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Front cover: A young *Varanus komodoensis* from Rinca island (Photograph by Rick J. Hodges)

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Deep Mitochondrial and Morphological Differentiation of *Hemidactylus persicus* Anderson, 1872 (Squamata: Gekkonidae) in Iran

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With currently 149 species, *Hemidactylus* Oken, 1817 is one of the most species-rich genera of the family Gekkonidae. In this study, 50 *Hemidactylus persicus* and *H. romeshkanicus* from southern Iran and three specimens of the newly described species *H. kurdicus* from north-eastern Iraq were screened using sequences of the mitochondrial 12S rRNA gene (approximately 400 bp) with two *H. hajarensis* as outgroups. In addition, 58 specimens were analysed morphologically using 25 mensural and six meristic characters. The genetic data recovered six well supported clades of *H. persicus* and *H. romeshkanicus* in southern Iran, which also showed morphological differentiation with the exception of specimens from Khuzestan and Fars provinces. Principal Coordinates Analysis (PCoA) and haplotype networks are compatible with our phylogenetic tree and morphological analyses. These findings highlight deep mitochondrial and morphological variation of *H. persicus* from Iran. Interestingly, our phylogenetic inference revealed that *H. romeshkanicus* should be regarded as a valid species, whereas *H. kurdicus* is not a distinct evolutionary lineage and synonymous with *H. romeshkanicus*.

Key words: Gekkonidae; Iranian plateau; Phylogeny; Radiation; Species complex

INTRODUCTION

The diverse herpetofauna of the Iranian plateau has been of interest to herpetologists, particularly with respect to ecology and zoogeography (e.g., Anderson, 1968; Hosseinzadeh et al., 2014a). Topographically, the Iranian plateau consists of a complex of mountain chains enclosing two main mountain ranges: the Elburz, which extends from north-west to north-east, and the Zagros, which extends from north-west to south-eastern Iran (Fisher, 1968). Descriptions of species using molecular tools resulted in the detection of cryptic taxa, and the raising of geographically isolated subspecies to the rank of species (Ahmadzadeh et al., 2013; Ficetola et al., 2013). However, further molecular and integrative studies are necessary in order to gain a more complete understanding of the Iranian herpetofauna.

With 149 recognised species (Uetz et al., 2018), the genus *Hemidactylus* Oken, 1817 is one of the most species-rich genera of the family Gekkonidae. It is globally distributed in tropical and subtropical regions. Four species of *Hemidactylus* have been reported from Iran: *Hemidactylus persicus* Anderson, 1872, *H. robustus* Heyden, 1827, *H. flaviviridis* Rüppell, 1840, and *H. romeshkanicus* Torki 2011 (Anderson, 1999; Bauer et al., 2006; Torki et al., 2011; Šmíd et al., 2014; Hosseinzadeh et al., 2014b). *Hemidactylus persicus* is distributed in the northern Arabian Peninsula, southern Iran, Iraq, Kuwait, Pakistan and India (Sindaco & Jeremčenko, 2008; Carranza & Arnold, 2012; Khan, 2013; Castilla et al., 2013; Šmíd et al., 2014). Molecular studies of Iranian *H. persicus* have shown a high level of genetic differentiation (Carranza & Arnold, 2012; Šmíd et al., 2013). Recently, a new species, *H. kurdicus*, has been reported from the oak woodlands of Zagros forest steppe of Qara Dagh Mountains, Sulaimani, north-eastern Iraq (Safaei-Mahroo et al., 2017).

The occupation of Iran by *H. persicus* in different climates and habitats along with deep intraspecific variation suggests that it might comprise a species complex. According to Torki et al. (2011), *H. romeshkanicus* is endemic to Iran, inhabiting western slopes of the Zagros Mountains and southern Lorestan. According to Šmíd et al. (2014), the species probably belongs to the arid clade together with its sister taxa *H. persicus*, *H. robustus* and *H. turcicus*. Here, we study the genetic variability of *H. persicus* across its entire range in the Iranian Plateau using 12S rRNA mtDNA sequences, together with multivariate analyses of mensural and meristic characters. Further, we evaluate the validity of *H. romeshkanicus* using these methods.

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METHODS

Fifty-three *Hemidactylus* were included in the phylogenetic analyses which were procured from collections (Collection of The Biology Department of Shiraz University (CBSU), Zoological Museum of University of Tehran (ZUTC), Department of The Environment of Hormozgan Zoological Collection (DHZC), Farhang Torki Herpetology Museum (FTHM), Collection of The California Academy of Sciences: (CAS), Centre for Ecological Sciences, Bangalore, India (CES), Museum of Vertebrate Zoology, Berkeley (MVZ), National Museum, Prague (NMP)). Morphological characters of specimens collected from the type locality of *H. romeshkanicus* were compared with the holotype from Zoologisches Museum of Berlin (ZMB). Other samples were obtained from recent expeditions and have been deposited in the Sabzevar University Herpetological Collection (SUHC), the Zoological Museum of Ferdowsi University of Mashhad (ZMFUM) and the Zoological Museum of University of Birjand (ZMUB) with appropriate sampling permission from the Department of Environment of Iran (see Table 1, Fig. 2). Specimens were euthanised with chloroform and tissues extracted and fixed in 75% ethanol. In total, 42 *H. persicus* were sequenced for this study. A further eight and three sequences of *H. persicus* and *H. kurdicus*, respectively, and two of *H. hajarensis* (used as outgroup) were downloaded from GenBank. We used 12Sa and 12Sb primers (Kocher et al., 1989) to amplify a section (approximately 400 bp) of the mitochondrial 12S ribosomal RNA gene. Sequences were imported to BioEdit, version7.0.9.0 (Hall, 1999), aligned using ClustalW multiple alignment, and adjusted by hand. A distance matrix using uncorrected p-distances was calculated with MEGA, version 5 (Tamura et al., 2011). Two phylogenetic analyses were performed: Maximum Likelihood (ML) and Bayesian Inference (BI). We choose GTR+I+G as the best-fitting model of nucleotide substitution based on the Akaike Information Criterion as implemented in ModelTest, version 3.7 (Posada & Crandall, 1998). The maximum likelihood (ML) tree was produced using RAxML v 7.0.3 (Stamatakis, 2006). To test the robustness of the nodes we ran 1000 bootstrap pseudo-replications under ML (Templeton et al., 1992). Bayesian analyses were performed in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Four Markov Chain Monte Carlo analyses (MCMC) were run simultaneously for 10 million generations and the first 1,000,000 trees (as a conservative 'burn-in') were discarded. Posterior probabilities for nodes were calculated from the remaining trees using a majority-rule consensus analysis. Clades are regarded as strongly supported if they have bootstrap values higher than 70% in ML, or posterior probabilities (pp) of 95% or above in the Bayesian analysis (Hillis et al., 1993). To visualise the number of specimens sharing certain haplotypes, haplotype networks of the 12S were constructed using the TCS software package (Clement et al., 2000). This program estimates the number of mutational steps by which pairwise haplotypes differ and computes the probability of parsimony for pairwise differences until the probability exceeds 0.95 (Templeton et al., 1992). To further evaluate relationships among populations of *H. persicus* we performed a Principal Coordinate Analysis (PCoA) using GenAlEx v.6.5 (Peakall & Smouse, 2006). We used the same software to perform a Mantel test to examine the correlation between geographic and genetic distances based on point localities in the populations of *H. persicus* (Jensen et al., 2005). An analysis of molecular variance (AMOVA) was performed to evaluate the population structure and mutational differences between the loci in different populations using GenAlEx v.6.5 (Peakall & Smouse, 2006). Additionally, to calculate the genetic differentiation fixation index, the partitioning of among-group genetic variation (PhiPT) values were calculated in order to examine the distribution of differences within and between populations using GenAlEx v.6.5 (Peakall & Smouse, 2006).

Fifty-eight specimens of *H. persicus* were examined morphologically, including 29 males and 29 females. All specimens were studied for 25 mensural and six meristic characters following Kluge (1969), Vences et al. (2004), Busais & Joger (2011), and Carranza & Arnold (2012, see supplementary section; Table 2).

Statistical analysis was performed with SPSS 16.0 and PAST v. 2.17c (Hammer et al., 2001). The multivariate canonical variate analysis (CVA) was conducted on the transformed matrix to determine if individuals would be assigned to the correct population group based on morphological measurements.

RESULTS AND DISCUSSION

A total of 399 characters of the 12S rRNA gene were used in the phylogenetic analyses, of which there were 44 parsimony-informative characters (224 invariant or monomorphic sites and 44 variable or polymorphic sites). The proportion of invariable sites, I = 0, for among-site rate variation followed a gamma distribution, with the shape parameter a = 0.2402. The frequencies of nucleotides were: freq A = 0.3285, freq C = 0.3047, freq G = 0.1913, freq T = 0.1755. Both methods (ML and BI) gave very similar results and showed only minor differences, at the base of the trees, where relationships had little support (Fig. 1). The phylogeny recovered six well-supported clades comprising the following populations: Clade A from south-west Iran (Behbahan city, East of Khuzestan Province); south-east Iran (Lipar, Jod Village and Bazman, Sistan and Baluchistan), extreme south-west Iran (Mahshar, Khuzestan Province); Clade B_1 from south Iran (south of Lorestan, Romeshkan, Pole-e-Dokhtar); north-east Iraq (western border of the Zagros forest steppe in south-western Sulaimani, Kurdistan region); Clade B_2 from south-west Iran (northern and central Khuzestan; western Ilam); Clade C_1 from south Iran (Bushehr and southern Fars Province); Clade C_2 from central Iran (Kerman and northern Fars Province); and Clade C_3 from south Iran (central and eastern Fars Province; south-eastern Khuzestan, Fig. 1). Uncorrected genetic distances ranged between 0.000 and 0.008 and between 0.026 and 0.097 within and between clades of *H. persicus*, respectively (Table 3). There is no genetic distance between *H. kurdicus* and clade B_1

Table 1. Details of studied specimens of *H. persicus* and *H. romeshkanicus*. The abbreviations refer to: Collection of The Biology Department of Shiraz University (CBSU), Zoological Museum of University of Tehran (ZUTC), Department of The Environment of Hormozgan Zoological Collection (DHZC), Farhang Torki Herpetology Museum (FTHM), Collection of The California Academy of Sciences (CAS), Centre for Ecological Sciences, Bangalore, India (CES), Museum of Vertebrate Zoology, Berkeley (MVZ), National Museum, Prague (NMP), Sabzevar University Herpetological Collection (SUHC), Zoological Museum of Ferdowsi University of Mashhad (ZMFUM) and Zoological Museum of University of Birjand (ZMUB). M. = Morphological study, G. = Genetic study.

Species	Voucher Code	Locality; number in Figure S_1	GenBank Accession No	Source	Type of Study
H. romeshkanicus	SUHC 1153	40 Km east of Haftgel, Iran;1	MG744524	This study	M., G.
H. romeshkanicus	SUHC 1154	40 Km east of Haftgel, Iran;1	MG744525	This study	M., G.
H. romeshkanicus	SUHC 1155	40 Km east of Haftgel, Iran;1	MG744526	This study	M., G.
H. romeshkanicus	SUHC 1156	40 Km east of Haftgel, Iran;1	MG744527	This study	M., G.
H. persicus	SUHC 1222	5Km west of Dayyer, Iran;2	MG744529	This study	M., G.
H. persicus	SUHC 1223	5Km west of Dayyer, Iran;2	MG744530	This study	M., G.
H. persicus	SUHC 1414	Nourabad, Iran;3	MG744531	This study	G.
H. persicus	SUHC 1415	Nourabad, Iran;3	MG744532	This study	G.
H. persicus	SUHC 1425	Nourabad, Iran;3	MG744535	This study	M., G.
H. persicus	SUHC 1433	Nourabad, Iran;3	-	This study	M.
H. romeshkanicus	SUHC 3622	Masjedsolyeman, Iran;4	MG744539	This study	G.
H. romeshkanicus	SUHC 3623	Masjedsolyeman, Iran;4	MG744540	This study	M., G.
H. romeshkanicus	SUHC 3624	Masjedsolyeman, Iran;4	MG744541	This study	M., G.
H. romeshkanicus	SUHC 3625	Masjedsolyeman, Iran;4	MG744542	This study	G.
H. persicus	SUHC 3643	Ahram mountain, Iran;5	-	This study	M.
H. persicus	SUHC 3644	Ahram mountain, Iran;5	-	This study	M.
H. persicus	SUHC 3645	Ahram mountain, Iran;5	-	This study	M.
H. persicus	SUHC 3693	Khabr national park, Iran;6	MG744544	This study	M., G.
H. persicus	SUHC 3694	Khabr national park, Iran;6	MG744551	This study	M., G.
H. persicus	SUHC 3696	Khabr national park, Iran;6	MG744545	This study	M., G.
H. persicus	SUHC 3691	Ahram mountain, Iran;5	MG744543	This study	G.
H. persicus	ZMFUM 10005	Gakal Cave, Gachsaran,Iran;7	MG744548	This study	M., G.
H. romeshkanicus	ZMFUM10001	Izeh,Iran;8	MG744515	This study	M., G.
H. romeshkanicus	ZMFUM10002	Izeh,Iran;8	MG744522	This study	M., G.
H. romeshkanicus	ZMFUM10003	Izeh,Iran;8	MG744523	This study	M., G.
H. persicus	CBSU R082	25km NW of Lamerd,Iran;9	-	This study	M.
H. persicus	CBSU R083	25km NW of Lamerd,Iran;9	-	This study	M.
H. persicus	ZMFUM10007	Varavi, Iran;10	MG744547	This study	M., G.
H. persicus	ZMFUM10008	Varavi, Iran;10	MG744549	This study	M., G.
H. persicus	ZMFUM10009	Varavi, Iran;10	MG744550	This study	M., G.
H. persicus	ZMFUM10010	Behbahan,Iran;11	-	This study	M.
H. persicus	ZMFUM10011	Behbahan,Iran;11	MG744546	This study	M., G.
H. persicus	CBSU 8071	GoohGorm Jahrum,Iran;12	-	This study	M.
H. persicus	CBSU 8068	GoohGorm Jahrum,Iran;12	-	This study	M.
H. persicus	CBSU 8091	GoohGorm Jahrum,Iran;12	-	This study	M.
H. persicus	CBSU 8083	GoohGorm Jahrum,Iran;12	-	This study	M.
H. persicus	CBSU 4217	Jahrum,Iran;13	-	This study	M.
H. persicus	CBSU 8055	Kazeron,Iran;14	-	This study	M.
H. persicus	CBSU 8056	Shiraz, Iran;15	-	This study	M.
H. persicus	CBSU 5395	Shiraz, Iran;15	-	This study	M.
H. persicus	CBSU R111	Gachsaran,Iran;16	-	This study	M.
H. romeshkanicus	SUHC 3788	Masjed soleman,Iran;17	MG744553	This study	M., G.
H. romeshkanicus	SUHC 3784	Pole-e-dokhtar,Iran;18	MG744555	This study	M., G.
H. romeshkanicus	SUHC 3786	Pole-e-dokhtar,Iran;18	MG744554	This study	M., G.
H. romeshkanicus	SUHC 3789	Pole-e-dokhtar,Iran;18	-	This study	M.
H. persicus	SUHC 3785	Mehran,Iran;19	-	This study	M.
H. persicus	SUHC 2097	Bazman,Iran;20	MG744520	This study	G.
H. romeshkanicus	ZMFUM 10024	Romeshkan, Lorestan, Iran;21	MG744556	This study	M., G.
H. romeshkanicus	ZMB 75020	Romeshkan, Lorestan, Iran;21	-	Torki et al., 2011	M.
H. persicus	SUHC 1558	Jahrom,Iran;22	MG744536	This study	M., G.
H. persicus	SUHC 1974	Marvdasht,Iran;23	MG744538	This study	M., G.
H. persicus	DHZCH132	Qeshm island, Iran;24	-	This study	M.
H. persicus	ZUTC R.1256	Bibi Hakemieh, KohgiloyehvaBoyerahmad, Iran;25	-	This study	M.
H. persicus	ZUTC R.1222	Bibi Hakemieh, KohgiloyehvaBoyerahmad, Iran;25	-	This study	M.
H. persicus	ZUTC R.1234	Bibi Hakemieh, KohgiloyehvaBoyerahmad, Iran;25	-	This study	M.
H. persicus	ZUTC R.1476	Jod Village, Sistan and Baluchistan, Iran;26	MG744552	This study	M., G.
H. persicus	SUHC 451	10 Km East of Evaz,Iran;27	-	This study	M.
H. persicus	SUHC 1787	10 Km East of Evaz,Iran;27	-	This study	M.
H. persicus	SUHC 1416	Parishan region,Iran;28	MG744533	This study	G.
H. persicus	SUHC 1421	Parishan region,Iran;28	MG744534	This study	G.
H. persicus	SUHC 1837	Darab,Iran;29	MG744537	This study	G.
H. persicus	SUHC 1211	Bushehr,Iran;30	MG744528	This study	G.
H. persicus	CBSU R004	Kazeron,Iran;31	-	This study	M.
H. persicus	CBSU B636	Kazeron,Iran;31	-	This study	M.
H. persicus	ZMUB 41	Behbahan,Iran;11	MG744516	This study	M., G.
H. persicus	ZMUB 42	Behbahan,Iran;11	MG744517	This study	M., G.
H. romeshkanicus	ZMUB 43	Mehran,Ilam,19	MG744518	This study	M.,G.
H. romeshkanicus	ZMUB 44	Mehran,Ilam,19	MG744519	This study	M.,G.
H. persicus	ZMFUM10004	Farur Island,Iran;36	MG744521	This study	G.
H. persicus	MVZHERP234385	Lipar Village, Sistan and Baluchistan, Iran;32	JQ957077	Šmíd et al.,2013	G.
H. persicus	FTHM005000	Mahshahr,Iran;33	JQ957074	Šmíd et al.2013	G.
H. persicus	FTHM005001	Mahshahr,Iran;33	JQ957075	Šmíd et al.2013	G.
H. romeshkanicus	FTHM005100	Bushehr,Iran;34	JQ957076	Šmíd et al.2013	G.
H. persicus	NMP6V74807/1	Booreki,Iran;35	KC818691	Šmíd et al.,2013	G.
H. persicus	NMP6V74807/2	Booreki,Iran;35	KC818690	Šmíd et al.,2013	G.
H. kurdicus	CAS 262258	Kurdistan Region, Iraq;37	MG549189	Safaei-Mahroo et al.,2017	G.
H. kurdicus	CAS 262259	Kurdistan Region, Iraq;37	MG549190	Safaei-Mahroo et al.,2017	G.
H. kurdicus	CAS 262260	Kurdistan Region, Iraq;37	MG549191	Safaei-Mahroo et al.,2017	G.
H. persicus	CES 08027	NabhDongar, Rajasthan, India;38	KC735107	Bansal and Karanth, 2013	G.
H. persicus	CES 1_08027	NabhDongar, Rajasthan, India;38	HM595701	Bansal and Karanth, 2010	G.
H. hajarensis	CAS 227612	Tanuf,Oman;39	DQ120337	Carranza and Arnold,2006	G.
H. hajarensis	CAS 227614	Tanuf,Oman;39	DQ120338	Carranza and Arnold,2006	G.

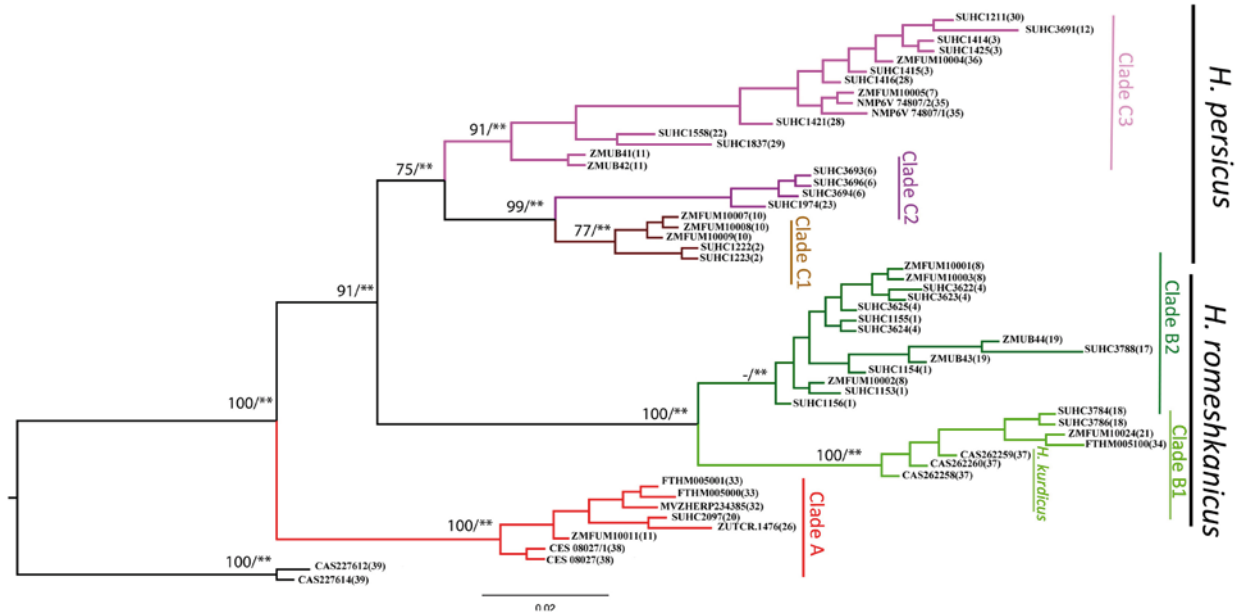

Figure 1. Bayesian 12S rRNA tree. Posterior probability and ML bootstrap values are indicated in star symbol (>99% (**), >95% (*)) and number on each branch of phylogenetic tree, respectively. Number in parenthesis showed locality of the specimens according to figure $S_{1.}$

(*H. romeshkanicus*) (0.000). Apart from this case, the lowest genetic distance was found between clades B_1 (*H. romeshkanicus*) and B_2 (0.026). The highest genetic distance was found between clades B_1 (*H. romeshkanicus*) and A as well as *H. kurdicus* and A (0.097). The most genetically divergent group was clade A, being sister to all other *H. persicus* clades. Haplotype network analyses revealed 18 haplotypes including five haplotype networks and three unique haplotypes recovered by TCS. Specimens of *H. kurdicus* with *H. romeshkanicus* formed the same haplotype (Fig. 3).

Principal Coordinate Analysis (PCoA) distinguished six groups of individuals along discriminate axes 1 and 2, which accounted for 61.91 % and 25.26 % of the genetic variation, respectively (Fig. 4). Along the first axis, clade A, separated from clades *H. romeshkanicus*, B_2 and C_1, while the second axis resolved the other clades, but clade C_2 and C_3 are very close to each other. Genetic distances were positively correlated with geographic distances among six population (Rxy=0.003). The AMOVA analyses revealed that more genetic variation within populations (60%) was observed than among the six populations of *H. persicus* (40%). The largest PhiPT value was between clades A and C_1 (0.666), with the smallest value between clades B_1 and B_2 (-0.044). Clade B_1 represents *H. romeshkanicus*. The PhiPT distances between clades B_1 (*H. romeshkanicus*) and B_2 (-0.044), and between clades C_1 and C_2 (0.138) were not statistically significant.

There was no significant sexual dimorphism in *H. persicus*, excluding the number of preanal pores, which are only present in males. Morphology divided the individuals of *H. persicus* and *H. romeshkanicus* into six groups according to the clades in the 12S rRNA topology. Morphological character summaries are shown in Table 4. CVA analyses of meristic and morphometric characters showed that clades A and *H. romeshkanicus* are fully differentiated from other groups, clades C_1 and C_2 are distinct from other groups, and clades C_3 and B_2 overlap with each other (Table 3, Fig. 5). The holotype of *H. romeshkanicus* falls within clade B_1, hence forth the *H. romeshkanicus* clade. Of thirty-one studied variables, SED/SVL, IO2/SVL and EEd/SVL had the highest CV1 and CV2 loadings (Table 5).

According to Vasconcelos & Carranza (2014), uncorrected genetic distances of up to 5.7% in 12S rRNA are considered to reflect high levels of genetic differentiation between different populations of *Hemidactylus* species. Interestingly, *H. kurdicus* shares the same haplotype with specimens of clade B_1 (*H. romeshkanicus*) (without genetic distance, 0.000). There is also little genetic differentiation between clade B_2 from Khuzestan and Ilam provinces and clade B_1 (*H. romeshkanicus*) from Lorstan province and Sulaimani, north-eastern Iraq, suggesting that these clades represent the same species at however high mitochondrial level of variation. Generally, *H. kurdicus* is not a distinct evolutionary lineage and synonymous with *H. romeshkanicus*, which has been described first by Torki et al. (2011). Taken together, five clades with significant genetic distances and eighteen different haplotypes are found within *H. persicus* of Iran, with *H. romeshkanicus* forming a distinct clade with a unique haplotype. However, unique haplotype networks according to defined clades probably imply the presence of isolated populations without gene flow. In addition, six haplotypes occur in clade C_3 that include all individuals from Fars Province, with the exception of specimens from mountainous areas in the north, which are included in clade C_2, and lowland regions in southern Fars which are assigned to clade C_1. With respect to different geographical conditions, three clades of *H. persicus* exist in Fars Province that show high genetic variation and most likely long-term

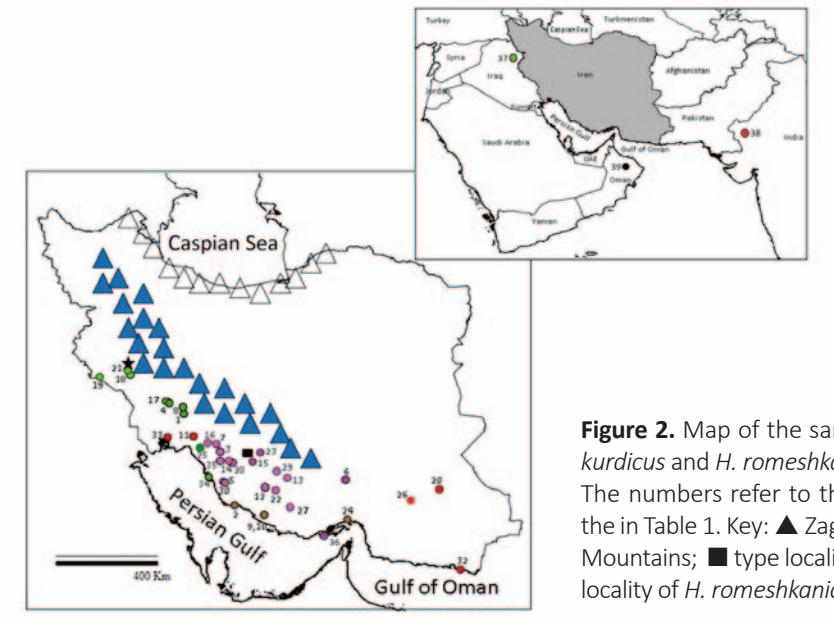

Figure 2. Map of the samples of *H. persicus*, *H. kurdicus* and *H. romeshkanicus* used in this study. The numbers refer to the samples indicated in the in Table 1. Key: ▲ Zagros Mountains; Δ Elburz Mountains; ■ type locality of *H. persicus*; ★ type locality of *H. romeshkanicus*.

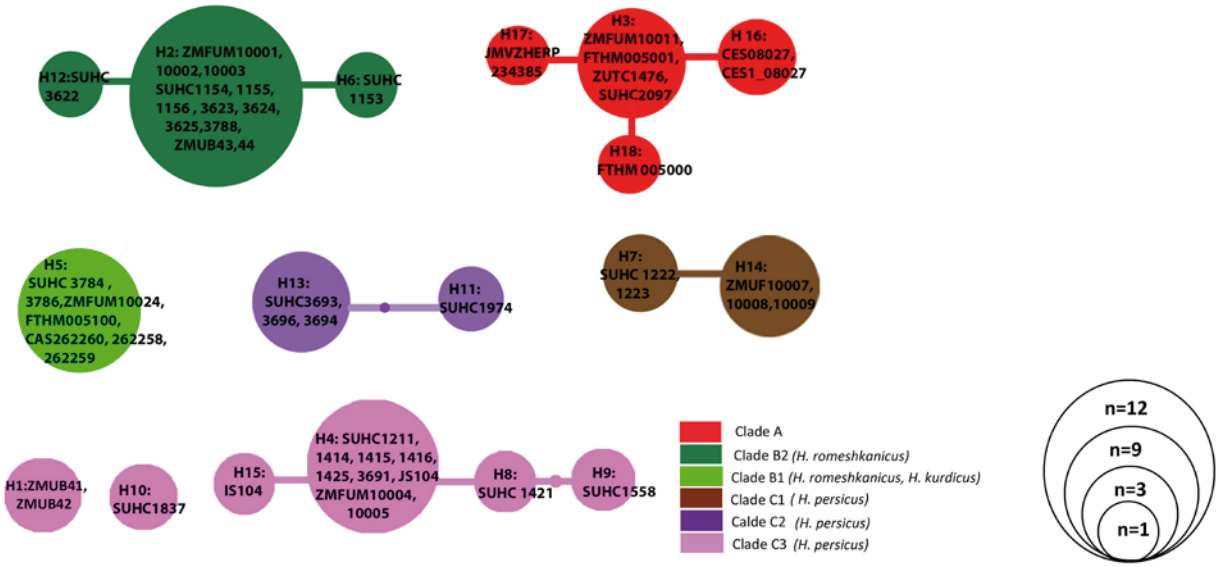

Figure 3. Haplotype networks constructed with statistical parsimony based on 399 bp of the mitochondrial 12S ribosomal RNA gene of *H. persicus*, *H. kurdicus* and *H. romeshkanicus* (50 individuals). Each circle represents one haplotype; size of circles is proportional to haplotype frequency.

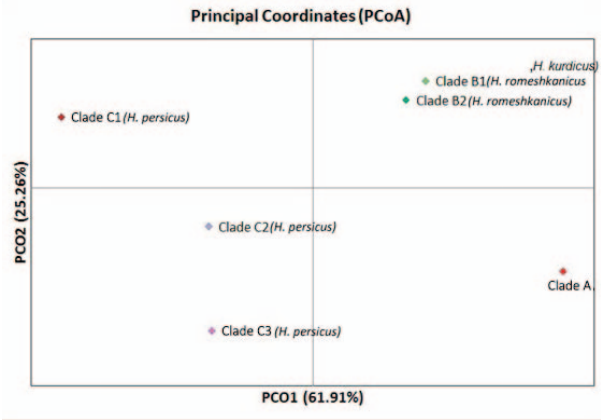

Figure 4. Principal coordinates analysis of five populations of *H. persicus*, *H. kurdicus* and *H. romeshkanicus*.

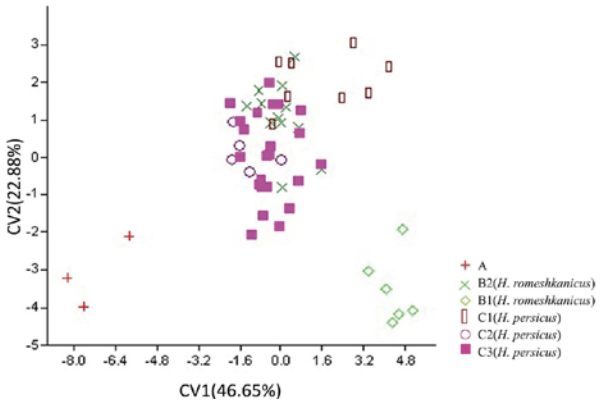

Figure 5. Multivariate morphological analyses of the five populations of *H. persicus* and *H. romeshkanicus*.

isolation. Morphologically, many external features of *Hemidactylus* species appear quite plastic, often varying within and between species (Carranza & Arnold, 2006). As a result, morphological characters may not be able to differentiate populations. Genetic distances suggest that clade C is characterised by deep interspecific variation between three main local populations from southern Iran (Bushehr and southern Fars Province; Clade C_1), central Iran (Kerman and northern Fars Province; Clade C_2), and southern Iran (central and eastern Fars Province; south-eastern Khuzestan; Clade C_3). Research is currently ongoing to clarify the phylogenetic relationships of *H. persicus* complex with more mitochondrial and nuclear genes.

The results derived from PCoA are compatible with our phylogenetic tree and morphological analyses. The individuals of clades C_2 and C_3 are closer in PCoA, PhiPT and morphological analyses, whereas in the phylogenetic tree clades C_3 and C_1 and clades C_1 and C_2 are closer than clades C_2 and C_3. The inconsistent results of clades C_2 and C_3 might be related to short geographic distances between the two clades which influence the PCoA, PhiPT and morphological analyses. The Mantel test showed a significant correlation with geographic and genetic distance, indicating that populations of *H. persicus* show a pattern of isolation by distance, which is usually explained by gene flow (Rousset, 1997). According to Šmíd et al. (2013), the long presence of *H. persicus* in Iran has resulted in high levels of intraspecific differentiation within the Iranian populations. Iran has two main mountain ranges that have played a significant role in the distribution, isolation and separation of reptile species; the Elburz Mountains that run from north-west to north-east and the Zagros Mountains that range from north-western to south-eastern Iran (Fisher, 1968; Macey et al., 1998). The formation of the Zagros Mountains began by the collision of the Arabian lithospheric plate moving in a north-easterly direction with the Eurasian landmass, which took place from the Oligocene to the Miocene 35 – 20 million years ago (Ma) (Mouthereau, 2011). According to Šmíd et al. (2013), the oldest reported dispersal of *Hemidactylus* from Arabia onto the Iranian Plateau occurred 13.1 Ma when the ancestor of *H. persicus* colonised Iran. The closest relatives of the Iranian *H. persicus* are found in UAE and northern Oman including *H. luqueorum* and *H. hajarensis* which are sister taxa of *H. persicus*. Dispersal therefore occurred most probably via the Gomphotherium land bridge connecting the Arabian and Anatolian plates approximately 18 Ma (Gardner 2009; Šmíd et al., 2013).

Geological events have led to the formation of different habitats and climatic conditions, separating the mountain regions from the Mesopotamian lowland populations and undoubtedly influencing the radiation, isolation, and differentiation of the Iranian herpetofauna (Wischuf & Fritz, 1996; Hrbek & Meyer, 2003; Feldman & Parham 2004; Rastegar-Pouyani et al., 2010). It seems likely that the ancestor of *H. persicus* penetrated the Iranian plateau from the south-west (basic dichotomy on the tree; clade A) and then dispersed to the more eastern parts (Gardner, 2009; Šmíd et al., 2013). Two samples from India grouped with individuals of clade A, suggesting an eastward distribution from south-western Iran to India. The seven samples from Šmíd et al. (2013) are dispersed in our phylogenetic tree, including three samples from Brooki (Fars Province, Iran) that are located in clade C_3; one sample from Bushehr that is located in the *H. romeshkanicus* clade; three samples including one from Lipar village (Sistan and Baluchistan Province, Iran) and two others from Mahshar (extreme south-western Iran) are placed in clade A. The latter three samples

Table 2. The mensural and meristic characters used in this study.

Characters	Definition
SVL	Maximum snout to vent length (from tip of snout to cloacal aperture)
HW	Head width (at the widest point of head)
HH	Head height (from occiput to underside of jaws)
HL	Head length(from tip of snout to the reteroarticular process of jaw)
CL	caudal length (from posterior edge of cloaca to tip of tail)
IO1	anterior interorbital distance (distance between left and right supracilary scale rows at anteriormost point of eyes)
IO2	posterior interorbital distance (distance between left and right supracilary scale rows at posterior-most point of eyes)
SL	supralabial scales (right)
IL	Infralabial scales (right)
4th SC	Scansors under 4th toe(Counts the sub digital lamellae in a single row of scales from the base of toe to the tip of the 4th toe)
1st SC	Scansors under 1st toe (Counts the sub digital lamellae in a single row of scales from the base of toe to the tip of the 1st toe)
OD	Orbital diameter (from greatest diameter of orbit)
EED	Eye to ear distance (from anterior edge of ear opening to posterior corner of eye)
SED	Snout to eye distance (from anterior point of eye to tip of snout)
DS	No. of dorsal scales (Counts the mid-way scales between the fore and hind limbs)
VS	No. of ventral scales (Counts the transverse row across the belly that includes the greatest number)
HLS	HL/SVL
HWS	HW/SVL
HHS	HH/SVL
OS	OD/SVL
O1S	IO1/SVL
O2S	102/SVL
ES	EED/SVL
SS	SED/SVL
HWH	HW/HL
HHH	HH/HL
HWHH	HW/HH
OH	OD/HL
EH	EED/HL
SH	SED/HL
O1H	IO1/HL

Table 3. Average uncorrected genetic distances (p-distance) between and within individual clades of *H. persicus*, *H. kurdicus* and *H. romeshkanicus* from the Iranian plateau based on 399 bp fragment of 12SrRNA.

Population	*H. kurdicus*	Clade B_2	Clade C_3	Clade A	Clade C_1	Clade C_2	Clade B_1	Within clades
H. kurdicus								0.000
Clade B_2	0.026							0.001
Clade C_3	0.088	0.085						0.008
Clade A	0.097	0.094	0.065					0.003
Clade C_1	0.076	0.080	0.044	0.054				0.002
Clade C_2	0.090	0.086	0.054	0.076	0.041			0.004
Clade B_1	0.000	0.026	0.088	0.097	0.076	0.090	0.000	0.000

Table 4. Descriptive parameters of 25 metric and six meristic characters including maximum, minimum, mean, and standard error in the studied clades of *H. persicus* and *H. romeshkanicus*.

Population	Clade A (n=3)		Clade B_2 (n=13)		Clade B_1 (n=6)	
Characters	Mean ± std. Error	Range	Mean ± std. Error	Range	Mean ± std. Error	Range
SVL	59.02±7.21	51.81-66.23	60.97±2.93	42.31-72.73	64.48±2.81	55.65-73.06
HW	12.23±1.54	10.69-13.78	11.96±0.64	7.72-14.06	13.01±0.44	12.05-14.49
HH	6.55±1.04	5.51-7.59	5.88±0.34	3.88-7.43	6.94±0.63	4.83-9.38
HL	15.93±0.73	15.20-16.66	17.8±0.80	12.95-20.73	19.63±0.89	16.42-22.47
CL	-	-	69.22±4.84	53.07-81.05	86.00	86.00-86.00
IO1	4.97±0.26	4.71-5.23	4.49±0.30	2.68-5.65	4.81±0.23	3.89-5.53
IO2	6.68±0.82	5.86-7.51	6.69±0.49	3.21-8.30	7.75±0.34	6.83-8.73
SL	10	10-10.00	11.09±0.16	10.00-12.00	12.66±0.66	11.00-15.00
IL	8.5±0.5	8.00-9.00	8.54±0.2	8.00-10.00	9.66±0.49	8.00-11.00
OD	3.55±0.62	2.93-4.18	4.04±0.31	2.54-6.49	4.51±0.23	3.68-5.36
EED	4.92±0.73	4.19-5.65	4.72±0.23	3.00-5.48	4.82±0.29	3.97-6.00
SED	6.57±0.83	5.74-7.40	6.72±0.33	5.14-8.52	6.98±0.51	4.98-8.66
DS	43±1	42.00-44.00	43.1±2.01	32.00-50.00	45.60±4.54	32.00-60.00
VS	43.5±7.5	36.00-51.00	43.3±2.28	27.00-53.00	40.00±1.84	34.00-46.00
1st SC	6.5±1.5	5.00-8.00	8.63±0.36	6.00-10.00	9.66±0.33	9.00-11.00
4th SC	12±2	10.00-14.00	12.45±0.15	12.00-13.00	13.00±0.25	12.00-14.00
CL/SVL	-	-	1.16±0.03	0.97-1.25	1.2113±0	1.21-1.21
HL/SVL	0.27±0.02	0.25-0.29	0.29±0.004	0.27-0.32	0.30±0.003	0.30-0.32
HW/SVL	0.20±0.0008	0.21-0.21	0.19±0.006	0.14-0.21	0.20±0.006	0.18-0.22
HH/SVL	0.11±0.004	0.11-0.11	0.09±0.003	0.07-0.11	0.10±0.008	0.07-0.13
OD/SVL	0.05±0.003	0.06-0.06	0.06±0.005	0.06-0.12	0.06±0.001	0.07-0.07
IO1/SVL	0.08±0.005	0.08-0.09	0.07±0.002	0.06-0.09	0.07±0.003	0.07-0.09
IO2/SVL	0.11±0.0001	0.11-0.11	0.1±0.004	0.08-0.13	0.12±0.002	0.11-0.13
EED/SVL	0.08±0.002	0.08-0.09	0.07±0.001	0.07-0.09	0.07±0.003	0.06-0.09
SED/SVL	0.11±0.0004	0.11-0.11	0.11±0.005	0.09-0.16	0.10±0.008	0.07-0.13
HW/HL	0.76±0.061	0.70-0.83	0.67±0.01	0.58-0.76	0.66±0.01	0.61-0.74
HH/HL	0.40±0.046	0.36-0.46	0.33±0.01	0.27-0.41	0.35±0.02	0.24-0.43
HW/HH	1.87±0.06	1.82-1.94	2.04±0.06	1.80-2.60	1.93±0.13	1.54-2.49
OD/HL	0.22±0.02	0.19-0.25	0.22±0.01	0.20-0.41	0.22±0.005	0.21-0.25
EED/HL	0.30±0.03	0.28-0.34	0.26±0.006	0.23-0.30	0.24±0.01	0.21-0.28
SED/HL	0.41±0.03	0.38-0.44	0.37±0.01	0.34-0.54	0.35±0.02	0.22-0.41
IO1/HL	0.31±0.002	0.31-0.31	0.25±0.01	0.21-0.31	0.24±0.01	0.21-0.30
IO2/HL	0.41±0.03	0.39-0.45	0.37±0.02	0.25-0.50	0.39±0.01	0.37-0.45

Population	Clade C_1 (n=8)		Clade C_2 (n=5)		Clade C_3 (n=23)	
Characters	Mean ± std. Error	Range	Mean ± std. Error	Range	Mean ± std. Error	Range
SVL	54.63±1.37	50.01-61.06	49.38±4.95	35.72-62.35	59.67±1.15	51.40-68.63
HW	10.63±0.22	9.70-11.68	9.45±0.94	6.78-11.80	11.72±0.25	9.75-14.27
HH	4.96±0.21	4.09-5.70	4.10±0.51	2.65-5.53	5.79±0.20	4.17-7.35
HL	16.15±0.41	14.56-17.76	14.95±1.07	11.77-17.63	17.33±0.34	14.00-19.90
CL	57.74±4.68	44.87-65.75	48.5±5.29	43.21-53.80	71.25±4.05	46.73-90.14
IO1	4.22±0.14	3.72-5.01	3.92±0.33	3.15-5.03	4.36±0.09	3.76-5.15
IO2	6.03±0.23	5.13-6.98	5.53±0.82	3.20-7.90	6.33±0.19	4.79-8.05
SL	11.50±0.32	10.00-13.00	11.40±0.50	10.00-13.00	11.57±0.28	9.00-15.00
IL	9.00±0.32	8.00-11.00	9.0±0.00	9.00-9.00	8.95±0.17	8.00-11.00
OD	3.39±0.14	3.01-4.08	3.37±0.18	2.77-3.75	3.76±0.11	2.60-4.72
EED	4.20±0.14	3.70-5.07	3.65±0.37	2.55-4.58	4.45±0.12	3.58-6.03
SED	5.81±0.12	5.25-6.42	5.64±0.43	4.25-6.82	6.38±0.13	5.41-7.63
DS	41.87±2.07	33.00-48.00	38.2±2.47	30.00-45.00	46.41±2.26	35.00-78.00
VS	42.87±0.91	40.00-47.00	40.40±1.69	36.00-46.00	43.40±1.17	31.00-53.00
1st SC	8.75±0.45	7.00-11.00	9.20±0.20	9.00-10.00	8.19±0.11	7.00-9.00
4th SC	13.25±0.25	12.00-14.00	12.6±0.50	11.00-14.00	11.90±0.15	11.00-13.00
CL/SVL	1.06±0.07	0.86-1.18	1.27±0.06	1.21-1.33	1.159±0.05	0.83-1.35
HL/SVL	0.29±0.003	0.28-0.31	0.30±.0009	0.28-0.33	0.29±0.003	0.27-0.33
HW/SVL	0.19±0.002	0.18-0.20	0.20±0.011	0.18-0.24	0.19±0.004	0.12-0.21
HH/SVL	0.09±0.003	0.08-0.10	0.08±0.006	0.07-0.11	0.09±0.003	0.08-0.12
OD/SVL	0.06±0.001	0.06-0.07	0.06±0.003	0.06-0.08	0.06±0.001	0.05-0.07
IO1/SVL	0.07±0.003	0.07-0.10	0.08±0.003	0.07-0.09	0.07±0.001	0.06-0.09
IO2/SVL	0.11±0.003	0.09-0.12	0.11±0.009	0.08-0.13	0.10±0.002	0.08-0.13
EED/SVL	0.07±0.002	0.07-0.09	0.07±0.0007	0.07-0.08	0.07±0.001	0.06-0.09
SED/SVL	0.10±0.001	0.10-0.11	0.11±0.003	0.11-0.13	0.10±0.004	0.10-0.12
HW/HL	0.65±0.01	0.60-0.69	0.62±0.02	0.58-0.69	0.67±0.009	0.57-0.77
HH/HL	0.30±0.009	0.26-0.33	0.27±0.02	0.23-0.35	0.33±0.01	0.25-0.41
HW/HH	2.16±0.08	1.89-2.58	2.34±0.11	1.96-2.56	2.05±0.06	1.63-2.61
OD/HL	0.20±0.005	0.19-0.24	0.22±0.004	0.21-0.24	0.21±0.003	0.18-0.24
EED/HL	0.26±0.007	0.23-0.29	0.24±0.007	0.22-0.26	0.25±0.006	0.20-0.31
SED/HL	0.36±0.006	0.33-0.39	0.37±0.006	0.36-0.40	0.35±0.01	0.03-0.41
IO1/HL	0.26±0.01	0.23-0.32	0.26±0.009	0.23-0.29	0.25±0.005	0.21-0.29
IO2/HL	0.37±0.01	0.31-0.40	0.36±0.03	0.24-0.45	0.36±0.009	0.29-0.47

Table 5. Factor loadings of canonical variate analysis (CVA) of 31 morphological characters for the 58 specimens of *H. persicus* and *H. romeshkanicus*.

Characters	CV_1	CV_2
SVL	39.705	26.865
HW	29.252	18.666
HH	41.437	28.425
HL	45.242	30.248
IO1	41.148	44.035
IO2	41.194	16.873
OD	54.858	11.709
EED	43.834	40.644
SED	32.829	19.96
HL/SVL	-772.13	-107.82
HW/SVL	-5.962	-7.9426
HH/SVL	-308.6	-118.62
OD/SVL	-931.89	-1818.7
IO1/SVL	-279.09	1072.1
IO2/SVL	1699.7	18.347
EED/SVL	-1404.8	701.88
SED/SVL	2778.4	579.07
HW/HL	251.45	206.15
HH/HL	93.773	-82.777
HW/HH	34.986	9.873
OD/HL	96.726	811.84
EED/HL	387.34	-409.82
SED/HL	-654.89	-34.759
IO1/HL	104.97	-578.45
IO2/HL	-459.2	183.4
SL	18.386	-4.128
IL	-12.804	-4.4937
DS	1.2861	7.2884
VS	7.6616	-0.19007
1st SC	14.834	-2.5593
4th SC	38.408	21.415
Eigenvalue	5.853	2.871
Accumulated percentage of variability	46.65	22.88

of *H. persicus* have a basal position in the phylogenetic tree relative to the other samples. The topology and phylogenetic positions of the Persian gecko of Šmíd et al., (2013) is consistent with their position in our data.

The results support the validity of *H. romeshkanicus* using morphological and molecular data. Interestingly, our phylogenetic inference revealed that *H. kurdicus* shares haplotypes with *H. romeshkanicus*. The new reported species is not representing a distinct evolutionary lineage and is synonymous with *H. romeshkanicus*. Hence, *H. romeshkanicus* is no longer endemic to Iran, expanding the distribution from the type locality (Iran, south of Lorestan, Romeshkan, Pole-e-Dokhtar) and the Khuzestan and Ilam provinces (all locations of specimens from clade B_2) to Iraq (south-western Sulaimani, Kurdistan region). Furthermore, the habitat of the two species is identical, representing by in oak woodlands of the Zagros forest steppe on western slopes separated only by the political border (Torki et al., 2011; Safaei-Mahroo et al., 2017). Hence, distribution of the species might be extended to central Iraq. Probably, the species is synonymous with previous described species of Iraq, *Hemidactylus bornmuelleri* Werner, 1895 that has been considered a synonym of *H. persicus* by Smith 1935. However, there is a need to collect specimens of Persian gecko from different regions of Iraq for final conclusion. These findings highlighted deep mitochondrial and morphological variations between different populations of the Persian gecko in Iran. Eventually, three definite species based on the molecular clades could be recognised: clade C corresponds to type locality of *H. persicus* and we therefore use the name *H. persicus* for this clade; clade B with the name *H. romeshkanicus*; and clade A, which might further represent a new cryptic species. Describing new species and studying variation in all populations is ongoing with additional loci for shedding more light on the clades of *H. persicus* in a further study.

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Corticosterone measurement in Komodo dragon shed skin

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The analysis of corticosterone (CORT), the main glucocorticoid in reptiles, via blood or faeces provides an index of hormone concentrations over a relatively short time period. Unlike these conventional matrices, snake shed skin is supposed to incorporate circulating CORT over the period of skin growth, thus reflecting long-term retrospective levels of the hormone. The present study aimed to assess the feasibility to extract CORT from shed skin of Komodo dragon and biochemically validate the quantification of the hormone by enzyme immunoassay (EIA). Additionally, possible sources of variation in shed skin CORT that could reflect biological variation were examined (sex, age, body region and season of ecdysis). Results of the biochemical validation showed that CORT can be reliably measured in shed skin of Komodo dragon by EIA through the presented methodology. Males presented statistically higher levels of CORT than females, and when accounting for males' seasonal differences, concentrations decreased significantly from spring to summer. Juveniles showed higher CORT values than adults, however, results should be interpreted with caution since the model revealed that date of ecdysis was significantly influencing CORT levels. Besides that, concentrations of CORT were not influenced by body region. Overall, the present study demonstrates a potential biological source of variation in shed skin CORT concentrations due to sex, age and season of skin ecdysis. Combined with other indicators, detection of CORT concentrations in shed skin could allow a systematic control of Komodo dragon's physiology, offering a useful tool for zoo management and providing key data for the species conservation.

Key words: Chronic stress; Ecdysis; Glucocorticoid; Saurian

INTRODUCTION

The secretion and regulation of glucocorticoids (GCs) through the hypothalamic-pituitary-adrenal (HPA) axis is a fundamental endocrine response to stress (Moberg & Mench, 2000; Sapolsky, 1992). These hormones have glucoregulatory functions and mobilise energy reserves to re-establish homeostasis (Turner et al., 2012). Acute increases in GCs, such as after a severe storm or pursuit by a predator, can trigger a suit of physiological and behavioural changes that facilitate survival (Johnstone et al., 2012; Dantzer et al., 2014). Nevertheless, long-term repeated or prolonged activation of the HPA axis and high levels of these hormones, known as chronic stress, can have detrimental effects on reproduction, the immune system, growth and the general health condition (Moberg & Mench, 2000; Reeder & Kramer, 2005). While there are many environmental factors that can cause either acute or chronic stress responses, several intrinsic factors related to the animal's biology can also influence GC levels (Cockrem, 2013; Busch & Hayward, 2009). Documenting how these biological attributes (e.g. species, body condition, age, sex) influence the stress responses is imperative for properly interpreting how environmental challenges or anthropogenic disturbances impact on animals' physiology (Dantzer et al., 2014; Busch & Hayward, 2009).

In reptiles, most stress-related studies use blood samples to measure corticosterone (CORT) (Tokarz & Summers, 2011), the main GC in reptiles (Cockrem, 2013). However, this method has important limitations. Circulating CORT levels can vary significantly over short periods of time, such as in response to capture and handling, or due to the circadian cycle (Romero & Reed, 2005). To solve these issues, some studies have tested the use of faeces as a non-invasive matrix to evaluate CORT concentrations (Ganswindt et al., 2014; Halliday et al., 2015; Kalliokoski et al., 2012; Rittenhouse et al., 2003). Levels of CORT in faeces have been successfully correlated to circulating hormone levels (Halliday et al., 2015) and also to ethological indicators of reptile stress (Kalliokoski et al., 2012). Corticosterone levels assessed through both blood and faeces are, however, indicative of relatively short-term activity of the HPA axis. Recently, the assessment of CORT concentrations in snake shed skin has been suggested to inform on the long-term HPA axis activity in a variety of species, including African house snake, Eastern Massasauga rattlesnake, Dumeril's boa and European asp (Carbajal et al., 2014a; Berkvens et al., 2013). The advantages of this sample type over traditional ones are that collection is non-invasive, samples are comparatively simple to obtain and easy to store at ambient temperatures. The

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Figure 1. Collection of shed skin samples from a Komodo dragon. Samples were collected under protected contact using tweezers.

most significant improvement by analysing shed skin CORT concentrations is, indeed, that the window of hormone detection is expected to be extended to weeks or even months compared to the other conventional matrices (Berkvens et al., 2013). As hypothesised, CORT is deposited in the new epidermal layer during its differentiation and keratinisation, and possibly also while it is in place (Berkvens et al., 2013). Accordingly, shed skin CORT concentrations should reflect circulating CORT levels secreted at least since the previous moulting (ecdysis). Therefore, the measurement of shed skin CORT concentrations can be an excellent non-invasive method to assess long-term secretion of steroid hormones for both wild and captive animals, providing useful data for any conservation programme or for captive species management. However, as CORT can vary due to life-history differences or seasonal factors between individuals, validation of this matrix deserves more detailed evaluation.

The Komodo dragon (*Varanus komodoensis*), the world's largest living lizard (Auffenberg, 1981), endemic to five tiny islands in Eastern Indonesia (Purwandana et al., 2014). The species faces a variety of ecological and anthropogenic stressors, such as the loss of their natural habitat or the increasing human population and distribution, being classified as vulnerable by the IUCN (2014). Studies on free living Komodo dragons are mainly focused on the assessment of demographic parameters, such as population size and distribution (Jessop et al., 2007; Purwandana et al., 2014, 2016). When using captive individuals, most of the studies look at management issues, such as the breeding and husbandry of this species (Ley et al., 2006; Sunter, 2008; Trooper et al., 2004). Research on the endocrine activity can improve our understanding of animal biology since hormones affect many body tissues (Kersey & Dehnhard, 2014). Nevertheless, until the present, only one study has assessed the CORT stress response in a species of monitor lizards (Jessop et al., 2015), while the Komodo dragons' endocrinology remains largely unknown. The analysis of shed skin CORT concentrations in Komodo dragons could be of particular interest for non-invasively assessing stress-related hormones and thus gain further insights into the physiology of this species. Accordingly, the present study aimed to: (i) assess the feasibility to extract CORT from shed skin of this endangered species and biochemically validate the quantification of shed skin CORT concentrations by enzyme immunoassay (EIA); and (ii) to examine sources of variation in shed skin CORT that may reflect biological variation (sex, age, body region and season of ecdysis).

METHODS

Animals were managed following the principles and guidelines of the Ethics Committee of Parc Zoològic de Barcelona.

Animals and shed skin sampling

Shed skin samples were collected between March and October 2014 from five Komodo dragons; two adult males, one adult female and two juvenile females, housed at Barcelona Zoo (Spain). Over the period of the study, 39 shed skins were collected with an interval time of 10.56 ± 16.29 days (mean ± SD) between sheds and stored at room temperature. Shed skin was collected with tweezers during training sessions under protected contact (Fig. 1). This technique allowed the collection and identification of shed skin samples originated from three different body regions (head, body and limbs) of each individual (Fig. 1).

Hormone extraction and analysis

Extraction procedures were performed during the following two weeks after all the samples had been collected. The extraction methodology was performed using a methanol-based technique modified from Berkvens et al. (2013). Each sample was placed into a 15-mL conical tube and first washed with distilled water to remove sand or small stones adhered at the shed skin. Distilled water was added to the conical tube until samples were completely covered by the liquid and vortexed vigorously for 2 min. Samples were allowed to dry for 48 h on a paper tissue at room temperature (20 - 25 ˚C).

Afterwards, samples were washed with 70% methanol to remove possible external sources of CORT coming from blood or faeces. Once samples were completely dried, they were minced with a ball mill (MM200 type, Retsch, Germany) for 2 minutes at 25Hz. For steroid extraction, between 50 and 100 mg of each powdered sample were incubated with 6 mL of 80% methanol (Scharlab, Spain) for 18 h at 30˚C with gentle shaking (G24 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc., Edison, USA). Following extraction, samples were centrifuged at 1750 x g for 15 min and 1.5 mL of the supernatant was transferred to a new aliquot. Samples were then placed in an oven at 38˚C. Dried extracts were reconstituted with 0.2 mL of EIA buffer provided by the EIA kit. This reconstitution volume was selected following previous hormone extraction methodologies in other keratinous samples performed by our laboratory (feathers: Carbajal et al., 2014b, Monclús et al., 2017; hair: Tallo-Parra et al., 2015) and immediately stored at -20˚C until analysis.

Hormone assay & biochemical validation of the EIA

Corticosterone concentrations from shed skin extracts and all the validation tests were determined by using competitive EIA kits (#402510 Neogen® Corporation Europe, Ayr, UK). Manufacturer reported cross-reactivity as follows: Deoxycorticosterone = 38%, 6-hydroxycorticosterone = 19%, progesterone = 5.1%, tetrahydrocorticosterone = 2.7%, prednisolone = 1.5%, cortisol = 1.1%, pregnenolone = 0.85%, 11-epicorticosterone = 0.78%, cortisone = 27%, 21-desoxycortisol = 0.24%, d-aldosterone = 0.13%, testosterone = 0.12%, 17 α-hydroxyprogesterone = 0.12%, prednisone = 0.10%, dexamethasone = 0.03%, cholesterol < 0.01%.

The biochemical validation of the EIA was carried out by following the criteria for an immunological validation: specificity, accuracy, precision and sensitivity (Reimers & Lamb, 1991). Extracts from 10 random samples were pooled for the assay validation. Intra-assay coefficient of variation (CV), and thus precision, was calculated by running samples by duplicate. The specificity was evaluated with the linearity of dilution, determined by using 1:1, 1:2, 1:5 and 1:10 dilutions of the pool with EIA buffer. Specificity was also evaluated with a parallelism test by comparing two different calibration curves (the standard curve and a pool curve created with serial dilutions following the standard curve pattern). To test for accuracy, different volumes of the pool were spiked with different volumes of standard steroids of known concentrations (0.22, 2.13 and 7.81 ng CORT/ml). The spike recovery was assessed measuring the final recovery of the known amounts of CORT added to the sample pools. The sensitivity of the test was given by the smallest amount of hormone concentration detected.

Statistical analysis

Data were analysed using R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) with a *P*-value below 0.05 as a criterion for significance.

For the biochemical validation, Pearson's Product Moment correlation was used to evaluate the correlation between obtained and expected values from serial dilutions and from spiked pool extracts with hormone standards. The test was also applied to calculate the relationship of the parallelism between the standards and the serially diluted pool extract.

The assumption of normality was checked using a Shapiro–Wilk test and concentrations were log-transformed to achieve normality. A linear mixed model with all data could not be performed since samples were not uniformly distributed among the independent variables of study. Accordingly, three separate linear mixed models, with Komodo subjects as a random factor to control for pseudo-replication, were used. Model 1 accounted for sex differences in shed skin CORT concentrations employing samples from adult Komodo dragons collected during spring. Model 2 tested for age effects on CORT levels from adult and juvenile females. Finally, model 3 tested for seasonal patterns in shed skin CORT concentrations from adult male samples. Shed skins from three different body regions were obtained throughout the study, therefore, this variable was included as a fixed factor in each of the three separate models. Additionally, date of ecdysis was included as covariate in each model. Selection of the best fit model was performed based upon Aikake's Information Criteria corrected for small sample size (AICc). We calculated AICc, ΔAICc (difference between each model's AICc and that of the lowest model) and Akaike weights. Candidate set models were chosen for which ΔAIC ≤ 2.

RESULTS

Biochemical validation of the EIA

The intra-assay coefficient of variation was 4.08 ± 3.35 % (mean ± S.D.). The obtained and expected corticosterone concentrations were significantly correlated (r = 0.99, P < 0.01; Fig. 2). In the spike-and-recovery test, hormone standards spiked with the pool presented a mean recovery percentage of 103.77 ± 11.82 % (mean ± S.D.) and obtained and expected values were significantly correlated (r = 0.90, P < 0.01). Corticosterone concentrations from the standard curve and the pool curve obtained in the parallelism test showed correlation (r = 0.99, P < 0.01; Fig. 3). The sensitivity of the assay was 0.22 pg CORT/mg shed skin.

Corticosterone concentration in shed skin

Corticosterone was detectable in all shed skin samples (Table 1). Shed skin CORT concentrations varied significantly between males and females (Table 2). When testing for age variances on shed skin CORT concentrations, juveniles presented significantly higher CORT levels than adult Komodo dragons although date of ecdysis showed an influence on shed skin CORT concentrations (Table 3). Season had a significant effect on CORT concentrations, with higher levels observed on skins shed during spring (Table 4). No influence of body region on shed skin CORT concentrations was detected in any of the models.

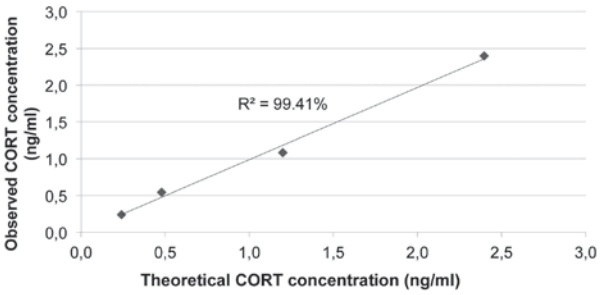

Figure 2. Correlation between observed and theoretical cortisol concentrations obtained in the dilution test (Pearson's correlation; $r = 0.99$, $P < 0.01$).

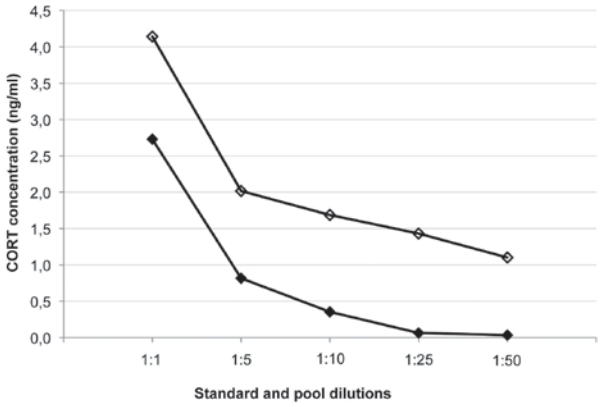

Figure 3. Parallelism relation between lines from the standard (black diamonds) and sample pool (white diamonds) curves obtained in the parallelism test (Pearson's correlation; $r = 0.99$, $P < 0.01$).

Table 1. Distribution of CORT concentrations (pg/mg) in Komodo dragon shed skin obtained for the four potential sources of variation analysed.

Variable	Level	# Samples	Mean ± S.D.	Median	Range
Sex	Female	11	11.10 ± 7.50	11.80	0.94 - 23.74
	Male	10	31.23 ± 19.70	25.78	16.9 - 76.37
Age	Adult	11	11.10 ± 7.50	11.80	0.94 - 23.74
	Juvenile	5	58.80 ± 18.50	53.66	33.73 - 78.37
Season	Spring	10	31.23 ± 19.70	25.78	16.9 - 76.37
	Summer	13	12.45 ± 7.02	11.87	4.22 - 25.58
Body	Head	10	21.87 ± 20.83	17.10	4.32 - 76.37
region	Body	14	17.87 ± 11.93	16.00	4.63 - 53.66
	Limbs	15	28.09 ± 26.16	25.58	0.94 - 78.37

DISCUSSION

Although the importance of studying threatened species endocrinology is evident, such studies are difficult to perform compared to studies on lab or domestic animals. One of the biggest challenges is that the access to samples is limited, even when using captive individuals. In addition, the invasiveness of most sampling methodologies hinders even more the study of these species physiology. In this paper we present a non-invasive and easy to obtain tool to study an emblematic threatened species which can give insight, for the first time, into their endocrine activity. Results of the current study show that CORT concentrations can be reliably measured in shed skin of Komodo dragons. To our knowledge, this is the first study reporting successful measurements of CORT levels in shed skin of any saurian. The sample processing and hormone extraction method presented here enabled the detection of CORT levels in all Komodo dragon samples. In addition, the detection of this hormone was biochemically validated, demonstrating the suitability of a commercial EIA kit in the quantification of CORT in samples processed through the stated methodology. As affirmed by Buchanan & Goldsmith (2004), this step is of primary importance when new techniques to measure steroid hormones are being developed.

In reptiles, as observed in other vertebrates, sex, age, body condition, health, season and the reproductive state can be sources of variation of the stress response (Moore & Jessop, 2003). We aimed to examine the effect of some of these factors on shed skin CORT concentrations with an attempt to explore the complex interaction between long-term levels of CORT and the physiological state of Komodo dragons. Sex, age and season of skin ecdysis had an effect on shed skin CORT concentrations. In contrast, no effect of the body region was observed.

Although sexual variation in the adrenocortical response has already been documented in different reptile species (Moore & Jessop, 2003), such information on Komodo dragons had never been addressed until the present. Here, we determined for the first time significant differences in shed skin CORT concentrations between adult males and females of Komodo dragon. Grassman & Hess (1992) also described sex differences in plasma CORT of six-lined racerunner (*Cnemidophorus sexlineatus*), detecting seasonal trends in those differences. These authors observed a decrease in circulating CORT levels from spring to summer only in males, concurring with the period of reproductive activity. To test for sex differences in the present study, only samples shed in spring could be used, hindering the detection of a potential seasonal connection with the sexual variation observed. Interestingly, we also observed seasonal differences on male shed skin CORT concentrations; levels declined significantly from spring to summer, a pattern similar to the one described in racerunners (Grasmann & Hess 1992) and in Texas horned lizards (*Phrynosoma cornutum*) (Wack et al., 2008). Reptiles can display fluctuations in circulating levels of CORT during the reproductive season (Moore & Jessop 2003), thus the decrease in male shed skin CORT concentrations could be partly influenced by the breeding period of the species. Accounting for the same seasonal fluctuations in females, in parallel with assessment of other reproductive indices, would be of interest to clarify the potential link of shed skin CORT concentrations with the reproductive activity of the species. The present study provides the first evidence that CORT levels detected on Komodo dragons shed skin could be influenced by the

Table 2. Model 1 selection and final model output for linear mixed models studying the effect of sex in SSCC of Komodo dragons.

Model	*k*	AICc	Δ AICc	wi
SSCC ~ Sex	**4**	**62.9**	**0.00**	**0.65**
SSCC ~ null	3	65.3	2.42	0.19
SSCC ~ Sex + Date	5	66.0	3.11	0.14
SSCC ~ Sex + Body region + Date	7	71.8	8.90	0.01
SSCC ~ Sex + Body region + Date + Sex*Body region	9	72.5	9.65	0.00
Parameter	**Estimate**	**S.E.**	**t (df)**	***P*-value**
Intercept	2.02	0.25	7.96 (21)	<0.01
Sex (male)	1.28	0.37	3.48 (21)	<0.01

Abbreviations: k is the number of parameters in the model; AICc is the bias-corrected Akaike's information criterion value; ΔAICc is the difference between each model and the top model; wi are the model weights.

Table 3. Model 2 selection and final model output for linear mixed models studying the effect of age in SSCC of juvenile and adult Komodo dragons.

Model	*k*	AICc	Δ AICc	wi
SSCC ~ Age + Date	**5**	**47.5**	**0.00**	**0.95**
SSCC ~ Age	4	53.7	6.19	0.043
SSCC ~ null	3	57.5	10.03	0.01
SSCC ~ Age + Body region + Date	7	59.7	11.58	0.00
SSCC ~ Age + Body region + Date + Age*Body region	9	76.9	29.35	0.00
Parameter	**Estimate**	**S.E.**	**t (df)**	***P*-value**
Intercept	2.02	0.27	7.49 (16)	<0.01
Age (juvenile)	2.01	0.49	4.13 (16)	<0.01
Date	0.06	0.02	3.86 (16)	<0.01

Abbreviations: k is the number of parameters in the model; AICc is the bias-corrected Akaike's information criterion value; ΔAICc is the difference between each model and the top model; wi are the model weights.

Table 4. Model 3 selection and final model output for linear mixed models studying the effect of the season in SSCC of Komodo dragons.

Model	*k*	AICc	Δ AICc	wi
SSCC ~ Season	**4**	**49.0**	**0.00**	**0.77**
SSCC ~ Season + Date	5	52.0	3.09	0.16
SSCC ~ null	3	54.0	5.05	0.06
SSCC ~ Season + Date + Body region	7	59.5	10.55	0.00
SSCC ~ Season + Date + Body region + Season*Body region	9	66.0	17.08	0.00
Parameter	**Estimate**	**S.E.**	**t (df)**	***P*-value**
Intercept	3.31	0.18	18.61 (23)	<0.01
Season (summer)	- 0.95	0.24	- 4.39 (23)	<0.01

Abbreviations: k is the number of parameters in the model; AICc is the bias-corrected Akaike's information criterion value; ΔAICc is the difference between each model and the top model; wi are the model weights.

season when ecdysis occurred. Results obtained here provide a further intriguing prospect for future research on the influence that reproduction can have on Komodo dragon's CORT levels.

In addition, we found that shed skin CORT concentrations differed significantly between juveniles and adult females. As reported by using short-term GC measures, baseline and stressor-induced GC levels can vary among ages (Dantzer et al., 2014; Sopinka et al., 2015). A higher metabolic rate in juvenile Komodo dragons could result in higher shed skin CORT concentrations, as previously described in other reptile species by using conventional matrices (Gregory et al., 1996; Jessop et al., 2000). Importantly, the model also detected that date of ecdysis was influencing shed skin CORT concentrations, possibly since all juvenile samples has been shed on the same day. In order to reliably confirm that juveniles differ from adults in shed skin CORT concentrations, further research should include a larger sample size shed at different periods.

Despite differences expected among body regions, we found no evidence of an effect on shed skin CORT concentrations. Previous research on snake's shed skin described higher CORT concentrations in tail sections than in the head and middle sections (Berkvens et al., 2013). Komodo dragons shed skin differs in thickness and shape of scales among body regions, but presumptively, it did not have an effect on CORT levels.

In conclusion, this paper demonstrates, for the first time, that CORT can be successfully measured in shed skin of Komodo dragon with the methodology presented above. Importantly, results demonstrate a potential biological source of variation in shed skin CORT concentrations due to the sex, age and season of skin ecdysis. The analysis of GC in shed skin may be the only method available to obtain a long-term and retrospective measurement of hormonal levels in reptiles. Combined with other indicators, detection of CORT concentrations in shed skin could allow a systematic control of Komodo dragon's physiology, offering a useful tool for zoo management and providing key data for the species conservation.

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Temporal trends in agile frog *Rana dalmatina* numbers: results from a long- term study in western France

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Reports of amphibian declines have highlighted the urgent need for long-term data sets to increase understanding of population changes. To detect population changes in the agile frog *Rana dalmatina* in Vendée, western France, counts were made of spawn masses over 16 years and road mortalities over 13 years. Long-term trends were evaluated using regression analysis of the logarithmic transforms of annual mortalities and egg masses as dependent variables against year as the independent variable. Tests of the regressions against a 0 hypothetical coefficient, indicative of population stability, gave coefficients that were positive for road mortalities and negative for spawn counts. However, neither was significantly different from 0, indicating a stable population. Further analysis using jackknifing produced a series of pseudo-regression coefficients, which agreed with the true regressions. Results from both datasets were therefore congruent and indicated wide annual fluctuations, with a major increase in numbers between 2009 and 2014. Data from spawn deposition in a recently established pond suggested that the presence of invasive crayfish *Procambarus clarkii* influenced both deposition sites and long-term population changes.

Key words: Amphibians, *Rana dalmatina*, long-term populations, spawn counts, road mortalities.

INTRODUCTION

Amphibians are widely reported as the vertebrate class most affected by the worldwide biodiversity decline (e.g. Blaustein et al., 1994; Alford & Richards, 1999; Hitchings & Beebee, 1997; Houlahan et al., 2000; Fischer, 2000; Cushman, 2006). No single contributing factor has been identified in the decline, although multifaceted interactions have been proposed (e.g. Collins & Storfer, 2003). These include time lags in density dependent responses leading to chaotic dynamics (e.g. Wilbur, 1990), climatic effects and habitat alterations by humans (e.g. Hartel, 2005; Gollmann, et al., 2002). Given the role of amphibians as both predator and prey in many ecosystems, insight into changes in their population status provides key ecological information. Current knowledge indicates frequent widespread population fluctuations with high increases in certain years, offset by moderate decreases over periods of several years (Pechmann et al., 1991; Pechmann and Wilbur 1994; Meyer et al. 1998). This suggests that short-term monitoring may introduce a degree of error when attempting to evaluate trends and hence, longer time series are required.

The primary objective of this study was to examine for changes in the numbers of agile frog *Rana dalmatina*, a species found across Europe and listed as under threat in Appendix II of the Bern Convention. It is an example of a pond-breeding amphibian that moves widely across the landscape. Population studies in several regions of Europe including Romania (Hartel, 2008a; 2008b), Sweden (Stromberg, 1995), Austria (Gollmann et al., 1998, 2002), Greece (Sofianidou et al., 1983) and France (Combes et al., 2018) have shown that numbers fluctuate widely but none showed evidence of major long-term declines. There have been few studies of *R. dalmatina* in fragmented landscapes (Wederkinch, 1988), environments that are universally implicated in the biodiversity decline (e.g. Fischer, 2000). This paper describes changes in *R. dalmatina* numbers in a fragmented habitat in western France that includes areas of extensive agriculture bisected by hedgerows, woodland and urban areas. Additionally, in the agricultural areas, fertilisers and pesticides are applied annually, including during the breeding season (Fig. 1) and have been implicated as contributing to population decline (Beebee & Griffiths, 2005).

The data are derived from two sources; counts of spawn masses and road mortalities. Egg mass counts have been used in previous studies of *R. dalmatina* and employed as proxies to estimate the size of breeding populations. As the spawn moves to the pond surface (see example in Meek, 2012b), it is relatively easy to count compared to breeding females (e.g. Pechmann & Wilbur, 1994: Grant et al., 2005) and so reduces observer error. In addition, these frogs are frequently killed on roads and in the study locality, second only to *Bufo bufo* in this respect (Meek, 2012a). However, they differ from sympatric

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Figure 1. Google Earth map of the study locality showing sampling areas for spawn masses and surrounding habitat consisting of agricultural fields, hedgerows, woodland and urban areas. Continuous lines indicate ditches adjacent to hedgerows or woodland, broken lines ditches alongside roads. New Pond and Old Pond locations are also highlighted.

amphibians in that the peak period for road mortalities is during the summer months and hence appear not to be primarily associated with migration (Meek, 2012a). Road mortalities have been used previously to estimate population changes in amphibians (e.g. Meyer et al. 1998), snakes (Capula et al., 2014; Rugiero et al., 2018), and mammals (e.g. Mallick et al., 1998; Baker et al., 2004; Widenmaier & Fahrig, 2006). Road mortalities give only an index of abundance but are independent of spawn count and avoids double counting and autocorrelation.

METHODS

The study area (46°27`N;1°53`W) is a fragmented landscape dominated by agriculture that had experienced little or no major changes in land use during the study period from 2003 to 2018. The climate is mild oceanic (June, July and August monthly mean air temperature = 26˚C; November through to February monthly mean = 10.2˚C), with a period of high precipitation usually falling from October until January (monthly mean = 85.7 mm). During the summer months of June, July, and August when rainfall is low (monthly mean = 51.3 mm) it is normal for all but the largest water bodies to dry up. Figure 1 shows a map of the area where spawn masses were counted with key areas identified.

Spawn is readily visible rising to the surface of the water within days of deposition (see examples in Meek, 2012b). Daily counts were made by a single observer each spring beginning February 2003 along the edge of ditches and ponds until the end of March 2018 after which no spawn masses were deposited. Annual peak counts were derived from the maximum number of spawn masses counted at a site in a given year. Spawn deposition was recorded in three ditches and two ponds in a woodland/wetland area on the edge of the village of Chasnais (Fig. 1). Total ditch length, using the measuring tool in Google Earth, was 1,377 m of which 1,081 m was at the roadside and 296 m next to hedgerow or woodland. New Pond (see Fig. 1) had an approximate surface area of 945 m^2 and was created during the summer/autumn of 2009, with the first spawn recorded in February 2010. The smaller pond (Old Pond) with a surface area of 64.1 m^2 had been established prior to 2003.

Data on road mortalities were collected on roads (total distance ≈16 km) between a wetland area close to the village of St Denis du Payre and the wetland next to Chasnais. The distance between the two is approximately 6 km (see Meek, 2012a for a schematic view). Surveying for road mortalities commenced in January 2005 and was undertaken between four and six times every month throughout each year until December 2017 in both study localities. Surveys were carried out by a single observer on a bicycle travelling at 5–10 km/hour. Road traffic volume increased slightly during the 13 year period from initial surveying 2005–2017 (see Meek (2012a) for traffic volumes).

Statistical analysis

Road mortalities and spawn masses were tested for departures for equality of annual counts using a *G*-test goodness-of-fit at n-1 d.f.. This gives the expected annual probability for spawn mass across 16 years of sampling as 1/16 = 0.0625 and for 13 years of road mortality data

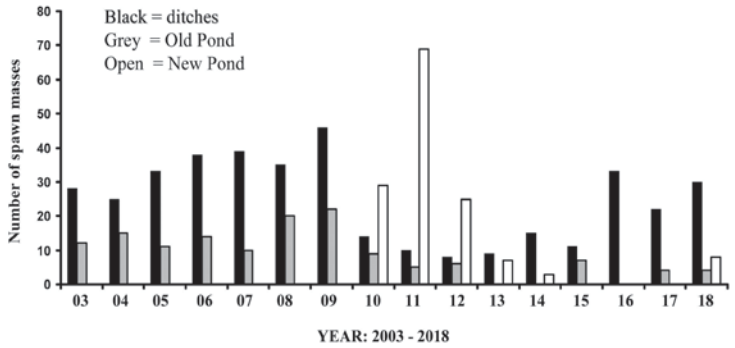

Figure 2. Changes in annual spawn counts in ditches (black bars), Old Pond (grey bars) and New Pond (open bars). See text for further details.

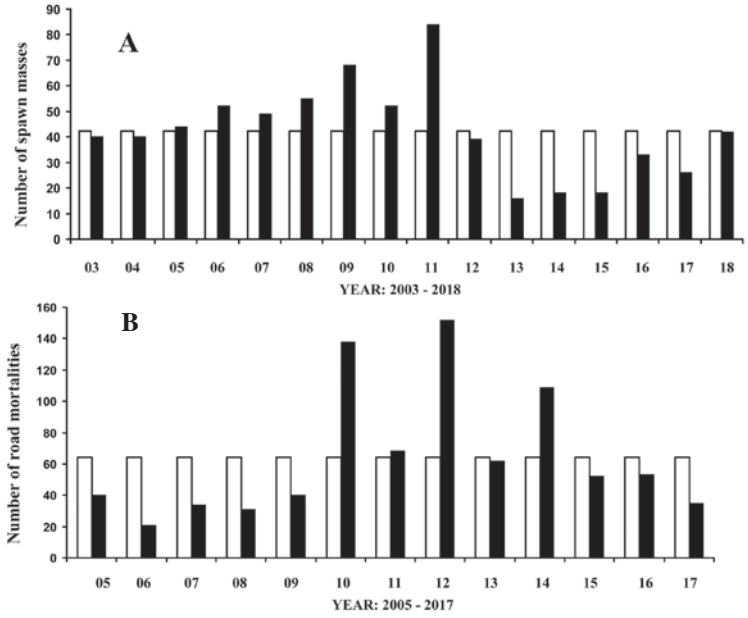

Figure 3. Histograms showing annual numbers of spawn mass **(A)** and road mortalities **(B)** as black bars. Open bars represent expected frequencies under a null hypothesis of equality of year counts. See text for further details.

as 1/13 = 0.077. The data for road mortalities were not normally distributed (Anderson-Darling; a^2 = 0.996, P = 0.008). Therefore to compare variation in annual counts of road mortalities with spawn masses, the data sets were subjected to a Leven's test for homoscedasticity. This is less sensitive to departures from normality and considers the distances of the observations from their sample medians. The test is robust for smaller samples (Box & Jenkins, 1976) and rejects equality of variance when

$$W > F\alpha_{,\ k\text{-}1,\ N\text{-}k}$$

where W is the test statistic, $F\alpha_{,\ k\text{-}1,\ N\text{-}k}$ the upper critical value of the F-distribution k-1 and N-k degrees of freedom with significance α.

To identify long-term trends in population parameters, regression analysis was applied to the logarithms of annual mortalities and spawn masses as dependent variables with year as the independent variable giving

$$\log_e N = b + m * \text{year},$$

where $\log_e N$, represents either numbers of spawn masses or road mortalities, m the regression coefficient and b the y-intercept. The null hypothesis is that $\log_e N$ is stable when m = 0; significant departures from m indicate population change. Departures from 0 were evaluated using a t-test at n-2 d.f. (Bailey, 1995). Since unusually high or low year counts may have an inordinate effect on m, a test for influence function (Gotelli & Ellison, 2004) to estimate the errors of the true regression coefficients was made using jackknifing (Sahinler & Topuz, 2007). This method has the advantage of giving exact repeatable results by systematically removing one-year data sets from the sample. Regression analysis was re-applied to produce a series of pseudo-m values that were then compared against the true coefficients.

RESULTS

A total of 836 road mortalities were found in the surrounding area between 2005 and 2017, consisting of 338 large adults and 498 smaller frogs (*mean* number per year = 64.3±42.2). Sexing adults was usually not possible

Table 1. Regression analysis of the logarithms of temporal changes in annual numbers of frog spawn masses or road mortalities as dependent variables against year as an independent variable. The regression coefficient *m* is shown with standard errors. The *t*-tests and P-values are derived from tests of the true coefficients against a hypothetical of *m* = 0, which would indicate long-term population stability. For comparison, data for *R. dalmatina* given by Stromberg (1995) and Hartel (2008b) has been subject to the same analysis. Length of the study in years is shown as n and actual numbers of spawn masses or road mortalities as Σn. See text for further details.

	m	±	*t*	p	n	source	∑n	Reference
France	- 0.048	0.023	1.98	0.07	16	Spawn counts	678	This study
France	0.03	0.04	0.87	0.40	13	Rd/mortalities	836	This study
Sweden	0.067	0.037	1.18	0.10	12	Spawn counts	1692	Stromberg (1995)
Hungary	- 0.013	0.03	0.46	0.66	11	Spawn counts	4484	Hartel (2008b)

due to carcass degradation due to the time present on roads. Snout to vent lengths ranged from 18 and 92 mm (mean = 49.7±14.1) with the distribution skewed towards smaller individuals (*s* = 0.34, a^2 = 2.19 P<0.0001). Spawn masses totalled 678 between 2003 and 2018; 397 in 3 ditches, 139 in Old Pond and 141 in New Pond; hence the majority (58.6%) were found in ditches (Fig. 2).

Annual road mortalities differed significantly from equal year counts *G* = 304.09, df =12, P<0.0001). The regression of log annual road mortalities against year gave a coefficient of *m* = 0.03±0.04, which did not differ significantly from 0 (*t* = 0.87, p = 0.40) suggesting general long-term population stability. *Mean* of the tests for pseudo-*m* was 0.03±0.038, which was in agreement with the true *m*. The test for influence function of road mortalities highlighted 2012 and 2010 as unusually high observations, 2.41 and 2.2 times greater than expected respectively (Fig. 3b). None of the pseudo-*m* values exceeded the 95% confidence interval (P-values 0.06–0.67).

Spawn mass counts differed significantly from annual regularity (*G* = 120.9 P<0.0001, d.f. = 15). Regression analysis of log spawn masses against year gave a negative coefficient -0.048±0.023 that, although not significantly different from 0, was close to the 95% confidence interval (*t* = 1.98, P = 0.07). Jackknifing produced a *mean* pseudo-*m* of 0.046±0.06 with the test for influence function indicating 2011 as unusually high (x2.0) and 2013 as unusually low (÷2.64) years (Fig. 3a). Re-analysis after first removing 2011 and then 2013, gave marginally significant results (P = 0.04 in both). This represents 12.5% of the 16 samples and suggests a possible moderate long-term decline in numbers. Variances of annual road mortalities and annual spawn mass counts were not significantly different (*W* = 3.3, p = 0.08).

DISCUSSION

Road mortality and egg mass counts showed similar levels of population fluctuation and long-term trends. Spawn mass counts were closer to the 95% interval in the regression and might suggest moderate population decline but the jackknife analysis supported the true regression. Changes in population levels indicating decline in amphibians have been misinterpreted in earlier studies, especially when data sets are gathered over limited time periods and populations fluctuate widely (Pechmann et al., 1991). For example, Pechmann & Wilbur (1994) suggested that amphibian populations are likely to be in decline most of the time, followed by short increases after a period of high recruitment (but see Alford & Richards, 1999). This agrees with the general pattern of change found here and with *R. dalmatina* in other areas where wide annual variation appears to be the norm, including in France (e.g. Combes et al., 2018). Application of coefficients of variation (Cv = δ/*mean*, where δ is the standard error; Scherer & Tracey, 2011) to other *R. dalmatina* time series gave 0.5 for Sweden (Stromberg, 1995) and 0.29 for populations in Romania (Hartel, 2008b). This compares to 0.46 for egg mass counts and 0.64 for road mortalities in the present study indicating similar levels of fluctuation and long-term trends (Table 1). Values ranging from 0.29 to 1.29 have been found in North American time series for anuran egg masses cited in Scherer & Tracey (2011).

Breeding phenology showed little change during the study period and Combes et al. (2018) found no effect of precipitation and temperature on egg-clutch abundance during the active period leading up to reproduction. However, climate data from the weather station at La Rochelle-Le Bout Blanc (around 25 km from the study locality) indicated rainfall from 2009 – 2011 was higher than average, which is in good agreement with high frog numbers from 2009–2014. The subsequent years of a general decline in frog numbers could involve these high densities, since increases in intra-specific competition in larvae may impact on metamorphosis (Scott, 1990; Scott 1994). Periods of extreme cold can increase embryo mortality by encasing floating egg masses in ice. This was observed during February 2012 (Meek, 2012b) and 2018. Estimates of larval mortalities in two spawn masses in a period of freezing during 2012 that lasted for several weeks indicated survivorship of 2.7 and 3.4% (Meek, 2012b) but inspection of several ice-impacted spawn during a 7 day freezing in 2018 indicated fewer eggs were affected, especially those positioned lowest in the egg mass.

Frequent cohort failure in amphibians is often connected to pond hydroperiod and cited as an underlying cause of population fluctuation (Beebee & Griffiths, 2005; Denoel & Ficetola, 2008). Extensive dry periods with low precipitation, especially during

February, may impact on the survival of *R. dalmatina* larvae through ditch drying. This has been observed in other *R. dalmatina* populations (Hartel 2005; 2008b). Throughout the study period water was present in the ditches until around June /July but from 2016 through to 2017, low precipitation resulted in early ditch drying. Additionally, topsoil from agricultural fields (see Fig. 1) drifting into ditches during dry summers and bank burrowing activities of Coypu (*Myocastor coypus*) result in ditch silting and early drying. Larval survival was more likely affected by hydroperiod in ditches than in deeper ponds where reasonable levels were usually present later into summer.

In other areas of France, introduced predators have been cited as partly influencing the reproduction dynamics of *R. dalmatina* (Combes et al., 2018), which is in agreement with the present study. Spawn mass number decline in New Pond were likely due to the presence of alien crayfish *Procambarus clarkii*, which is known to impact on adult and especially the larvae of *R. dalmatina* and other amphibians (Ficetola et al., 2011). The decline in spawn mass numbers began 2012 and continued to 2015 when they reached zero. This trend coincided with first sightings of *P. clarkii* in New Pond during 2012 with numbers peaking in 2015. However, breeding female *R. dalmatina* returned in small numbers in spring 2018 (see Fig. 3) coinciding with *P. clarkii* absence from at least 2017. The return of algae and some macrophytes, which are heavily grazed by *P. clarkii* and mostly not present during peak numbers, suggests the decline/absence was real. Although its natural dispersal capability has been cited as low (e.g. Geiger et al., 2005), *P. clarkii* fulfils the criteria of Article 4(3) of Regulation (EU) No 1143/2014 of the European Parliament—a species wide spread in Europe and impossible to eradicate in a cost-effective manner (Souty-Grosset et al., 2016). However, an adaptive trait in *R. dalmatina* may be wide foraging behaviour (up to 1,100 m from the egg deposition site; Blab, 1986) and lack of site fidelity (Waringer-Loschenkohl, 1991; Gollmann et al., 2002; Ficetola et al., 2006) enabling breeding site selection and avoidance of *P. clarkii*. Five out of nine European species of anurans showed behaviour changes in response to *P. clarkii* presence, signalled by chemical cues from predated or injured conspecifics (Nunes et al., 2013). Removal of alien species, for example introduced goldfish (*Carassius auratus*), successfully restored *Triturus carnifex* populations in Central Italy (Mori et al., 2016) and trout removal enabled rapid recovery of *Rana muscosa* in North America (Vredenberg, 2004).

A potential weakness in any long-term study of amphibian populations concerns funding limitations and time factors involved in field work. There is also the question of whether or not the study populations form a series of meta-populations with boundaries that are difficult to delimit. Wide foraging and lack of breeding site fidelity suggests that individual *R. dalmatina* may reproduce in areas outside the study locality in certain years. However, the data for road mortalities were collected up to 6 km from the spawning area and when split into an approximate 50/50 east–west geographic, were congruent in both fluctuations and long-term trends. Hence, breeding aggregations may have some continuity and are valuable as sampling sites. Continual monitoring of spawn mass and road mortality in addition to collecting potentially important environmental/ climatic data alongside monitoring to try and identify population drivers remains a useful tool in monitoring population trends (Temple & Cox, 2009).

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Effects of chronic corticosterone increases on the maternal behaviour of the prairie skink, *Plestiodon septentrionalis*

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Maternal care behaviour is rare in reptiles and the hormonal control of this behaviour is less well known than in other vertebrates. The steroid hormone, corticosterone, suppresses maternal behaviour in avian species. We investigate whether corticosterone similarly affects maternal behaviour of a lizard. We artificially elevated corticosterone in female prairie lizards, *Plestiodon septentrionalis*, during egg brooding and assessed effects on maternal behaviour (versus females receiving a vehicle control). The application of exogenous corticosterone significantly decreased the amount of time that females spent in contact with their eggs. These results suggest that, as in birds, corticosterone acts to reduce maternal behaviours in reptiles. This provides important insight into the hormonal control of, and effects of stress on, parental care in reptiles.

Key words: Brooding, stress, eggs, hormone, lizard, parental care, reptile

INTRODUCTION

Parental behaviour in reptiles appears to be relatively rare but does exist. Parental care is widespread in crocodilians (Somma, 2003) and, although there have been relatively few studies of parental care in squamates, there is evidence of nest defence in skinks (Huang & Pike, 2011a,b), shivering thermogenesis in pythons (Aubret et al., 2005) and the suggestion that skinks and pythons actively maintain appropriate moisture levels within their nest (Somma & Fawcett, 1989; Lourdais et al., 2007). Females of some viperid (Hoss & Clark, 2014; Greene et al., 2002) and scincid species (Langkilde et al., 2007; O'Connor & Shine 2004) also remain associated with their young and provide them care and protection. While these studies have advanced our knowledge of reptilian parental behaviours, there are few studies addressing the hormonal control of this behaviour (but see Hoss et al., 2014; Lind et al., 2017).

There has been a great deal of research on the hormonal control of parental behaviour in birds, where parental care is nearly universal. It is generally accepted that prolactin (PRL) induces parental behaviour in birds (Buntin et al., 1991) and recent studies have revealed that PRL can interact with the stress hormone corticosterone (CORT), suggesting a possible role of CORT in parental behaviour. Specifically, increased CORT can cause a decrease in PRL, which leads to decreased parental effort and in extreme cases, nest abandonment (Chastel & Lormee, 2005; Chastel et al., 2005; Groscolas et al., 2008; Angelier et al., 2009). Reptiles are closely related to birds and so CORT may similarly affect parental care in reptiles.

We test the effect of CORT on maternal care behaviour in the Northern Prairie Skink, *Plestiodon septentrionalis*, a small lizard (70 mm SVL) that occurs from Kansas to Canada. *Plestiodon septentrionalis* and closely related species are known to exhibit parental care in the form of egg brooding (Breckenridge, 1943; Noble & Mason, 1993; Somma, 2003). During egg brooding, the female remains with the eggs until hatching, exhibiting near constant nest attendance (Breckenridge, 1943). During this time, the female may reposition eggs within the nest, retrieve displaced eggs, or coil her body around the eggs (Table 1). The female may even consume eggs that become infected with fungus in order to prevent the infection spreading to the other eggs (Somma, 1989). We experimentally elevated plasma CORT of female *P. septentrionalis* twice daily during brooding and quantified effects on maternal brooding behaviour.

MATERIALS AND METHODS

Study species

Eight female and eight male skinks were captured from Pawnee and Douglas Counties, Nebraska in April and May 2012. Reproductive maturity was determined by orange mating colouration on the neck in males and SVL above 60 mm in females (Ballinger et al., 2010). All skinks were returned to the laboratory at the University of Nebraska

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Table 1. Descriptions of maternal brooding behaviours recorded in this study (from Somma & Fawcett, 1989)

Name of behaviour	Description
Positional Behaviours	
Contact	Female positions herself in contact with the eggs.
Coiling	Female tightly coils around eggs.
No Contact	Female is under the shelter but is not in contact with the eggs.
Not Present	Female is absent from the shelter.
Active Behaviours	
Biting	Female bites an egg.
Digging	Female excavates the nest or area around it using her forelimbs.
Nudging	Female nudges an egg but does not change its position.
Pushing	Female changes the position of an egg without overturning it.
Rolling	Female changes the position of an egg by overturning it.

at Omaha. Each female was temporarily housed with a single male for mating. Once females were gravid, as indicated by abdominal swelling, males were removed from the enclosure. Enclosures consisted of 37.85 litre aquariums filled with 2,000 grams of commercial topsoil mixed with 500 grams of sand. Each enclosure contained a translucent red acrylic plate (15 x 15.5 cm, L x W) for shelter and for females to nest beneath. These plates allowed us to view females through the shelters using night vision cameras without disturbing them. Room lighting was on from 0600h to 2000h. Ultraviolet lighting (R-Zilla UVA/UVB 48") in reflective hoods was hung 1.5 metres above enclosures and 50-watt heat lamps were positioned over one side of each enclosure to allow the lizard to thermoregulate. Lizards were fed to satiation daily and water was available ad libitum.

Experimental design

Females were randomly assigned to one of two treatments, CORT application or vehicle control, which they received each day at 0800h and 1400h. CORT application began the day after oviposition. The CORT application treatment consisted of 45ug CORT dissolved in 4.5ul of sesame oil and the control treatment consisted of 4.5ul of sesame oil (n=4 for each treatment group). Treatments were applied to the lizard's backs using a pipette. Females were not handled during this process. If females were on the nest, the shelters were lifted slightly to facilitate application. The lipophilic nature of lizard skin means that the oil or oil/hormone mixture is quickly absorbed (Belliure & Clobert, 2004). This treatment method has been previously used to temporarily increase plasma CORT levels in reptiles (Knapp & Moore, 1997; Cote et al., 2006; Trompeter & Langkilde, 2011). A pilot study revealed that this CORT application regime resulted in elevated plasma CORT concentrations for *P. septentrionalis* at 6 hrs post application (baseline mean = 39.1 ng/ml, post CORT-application mean = 67.2 ng/ml; repeated measures ANOVA: $F_{1,9} = 4.91$, $p=0.054$; A. Anton & T. Langkilde, unpublished data). Soil samples were carefully taken from the edge of each nest at 0830h, after females had nested. This rarely scared females off the nest. These were analysed for moisture content and water was added to the nest as necessary to maintain these at 20% moisture. Video trials began at 0930h the day after the females had laid their eggs and ended at 1330h each day. Two camcorders simultaneously recorded behaviour of two randomly selected females for one hour, and were then moved to another pair of females. Each female was video recorded for one hour per day and the order in which the females were recorded was rotated each day. Video trials continued daily until the female's eggs were determined either unviable, as indicated by a shrivelled or mouldy appearance, or had hatched. The number of eggs that successfully hatched was recorded for each female.

Analysis

Video recordings were analysed using Windows Media Player 12 (Microsoft, Redmond, WA). Videos were scored blind to treatment. Two types of behaviours were quantified: positional and active (as per Somma & Fawcett, 1989). Positional behaviours describe the female presence and position in relation to the eggs within the nest and have been suggested to alter moisture loss and gain of eggs (Somma & Fawcett, 1989) (Table 1). Active behaviours are those in which an individual physically interacted with the nest or eggs (Table 2). Both types of behaviour were recorded using presence-absence sampling at 30-second intervals. The total observations of each behaviour were weighted for the number of observations made and used in analyses. Data are presented in figures as percentage of observations a female exhibited for each behaviour. Data were analysed using separate Mann-Whitney U tests in Minitab 17 (Minitab Inc., State College, PA) with the weighted occurrence of each behaviour as the dependent variable and treatment as the factor.

RESULTS

Positional Behaviours

CORT-treated females tended to spend less time present at the nest and, when on the nest, were less likely to be in contact with the with the eggs and spent less time coiled around the eggs than did females in the control group (Table 2, Figs. 1 and 2). For all other positional behaviours, no significant effect of CORT treatment was found (Table 2).

Active Behaviours

No significant effects of CORT treatment were found between control and experimental groups for any of the five active behaviours (Table 2).

DISCUSSION

We found effects of application of CORT on maternal-care behaviour; females treated with CORT after oviposition tended to spend more time away from the nest. When CORT-treated females were at the nest, they spent

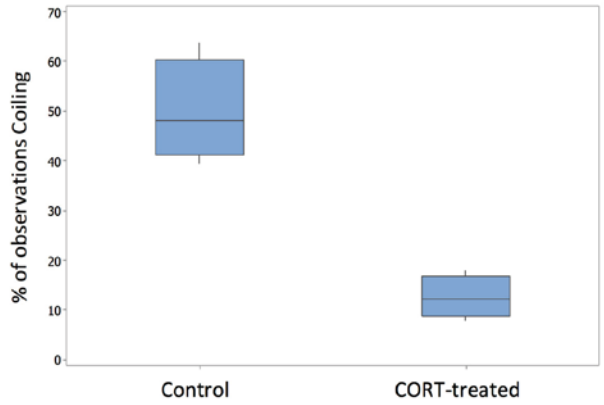

Figure 1. The percentage of observations in which female *P. septentrionalis* in the CORT-application and control treatment were coiled around their eggs (n = 4 per treatment). The box encompasses the 1st to 3rd quartile of the samples. The line within the box indicates the median value. The top and bottom of each vertical line represents the maximum and minimum values.

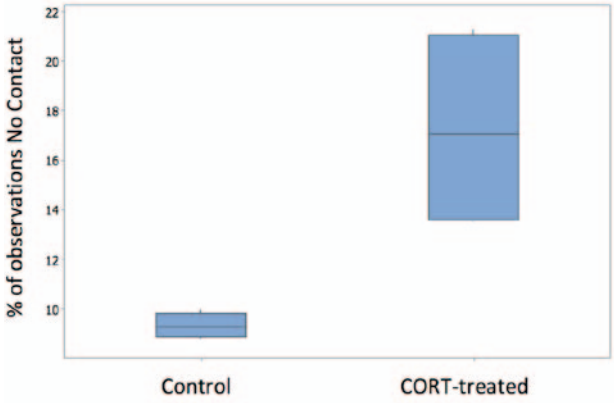

Figure 2. Percentage of observations in which female *P. septentrionalis* in the CORT-application and control treatment were within the nest but not in contact with their eggs (n = 4 per treatment). The box encompasses the 1st to 3rd quartile of the samples. The line within the box indicates the median value. The top and bottom of each vertical line represents the maximum and minimum values.

Table 2. The results of Mann-Whitney tests for the effect of treatment (application of corticosterone versus vehicle control) on behaviour of *P. septentrionalis*. n = 4 per treatment. Significant results are indicated by bold text.

Name of behaviour	Z	P
Positional Behaviours		
Contact	0.13	0.19
Coiling	-0.36	**0.03**
No Contact	0.08	**0.03**
Not Present	0.16	0.06
Active Behaviours		
Biting	>0.001	0.67
Digging	0.01	0.66
Nudging	-0.01	0.67
Pushing	0.01	0.89
Rolling	0.01	0.31

significantly less time in any contact with their eggs and less time coiled around the eggs. If the primary function of maternal care in *P. septentrionalis* is to maintain moisture levels as has been previously suggested (Somma & Fawcett, 1989), then less time spent in the nest in contact with the eggs could lead to detrimental conditions and lower hatching success. In our experiment, despite differences in female behaviour, hatching success was low in both the treatment and control groups (only two control females had at least one egg hatch and no eggs from CORT-treated females hatched), possibly due to the soil composition of our artificial nests, and so we were unable to determine the consequences of this change in behaviour for offspring. It is also possible that the nesting substrate and low viability of eggs could have impacted maternal behaviour. Future research should test for additional CORT-associated changes in maternal-care behaviour and for implications of these changes for the females (e.g., effects on body condition) and their offspring (e.g., survival, body condition).

Our results suggest that increases in CORT may decrease parental behaviour in reptiles, as it does in birds (Groscolas et al., 2008; Angelier et al., 2009). CORT has been implicated in the natural cessation of maternal care in cottonmouth snakes (Hoss et al., 2014). This effect may also be mediated by an interaction between CORT and the hormone prolactin, which is primarily responsible for the induction and maintenance of parental behaviour in birds (Buntin et al., 1991), but the mechanism is currently unknown. In species of reptile that exhibit parental care, elevations in CORT induced by anthropogenic activities such as urbanisation (French et al., 2008), pollution (Wikelski, 2001), tourism (Romero & Wikelski, 2002; French et al., 2010) or the introduction of non-native species (Graham et al., 2012) may thus negatively affect parental behaviour and therefore reproductive success.

Even with a small sample size, significant effects of CORT on maternal care behaviour of *P. septentrionalis* were detected, indicating that future studies on this phenomenon would be useful. Further research on the relationship between CORT and maternal care in this and other reptile species would shed important light onto the hormonal controls of this behaviour, and possible implications of increased stress.

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Genetic diversity of common toads (*Bufo bufo*) along the Norwegian coast: disjunct distribution of locally dominant haplotypes

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Little is known about the phylogeographic history of amphibian populations along the western Fennoscandinavian coast. In the present study, we focus on the common toad (*Bufo bufo*) and document the spatial distribution of mitochondrial DNA (cytb) haplotypes at 20 localities along its coastal Norwegian range. Two common haplotypes (out of eight haplotypes in total) were represented by 142 out of the 154 (92%) investigated individuals. However, they were shared at only three localities and clustered at two separate geographic regions each. The most common haplotype (55% of individuals) has previously been found to be abundant across central and eastern Europe, whereas the second most common haplotype (37% of individuals) has so far only been recorded in Sweden. The disjunct distribution of genetic lineages is in line with an assumption that the Norwegian coastline was postglacially colonised both from the south as well as across mountain passes from the east. Our data support previous studies on the phylogeography of Fennoscandinavia that revealed that post-glacial recolonisation patterns led to a pronounced spatial structure of local populations.

Key words: phylogeography, Fennoscandinavia, cytb, mitochondrial DNA, Bufonidae

INTRODUCTION

The historical biogeography of Europe is markedly shaped by glacial cycles. While initial seminal studies identified the Mediterranean peninsulas as the most important refugia during cold periods (Hewitt, 1996; 2000), subsequent research revealed a more complex picture with further important refugial areas in central and eastern Europe (e.g. Stewart & Lister, 2001; Babik et al., 2004; Schönswetter et al., 2005). Fennoscandinavia in northern Europe is generally characterised by a post-glacial recolonisation of lineages that arrive either via existing land connections from the east or via the Baltic Sea, leading to secondary contact zones between two distinct sets of populations for some species (e.g. Taberlet et al., 1995; Kontula & Väinölä, 2001). In addition, specific islands along the Norwegian coastline may represent isolated northern refugia for plants and mammals (Fedorov & Stenseth, 2001; Printzen et al., 2003; Brunhoff et al., 2006).

Due to their low vagility and water dependency, amphibians are particularly spatially structured and are therefore excellent models for phylogeographic and conservation genetic investigations (Beebee, 2005; Zeisset & Beebee, 2008; McCartney-Melstad & Shaffer, 2015). The most comprehensive studies on the phylogeography of Fennoscandinavian amphibians were conducted on brown frogs (*Rana temporaria* and *R. arvalis*), which confirmed a post-glacial recolonisation both via the Baltic Sea as well as from the east (Palo et al., 2004; Knopp & Merilä, 2009; Cortázar-Chinarro et al., 2017). Other anurans such as the pool frog (*Pelophylax lessonae*) only reside in southern Fennoscandinavian areas, where they represent distinctly northern clades (e.g. Zeisset & Hoogesteeger, 2018).

The common toad *Bufo bufo* (Linnaeus 1758, Anura, Bufonidae) is widely distributed from Western Europe to Asia and is among the European amphibians with the northernmost occurrences (Sillero et al., 2014). However, while its species delimitation and phylogeography has recently attracted significant attention at the continental scale (e.g. García-Porta et al., 2012; Recuero et al., 2012; Arntzen et al., 2017), the specific biogeographic history of *B. bufo* in Fennoscandinavia has received little attention so far. In Norway, *B. bufo* generally occurs in the south of the country as well as along the western coastline, where it reaches 68.01° in latitude; it is currently absent in inland areas of higher altitudes (Pedersen & Dolmen, 1994; Artsdatabanken, 2015; Roth et al., 2016; Syvertsen, 2016). The aim of the present study is to determine the spatial genetic structure of *B. bufo* populations along the Norwegian coast using mtDNA cytochrome b (cytb) haplotypes and to integrate the revealed patterns of variation into previously published haplotype distributions across the species' range. In particular, we investigate whether the Norwegian coast is inhabited by

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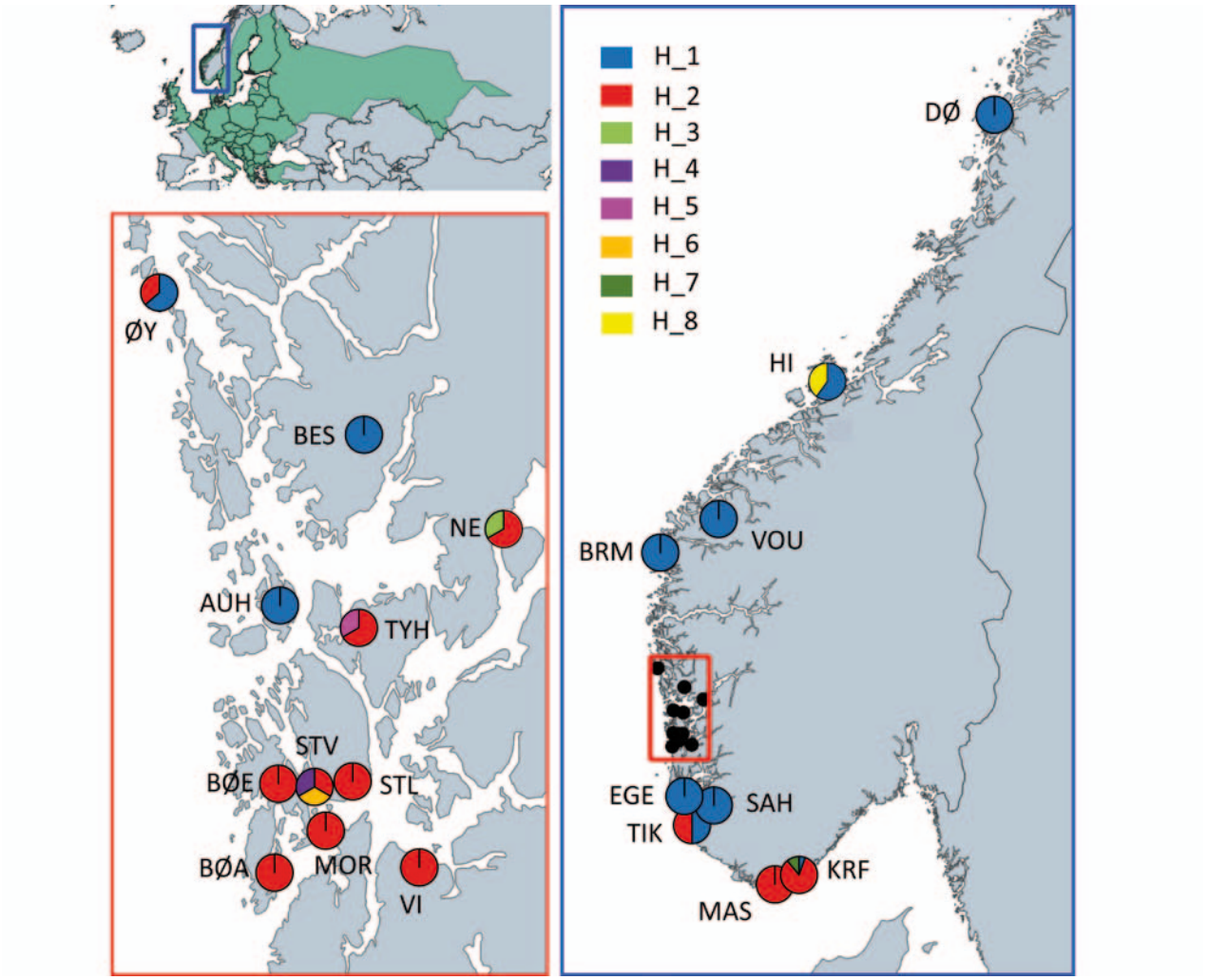

Figure 1. Sampling localities and haplotype distribution of *B. bufo* in Norway. Top left: Distribution range of *B. bufo*. For more details on sampling localities and haplotypes see Tables 1 and 2

a single or several genetic lineages attributable to post-glacial recolonisation routes (the latter was observed for other anurans at a wider Fennoscandinavian scale: Palo et al., 2004; Knopp & Merilä, 2009; Cortázar-Chinarro et al., 2017). We also investigate whether a post-glacial recolonisation from southern refugia is reflected in a south-north decline in genetic variation, which is expected to arise through serial genetic bottlenecks during range expansion (Hewitt, 1996).

MATERIAL AND METHODS

Field work was conducted in spring 2007 and 2008, broadly across the entire distribution of *B. bufo* along the Norwegian coast (Fig. 1). In total, 154 samples across 20 localities spanning over 1000 km of latitude were included. Eleven localities were situated on the mainland and nine localities were situated on offshore islands; for two localities (Sandness, SAH, and Hitra, HI), 2 or 3 populations at a geographic distance not exceeding individual migration capabilities (about 2 km) were pooled (Table 1, Fig. 1). DNA was collected from toe clips (opportunistically from fresh roadkills and deceased individuals) and skin swabs from adults using cotton buds, supplemented with a maximum of two sacrificed tadpoles for each locality. Sample size per locality ranged between 1 and 21; to maximise geographic coverage under logistic constraints, numbers were generally lower when localities were at closer geographic proximity (such as around the city of Bergen, see the insert in Figure 1). Samples were stored in 96% ethanol immediately in the field.

Total genomic DNA was extracted using the Purelink DNA isolation kit according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). A 722bp long fragment of the mtDNA cytb region (excluding primers) was amplified using PCR primers described in Recuero et al. (2012) (F: ATCTACCTTCACATCGGACGAG, R: AGTTTRTTTTCTGTGAGTCC). PCR was performed in 25 µl reaction volumes containing 50 ng DNA, 1x Taq DNA polymerase buffer, 2 mM MgCl2 (25 mM; Fermentas, MBI), 0.2 mM dNTPs (0.05 mM each; Fermentas, MBI), 0.5 U Taq DNA polymerase (Fermentas, MBI) and 0.1 µM of each primer. The amplification conditions were 2 min at 96°C, followed by 37 cycles of 30 s at 94°C, 45 s annealing at 53°C, and 1 min 30 s at 72°C, followed by a final 5 min at 72°C. PCR products were purified using the Purelink PCR purification kit (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's protocol and visualised by electrophoresis in 1.5 % agarose gels

Table 1. Sampling localities and codes, sample size, locality coordinates and sampling dates for *B. bufo* samples collected along the Norwegian coast.

Localities	Code	Sample Size	Coordinates	Date
Kristiansand, Flekkerøy	KRF	18	58.0692N 7.9939E	30.04.08
Mandal, Skjernøy	MAS	21	57.9946N 7.5234E	01.05.08
Time, Kvernaland	TIK	4	58.7855N 5.7348E	29.04.08
Sandness, Hommarsåk	SAH	14	58.9488N 5.8904E; 58.942083 5.9415E	02.05.08
Egersund, Egerøy	EGE	18	59.0175N 5.7803E	29.04.08
Vindfjord	VI	1	59.6538N, 5.6744E	02.05.07
Moster, Revsnes	MOR	1	59.7037N, 5.3937E	02.05.07
Bømlo, Andal	BØA	3	59.6328N, 5.2361E	03.05.07
Stord, Landathørn	STL	3	59.7772N, 5.4685E	02.05.07
Stord, Vikånes	STV	3	59.7765N 5.3678E	25.04.07
Bømlo, Ekhornsaeter	BØE	2	59.7841N 5.2553E	21.04.07
Tysnes, Heie	TYH	3	60.0172N 5.4853E	17.05.07
Austevoll, Huftarøy	AUH	1	60.0418N 5.2651E	17.05.07
Nes	NE	3	60.1656N 5.9369E	01.05.08
Bergen, Samdalsvatnet	BES	1	60.3035N 5.5061E	01.05.08
Øygarden	ØY	11	60.5144N 4.9035E	02.06.08
Bremanger, Myrevatnet	BRM	6	61.8039N 4.9540E	11.05.08
Volda, Ullalandsvanet	VOU	8	62.1126N 6.2320E	11.05.08
Hitra	HI	15	63.5165N 9.0323E; 63.4841N 8.8846E; 63.4834N 8.7855E	12.05.08
Dønna	DØ	18	66.0904N 12.5148	13.05.08

stained with Safe ViewTM Nucleic Acid Stains (abm) and photographed with a Vilber Loumart gel documentation and visualisation system. Sequencing was carried out single-stranded by Macrogen Corporation (Netherlands) using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI 3730XL capillary sequencer.

Together with the 154 new sequences generated in this study, we also considered further cytb sequences previously deposited in the GenBank database (Genbank UID: 353256652, Recuero et al., 2012, n = 325). Sequences were aligned using Clustal W (Thompson et al., 1994) in BioEdit ver 7.1.3.0 (Hall, 1999). Haplotype numbers (h), haplotype diversities (Hd), and nucleotide diversities (π, Nei, 1987) were estimated using DNAsp ver. 5.10 (Rozas et al., 2003). To investigate genetic structuring in Norway, we performed an Analysis of Molecular Variance (AMOVA, Excoffier et al., 1992) based on alternative grouping of populations using Arlequin 3.5 (Excoffier & Lischer, 2010). Significance was determined based on 10000 permutations and we show the five population groupings explaining the highest amount of variation. To quantify genetic differences between populations, pairwise genetic distances values between Norwegian populations (FST) were calculated also in Arlequin, using sequential Bonferroni corrections for adjustment of statistical levels of multiple tests (Holm, 1979). Principal component analysis (PCA) performed using GenAlex ver 6.501 (Peakall & Smouse, 2012) for the eight populations with n > 8 (DØ, EGE, HI, KRF, MAS, ØY, SAH, and VOU) revealed whether principle components characterising genetic properties of populations are reflected in their spatial settings. To illustrate the population share across haplotypes, and to distinguish between ancestral and derived haplotypes, Network 4.6.1.3 (Fluxus Technology Ltd.-http://www.fluxus-engineering.com/sharenet.htm) was used to compile a median-joining (MJ) network. The best substitution model selected by the Akaike information criterion (AIC) was TrN+G implemented in MODELTEST 3.7 (Posada & Crandall, 1998). Whether genetic variation of *B. bufo* populations in Norway is subject to non-random processes such as selection or demographic population contraction or expansion was determined using Tajima's selective neutrality as well as Fu and Li's neutrality test using DNAsp (Tajima, 1989; Fu & Li, 1993).

RESULTS

All newly produced sequences have been deposited in Genbank (accession numbers KX230483- KX230490 for Haplotypes 1-8). Among these sequences, we found 715 monomorphic and seven segregating sites across the 154 *B. bufo* individuals, with four singleton variable sites and three parsimony informative sites defining eight haplotypes. The majority of individuals were represented by two haplotypes (Haplotype 1: 55% of individuals, Haplotype 2: 37% of individuals), which were

Table 2. Haplotype distributions across *B. bufo* sampling localities in Norway. h: number of haplotypes; *Seven polymorphic sites (invariable sites excluded)

	KRF	MAS	TIK	SAH	EGE	VI	MOR	BØA	STL	STV	BØE	TYH	AUH	NE	BES	ØY	BRM	VOU	HI	DØ	*H**
H_1	1		2	14	18								1		1	7	6	8	9	18	AGGATAT
H_2	15	21	2			1	1	3	3	1	2	2		2		4					AGGACAT
H_3														1							AAGACAT
H_4										1											AGGGCAT
H_5												1									AGGATGT
H_6										1											GGGACAT
H_7	2																				AGAACAT
H_8																			6		AGGATAC
h	3	1	2	1	1	1	1	1	1	3	1	2	1	2	1	2	1	1	2	1	

Table 3. Analysis of Molecular Variance (AMOVA) of *B. bufo* samples distributed in Norway. *: $p < 0.05$; **: $p < 0.001$. Groupings are shown in descending amount of overall variation explained, and different groups are separated with the symbol ●. Va: Percentage variation, F_{SC}: differences between localities within groups, F_{ST}: genetic differences within localities, F_{CT}: genetic differences between groups.

Group	Va	F_{SC}	F_{ST}	F_{CT}
1. KRF, MAS●TIK, SAH, EGE●VI, MOR, BØA●STL, STV, BØE●TYH, AUH, NE, BES ● ØY, BRM, VOU, HI, DØ	60.32	0.33**	0.73**	0.60**
2. KRF, MAS●TIK, SAH, EGE●VI, MOR, BØA, STL, STV, BØE●TYH, AUH, NE, BES, ØY ● BRM, VOU, HI, DØ	60.17	0.31**	0.73**	0.60**
3. KRF, MAS●TIK, SAH, EGE●VI, MOR, BØA, STL, STV, BØE●TYH, AUH●NE, BES, ØY ● BRM, VOU●HI●DØ	60.09	0.28**	0.71*	0.60*
4. KRF, MAS●TIK, SAH, EGE●VI, MOR, BØA●STL, STV, BØE●TYH, AUH ● NE, BES ● ØY, BRM, VOU, HI, DØ	60.03	0.33**	0.73**	0.60**
5. KRF, MAS●TIK, SAH, EGE●VI, MOR, BØA●STL, STV, BØE●TYH, AUH, NE, BES, ØY ● BRM, VOU, HI, DØ	59.91	0.38**	0.73**	0.60**

Figure 2. Principal Coordinates Analysis of eight *B. bufo* populations based on F_{ST} values for cytb sequences. Percentages explaining variation by axes are shown; only localities with a minimum of eight sequenced haplotypes are considered.

shared in only three localities (KRF, TIK, ØY; Fig. 1, Table 2). Remarkably, both haplotypes were clustered at two separate regions across our study area. Haplotype 1 was dominant in two populations south of Stavanger (SAH and EGE) as well as in the northernmost populations (AUH, BES, ØI, BRM, VOU, HI, and DØ), whereas Haplotype 2 predominated in the southernmost localities (KRF and MAS) as well as around the city of Bergen (VI, MOR, BØA, STL, BØE, TYH). Four further haplotypes were represented by a single individual across three localities, in addition to two site-specific haplotypes represented by two and six individuals each, respectively (Table 2).

Corresponding to haplotype numbers and distributions, overall measures of haplotype and nucleotide diversities in Norway were estimated as 0.560 and 0.001, respectively. Tajima's D was not significantly negative ($D = -1.08$, $p > 0.05$), suggesting a lack of selection on the cytb locus across all Norwegian populations. Fu and Li's test was however significantly negative ($D = -2.54$, $p < 0.05$; $F = -2.42$, $p < 0.05$), suggesting either positive selection or population expansion of Norwegian populations. Genetic differentiation among localities was overall high (F_{ST}: 0.71, $p<0.001$, see Supplementary Materials, Table 1 for pairwise comparisons between populations). The disjunct distribution of major haplotypes resulted in main principle components which do not represent geographic settings (Fig. 2). The first axis explained > 99% of the observed variation and distinguished all populations fixed for Haplotype 1 (SAH, EGE, VOU and DØ) from populations dominated by or fixed for Haplotype 2 (KRF, MAS, ØY and HI). The five most likely groupings revealed by the AMOVA explained at least 59.91% of the observed variation (Table 3). The highest support was found for a grouping that identified six population clusters, three of which corresponded to the distribution of the two common haplotypes as outlined above; the remaining three clusters encompassed the populations around Bergen which are represented by low sample sizes. Other groupings comprised at least five clusters, and all groupings contained two identical clusters comprised of the five southernmost localities (Table 3). The haplotype network presents Haplotype 2, which comprises the two southernmost populations as well as the populations around Bergen, as ancestral (Fig. 3).

Integrating our findings into existing knowledge of mtDNA cytb haplotype distribution across the range of *B. bufo* (Recuero et al., 2012) revealed that the common Haplotype 1 is widespread in eastern Europe, whereas our second-most common Haplotype 2 as well as

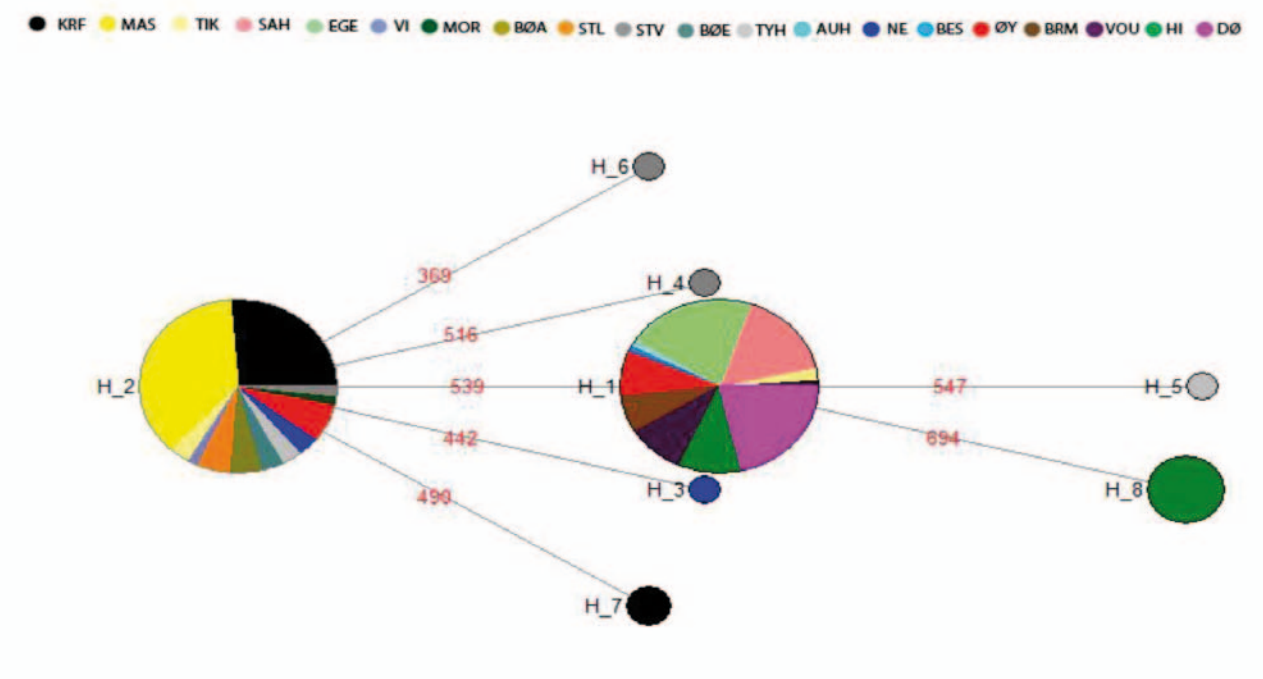

Figure 3. Median Joining Haplotype Network of *B. bufo* cytb sequences distributed along the Norwegian coast. Nucleotide positions of mutated sites are showed as numbers; shared haplotypes are divided into colours representing populations.

Haplotype 8 (represented by six individuals at a second northernmost locality) have previously been found only in Sweden (Supplementary Materials, Table 2). Our five rare haplotypes (Haplotypes 3-7, represented by one or two individuals each) have not previously been found elsewhere in the species' range.

DISCUSSION

Our findings from coastal Norwegian mtDNA haplotypes in *B. bufo* are generally in line with previous studies on Fennoscandinavian amphibians, which revealed pronounced spatial genetic structuring linked to a post-glacial recolonisation which took place from different directions (Palo et al., 2004; Knopp & Merilä, 2009; Cortázar-Chinarro et al., 2017). The significant diversity of haplotypes harboured by the study area also conforms to a previous spatially more-restricted study, which revealed high levels of genetic variation at the level of nuclear microsatellite markers (Roth & Jehle, 2016). Our study is, however, hampered by low sample sizes for some of the localities, which, jointly with their rather uneven spatial distribution, precludes a fine-scale capture of genetic variation at local levels. Four of the eight encountered haplotypes are represented by a single individual and have not been previously found elsewhere in Europe. As we were unable to repeat amplification and sequencing of these samples we cannot entirely rule out the possibility that these four haplotypes may be the result of PCR or sequencing errors, but consider it unlikely. Although introductions of *B. bufo* to offshore islands are occasionally reported (Dolmen & Seland, 2016), we assume that our populations are generally of natural origin (see also Roth & Jehle, 2016).

In contrast to previous work that covered larger areas in Sweden and Finland (e.g. Knopp & Merilä, 2009; see also e.g. Taberlet et al., 1995 for an early study on mammals), we did not reveal a single zone dividing northern populations from their southern counterparts. It is noteworthy that Haplotype 1, represented by more than half of our study individuals and common in central and eastern Europe, has not previously been found among eight *B. bufo* individuals from Sweden (Recuero et al., 2012). This suggests that this haplotype represents a genetic lineage that might have colonised the Norwegian coast from the Baltic Sea, an area that is also at closer geographic proximity to our study area than eastern land bridges into Fennoscandinavia. Haplotypes 2 and 8, together comprising 41% of our study individuals, have previously been found in adjacent Sweden but so far remained unreported elsewhere across the *B. bufo* range (Recuero et al., 2012). We were however unable to draw any conclusive inferences about their origins compared to the other haplotypes observed, as Haplotype 2 was characterised as ancestral whereas Haplotype 8 was characterised as distal in the MJ network. Denser sampling and a consideration of additional mitochondrial and nuclear loci is desirable to reveal whether Fennoscandinavia is inhabited by unique genetic lineages of *B. bufo*.

Amphibian species residing in Norway are generally distributed across southern parts of the country as well as along the coast (e.g. Dolmen, 1982; Sillero et al., 2014). The significant signature of population expansion based on Fu and Li's neutrality test is generally in line with the hypothesis that *B. bufo* recolonised our study area after cold periods. Our study did however not reveal a gradual reduction of genetic variation towards northern regions in line with the assumption that recolonisation took place only uni-directionally from south to north. That the post-glacial recolonisation did not take place exclusively northward is also supported by the disjunct alternating

distribution of the two dominant haplotypes, which, due to their fixed nature in the majority of localities where they occur, is unlikely caused by for example incomplete lineage sorting. Mutations arising during expansions would also lead to range-edge haplotypes being distally positioned in a haplotype network. This is the case for Haplotype 8, which is only found towards the northern edge of the *B. bufo* range (HI).

Based on distribution data of urodeles, Dolmen (1982) already suggested that amphibians might have reached the central Norwegian coast also from Jämtland in Sweden through mountain passes during temporarily more favourable climatic conditions during the Atlantic (5500-300 B.C.) and/or sub-boreal (3000 – 500 B.C.) age (see also Gislen & Kauri, 1959, as well as, for example, O'Brien et al. (2015) for another range expansion by a Northern European amphibian at similar spatial and temporal scales). Indeed, the distribution of Haplotypes 2 and 8 would be in line with the hypothesis that genetic lineages documented for Sweden reached central Norway around Bergen, as well as Hitra more than 500 km further north, across the currently unoccupied terrain of the Scandinavian mountain range. Wider sampling across Norway and Sweden is required to for example reveal why Haplotype 2 is also dominant at the two southernmost localities.

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SHORT NOTE

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Evidence of loggerhead sea turtle (*Caretta caretta*, Linnaeus, 1758) injuries caused by Rapido (beam) trawling in the Mediterranean

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The loggerhead turtle (Caretta caretta, Linnaeus, 1758) is the most abundant sea turtle species in the Mediterranean Sea, where commercial fishing appears to be the main driver of mortality. The North Adriatic Sea (central Mediterranean) is a major feeding habitat for turtles in the demersal stage. Its shallow and flat seabed is ideal for bottom-towed gears, making interactions with sea turtles and incidental catches unavoidable. We provide evidence of the impact of Rapido trawls (a type of beam trawl) on sea turtles through the analysis of the distinctive injuries sustained by four turtles.

Key words: Caretta caretta; Loggerhead turtle; Sea turtle-fisheries interaction, Rapido trawl, Sea turtle injuries, Mediterranean Sea.

The loggerhead turtle (*Caretta caretta*, Linnaeus, 1758) is the most abundant sea turtle species in the Mediterranean Sea (Casale & Margaritoulis, 2010; Lucchetti and Sala, 2010; Lucchetti et al., 2017) and a priority species listed in Appendix II/IV of the Habitats Directive, the cornerstone of Europe Union's nature conservation policy ("least concern" status; Casale, 2015). The main threats to marine turtle populations in the Mediterranean Sea are related to human activities such as incidental capture by fishing gears, degradation of habitats (mainly of nesting beaches), and marine litter (Margaritoulis et al., 2003). Incidental capture (or bycatch) is probably the most significant danger to sea turtles as well as to several other species worldwide. According to recent data, more than 50,000 turtle capture events are estimated to take place in Italian waters each year; of these, 10,000 are believed to result in death, sketching a more alarming scenario than expected based on earlier estimates (Lucchetti et al., 2017). The most harmful fishing gears are towed gears; in particular, a turtle bycatch hotspot has been identified in the Adriatic Sea (central Mediterranean Sea; Lucchetti & Sala, 2010; Lucchetti et al., 2016a). The shallow seabed and the rich benthic communities characterising this semi-enclosed basin provide a major feeding habitat for loggerhead sea turtles in the demersal stage, especially the populations nesting in Greece (Lazar et al., 2004; Zbinden et al., 2008).

The turtle rescue centres (MTRCs) operating in the Adriatic periodically collect stranded turtles exhibiting distinctive, regularly spaced carapace injuries that are commonly attributed to vessel propellers. In this study we propose a different explanation and provide evidence that at least some of them are caused by fishing gears such as Rapido trawls.

The Rapido trawl is a type of beam trawl (Fig. 1). It is mainly used in the Adriatic Sea to target flatfish (*Solea* spp., *Platichthys flesus*, *Psetta maxima*, *Scophthalmus rhombus*) in muddy inshore areas and, rarely, pectinids (*Pecten jacobaeus*) in sandy offshore grounds in the northern area of the basin. The modern Rapido gear resembles a toothed beam trawl. It consists of a box dredge (about 4 m wide, 120 kg in weight) rigged with

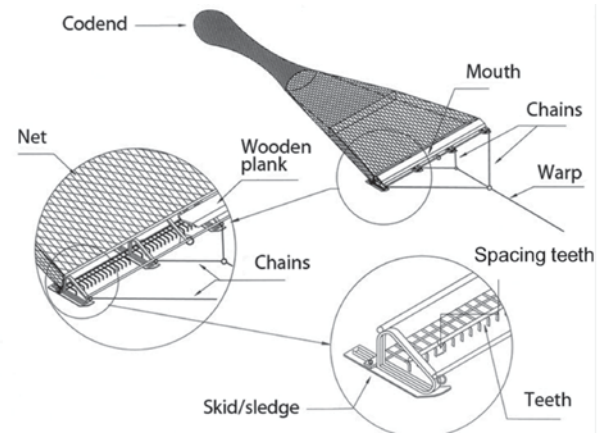

Figure 1. Rapido trawl: technical design and detail of tooth spacing.

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teeth (13-15 cm long) along the lower leading edge, and fitted with a codend to collect the catch (Giovanardi et al., 1998). The gear is towed at high speed (7-8 knots); a spoiler prevents it from rising off the bottom (Pranovi et al., 2000). A commercial vessel typically tows four sets of gears simultaneously. The Rapido trawls used in Adriatic fisheries share the same key characteristics, i.e. tooth spacing, gear dimension, and net length (Fig. 1).

Of the four injured specimens of *C. caretta* described in this study, two were retrieved by the local MTRCs – Fondazione Cetacea (Riccione, North Adriatic Sea) and Legambiente (Manfredonia, South Adriatic Sea) and two by a research institute, the National Research Council (CNR) of Ancona (Central Adriatic Sea) in winter 2016-2017. Whereas three were stranded, the fourth was incidentally caught by a Rapido trawler. Two died at the MTRC from deep bowel perforations a few days after being rescued.

The four specimens were measured (Table 1). All were sub-adults with a curved carapace length of 42 to 60 cm. All bore from two to four regularly spaced wounds on the carapace (Fig. 2). The space between wound pairs was measured. To establish whether the injuries could have been caused by a Rapido trawl, the space between the rake teeth was measured in four Rapido trawls randomly selected from different fishing vessels. All measurements were performed with both a calliper and a tape measure. To minimise errors, each measurement was also recorded with a photograph. Photographs were processed with ImageJ software (Rasband, 2010) to measure the distance between two consecutive wounds and two consecutive rake teeth.

Descriptive statistics (mean and standard deviation) were computed. Data analysis with one-way analysis of variance (ANOVA) showed that there was no significant difference in wound spacing among the four turtles ($p = 0.598$), in rake tooth spacing among the four trawls ($p = 0.142$), or between wound spacing (80.39 ± 6.26 mm) and tooth spacing (79.92 ± 5.71 mm) ($p = 0.848$). The fact that the individual incidentally caught by the trawler presented the same wounds as the other three (Fig. 2) reinforced the hypothesis that all four specimens had been injured by a Rapido rake.

Information on sea turtle bycatch by Rapido trawls is still scarce and unreliable (Lucchetti et al., 2017). However, considering that around 70 vessels operate in the central-northern Adriatic Sea for a total of 130 fishing days per year, and that a Rapido trawler can explore a wide area (ca. 224,000 m^2 per hour) in a single fishing day, interactions with sea turtles are highly likely, especially in winter, when turtles forage near the coast in the same grounds exploited by trawlers (Lucchetti et al., 2016b). As shown by our data, the high towing speed of this gear can injure sea turtles severely and even cause their death.

Interactions between sea turtles and towed gears similar to the Rapido trawl are a major conservation issue in some fisheries, such as the sea scallop dredge in the US, where bycatch is well documented. Although some bycatch-reducing devices have already been tested and adopted (i.e. in US; Haas et al., 2008), the size and

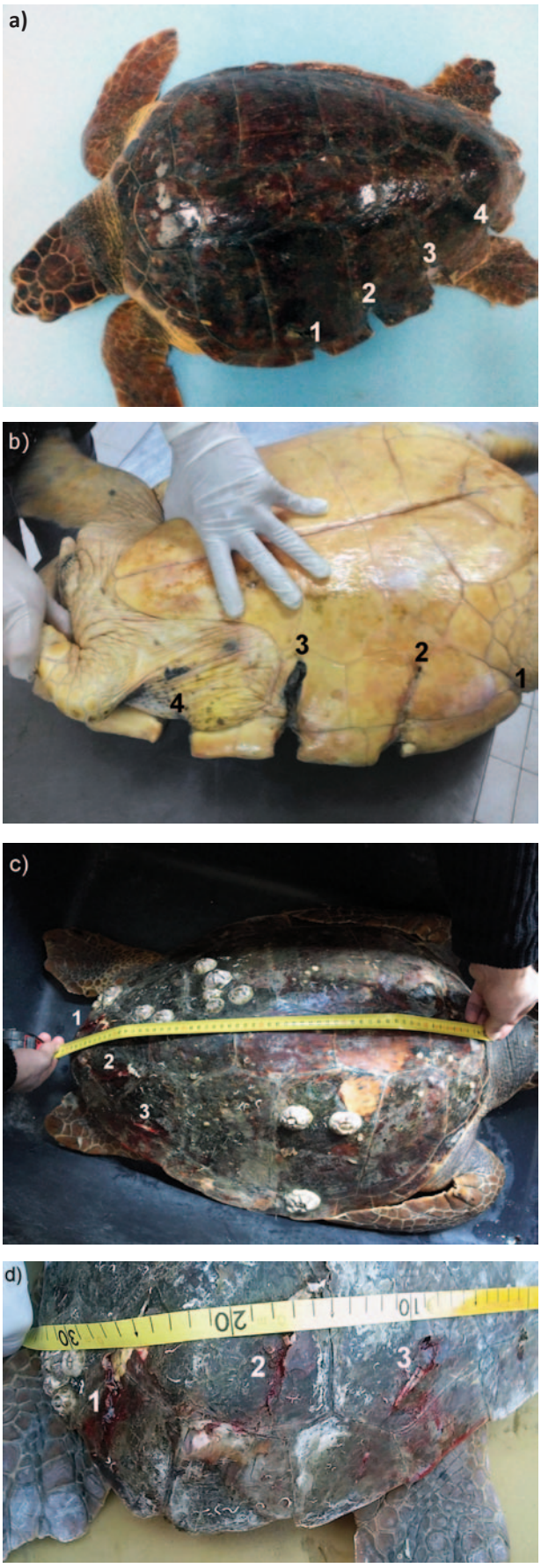

Figure 2. (Top) One of the three stranded sea turtles presenting regularly spaced wounds: **a)** dorsal view, **b)** ventral view. (Bottom) Sea turtle incidentally caught by a Rapido trawler and detail of wounds: **c)** dorsal view, **d)** close up view of the three wounds.

configuration of Rapido trawls (especially its teeth and limited height) and the non-sedentary nature of target species hamper their use in this fishery. Given that the Rapido trawl is banned in the rest of the Mediterranean, management measures, at least spatial and temporal closed areas, should be adopted also in Italy.

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