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Comprehensive DNA barcoding of the herpetofauna of Germany

O. HAWLITSCHEK,*† J. MORINIÈRE,* A. DUNZ,* M. FRANZEN,* D. RÖDDER,‡ F. GLAW* and G. HASZPRUNAR*

*Zoologische Staatssammlung (ZSM-SNSB), Münchhausenstrasse 21, 81247 München, Germany, †Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Passeig Maritim de la Barceloneta 37, 08003 Barcelona, Spain, ‡Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, 53113 Bonn, Germany

Abstract

We present the first comprehensive DNA barcoding study of German reptiles and amphibians representing likewise the first on the European herpetofauna. A total of 248 barcodes for all native species and subspecies in the country and a few additional taxa were obtained in the framework of the projects 'Barcoding Fauna Bavarica' (BFB) and 'German Barcode of Life' (GBOL). In contrast to many invertebrate groups, the success rate of the identification of mitochondrial lineages representing species via DNA barcode was almost 100% because no cases of Barcode Index Number (BIN) sharing were detected within German native reptiles and amphibians. However, as expected, a reliable identification of the hybridogenetic species complex in the frog genus *Pelophylax* was not possible. Deep conspecific lineages resulting in the identification of more than one BIN were found in *Lissotriton vulgaris*, *Natrix natrix* and the hybridogenetic *Pelophylax* complex. A high variety of lineages with different BINs was also found in the barcodes of wall lizards (*Podarcis muralis*), confirming the existence of many introduced lineages and the frequent occurrence of multiple introductions. Besides the reliable species identification of all life stages and even of tissue remains, our study highlights other potential applications of DNA barcoding concerning German amphibians and reptiles, such as the detection of allochthonous lineages, monitoring of gene flow and also noninvasive sampling via environmental DNA. DNA barcoding based on *COI* has now proven to be a reliable and efficient tool for studying most amphibians and reptiles as it is already for many other organism groups in zoology.

Keywords: conservation, habitats directive, *Pelophylax*, *Podarcis*, subspecies

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Introduction

Reptiles and amphibians play important roles in terrestrial and partly also in aquatic ecosystems, both in temperate and tropical environments (Fouquet *et al.* 2007; Vitt & Caldwell 2013). The present-day herpetofauna of Central Europe is the result of the recolonization of this area since the end of the last glaciation ca. 10 000 years ago (Joger *et al.* 2007). Today, amphibians and reptiles in Central Europe are threatened by many factors, including intensive agriculture as well as anthropogenic destruction, isolation and fragmentation of their habitats, and are therefore common marker species in environmental monitoring (Araújo 2003; Beebee & Griffiths 2005; Hajibabaei *et al.* 2011; Thomsen *et al.* 2012). Many species are protected by national laws and listed in the appendices of the EU Habitats Directive (The Council of the European Communities 1992). Other potential factors of threat are probably global warming (Rödder & Schulte 2010), changes in competition regimes due to anthropogenic range shifts (Schlaepfer *et al.* 2005; Heym *et al.* 2013), and – for amphibians – the chytrid disease (Ohst *et al.* 2013; Martel *et al.* 2014). Because of their sensitive responses to changes in climate and habitat, and as representative indicators of threatened habitat types (e. g. in the EU Habitats Directive), amphibians and reptiles play important roles in conservation management. Conservation management in Central Europe needs to regularly identify species of amphibians and reptiles in the field, which generally does not pose any problems to experts. However, there are cases in which reliable identification is much more difficult, for example roadkills, amphibian eggs, larvae and juveniles of closely related species such as newts (*Lissotriton* spp.) and brown frogs (*Rana* spp.), or when the distinction of subspecies or populations is

Correspondence: Oliver Hawlitschek, Fax: +49-89-8107-300; E-mail: oliver.hawlitschek@gmx.de

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necessary (Gassert *et al.* 2013). In these cases, a fast, reliable and cheap identification tool is required.

In the DNA barcoding approach, DNA sequences of a specific short and standardized genomic region are compared with a reference database (Savolainen *et al.* 2005; Hajibabaei *et al.* 2007). The standard genomic region used in DNA barcoding of animals is a 648-bp long fragment of the mitochondrial 5′ cytochrome c oxidase I (*COI*) gene (Hebert *et al.* 2003). *COI* has proven highly efficient and reliable in many animal groups and is regularly used for a variety of applications, such as biodiversity assessments (e.g. Nagy *et al.* 2012; Hawlitschek *et al.* 2013). DNA barcoding that has been shown to be highly efficient in identifying species (Ratnasingham & Hebert 2013), however, is of limited value in elucidating phylogenetic relationships (Vences *et al.* 2005a) and may sometimes 'disguise' cryptic species that cannot be identified by barcoding (Meyer & Paulay 2005; Hickerson *et al.* 2006).

In reptiles and even more in amphibians, DNA barcoding using *COI* remained difficult (Vences *et al.* 2005a, b) until specific primers and amplification protocols were developed (Che *et al.* 2012; Nagy *et al.* 2012; Xia *et al.* 2012). Therefore, most larger DNA barcoding studies of these groups were undertaken only very recently, such as the worldwide initiative of the Cold Code (Murphy *et al.* 2013) and other initiatives with regional ranges (Vences *et al.* 2012; Hawlitschek *et al.* 2013; Jeong *et al.* 2013; Perl *et al.* 2014).

This data release presents the results of a DNA barcoding study of the reptiles and amphibians of Germany, with a focus on the state of Bavaria. Bavaria is the largest of the German federal states with a terrestrial area of 70 000 km² and spans an altitudinal range from 100 to 2962 m a.s.l. At least 35 000 species of animals are recorded in Bavaria (Voith 2003), which corresponds to a significant portion of the Central European fauna. The Barcoding Fauna Bavarica (BFB: http://www.faunabavarica.de) aims at creating a DNA barcode library focusing on Bavarian animal species (e.g. Haszprunar 2009; Hausmann *et al.* 2011). These data are integrated with data from the German Barcode of Life (GBOL: http://www.bolgermany.de) project, which is conducted by a network of German natural history museums and other biodiversity research institutes. As current estimates of animal species numbers differ over a wide range, the German barcoding campaigns aim to create a reliable library of determined species, in order to make a contribution to the worldwide running iBOL initiative (Ratnasingham & Hebert 2007).

At the current state of taxonomy, 13 species of reptiles and 20 species of amphibians are recognized as native in Germany. In this study, we present DNA barcoding data on all of these species and of some species with introduced (or potentially introduced) populations in Germany, point out directions of future research, and discuss the application of DNA barcoding in the context of reptiles and amphibians in Central Europe.

Material and methods

Sampling, permits, ethics statement, terminology and taxonomy

We used museum samples from the tissue bank at the Zoologische Staatssammlung München (ZSM), Germany. We collected additional samples in the field, including cloacal and thigh swabs. We also included a sample of an unidentified exotic specimen found in the green houses of the Munich botanical garden. Newly collected samples were also deposited in the ZSM tissue bank. Further samples were obtained from the tissue bank of the Zoologisches Forschungsmuseum Alexander Koenig, Bonn (ZFMK). All tissue samples were stored in 96% ethanol. For some species and specimens, not only tissue samples but also voucher specimens were available (Table S1, Supporting information). Voucher specimens were euthanized using approved methods (e.g. anaesthesia with ketamine, followed by ketamine overdose) that do not require approval by an ethics committee according to national law. Collection permits were issued by the departments of environment of the governmental districts of the state of Bavaria, Germany. Samples were assigned to previously existing taxonomical units based on morphological identification of vouchers. Species of water frogs (*Pelophylax*) were identified following the methods described in Mayer *et al.* (2013). Species names follow the 'Amphibian Species of the World' database (6.0) (Frost 2014) with the exception of the Bufonidae, where we used the traditional genus name *Bufo* instead of *Epidalea* for *Bufo calamita* and *Pseudepidalea* or *Bufotes* for *B. viridis*). We do not conduct any new definition or delimitation of species or subspecies.

Laboratory procedures

For DNA extraction, muscle tissue of ethanol-preserved samples was collected and transferred into 96-well plates. For live specimens, we applied cloacal and thigh swabbing and transferred the mucus material to the exact same plates. These plates were then sent to the Canadian Center for DNA Barcoding (CCDB) for standardized and high-throughput DNA extraction, PCR amplification and Sanger sequencing (see http://www.ccdb.ca/resources.php). The amplified target region has a length of 658 bp, starting from the 5′ end of the mitochondrial cytochrome oxidase I (*COI*) gene, which includes the 648-bp barcode region (Hebert *et al.*

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2003). Data of successfully sequenced specimens were then uploaded into the Barcoding of Life database (BOLD: http://www.boldsystems.org).

Analyses

Sequence divergences for the barcode region (mean and maximum intraspecific variation and minimum genetic distance to the nearest-neighbour species) were calculated using the '*Barcode Gap Analysis*' tool on BOLD, employing the Kimura-2-Parameter (K2P) distance metric (Puillandre *et al.* 2012). The K2P model is used for comparability with other barcoding studies (Che *et al.* 2012; Nagy *et al.* 2012; Hawlitschek *et al.* 2013; Hendrich *et al.* 2015). MUSCLE (Edgar 2004) was applied for sequence alignment restricting analysis to sequences with a minimum length of 500 bp. A Taxon ID tree was created on BOLD using the neighbour-joining (NJ) method following alignment based on K2P distances. Barcoding trees produced in this way are not equipped with any support values for their nodes because they are meant for displaying barcode clusters, but not phylogenetic relationships. Only sequence distances are displayed. For the same reason, no outgroups were used, but barcode sequences of water frogs (*Pelophylax*) were blasted on GenBank to verify the identification. The '*BIN Discordance*' analysis on BOLD was used to reveal species clusters sharing a Barcode Index Number (BIN) or assigned to multiple BINs. A BIN (Ratnasingham & Hebert 2013) is a globally unique identifier for species based on DNA barcodes. In this analysis, sequences were grouped algorithmically in a 3-step online pipeline into clusters of very similar *COI* barcode sequences. These groups were considered operational taxonomic units (OTUs) and were then assigned a BIN. This system allows the verification of species identifications when taxonomic information is lacking. In previous studies with numerous insect species from Germany, most BIN numbers corresponded to single species as delineated by traditional taxonomy (Hausmann *et al.* 2013).

Results

We produced a total of 242 DNA barcodes of 500 bp or more (6 sequences were less than 500 bp) for all native species and subspecies currently known to occur in Germany. A single barcode sequence was obtained only for *Alytes obstetricans*, *Pelophylax esculentus*, *Lacerta bilineata* and *Natrix natrix helvetica*, and two or more DNA barcodes were obtained for all other taxa. *COI* amplification was successful for 80.8% of the 307 specimens submitted and for 100% of all species analysed. The highest success rates in PCR amplification and sequencing were achieved using the following primers and primer cocktails: C_LepFolF (Hajibabaei *et al.* 2006)/Nancymod2192 (Silva-Brandão *et al.* 2008), dgHCO-2198 (Meyer 2003)/dgLCO-1490 (Meyer 2003), C_FishF1t1 (Ivanova *et al.* 2007)/C_FishR1t1 (Ivanova *et al.* 2007), C_VF1LFt1 (Ivanova *et al.* 2007)/C_VR1LRt1 (Ivanova *et al.* 2007), Chmf4 (Che *et al.* 2012)/Chmr4 (Che *et al.* 2012). All primers were designed in collaboration with the iBOL team, Guelph, Canada, and sequences are available at the BOLD database.

A round tree based on all successfully amplified *COI* sequences is shown in Fig. 1. The barcodes allowed reliable and unambiguous species identifications of almost all species. Only sequences of the hybridogenetic water frog complex (genus *Pelophylax*) did not fit consistently with morphological identifications: sequences of frogs morphologically identified as *P. ridibundus* formed one cluster which however included among the 16 sequences each one sequence of a frog identified morphologically as *P. lessonae* and *P. esculentus*, respectively. The second *Pelophylax* cluster (14 sequences) included five sequences of *P. lessonae* and nine of *P. ridibundus* (Fig. 2). As expected, the reliable identification of these species by DNA barcodes was not possible (Plötner *et al.* 2008; Mayer *et al.* 2013).

The K2P distances of *COI* within families, genera and species are given in Table 1. This table shows that intraspecific genetic distances are very low within most species. In some species, such as *Rana temporaria* ($n = 8$) and *Bufo bufo* ($n = 11$), most haplotypes were found to be very similar and differing by only very few base pairs (0.16% and 0.46%, respectively) even if a relatively high number of samples were included in the analysis. Larger intraspecific distances were found in some other species (e.g. 2.6–5.84% in *Natrix natrix*), corresponding either to single divergent haplotypes or to clearly distinct clusters. 17 BINs were recognized within reptiles and 22 within amphibians. Most species were represented by exactly one BIN, with the following exceptions: *Podarcis muralis* (3 BINs, Fig. 3), *Natrix natrix* (3 BINs, Fig. 4A), *Lissotriton vulgaris* (2 BINs), *Pelophylax ridibundus* (2 BINs) and *Vipera berus* (2 BINs; Fig. 4B). On the other hand, *Emys orbicularis* + *E. trinacris* shared the same BIN. *E. trinacris* is not native to Germany but was included for comparison to *E. orbicularis* due to the putative abundance of allochthonous individuals of this species complex in Germany (Fig. 4C). The latter two species also displayed the lowest interspecific divergence in all reptiles studied (1.87%). The lowest interspecific divergence among the studied squamate reptiles was detected between the lizard species *Lacerta viridis* and *L. bilineata* (6.01%). A similar level of divergence was found between distinct clades within the *Podarcis muralis* complex (8.7%) and between two subspecies of grass snake (*Natrix n. natrix* and *N. n. helvetica*: 5.84%), whereas the maximum divergence

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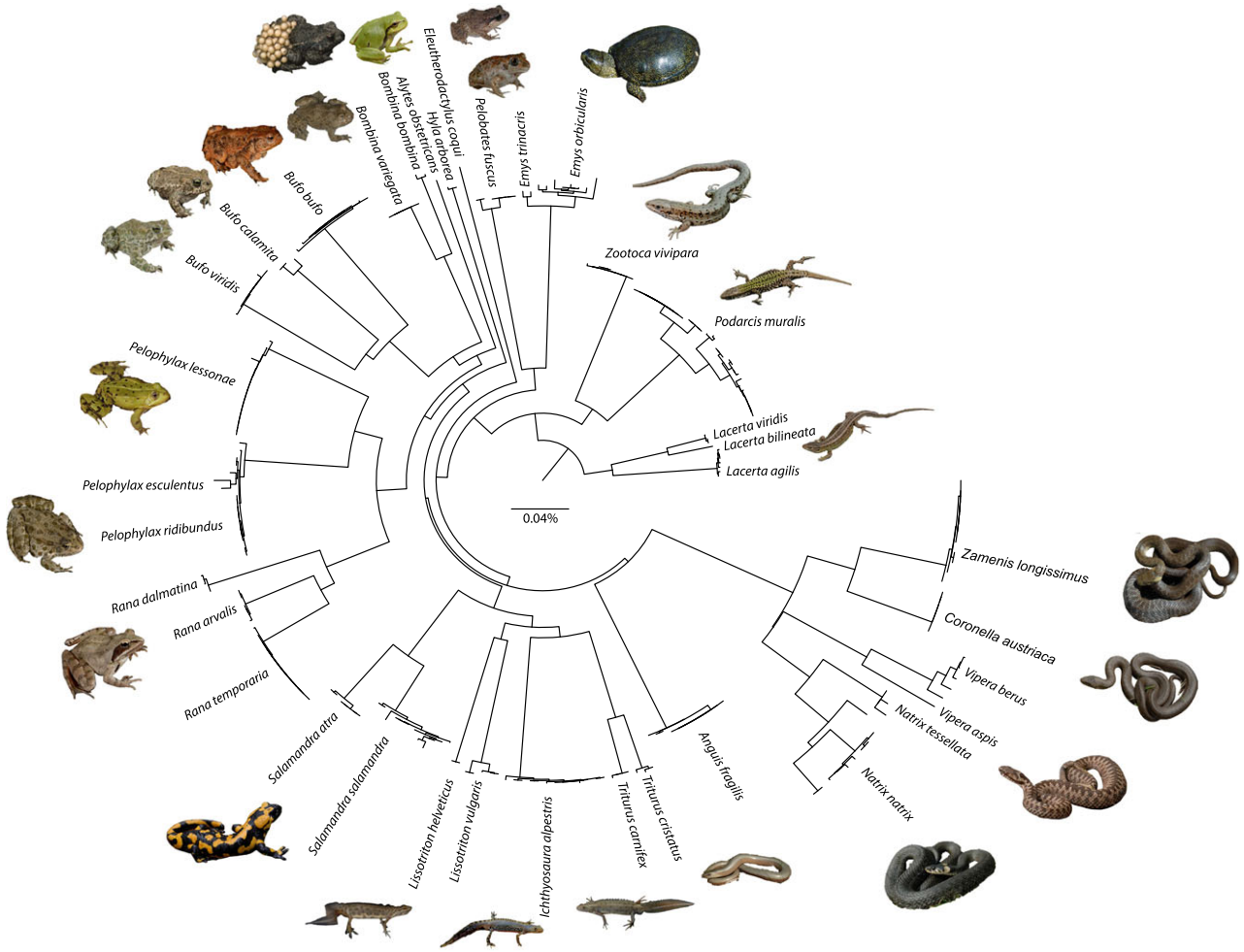

Fig. 1 Neighbour-joining tree based on *COI* barcodes of German amphibians and reptiles, created in BOLD. The round tree appearance was created in FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

between adder populations (*Vipera berus*) was distinctly smaller (1.61-2.83%). The two haplotypes (1.54% divergence) of the European common spadefoot (*Pelobates fuscus*) were detected within the same population (Fig. 4D), whereas the two haplotypes of the smooth newt (*Lissotriton vulgaris*) reflect an allopatric situation: one BIN was assigned to populations north of the Danube River, another one to populations south of the Danube (Fig. 4E). The two former subspecies of crested newts (*Triturus cristatus* and *T. carnifex*), now recognized as species (Macgregor *et al.* 1990), were found to differ by 8.08%. The haplotype of the only introduced *T. carnifex* population in Germany (at Isen, Bavaria) is almost identical to that from Salzburg (Austria), and the haplotypes of the fire salamander subspecies *Salamandra s. salamandra* and *S. s. terrestris* are only poorly differentiated (1.71%; Fig. 4F). Most Bavarian samples of the snake *Zamenis longissimus* were found to share the same haplotype with the only sample from an isolated population in Hesse. The tropical frog species discovered in greenhouses in the botanical garden of Munich was identified as *Eleutherodactylus coqui* by reverse taxonomy using the BOLD database.

Consequently, all 33 native species that were included in the analysis form clearly distinct clusters in the neighbour-joining (NJ) tree (Figs. 1, S1, Supporting information). All 22 genera are reflected correctly by clusters displayed in the tree, as are all 12 families. However, at a higher taxonomical level, the tree is no longer in concordance with currently accepted classifications (Frost 2014).

Discussion

The overall success rate of DNA sequencing and barcode generation of 80.8% is comparably high in contrast to the rates of some other groups. The success rates of related barcoding studies were 64% in orthopterans

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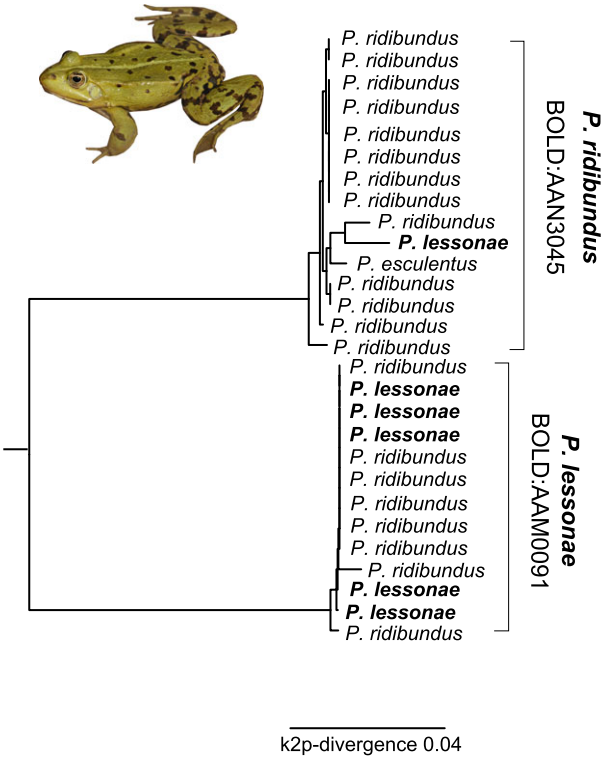

Fig. 2 The hybridogenetic *Pelophylax ridibundus/lessonae* species complex. DNA barcoding does not allow the distinction of the two parental species and the hybrid *P. esculentus*.

(O. Hawlitschek, unpublished data), 63% in beetles (Hendrich 2015), 83% in neuropterans (Morinière *et al.* 2014) and up to 100% in fresh lepidopterans (A. Hausmann, unpublished data). Many groups especially of arthropods were found to include clades in which species identification by DNA barcodes was not feasible because the *COI* marker did not provide sufficient resolution. This was the case in, for example, 10% of all German bees (Schmidt *et al.* 2015) and 11.4% of all German orthopterans (O. Hawlitschek, unpublished data). In contrast to these groups, 100% of the nonhybridogenetic German species of amphibians and reptiles are represented by clearly distinct barcode clusters that allow unambiguous identification to the species level. This is remarkable because the generation of sequences started in 2009, whereas efficient protocols for the DNA barcoding of reptiles and amphibians using *COI* were published only relatively recently (Che *et al.* 2012; Nagy *et al.* 2012; Xia *et al.* 2012).

The only case in which DNA barcoding does not serve as a reliable tool for species identification is the complex of *Pelophylax* water frogs. This complex consists of the two parental species *P. lessonae* and *P. ridibundus* and a hybridogenetic hybrid dubbed *P. esculentus*, which usually interbreeds with one of the parental species, but can also occur in pure hybrid populations comprising diploid and triploid individuals (Tunner 1973; Plötner *et al.* 1994). *P. esculentus* can be distinguished from the parental species, though with difficulties, by morphological and bioacoustical characters. They are therefore treated as a unit of conservational importance separate from their parental species (Plötner 2005). However, a distinction by mtDNA, such as in DNA barcoding, is not possible because *P. esculentus* carries the mitochondrial genome of either *P. lessonae* (in most cases) or *P. ridibundus* (Plötner *et al.* 2008).

As a consequence of cross-breeding via hybridization between the two parental species, mitochondrial alleles of *P. lessonae* can also be found in specimens identified as *P. ridibundus* by morphology or nuclear DNA (Fig. 2; see also Ohst 2008; Plötner *et al.* 2008). No alleles of *P. ridibundus* have been found in *P. lessonae* in any previous studies (J. Plötner, pers. comm. 2014), but Fig. 2 shows *P. ridibundus* alleles in the *P. lessonae* clade. Probably, the specimens here identified as *P. ridibundus* (in the *P. lessonae* cluster) are actually hybrids. This underlines the difficulty of identifying Central European water frogs with any morphological or genetic method.

As stated above in the results section, all other species and subspecies studied are represented by distinct clusters and BINs in the barcoding tree. While the genetic distances within some of these clusters are low and very similar, other clusters show substructures that do not correspond to any previously recognized taxa of species or subspecies level. The advance of molecular genetics in the last decades supported the detection and consolidation of a number of cryptic species among European reptiles and amphibians, such as the full recognition of the species in the complex of *Triturus* newts (Macgregor *et al.* 1990), the resurrection of *Lacerta bilineata* Daudin 1802; as a taxon distinct from *L. viridis* (Laurenti 1768) (Rykena 1991; Amann *et al.* 1997), and the description of *Emys trinacris* Fritz, Fattizzo, Guicking, Tripepi, Pennisi, Lenk, Joger & Wink, 2005; as a taxon distinct from *E. orbicularis* (Linnaeus 1758) (Fritz *et al.* 2005). In our barcoding tree, the *COI* distance between *Lacerta bilineata* and *L. viridis* is 6.01%, with all samples of *L. viridis* sharing the same haplotype ($N = 3$). Furthermore, Stöck *et al.* (2006) detected a number of divergent mitochondrial lineages within *Bufo viridis* and tentatively used the name *B. variabilis* for a widespread lineage also inhabiting Germany. We tentatively consider these divergences to be deep conspecific lineages and maintain the name *B. viridis* for all German lineages of the complex.

For several species, our tree shows splits into two clusters in a pattern similar to that of *Lacerta bilineata* and *L. viridis*, albeit much shallower. In *Lissotriton vulgaris* (distance between clusters 1.54%, within clusters ≤0.2%, $n = 2 + 3$) and *Natrix natrix* (maximum intraspecific distance 5.84%, $N = 9$), the split is reflected by the assigning

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Table 1 K2P distances of *COI* within families, genera and species studied

Species	Country	BIN	*N*	I_{min}	I_{max}	Nearest neighbor	DNN
Anura							
Alytidae							
Alytes obstetricans	DE	BOLD:AAJ2048	1	0.45	0.74	*Salamandra salamandra*	15.89
Bombinatoridae							
Bombina bombina	HU	BOLD:AAD1964	2	0.46	0.46	*Bombina variegata*	10.19
Bombina variegata	DE	BOLD:AAD4416	5	0	0	*Bombina bombina*	10.19
Bufonidae							
Bufo bufo	DE	BOLD:AAC2139	11	0.11	0.46	*Bufo calamita*	17.57
Bufo calamita	DE	BOLD:AAI8496	3	1.13	1.7	*Bufo viridis*	17.44
Bufo viridis	DE	BOLD:AAJ8500	6	0.14	0.35	*Bufo calamita*	17.44
Eleutherodactylidae							
Eleutherodactylus coqui	DE	BOLD:ACC1316	1	N/A	N/A	*Alytes obstetricans*	26.53
Hylidae							
Hyla arborea	DE	BOLD:AAN9979	3	0.29	0.48	*Bufo bufo*	24.27
Pelobatidae							
Pelobates fuscus	DE	BOLD:AAL6663	6	0.8	1.54	*Emys orbicularis*	24.59
Ranidae							
Pelophylax esculentus	DE	BOLD:AAN3045	1	N/A	N/A	*Pelophylax ridibundus*	0.46
Pelophylax lessonae	DE	BOLD:AAM0091	5	5.15	15.37	*Pelophylax ridibundus*	0
	DE	BOLD:ACM1278	1				
Pelophylax ridibundus	DE	BOLD:AAN3045	13	8.16	17.22	*Pelophylax lessonae*	0
	DE	BOLD:AAM0091	9				
Rana arvalis	DE	BOLD:AAL1420	5	0.16	0.32	*Rana temporaria*	10.42
Rana dalmatina	DE	BOLD:AAM0090	3	0.21	0.31	*Rana temporaria*	14.03
Rana temporaria	AT, DE	BOLD:AAL6095	12	0.03	0.16	*Rana arvalis*	10.42
Caudata							
Salamandridae							
Ichthyosaura alpestris	AT, DE	BOLD:AAC5105	13	0.23	0.61	*Salamandra salamandra*	19.93
Lissotriton helveticus	DE	BOLD:AAE8022	2	0	0	*Lissotriton vulgaris*	19.27
Lissotriton vulgaris	DE	BOLD:AAL6213	2	0.95	1.54	*Lissotriton helveticus*	19.27
	DE	BOLD:ACF1004	3				
Salamandra atra	AT, DE	BOLD:ACM1022	6	0.25	0.46	*Salamandra salamandra*	9.17
Salamandra salamandra	DE	BOLD:ACE6170	12	0.54	1.71	*Salamandra atra*	9.17
Triturus carnifex	AT, DE	BOLD:ACE8564	3	0.11	0.17	*Triturus cristatus*	8.08
Triturus cristatus	DE	BOLD:AAC3031	3	0.37	0.61	*Triturus carnifex*	8.08
Squamata							
Anguidae							
Anguis fragilis	DE	BOLD:AAK0900	12	0.2	0.52	*Bombina variegata*	21.69
Colubridae							
Coronella austriaca	DE	BOLD:AAL9606	5	0	0	*Zamenis longissimus*	12.3
Zamenis longissimus	DE. SL	BOLD:AAL5946	11	0.16	0.57	*Coronella austriaca*	12.3
Natricidae							
Natrix natrix	AT, DE	BOLD:AAL6710	8	2.6	5.84	*Natrix tessellata*	9.36
	DE	BOLD:ACM1720	2				
	DE	BOLD:AAX3380	1				
Natrix tessellata	DE, SL	BOLD:AAN4201	3	0.77	1.55	*Natrix natrix*	9.36
Viperidae							
Vipera aspis	FR	BOLD:ACM0956	1	N/A	N/A	*Vipera berus*	11.89
Vipera berus	DE	BOLD:AAW7158	4	1.61	2.83	*Vipera aspis*	11.89
	AT	BOLD:ACM2231	1				
Lacertidae							
Lacerta agilis	AT, DE	BOLD:AAL6669	7	0.29	0.61	*Lacerta viridis*	13.66
Lacerta bilineata	DE	BOLD:AAX0768	1	N/A	N/A	*Lacerta viridis*	6.01
Lacerta viridis	DE	BOLD:AAJ3146	3	0.1	0.15	*Lacerta bilineata*	6.01
Podarcis muralis	AT, DE	BOLD:AAL6640	15	3.13	8.7	*Zootoca vivipara*	18.14
	DE	BOLD:AAL6639	4				
	DE	BOLD:ACF0185	11				
	DE	BOLD:ACM2400	2				

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Table 1 (Continued)

Species	Country	BIN	*N*	I_{min}	I_{max}	Nearest neighbor	DNN
Zootoca vivipara	AT, DE	BOLD:AAL6569	9	0.09	0.31	*Podarcis muralis*	18.14
Testudines							
Emydidae							
Emys orbicularis	DE, FR, HU, IT, PL, SP	BOLD:AAF8183	8	1.15	2.51	*Emys trinacris*	1.87
Emys trinacris	IT	BOLD:AAF8183	2	0.77	0.77	*Emys orbicularis*	1.87

BIN, Barcode Index Number, an identification number for barcoding clusters recognized by BOLD within the species; *N*, number of barcode sequences; I_{min}, minimum intraspecific distance; I_{max}, maximum intraspecific distance. Nearest neighbour = most closely related species retrieved in the barcoding. DNN = Average genetic distance to the nearest neighbour.

of different BINs. Further species with intraspecific lineage diversity but without splitting into different BINs are *Bufo calamita* (distance between clusters 1.7%, within cluster 0%, $n = 1 + 2$), *Pelobates fuscus* (distance between clusters 1.54%, within clusters 0%, $n = 2 + 3$) and *Anguis fragilis* (distance between clusters 0.52%, within clusters 0%, $n = 1 + 2$). This lineage diversity may reflect biogeographical structures at the population level or populations containing different haplotypes, which are outside the scope of this study. Previous studies on the population genetics of *Bufo calamita* already traced a postglacial expansion from its Iberian refuge (Rowe *et al.* 2006; Beebee & Rowe 2008). A genetic structuring of populations across Europe was also shown for *L. vulgaris* (Weisrock *et al.* 2006). Our *COI* data also show a genetic structure in Bavarian *B. calamita*, but further sampling is necessary to allow for a comparison with previous results. Extensive studies have also been conducted on the phylogeography of *P. fuscus*, but these were mostly centred on southern and eastern European populations and have not detected any genetic structure similar to that shown in our data set (Borkin *et al.* 2001; Crottini *et al.* 2007; Litvinchuk *et al.* 2013). Similarly, extensive studies on the phylogeography of *A. fragilis* covered German populations only peripherically (Gvoždík *et al.* 2010, 2013). Against this background, the results of our

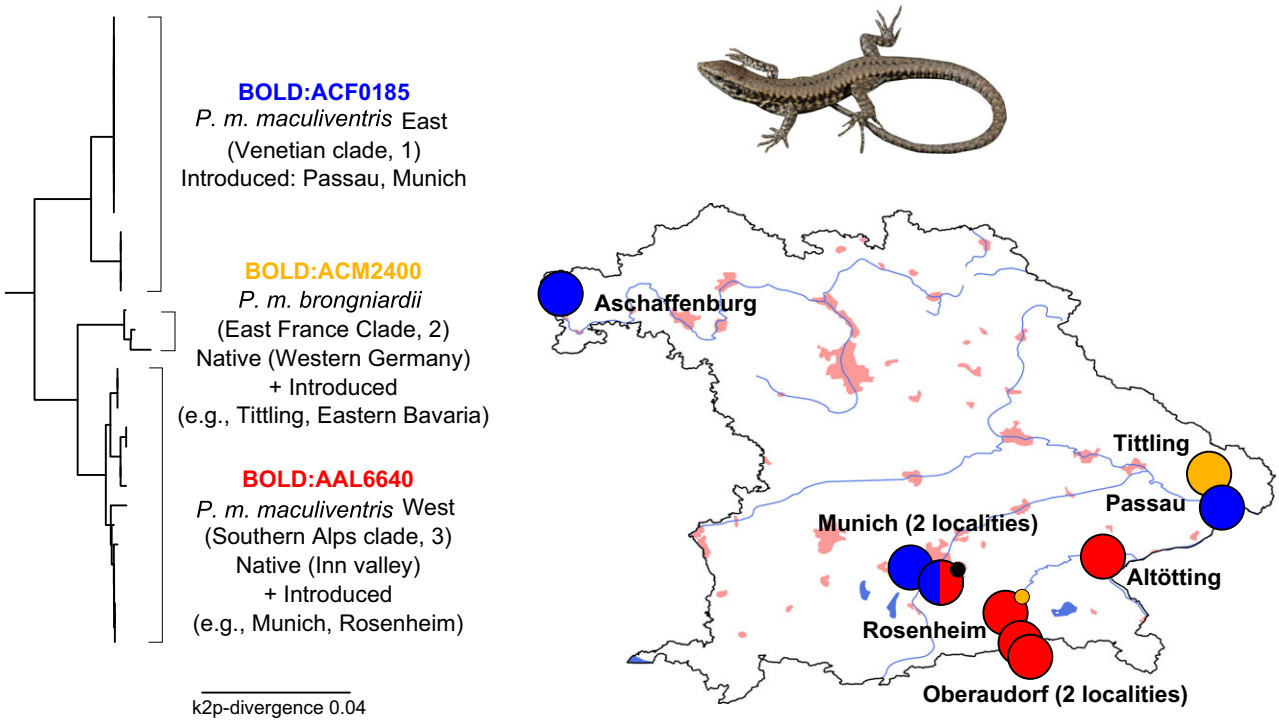

Fig. 3 Subspecies of the *Podarcis muralis* complex. DNA barcoding retrieves the clades proposed in Schulte *et al.* (2011, 2012a), but does not allow the distinction between autochthonous populations and introduced populations in Bavaria. Circles represent localities in the map of Bavaria, and fill colours refer to clades. The small inlay circles indicate that other clades were found at these localities in the previous studies cited above (Italian Marche lineage, black, in Munich, East France Clade in Rosenheim).

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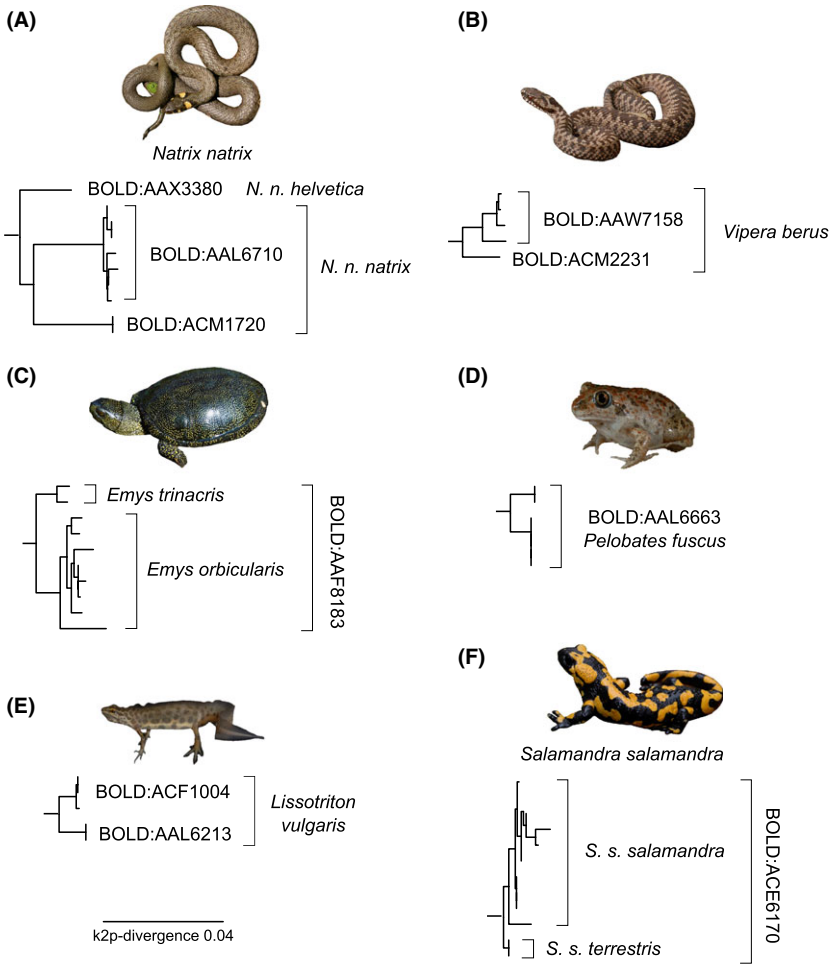

Fig. 4 Cases of discordance between current taxonomy and BINs. (A) High genetic divergences within *Natrix natrix*. The subspecies *N. n. helvetica* is distinguished by a separate BIN, but there were also two BINs retrieved within the nominal form. (B) Two BINs were retrieved within *Vipera berus*. (C) *Emys orbicularis* shares a BIN with *E. trinacris*. (D) Two distinct clades were detected within *Pelobates fuscus*, but they are not represented by different BINs. (E) Bavarian *Lissotriton vulgaris* are clustered in two BINs representing populations north and south of the Danube River. (F) The subspecies *Salamandra salamandra terrestris* forms a cluster distinct from the nominal form, but both subspecies share a BIN.

barcoding study suggest that the biogeography, phylogeny and possibly also taxonomy of German reptiles and amphibians may warrant further investigation.

In a number of other species, the genetic structure is more complex, and more than one distinct clade is found within a cluster. This is the case in *Salamandra salamandra* (maximum intraspecific distance 1.33%, $N = 9$), *Vipera berus* (maximum intraspecific distance 2.83%, $N = 5$) and *Emys orbicularis* (maximum intraspecific distance 2.51%, $N = 8$). All these species occupy large areas across Europe and partly Asia, and complex dynamics of recolonization of their ranges from multiple glacial refugia have been demonstrated. Large-scale phylogeographic studies have been conducted for *S. salamandra* (Steinfartz *et al.* 2000) and *V. berus* (Ursenbacher *et al.* 2006), but no exhaustive sampling of German populations was included in any of these studies. Our barcoding topology shows that genetically divergent populations are also present in Central Europe. These populations may be of high relevance for phylogeographic studies because Central Europe was the area that was recolonized latest after the end of the glaciations. Ursenbacher *et al.* (2006) suggested the hypothesis of a postglacial recolonization of Central Europe by *V. berus* from refugia in the Balkan and in the Carpathians, immigrating into Central Europe from the east to the west.

The genetic structuring in our barcoding topology may also reflect taxonomic implications. Kindler *et al.* (2013) showed that mtDNA of *N. natrix* did not correspond with the current subspecific taxonomy. The samples included in our study may therefore include both currently recognized subspecies and deep mitochondrial lineages without taxonomical relevance.

Deep mitochondrial lineages are also detected in our samples of *Emys orbicularis*. This species is represented by a monophyletic cluster including samples from many parts of Europe and forms the sister clade to *E. trinacris*. The *COI* distance between the two species of 1.87% is lower than the maximum intraspecific distance of *E. orbicularis* from various regions of Europe of 2.51%. The overall low genetic divergence at species level is possibly attributable to the rates of mitochondrial evolution that are very different between groups of reptiles (Nagy *et al.*

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2012; Hawlitschek *et al.* 2013). This may also be the reason why *E. trinacris* is not assigned a distinctive BIN, as the RESL algorithm assigns BINs based on clustering relative to neighbouring sequences. Nevertheless, the topology of our *COI* tree is in concordance with studies confirming the basal position of *E. trinacris* in relation to *E. orbicularis* from its entire Eurasian range (Fritz *et al.* 2005). As also thorough morphological studies (e.g. Fritz *et al.* 2006) have proven statistically significant differences between *E. orbicularis* and *E. trinacris*, the distinct taxonomic status of the latter taxon appears justified.

Wall lizards (*Podarcis muralis*) are comparably widespread and common in western Germany, where large native populations exist in the valleys of the rivers Rhein and Mosel and many of their tributaries. By contrast, Bavaria only houses one comparably small native population at the Inn valley close to the Austrian border (Schulte 2008). In addition, many introduced populations of different subspecies and genetic lineages exist all over Germany and the species is commonly characterized as invasive (at least 84 populations in 2011: Schulte *et al.* 2011, 2012a): the genetic integrity of native wall lizard populations may be threatened by hybridization with translocated non-native specimens (Schulte *et al.* 2012b), and other native lizard species may be negatively impacted (Heym *et al.* 2013). The distinction between native and introduced populations of wall lizards is therefore of high conservational importance (Bräu & Sacher 2009), but difficult or even impossible to accomplish by any methods applicable in the field (Schulte *et al.* 2011).

Our wall lizard topology forms three major clusters (Fig. 3) largely in concordance with the studies on cytochrome b sequences by Schulte *et al.* (2011, 2012a). (i) Venetian clade (*P. m. maculiventris* East): comprises all samples from the introduced populations of Passau, Aschaffenburg and some from Munich. Individuals from Passau and the adjoining valley of the Danube River represent the largest and oldest German introduced wall lizard population, which dates back to the first half of the 20th century (Sochurek 1982; Schulte *et al.* 2008); (ii) Eastern France clade (*P. m. brongniardii*): comprises samples from introduced populations from eastern Bavaria (Tittling) and from a native population in western Germany (Wachenheim); (iii) Southern Alps clade (*P. m. maculiventris* West): comprises native populations from the Inn valley (Oberaudorf, Kiefersfelden) and introduced populations of Rosenheim, Munich and Altötting. A distinction between native and introduced *COI* haplotypes was not possible.

Our barcoding topology of wall lizards furthermore confirms that some introduced populations are based on multiple introductions, as assumed in Schulte *et al.* (2011, 2012a). We detected at least two genetic lineages, that is the Southern Alps clade and the Venetian clade, in urban populations in Munich (Großmarkthalle/Südbahnhof, Aubing), whereas Schulte *et al.* (2011) identified individuals from the Central Italian Marche lineage at one of these localities. Moreover, Schulte *et al.* (2011) allocated non-native specimens from Rosenheim to the Eastern France lineage, whereas our samples from this locality clustered with native individuals belonging to the Southern Alps lineage. The comparison of our results with the previous studies cited above (i) shows that exhaustive sampling especially of urban populations of wall lizards may yield lineages belonging to more than one of the major clades. These populations could therefore be characterized as 'hot spots' of the mixture of clades introduced from many parts of Europe. Furthermore, (ii) it suggests that Central European wall lizard populations are in a highly dynamic state and that there are ongoing fast migrations between these 'hot spots'.

Our study highlights many potential applications for the DNA barcoding data of the German herpetofauna. As described above, barcodes of many species allow the identification of the geographical origin of the samples, at least at a broad resolution. This makes DNA barcoding an excellent tool for tracing natural and human-mediated migration activities of these animals. Some examples, such as allochthonous populations of *Podarcis* lizards (Schulte *et al.* 2012b) and *Pelophylax* frogs (Plötner 2005; Ohst 2008; Holsbeek & Jooris 2010), were already studied using mtDNA and nDNA markers. Increased human traffic across Europe and perhaps a changing climate will likely contribute to the increase of migrations of amphibians and reptiles at the population and species levels (Araújo *et al.* 2006; Meyerson & Mooney 2007). Such events can be detected early and traced easily with DNA barcoding once a database covering all Europe is installed, but also regional data sets – such as the one released here – allow the detection of divergent and possibly allochthonous haplotypes. The observation of changes in the distributions of animal species may be of conservational importance, such as in the case of *Podarcis* (Schulte *et al.* 2012b), and will also contribute to our understanding of biogeographical patterns and the ecological and climatic events that are responsible for their development.

The complete barcoding of the German herpetofauna will also serve as a starting point in an European context. As pointed out in a review of the distribution of European amphibians and reptiles (Sillero *et al.* 2014), the geographical ranges of many of these species are not well known, often because of insufficiently defined species boundaries and changes in taxonomy. DNA barcoding may help in delimiting the ranges of European species of amphibians and reptiles and of phylogeographic lineages within these species.

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So far, DNA barcoding has been mostly performed using traditional Sanger sequencing, likely because of the focus on nonmodel organisms, and of the lack of consensus on library preparation protocols (McCormack *et al.* 2013). However, there is also a range of applications that will require the implementation of high-throughput sequencing methods. Environmental sequencing, that is the sampling of DNA from the (mostly aquatic) medium without any contact with the actual organisms, was developed to a large extent based on amphibians (Hajibabaei *et al.* 2011; Thomsen *et al.* 2012). DNA barcodes will allow easy identification of such environmental samples making environmental barcoding a tool that will generate much new knowledge on the distribution and ecology of the allegedly well-known amphibian fauna of Central Europe. This is only one example that suggests that DNA barcoding data will be an important tool in organismic biology also in the future and that the investment into the production of DNA barcoding data is a sustainable one.

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Provided samples: O.H., M.F., D.R. and F.G.; conducted lab work: J.M. and A.D.; analysed the data: O.H. and J.M.; wrote the manuscript: O.H., J.M., M.F., F.G. and G.H.

Data accessibility

All data are accessible via the BOLD database in the project Fauna Bavarica – Herpetology (FBHER) (http://www.boldsystems.org/) under dx.doi.org/10.5883/DS-HERPGER. Information on the sequence data and GenBank Accession numbers are also given in Table S1, Supporting information.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Data of specimens and samples used in this study.

Fig. S1 Neighbor-Joining tree based on *COI* barcodes of German amphibians and reptiles, created in BOLD.

Appendix S1. GoogleEarth kml file with the localities of all samples from Germany.

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