sciences (Vácrátót).

Davison SE, Forman RTT (1982) Herb and Shrub Dynamics in a Mature Oak Forest: A Thirty-Year Study. Bulletin of the Torrey Botanical Club 109: 64–73. https://doi.org/10.2307/2484469

De Frenne P, Rodriguez-Sánchez F, Coomes DA, Baeten L, Verstraeten G, Vellend M, Bernhardt-Römermann M, Brown CD, Brunet J, Cornelis J, Decocq GM, Dierschke H, Eriksson O, Gilliam FS, Hédlm R, Heinkenn T, Hermy M, Hommel P, Jenkins MA, Kelly DL, Kirby KJ, Mitchell FJG, Naaf T, Newman M, Peterken G, Petřík P, Schultz J, Sonnier G, Van Calster H, Waller DM, Walther GR, White PS, Woods KD, Wulf M, Graae BJ, Verheyen K (2013) Microclimate moderates plant responses to macroclimate warming. Proceedings of the National Academy of Sciences 110: 18561–18565. https://doi.org/10.1073/pnas.1311190110

Degen T, Devillez F, Jacquemart A (2005) Gaps promote plant diversity in beech forests (Luzulo-Fagetum), North Vosges, France. Annals of Forest Science 62: 429–440. https://doi.org/10.1051/forest:2005039

Diamond JM (1969) Avifaunal equilibria and species turnover rates on the Channel Islands of California. Proceedings of the National Academy of Sciences 64: 57–63. https://doi.org/10.1073/pnas.64.1.57

Durak T (2010) Long-term trends in vegetation changes of managed versus unmanaged Eastern Carpathian beech forests. Forest Ecology and Management 260: 1333–1344. https://doi.org/10.1016/j.foreco.2010.07.026

Durak T (2012) Changes in diversity of the mountain beech forest herb layer as a function of the forest management method. Forest Ecology and Management 276: 154–164. https://doi.org/10.1016/j.foreco.2012.03.027

Falkengren-Grerup U, Tyler G (1991) Dynamic Floristic Changes of Swedish Beech Forest in Relation to Soil Acidity and Stand Management. Vegetatio 95: 149–158.

Fedrowitz K, Koricheva J, Baker SC, Lindenmayer DB, Palik B, Rosenvald R, Beese W, Franklin JF, Kouki J, Macdonald E, Messier C, Sverdrup-Thygeson A, Gustafsson L (2014) Can retention forestry help conserve biodiversity? A meta-analysis. Journal of Applied Ecology 51: 1669–1679. https://doi.org/10.1111/1365-2664.12289

Gálhidy L, Mihók B, Hagyó A, Rajkai K, Standovár T (2006) Effects of gap size and associated changes in light and soil moisture on the understorey vegetation of a Hungarian beech forest. Plant Ecology 183: 133–145. https://doi.org/10.1007/s11258-005-9012-4

Gilliam FS (2007) The ecological significance of the herbaceous layer in temperate forest ecosystems. BioSciene 57: 845–858. https://doi.org/10.1641/B571007

Gilliam FS (Ed.) (2014) The herbaceous layer in forests of eastern North America. Oxford University Press, New York.

Hermy M, Honnay O, Firbank L, Grashof-Bokdam C, Lawesson J (1999) An ecological comparison between ancient and other forest plant species of Europe, and the implications for forest conservation. Biological Conservation 91: 9–22. https://doi.org/10.1016/S0006-3207(99)00045-2

Hédl R (2004) Vegetation of beech forests in the Rychlebské Mountains, Czech Republic, re-inspected after 60 years with assessment of environmental changes. Plant Ecology 170: 243–265. https://doi.org/10.1023/B:VEGE.0000021681.83068.53

Hédl R, Kopecký M, Komárek J (2010) Half a century of succession in a temperate oakwood: from species-rich community to mesic forest. Diversity and Distributions 16: 267–276. https://doi.org/10.1111/j.1472-4642.2010.00637.x

Heilmann-Clausen J, Aude E, van Dort KW, Christensen M, Piltaver A, Veerkamp MT, Walleyn R, Siller I, Standovár T, Ódor P (2014) Communities of wood-inhabiting bryophytes and fungi on dead beech logs in Europe – reflecting substrate quality or shaped by climate and forest conditions? Journal of Biogeography 41: 2269–2282. https://doi.org/10.1111/jbi.12388

Heinrichs S, Winterhoff W, Schmidt W (2014) 50 Jahre Konstanz und Dynamik im Seggen-Hangbuchenwald (Carici-Fagetum) – Ein Vergleich alter und neuer Vegetationsaufnahmen aus dem Göttinger Wald. Tuexenia 34: 9–38.

Hofmeister J, Hošek J, Brabec M, Dvořák D, Beran M, Deckerová H, Burel J, Kříž M, Borovička J, Běťák J, Vašutová M (2014) Richness of ancient forest plant species indicates suitable habitats for macrofungi. Biodiversity and Conservation 23: 2015–2031. https://doi.org/10.1007/s10531-014-0701-y

Kelemen K, Mihók B, Gálhidy L, Standovár T (2012) Dynamic Response of Herbaceous Vegetation to Gap Opening in a Central European Beech Stand. Silva Fennica 46: 53–65. https://doi.org/10.14214/sf.65

Kelemen K, Kriván A, Standovár T (2014) Effects of land-use history and current management on ancient woodland herbs in Western Hungary. Journal of Vegetation Science 25: 172–183. https://doi.org/10.1111/jvs.12046

Kopecký M, Hédl R, Szabó P (2013) Non-random extinctions dominate plant community changes in abandoned coppices. Journal of Applied Ecology 50:79–87. https://doi.org/10.1111/1365-2664.12010

Kopecký M, Macek M (2015) Vegetation resurvey is robust to plot location uncertainty. Diversity and Distributions 21: 322–330. https://doi.org/10.1111/ddi.12299

Korpeľ S (1995) Die Urwälder der Westkarpaten. Gustav Fischer Verlag (Jena).

Kovács M (1968) Die Acerion pseudoplatani-Wälder (Mercuriali-Tilietum und Phyllitidi-Aceretum) des Mátra-Gebirges. Acta Botanica Academiae Scientiarum Hungaricae 14: 331–350.

Łysik M (2008) Ten years of change in ground-layer vegetation of European beech forest in the protected area (Ojcow National Park, South Poland). Polish Journal of Ecology 56: 17–31.

Łysik M (2009) A 13-year change in ground-layer vegetation of Carpathian beech forests. Polish Journal of Ecology 57: 47–61.

Martináková M, Martinák M (2012) Short-term changes in the vegetation of fir-beech natural forests in the Padva reserve. Acta Facultatis Forestalis Zvolen 54: 37–54.

Moore MR, Vankat JL (1986) Responses of the Herb Layer to the Gap Dynamics of a Mature Beech-Maple Forest. American Midland Naturalist 115: 336–347. https://doi.org/10.2307/2425870

Mountford EP, Savill PS, Bebber DP (2006) Patterns of regeneration and ground vegetation associated with canopy gaps in a managed beechwood in southern England. Forestry 79: 389–408. https://doi.org/10.1093/forestry/cpl024

Naaf T, Kolk J (2016) Initial site conditions and interactions between multiple drivers determine herb-layer changes over five decades in temperate forests. Forest Ecology and Management 366: 153–165. https://doi.org/10.1016/j.foreco.2016.01.041

Nordén B, Dahlberg A, Brandrud TE, Fritz Ö, Ejrnaes R, Ovaskainen O (2014) Effects of ecological continuity on species richness and composition in forests and woodlands: A review. Écoscience 21: 34–45. https://doi.org/10.2980/21-1-3667

Ódor P (2000) Description of the bryoflora and bryophyte vegetation of Kékes North Forest Reserve in Mátra mountains (N-Hungary). Kitaibelia 5: 115–123.

Ódor P, Standovár T (2001) Richness of bryophyte vegetation in near-natural and managed beech stands: The effects of management-induced differences in dead wood. Ecological Bulletins 49: 219–229.

Ódor P, Heilmann-Clausen J, Christensen M, Aude E, van Dort KW, Piltaver A, Siller I, Veerkamp MT, Walleyn R, Standovár T, van Hees AFM, Kosec J, Matočec N, Kraigher H, Grebenc T (2006) Diversity of dead wood inhabiting fungi and bryophytes in semi-natural beech forests in Europe. Biological Conservation 131: 58–71. https://doi.org/10.1016/j.biocon.2006.02.004

Peterken GF, Game M (1984) Historical Factors Affecting the Number and Distribution of Vascular Plant-species In the Woodlands of Central Lincolnshire. Journal of Ecology 72: 155–182. https://doi.org/10.2307/2260011

Rooney TP, Dress WJ (1997) Patterns of plant diversity in overbrowsed primary and mature secondary hemlock-northern hardwood forest stands. Journal of the Torrey Botanical Society 124: 43–51. https://doi.org/10.2307/2996597

Ross LC, Woodin SJ, Hester A, Thompson DB, Birks HJB (2010) How important is plot relocation accuracy when interpreting re-visitation studies of vegetation change? Plant Ecology & Diversity 3: 1–8. https://doi.org/10.1080/17550871003706233

Schmidt M, Kriebitzsch WU, Ewald J (Eds) (2011) Waldartenlisten der Farn-und Blütenpflanzen, Moose und Flechten Deutschlands. BfN, Bonn.

Šebesta J, Šamonil P, Lacina J, Oulehle F, Houška J, Buček A (2011) Acidification of primeval forests in the Ukraine Carpathians: vegetation and soil changes over six decades. Forest Ecology and Management 262(7): 1265–1279. https://doi.org/10.1016/j.foreco.2011.06.024

Soó R (1964–1980) Synopsis systematico-geobotanica florae vegetationesque Hungariae [Systematic and geobotanical handbook of the Hungarian flora and vegetation] I-VII. Akadémiai Kiadó, Budapest.

Standovár T, Ódor P, Aszalós R, Gálhidy L (2006) Sensitivity of ground layer vegetation diversity descriptors in indicating forest naturalness. Community Ecology 7(2):199–209. https://doi.org/10.1556/ComEc.7.2006.2.7

Taverna K, Peet RK, Phillips LC (2005) Long-term change in ground-layer vegetation of deciduous forests of the North Carolina Piedmont, USA. Journal of Ecology 93: 202–213. https://doi.org/10.1111/j.0022-0477.2004.00965.x

Tutin TG, Heywood VH, Burges NA, Valentine DH, Walters SM, Webb DA (Eds) (2010) Flora Europaea, Cambridge University Press, Cambridge.

Ujházy K, Križová E, Vančo M, Freňáková E, Ondruš M (2005) Herb layer dynamics of primeval fir-beech forests in central Slovakia. In Hamor F, Commarmot B (Eds) Natural forests in the temperate zone of Europe – Values and Utilisation Conference Proceedings (2005) Swiss Federal Research Institute WSL (Birmensdorf) and Carpathian Biosphere Reserve (Rakhiv). 142–143.

Ujházy K, Krizová E, Ondruš M, Vigoda M (2007) Short-term vegetation change in the fir-beech primeval forest (Badinsky prales, Central Slovakia). In Abrudan VI (Ed.) Forest and sustainable development. Proceedings of the biennial international symposium (2007) Editura Universităţii Transilvania (Braşov), 213–218

Ujházy K, Križová E, Glončák P, Benčať ová B, Nič J (2013) Tree species and management effect on herb layer species composition in mountain fir-beech forests of the Western Carpathians. In Kozak J, Ostapowicz K, Bytnerowicz A, Wyżga B (Eds) The Carpathians: Integrating Nature and Society Towards Sustainability. Springer-Verlag (Berlin-Heidelberg). https://doi.org/10.1007/978-3-642-12725-0_18

Van Der Maarel E (1996) Pattern and process in the plant community: Fifty years after A.S. Watt. Journal of Vegetation Science 7: 19–28. https://doi.org/10.2307/3236412

Vanhellemont M, Baeten L, Verheyen K (2014) Relating changes in understorey diversity to environmental drivers in an ancient forest in northern Belgium. Plant Ecology and Evolution 147: 22–32. https://doi.org/10.5091/plecevo.2014.921

Vera FWM (2000) Grazing ecology and forest history. CABI Publishing (Wallingford). https://doi.org/10.1079/9780851994420.0000

Verheyen K, Baeten L, De Frenne P, Bernhardt-Römermann M, Brunet J, Cornelis J, Decocq G, Dierschke H, Eriksson O, Hédl R, Heinken T, Hermy M, Hommel P, Kirby K, Naaf T, Peterken G, Petřík P, Pfadenhauer J, Van Calster H, Walther GR, Wulf M, Verstraeten G (2012) Driving factors behind the eutrophication signal in understorey plant communities of deciduous temperate forests. Journal of Ecology 100: 352–365. https://doi.org/10.1111/j.1365-2745.2011.01928.x

Whigham D (2004) Ecology if woodland herbs in temperate deciduous forests. Annual Review of Ecology, Evolution, and Systematics 35: 583–621. https://doi.org/10.1146/annurev.ecolsys.35.021103.105708

Wiegmann SM, Waller DM (2006) Fifty years of change in northern upland forest understories: identity and traits of „winner" and „loser" plant species. Biological Conservation 129: 109–123. https://doi.org/10.1016/j.biocon.2005.10.027

Williamson GB (1978) A comment on equilibrium turnover rates for islands. The American Naturalist 112: 241–243. https://doi.org/10.1086/283265

Woods KD, Hicks DJ, Schultz J (2012). Losses in understory diversity over three decades in an old-growth cool-temperate forest in Michigan, USA. Canadian Journal of Forest Research 42: 532–549. https://doi.org/10.1139/x2012-006

Supplementary material 1

Map showing the position of sampling plots in 1996 and 2013

Authors: Tibor Standovár, Soma Horváth, Réka Aszalós

Data type: specimens data

Supplementary material 2

Map showing canopy trees in the study area.

Authors: Tibor Standovár, Soma Horváth, Réka Aszalós

Data type: specimens data

Nature Conservation 17: 57–69 (2017)
doi: 10.3897/natureconservation.17.11582
http://natureconservation.pensoft.net

REVIEW ARTICLE

A peer-reviewed open-access journal
Nature Conservation
Launched to accelerate biodiversity conservation

Plant cover effect on Bolson tortoise (*Gopherus flavomarginatus* Legler 1959, Testudinidae) burrow use

Jorge Luis Becerra-López[1], Cristina García-De la Peña[2], Ulises Romero-Méndez[2], Aurelio Ramírez-Bautista[1]

1 *Instituto de Ciencias Básicas e Ingeniería, Laboratorio de Ecología de Poblaciones, Universidad Autónoma del Estado de Hidalgo. Carretera Pachuca-Tulancingo, Km. 4.5, Ciudad del Conocimiento, Colonia Carboneras, 42184 Mineral de la Reforma, Hidalgo, México* **2** *Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango. Av. Universidad s/n, Fraccionamiento Filadelfia, 35010 Gómez Palacio, Durango, México*

Corresponding author: *Aurelio Ramírez-Bautista* (ramibautistaa@gmail.com)

Academic editor: *F. Andreone* | Received 22 December 2016 | Accepted 4 March 2017 | Published 28 March 2017

http://zoobank.org/BDD70532-A481-4558-8828-E095B722B3BD

Citation: Becerra-López JL, García-De la Peña C, Romero-Méndez U, Ramírez-Bautista A (2017) Plant cover effect on Bolson tortoise (*Gopherus flavomarginatus* Legler 1959, Testudinidae) burrow use. Nature Conservation 17: 57–69. https://doi.org/10.3897/natureconservation.17.11582

Abstract

The Bolson tortoise, *Gopherus flavomarginatus*, occurs within a restricted geographical area in the Mexican Chihuahuan Desert. We analyzed the variation in surface microhabitat with relation to the burrow occupancy for this tortoise at the Mapimí Biosphere Reserve, Mexico. In summer 2010, we monitored burrow activity (active, inactive, or abandoned) and measured environmental factors that might influence the burrow's occupancy by tortoises (air temperature, relative humidity and substrate temperature, both inside and outside the burrow, and the plant cover around it). Discriminant analysis was used to identify the importance of these variables influencing burrow occupancy. Correlation and linear regression analyses were performed to quantify the relation between environmental factors in the sampled burrows.

Results. Sixty-one burrows were identified at the Tortugas locality. The first function's auto-value analysis indicates that this function explains 97.9% of the variation in burrow activity status; high occupancy scores were associated with low substrate temperature inside the burrow. Plant cover was inversely proportional to substrate temperature inside the burrow. These results suggest the importance the density of plants surrounding the tortoise's burrow as a key factor influencing the burrow microclimate and occupancy by the tortoises.

Conclusions. *Gopherus flavomarginatus* inhabits burrows, in part, based on microhabitat structure, with plant cover being a main factor influencing burrow occupancy. Our findings indicate that human land use and vegetation management are important for conserving Bolson tortoises, and for understanding habitat conditions necessary for the successful establishment of populations elsewhere.

Keywords
Gopherus flavomarginatus, burrow, plant cover, habitat, temperature, microclimate

Introduction

Research on the ecology of ectothermic organisms has established the importance of vegetation structure for their microhabitat selection (Hertz et al. 1994, Vitt et al. 1997, Litzgus and Brooks 2000, Bryant et al. 2002). Changes in vegetation produce variations in other microhabitat attributes, like light intensity, wind speed, air and soil temperatures (Pringle et al. 2003). Variation in these features influences thermoregulatory behaviors and activity levels in ectotherms (Adams and Decarvalho 1984, Huey 1982, 1991, Huey and Kingsolver 1989, Webb et al. 2005, Turbill et al. 2011), resulting in greater impact on species that are thermally sensitive to changes in habitat structure (Walther et al. 2002, Pringle et al. 2003).

Population ecology theory predicts that in a changing environment, a population can adapt to new conditions, migrate to a place that favors its survival, or become extinct (Pease et al. 1989, Pringle et al. 2003) if the species presents a capacity of dispersion and limited evolutionary responses (Allendorf and Luikart 2007). Long term studies have established relationships between changes in vegetation density and animal movements and extirpations of populations with small distributions (Fitch 1999, Pringle et al. 2003). For example, abundances of forest birds in New Hampshire decreased considerably over a period of 30 years causing local extinction of four species; the most important local factor affecting bird abundance was temporal change in forest vegetation structure (Holmes and Sherry 2001). Likewise, it has been reported that for *Gopherus polyphemus* in sites invaded by an introduced weed, tortoises avoided areas where weeds had formed a dense monoculture, suggesting that habitat selection increases isolating effect of habitat fragmentation on this tortoise (McCoy et al. 2013).

The Bolson tortoise, *Gopherus flavomarginatus* (Figure 1), is North America's least studied tortoise; it is considered as Vulnerable by IUCN Red List (2015), and has a geographical distribution restricted to the Mapimí Basin in the Mexican Chihuahuan Desert (Aguirre et al. 1984). This restricted distribution is likely due to specific habitat requirements (Aguirre et al. 1997), including constant temperatures and humidity levels provided by their burrows throughout the year, as *G. flavomarginatus* seems to have a limited thermoregulation capacity (Adest et al. 1989); Adult individuals of this species have a high fidelity to their burrow, spending about 95% of their life hibernating or aestivating within this structure, and remain only 5% outside of them during the summer season (Adest et al. 1989, Lovich and Daniels 2000, Daren-Riedle et al. 2008), and adult tortoises are unlikely to be naturally depredated (Treviño et al. 1995).

Therefore, if Bolson tortoise requires specific microclimatic conditions to inhabit burrows and survive, variations in microhabitat are expected to influence either their use or abandonment. An analysis of microhabitat variation is shown here in relation to the occupation of burrows of *G. flavomarginatus*. Our objectives included: 1) charac-

Figure 1. *Gopherus flavomarginatus* (Bolson tortoise).

terization of the environmental factors of air temperature, relative humidity, substrate temperature and pH; physical factors of width and height of burrows and 2) determine how these factors are related to plant cover and occupancy of burrows. This information can increase understanding of this species' response to variation among microhabitats, and support conservation efforts for this species.

Methods

The 100 hectare study site, Tortugas, is located in the south-central portion of the Mapimí Biosphere Reserve, in Mexico (26°00', 26°10'N and 104°10', 103°20'W; CONANP 2006) and within the region known as the Mapimí Basin (Figure 2). The reserve encompasses parts of the municipalities of Tlahualilo and Mapimí in the State of Durango, Sierra Jimenez in Chihuahua, and Sierra Mojada in Coahuila. The site is located at an altitude of 1000–1200 m in the lowlands up to 2000 m and provides numerous exposures of volcanic and chalky origin and sand dunes (Ramírez-Carballo and Pedroza-Sandoval 2011). A semi-hot desert climate prevails (2.8°C in winter to 36.3°C in summer), with an annual mean precipitation of 145.88 mm (CNA 2007) concentrated in summer (from June to September).

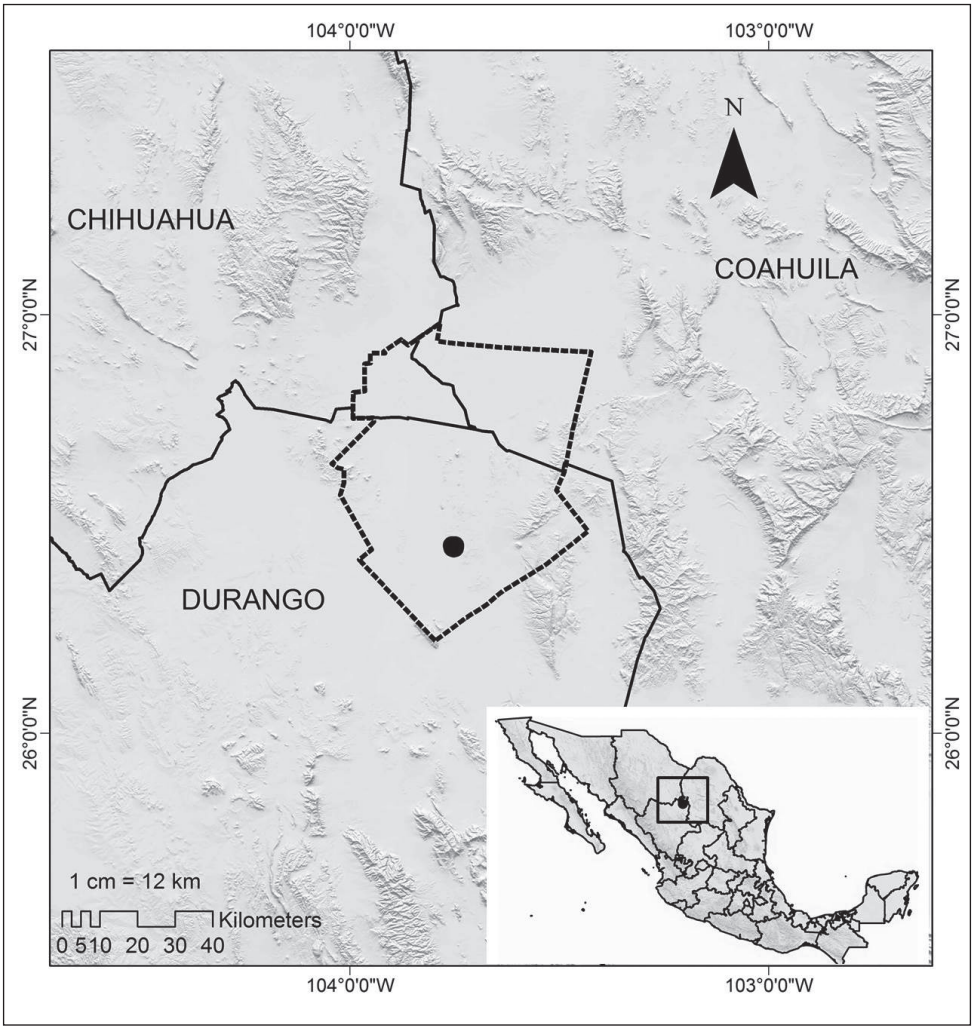

Figure 2. Study site. The black circle show Tortugas locality, in dotted lines is show The Mapimí Biosphere Reserve and continuous lines the state limits.

At Tortugas, we followed the monitoring protocol established by CONANP to find adult tortoise burrows (CONANP 2006). Burrow monitoring was performed during two consecutive days in summer (September, 2010). There was no rainfall before or after those two days. We classified burrow occupancy (active, inactive, or abandoned) based on measuring external characteristics according to Auffenberg and Franz (1982) and Cox et al. (1987). Accordingly, an active burrow shows foot or plastron prints on the access tunnel and the surrounding mound; the soil is loose, with little compaction. In an inactive burrow, no tortoise tracks are seen, and soil at the burrow's entrance and the mound looks compacted. Finally, an abandoned burrow entrance shows an accumulation of debris, such as branches, grass, cobwebs, and the soil of the mound is clearly compacted.

In every burrow, was measured microhabitat structure considering the variables width (W) and height (H) of the entrance and the substrate's pH 30 cm inside. Dataloggers (Datalogger USB-WK057, accuracy: ± 1.0) were used to measure environmental factors continuously, including air temperature (T_a) and relative humidity (RH) inside (30 cm depth) and outside (30 cm above surface) the burrow, except pH, all environmental data were recorded each hour for 24 hours; substrate temperature inside the burrow (T_s) was also recorded using dataloggers (in contact with the surface). Also, we measured plant cover (PC) using an ellipse area formula ($\pi \times a \times b/4$, where a = major axis and b = minor axis), within three meters of each burrow.

Discriminant analysis was used to determine which habitat and environmental factors differentiate burrows categorized by their occupancy status. Normality was not achieved (Kolmogorov-Smirnov tests; $P \leq 0.05$) and we transformed the continuous data (W, H, pH, T_a, RH, T_s) with the logarithmic formula ($X' = LOG10(X + 1)$), and PC with the arcsine formula ($X' = Arcsin\sqrt{X}$), according to Zar (1999). A *Post Hoc* test (LSD) was performed to identify differences among the averages of the three status groups. Lastly, correlation and linear regression analyses were performed to quantify the relation between significant environmental factors and PC in the sampled burrows and was plotted temporal variation of temperature. All statistical analyses were made using STATISTICA 10.0 (StatSoft 2011) software and considered statistically significant at $P \leq 0.05$.

List of abbreviations

W	burrow width
H	burrow height
T_{ai}	air temperature inside the burrow
T_{ao}	air temperature outside the burrow
RH_i	relative humidity inside the burrow
RH_o	relative humidity outside the burrow
T_{si}	substrate temperature inside the burrow
PC	plant cover
LSD	least significant difference
d.f.	degrees of freedom
SD	standard deviation

Results

We located and measured a total of 61 burrows at the Tortugas study site. There was significant difference in the T_{si} among the three types of burrows ($F = 32.40$, d.f. = 2, 58, $P < 0.001$; Table 1). *Post hoc* analysis (LSD) showed that abandoned burrows had higher T_{si} ($\bar{x}$ = 31.1°C, SD = 5.24) than active ($\bar{x}$ = 28.0°C, SD = 4.7) and inactive ($\bar{x}$ = 27.0°C, SD= 3.8) ones (Table 1).

Table 1. Descriptive statistics of environmental factors for active (n = 26), inactive (n = 7), and abandoned (n = 28) burrows, and means comparison tests among burrows categories (d.f. = 2, 58 for all cases). Air temperature inside the burrow (T_{ai}), air temperature outside (T_{ao}), relative humidity inside (RH_i), relative humidity outside (RH_o), substrate temperature inside (T_{si}).

Environmental factor/ Burrow´s status	Mean	Standard deviation	Min-Max	Wilks Lambda	F	P
T_{si} (°C)				0.472	32.40	<0.001
Active	28.00	4.72	18.0–37.0			
Inactive	27.00	3.82	24.0–35.0			
Abandoned	31.10	5.24	20.0–43.0			
T_{ai} (°C)				0.995	0.136	0.873
Active	33.74	7.80	15.5–48.1			
Inactive	34.92	6.86	22.0–40.5			
Abandoned	33.47	5.14	20.6–43.8			
T_{ao} (°C)				0.993	0.191	0.827
Active	33.40	7.51	14.8–44.0			
Inactive	34.90	7.15	22.2–41.9			
Abandoned	33.45	4.94	21.0–42.2			
RH_i (%)				0.964	1.090	0.343
Active	29.49	7.29	19.0–51.4			
Inactive	30.65	8.19	20.5–41.5			
Abandoned	34.55	14.37	19.0–75.0			
RH_o (%)				0.984	0.478	0.623
Active	21.48	6.37	14.0–37.1			
Inactive	20.68	5.91	13.4–28.8			
Abandoned	22.30	5.07	15.3–38.0			
pH				0.987	0.384	0.683
Active	7.07	0.57	6.0–8.0			
Inactive	7.0	0.0	7.0–7.0			
Abandoned	6.98	0.28	6.0–8.0			
LC (%)				0.979	0.633	0.535
Active	56.20	26.7	4.9–116.6			
Inactive	59.84	21.71	38.7 - 100			
Abandoned	55.2	21.4	19.8–86.8			
W (cm)				0.978	0.649	0.526
Active	30.73	12.79	14.0–61.0			
Inactive	24.71	8.63	13.0–38.0			
Abandoned	23.75	11.19	12.0–60.0			
H (cm)				0.909	2.915	0.062
Active	21.88	14.02	6.0–75.0			
Inactive	19.57	7.13	9.0–30.0			
Abandoned	15.10	8.12	1.0–46.0			

Results of discriminant analysis were as follows: the first function was statistically significant ($\lambda = 0.241$, $x^2 = 76.74$, d.f. = 18, $P < 0.001$; n = 61), while the second function was not ($\lambda = 0.942$, $x^2 = 3.25$, d.f. = 8, $P < 0.917$; n = 61). The first function's autovalue analysis indicates that this function explains 97.9% of the variation in burrow activity status, where T_{si} showed the higher scores (Table 2 and Figure 3).

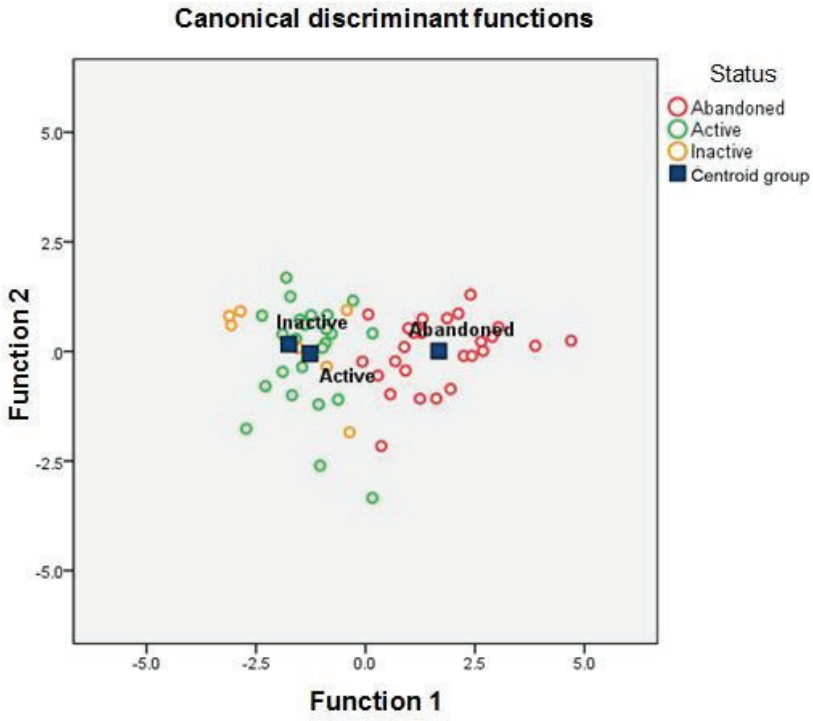

Figure 3. Distribution of the centroids for Bolson tortoise burrows during the summer season.

Table 2. Discriminant canonical function 1 scores with relation to burrow entrance width (W), height (H), air temperature inside the burrow (T_{ai}), air temperature outside (T_{ao}), relative humidity inside (RH_i), relative humidity outside (RH_o), substrate temperature inside (T_{si}), plant cover (PC), and substrate pH.

Environmental factors	Score
T_{si}	621*
H	-.185*
RH_i	.110*
RH_o	.073*
PC	.061
pH	-.054
W	.020
T_{ao}	.009
T_{ai}	-.004

An inverse relationship was observed between PC and T_{si} (y = -0.2181x + 41.504), indicating that the higher the plant cover around the burrow, the lower the substrate temperature inside it (Figure 4). Correlation and determination coefficients were high (R = 0.98, R^2 = 0.96, respectively); plant cover around the burrows influences 96% the

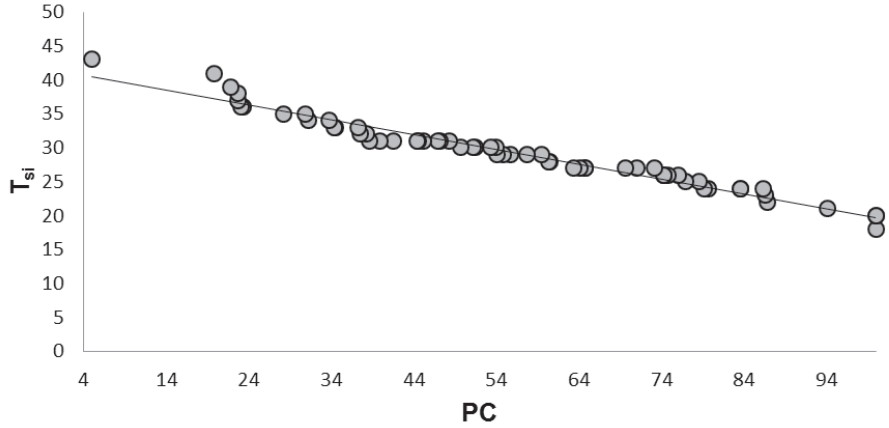

Figure 4. Relation between plant cover (PC) and inner burrow substrate temperature (T_{si}) for *Gopherus flavomarginatus* burrows.

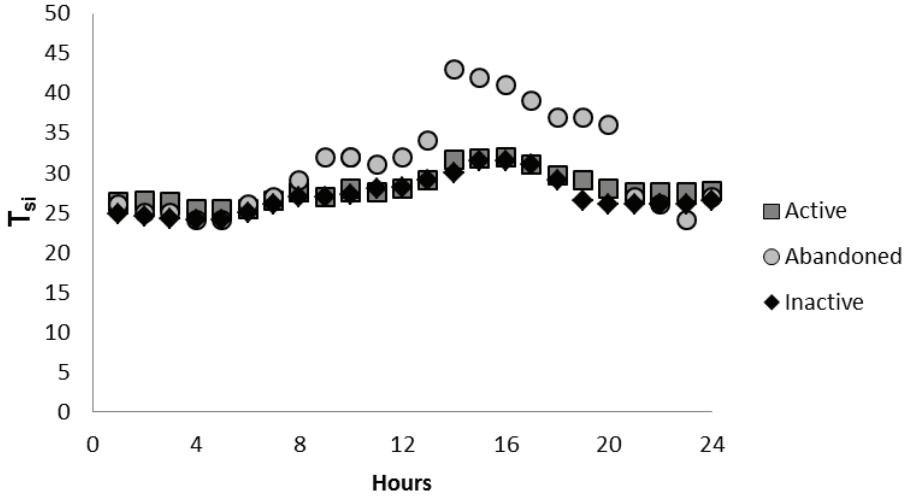

Figure 5. Temporal variation of temperature. T_{si} makes reference to temperature of substrate, squares indicate active burrows, circles make reference to abandoned burrows and rhombus show inactive burrows.

increase of substrate temperature inside the burrows. This relationship was found to be highly significant ($F = 1408.949$, d.f. = 1,59; $P < 0.001$), the temporal variation of temperature is shown in Figure 5.

Discussion

Adest et al. (1989) described individuals of this species emerging from their burrows at night as a response to increasing substrate temperatures inside their burrows (>34°C)

because of thermal delay (which it is described as the speed at which the temperature fluctuations penetrate the substrate (Körtner et al. 2008)). Also, they described that around 0700 hr at a distance of 15 cm inside the burrow, the substrate temperature is still above 31°C, while the temperature of an adult individual (7.3 kg) is below 30°C when beginning foraging bouts at the surface. These observations support a hypothesis that the substrate temperature inside *G. flavomarginatus* burrows influences its occupancy dynamics, increasing the possibility of abandonment when the substrate temperature inside these structures consistently is equal to or greater than 31°C.

Our analyses provided evidence that an increase in substrate temperature inside the burrows and their consequent abandonment at our Tortugas study site was correlated with vegetation cover at a scale of 3 m. Aguirre et al. (1984) described that the presence of *G. flavomarginatus* burrows at the Mapimí Biosphere Reserve seemed to be related to shrub type (*Larrea tridentata* (Coville 1893), *Prosopis glandulosa* (Torrey 1827) and grasslands (*Hilaria mutica* (Bentham 1881)). McCoy et al. (2006) and Waddle et al. (2006) reported that habitat quality reduction was the apparent explanation for the increase of abandoned burrows in *G. polyphemus* population in Florida, USA. Additionally, Aresco and Guyer (1999) stated that land cover changes around *G. polyphemus* burrows can result in their abandonment in certain habitats. Similarly, Boglioli et al. (2000), Hermann et al. (2002), Jones and Dorr (2004), Baskaran et al. (2006), and Ashton et al. (2008) all described that for other species of *Gopherus* the presence of burrows is associated with the vegetation, and that the permanent abandonment of these burrows seems to happen as a response to unfavorable habitat conditions.

Moreover, Huey (1982), Hertz et al. (1994), Vitt et al. (1997), and Bryant et al. (2002) mentioned that vegetation structure plays a key role in the activity, feeding, and distribution of some ectothermic organisms. These previous studies support our conclusion that the occupation of *G. flavomarginatus*' burrows are related to microhabitat structure, with vegetation cover being one of the main environmental factors that can affect habitat selection, this interaction of temperature and microhabitat is key to the species' survival. With predicted increasing temperatures as climate change effects become more pronounced in the deserts of America (Friggens 2012), this interaction will be critical over the coming years. On the other hand, we consider that relative humidity inside the burrows is important for Bolson tortoise. However, this variable showed low scores to discriminate activity status of the burrows and did not present significant differences when comparing between activity status; therefore, it was no possible to determine its influence on burrows occupation.

It is important to note that *G. flavomarginatus* might not have originated as a desert ecosystems species, they appeared toward the end of the Tertiary, so they could have spent more than 94% of their evolutionary history during the Quaternary (Pleistocene-Holocene) living in non-desert grasslands (Van Devender and Burgess 1985). Therefore, their current restriction to cool microclimates in their summer burrows could be an extension of a physiology geared to a cooler, more mesic climate. Consequently, it is likely that their thermal physiology and even their social behavior reflect more their burrow microhabitat than the surface environment of the Chihuahuan Desert (Adest et al. 1989).

Conclusions

Having in mind that vegetation cover is a key part of burrows occupancy dynamics for this species, preserving the plant life in regions where *G. flavomarginatus* might potentially colonize or be translocated to in and outside the Mapimí Biosphere Reserve is of critical importance. To achieve this, we need to conceptualize a dynamic reserve (as opposed to a static one that actually exists) that follows ecological succession processes on which this tortoise species survival seems to be strongly dependent.

Acknowledgments

To members of the Tlahualilo, Durango commonality and the Mapimí Biosphere Reserve administration for all the help and assistance provided to carry out this study; also we thank L. D. Wilson for reading and improving the manuscript.

References

Adams SR, De Carvalho MC Jr (1984) Rates of heat exchange in the ornate box turtle, *Terrapene ornata*. Comparative Biochemistry and Physiology 79A 3: 359–361.

Adest GA, Aguirre-León G, Morafka DJ, Jarchow JV (1989) Bolson tortoise (*Gopherus flavomarginatus*) conservation: I. Life history. Vida Silvestre Neotropical 2: 7–13.

Allendorf FW, Luikart G (2007) Conservation and the genetics of populations. Blackwell, London. http://www.wiley.com/Wiley CDA/WileyTitle/productCd-EHEP002672.html

Aguirre G, Morafka DJ, Adest GA (1997) Conservation strategies for the Bolson Tortoise, *Gopherus flavomarginatus*, in the Chihuahuan Desert. In: Van Abbema J (Eds) Proceedings: Conservation, Restoration, and Management of Tortoises and Turtles-An International Conference. Conference. New York Turtle & Tortoise Society, New York, 333–338. http// www.eaglemountainpublishing.com/?page=shop/flypage &product_id=268

Aguirre G, Adest GA, Morafka DJ (1984) Home range and movement patterns of the Bolson Tortoise, *Gopherus flavomarginatus*. Acta Zoológica Mexicana 1: 1–28.

Aresco MJ, Guyer C (1999) Burrow abandonment by Gopher Tortoises in slash pine plantations of the Conecuh National Forest. Journal of Wildlife Management 63: 26–35. http:// https://doi.org/10.2307/3802484

Ashton KG, Engelhardt BM, Brancifort BS (2008) *Gopher* tortoise (*Gopherus polyphemus*) abundance and distribution after prescribed fire reintroduction to Florida scrub and sandhill at Archbold Biological Station. Journal of Herpetology 42: 523–529. https://doi.org/10.1670/06-246.1

Auffenberg W, Franz R (1982) The status and distribution of the gopher tortoise (*Gopherus polyphemus*). In: Bury R (Ed.) North American Tortoises: Conservation and Ecology. US Fish and Wildlife Service (Washington, DC), Wildlife Research Report 13: 95–126.

Baskaran LM, Dale VH, Efroymson RA, Birkhead W (2006) Habitat modeling within a regional context: An example using gopher tortoise. American Midland Naturalist 155: 335–351. http://dx.doi.org/10.1674/0003-0031(2006) 155[335:HMWARC]2.0.CO;2

Bentham G (1881) Notes on gramineae. Journal of the Linnean Society, Botany 19: 93–94. https://doi.org/10.1111/j.1095-8339.1881.tb00355.x

Boglioli MD, Michener WK, Guyer C (2000) Habitat selection and modification by the gopher tortoise, *Gopherus polyphemus*, in Georgia longleaf pine forest. Chelonian Conservation and Biology 3: 699–705.

Bryant SR, Thomas CD, Bale JS (2002) The influence of thermal ecology on the distribution of three *Nymphalid butterflies*. Journal of Applied Ecology 39: 43–55. https://doi.org/10.1046/j.1365-2664.2002.00688.x

Bury RB, Morafka DJ, McCoy CJ (1988) Part I. Distribution, abundance, and status of the Bolson tortoise. In: Morafka DJ, McCoy CJ (Eds) The ecogeography of the Mexican Bolson tortoise (*Gopherus flavomarginatus*): derivation of its endangered status and recommendations for its conservation. Annals Carnegie Museum (Pittsburgh): 5–30.

CNA (Comisión Nacional del Agua) (2007) Datos climatológicos de las estaciones meteorológicas de La Soledad, Laboratorio del Desierto y La Flor. Información proporcionada por Comisión Nacional del Agua en archivo digital.

CONANP (Comisión Nacional de Áreas Naturales Protegidas) (2006) Programa de conservación y manejo Reserva de la Biosfera Mapimí (México): 182. http://www.conanp.gob.mx/programas/pdf/Anexo%202%20Protocolo%206%20RB%20Mapimi.pdf

Cox J, Inkley D, Kautz R (1987) Ecology and habitat protection needs of gopher tortoise (*Gopherus polyphemus*) populations found on lands slated for large-scale development in Florida. Florida Game and Freshwater Fish Commission Non-game Wildlife Program Technical Report 4: 69.

Coville FV (1893) Botany of the Death Valley expedition. A report on the botany of the expedition sent out in 1891 by the U.S. Department of Agriculture to make a biological survey of the region of Death Valley, California. Government Printing Office (Washington) 318.

Daren-Riedle JR, Averill-Murray C, Lutz CL, Bolen DK (2008) Habitat use by Desert Tortoises (*Gopherus agassizii*) on alluvial fans in the Sonoran Desert, South Central Arizona. Copeia 2: 414–420. https://doi.org/10.1643/CH-06-010

Fitch HS (1999) A Kansas snake community: composition and change over fifty years. Krieger, Malabar (Florida): 165.

Friggens MM, Warwell MV, Chambers JC, Kitchen SG (2012) Modeling and predicting vegetation response of western USA grasslands, shrublands, and deserts to climate change (Chapter 1). In: Finch DM (Ed.) Climate change in grasslands, shrublands, and deserts of the interior American West: a review and needs assessment. Gen. Tech. Rep. RMRS-GTR-285. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO, 1–20. https://www.fs.fed.us/rm/pubs/rmrs_gtr285.pdf

Hermann SM, Guyer C, Waddle JH, Nelms MG (2002) Sampling on private property to evaluate population status and effects of land use practices on the gopher tortoise, *Go-*

pherus polyphemus. Biological Conservation 108: 289-298. doi: http://dx.doi.org/10.1016/S0006-3207(02)00123-4

Hertz PE, Fleishman LJ, Armsby C (1994) The influence of light-intensity and temperature on microhabitat selection in *Anolis* lizards. Functional Ecology 8: 720–729. http://www.jstor.org/stable/2390231

Holmes RT, Sherry TW (2001) Thirty-year bird population trends in an unfragmented temperate deciduous forest: importance of habitat change. The Auk 118: 589–609. https://doi.org/10.1642/0004-8038(2001)118[0589:TYBPTI]2.0.CO;2

Huey RB (1982) Temperature, physiology, and the ecology of reptiles. In: Gans C, Pough FH (Eds) Biology of the Reptilia, Academic Press, New York, 12: 25–91.

Huey RB (1991) Physiological consequences of habitat selection. American Naturalist 137: 91–115. https://doi.org/10.1086/285141

Huey RB, Kingsolver JG (1989) Evolution of thermal sensitivity of ectotherm performance. Trends in Ecology and Evolution 4: 131–135. https://doi.org/10.1016/0169-5347(89)90211-5

IUCN (International Union for Conservation of Nature) (2015) Red List of Threatened Species. http://www.iucnredlist.org/details/9402/0 [Accessed: 09 November 2015]

Jones JC, Dorr B (2004) Habitat associations of gopher tortoise burrows on industrial timberlands. Wildlife Society Bulletin 32: 456–464. https://doi.org/10.2193/0091-7648(2004)32[456:HAOGTB]2.0.CO;2

Körtner G, Pavey CR, Geiser F (2008) Thermal biology, torpor and activity in free-living mulgaras in arid zone Australia during the winter reproductive season. Physiological and Biochemical Zoology 81: 442–451. https://doi.org/10.1086/589545

Litzgus JD, Brooks RJ (2000) Habitat and temperature selection of *Clemmys guttata* in a northern population. Journal of Herpetology 34:178–185. https://doi.org/10.2307/1565413

Lovich JE, Daniels R (2000) Environmental characteristics of desert tortoise (*Gopherus agassizii*) burrow locations in an altered industrial landscape. Chelonian Conservation and Biology 3: 714–721. http://www.dmg.gov/documents/PAP_Envrnmntl_Chrctrstcs_DT_Brrw_Lctns_in_Altrd_Indstrl_Lndscpe_Lovich_Daniels_012301.pdf

McCoy ED, Mushinsky HR, Lindzey J (2006) Declines of the gopher tortoise on protected lands. Biological Conservation 128: 120–127. http://www.sciencedirect.com/science/journal/00063207/128/1

McCoy ED, Basiotis KA, Connor KM, Mushinsky HR (2013) Habitat selection increases the isolating effect of habitat fragmentation on the gopher tortoise. Behavioral Ecology and Sociobiology 67.5: 815–821. https://doi.org/10.1007/s00265-013-1505-x

Pease C M, Lande R, Bull JJ (1989) A model of population growth, dispersal and evolution in a changing environment. Ecology 70: 1657–1664. https://doi.org/10.2307/1938100

Pringle RM, Webb JK, Shine R (2003) Canopy structure, microclimate, and habitat selection by a nocturnal snake, *Hoplocephalus bungaroides*. Ecology 84: 2668–2679. https://doi.org/10.1890/02-0482

Ramírez-Carballo H, Pedroza-Sandoval A (2011) Evaluación participativa de la degradación del suelo en la reserva de la biósfera de Mapimí, Durango, México. Revista Chapingo Serie Zonas Áridas 6: 247–254. http://www.redalyc.org/articulo.oa?id=62921030009

Treviño E, Morafka DJ, Aguirre-León G (1995) Morphological distinctiveness of the northern population of the Bolson tortoise, *Gopherus flavomarginatus*. Publicaciones de la Sociedad Herpetológica Mexicana.

StatSoft Inc (2011) STATISTICA (data analysis software system), version 10. http://www.statsoft.com

Torrey J (1827) Some account of a collection of plants made during a journey to and from the Rocky Mountains in the summer of 1820. In: James EP (Ed.) Annals of the Lyceum of Natural History of New York. New York, 161–254.

Turbill C, Bieber C, Ruf T (2011) Hibernation is associated with increased survival and the evolution of slow life histories among mammals. Proceedings of the Royal Society of London Series B. Biological Sciences 278: 3355–3363. https://doi.org/10.1098/rspb.2011.0190

Van Devender TR, Burgess TL (1985) Late Pleistocene woodlands in the Bolson de Mapimí: a refugium for the Chihuahuan Desert biota? Quaternary Research 24: 346–353. https://doi.org/10.1016/0033-5894(85)90056-0

Vitt LJ, Zani PA, Lima ACM (1997) Heliotherms in tropical rain forest: the ecology of *Kentropyx calcarata* (Teiidae) and *Mabuya nigropunctata* (Scincidae) in the Curua-Una of Brazil. Journal of Tropical Ecology 13: 199–220. https://doi.org/10.1017/s0266467400010415

Waddle JH, Mazzotti FJ, Rice KG (2006) Changes in abundance of gopher tortoise burrows at Cape Sable, Florida. Southeastern Naturalist 5: 277–284. http://dx.doi.org/10.1656/1528-7092(2006)5[277:CIAOGT]2.0.CO;2

Walther GR, Post E, Convey P, Menzel A, Parmesan C, Beebee TJC, Fromentin JM, Hoegh-Guldberg O, Bairlein F (2002) Ecological responses to recent climate change. Nature 416: 398–395. https://doi.org/10.1038/416389a

Webb JK, Shine R, Pringle RM (2005) Canopy removal restores habitat quality for an endangered snake in a fire suppressed landscape. Copeia 4: 894–900. http://dx.doi.org/10.1643/0045-8511(2005)005[0894:CRRHQF]2.0.CO;2

Zar JH (1999) Biostatistical Analysis (4th edn). Prentice Hall, 947.