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On the origin of the recent herpetofauna of Sicily: Comparative phylogeography using homologous mitochondrial and nuclear genes

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ABSTRACT

In contrast to oceanic, continental islands are expected to show less diversification and endemism and thus phylogeographic signatures of multiple colonization events from adjacent continents due to episodic connections by sea level changes. In order to test this situation for the herpetofauna of Sicily, we here focus on three amphibian and four reptile species-groups and investigate their phylogeographic relationships across the Sicily and Messina straits, where Plio-Pleistocene marine transgressions shortened the distances between (or connected) Sicily, North Africa and/or the Italian (Apennine) Peninsula. Using a multi-species, multi-marker phylogeographic approach (mitochondrial cytochrome *b*; 16S rDNA, nuclear intron of tropomyosin), we apply Bayesian and Maximum Likelihood phylogenetic methods and haplotype networks to examine the phylogenies, and to estimate divergence times from molecular data using the program BEAST. We recognize three colonization patterns: (i) Plio-Pleistocene colonization of Sicily from North Africa for the skinks *Chalcides chalcides* (1.8 Mya) and *Chalcides ocellatus* (0.61 My), (ii) Pleistocene colonization from the Italian Peninsula for the anurans *Pelophylax* spp. (0.81 Mya) and *Bufo bufo* (late Pleistocene), and (iii) recent (late Pleistocene to Holocene), natural or man-mediated out-of-Africa dispersal for the anuran *Discoglossus pictus* and out-of-Africa human introduction for the gekkonid lizards *Tarentola mauritanica* and *Hemidactylus turcicus*. The Sicilian herpetofauna shows phylogeographic signatures as typical of continental islands, with limited diversification and endemism. Colonization by terrestrial amphibians and reptiles from adjacent continents appears shaped by interactions of the active geo-marine history along with species' ecology and human intervention, including a widely neglected faunal contribution from Africa. On some small islands and in Tunisia, we found isolated local populations significant for conservation. Our results underline how only multispecies approaches involving ecologically diverse taxa are able to reveal the complexity of faunal contributions to large continental islands like Sicily.

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1. Introduction

In contrast to oceanic ones, continental islands are expected to show less diversification and endemism because they represent environmental and biological detachments of the mainland due to episodic connections by sea level changes (Williamson, 1981; Whittaker and Fernandez-Palacios, 2007; Cody, 2006; Johnson et al., 2012; Fabre et al., 2012). However, for specific, relatively old (continental) islands, origin and/or timing of colonization by organisms are of high interest to understand their biogeographic history and the underlying evolutionary processes.

The geological past and biogeography of the Mediterranean and its continental islands was first imprinted by the final Miocene period, the Messinian (7.25–5.33 Mya), which isolated the Mediter-

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ranean Sea from the Atlantic and created salt lakes and deserts, serving as "near-land-bridges" (Krijgsman et al., 1999; lower sea level facilitating faunal exchanges) for terrestrial fauna between Africa, Europe and most islands ("Messinian crisis"; Krijgsman et al., 1999). Messinian exchange ended with a dramatic flooding through the Strait of Gibraltar (5.33 Mya; Garcia-Castellanos et al., 2009), leading to the probably oldest formation of islands in the recent Mediterranean. However, during the following Plio-Pleistocene glacial cycles, sea level changes (Rohling et al., 1998) brought North-African and European coasts closer again (Thiede 1978; Krijgsman et al., 1999), with episodes of potential faunal exchange on islands.

The resulting complex Messinian and Plio-Pleistocene phylogeographic relationships of North-African and Southwest-European (Iberian) faunas have been widely studied (e.g. Busack, 1986; Carranza et al., 2006b; Fromhage et al., 2004; Busack and Lawson, 2008). In contrast, phylogenetic history and biogeography and their timing in Sicily were much less examined. From early Pliocene to early Pleistocene, Sicily consisted of two islands (equivalent to modern north-central and south-eastern portions; van der Geer et al., 2010; Guglielmo and Marra, 2011). The Strait of Sicily was formed at the end of the Messinian, when the last well-documented connection between African mainland and a landmass that became part of Sicily was flooded. While Sicily and Tunisia are at present approximately 140 km apart, Pleistocene sea levels of −120 m (Thiede, 1978; Rohling et al., 1998; Dorale et al., 2010) have brought African and Sicilian coasts closer than ~50 km. "Stepping stone islands" (Stöck et al., 2008a) may have facilitated terrestrial animals in overcoming this sea barrier. This geological history has been documented by close faunal relationships in co-occurring taxa between N-Africa (from now: N-Africa) and Sicily from single animal species groups (e.g. Zangari et al., 2006; Giovannotti et al., 2007; Stöck et al., 2008a; Kornilios et al., 2010; Carranza et al., 2008), and between the Italian Peninsula (from now "IP") and Sicily (Giovannotti et al., 2007; Colliard et al., 2010; Stöck et al., 2008a,b; Kindler et al., 2013; and refs. therein). Several other hypotheses on the colonization patterns of Sicily remained partly speculative; mostly based on taxonomic assignments and limited palaeontological data (cf. SIB, 2011). However, comparative molecular-based estimations of times of divergence with sufficient samples sizes from N-Africa, Sicily and the IP are scarce, and for Sicily no multispecies study on the timing of the origins of the terrestrial herpetofauna has been undertaken. In doing so, we expect to find single or multiple dispersals across the Strait of Sicily (out-of-Africa) and/or the region that formed the Italian (Apennine) Peninsula (out-of-Italy). Assuming no overseas dispersal, relationships could be explained by three major scenarios: (i) a Messinian land-connection (>5.3 Mya); (ii) post-Messinian (=Plio-Pleistocene) "near-land-bridges" or "stepping stone islands" at low sea levels (<5.3 Mya to 10 kya); (iii) recent (Holocene) human introductions (<9 kya). As typical of continental islands, for any given species, scenarios might also be more complex, involving both Plio-Pleistocene out-of-Africa and Pleistocene out-of-Italy events, as shown for green toads (Stöck et al., 2008a; Colliard et al., 2010).

2. Material and methods

2.1. Sampling

In order to examine potentially complex patterns, we use homologous mtDNA and nuDNA markers from three terrestrial amphibian and four reptile 'species-groups' (term used throughout the paper to circumscribe more or less closely related taxa without implications on taxonomy or depth of relationships). For several of these species-groups we analysed a greater number of samples from different, and often more, localities in the target region than previous studies. Sampling was undertaken to fulfill four major prerequisites: species-groups should (i) be represented in all three regions of interest (Italian mainland, Sicily and N-Africa) or at least on Sicily and one of the other two, (ii) display different ecologies and susceptibilities to man-mediated introduction, expected to reflect a spectrum of colonization histories of Sicily; (iii) include as many sampling localities as possible, especially on Sicily, and (iv) include samples from the islands in the Strait of Sicily, if present.

Species-groups selected for analyses (distributions: Figs. 1–3) were painted frogs (*Discoglossus pictus* Otth, 1837, Anura, Discoglossidae); common toads (*Bufo bufo* (Linnaeus, 1758) and *B. spinosus* Daudin, 1803, Anura, Bufonidae); Palearctic water frogs (*Pelophylax* kl. *hispanicus* (Bonaparte, 1839), *P. bergeri* (Günther, 1985) and *P. saharicus* (Boulenger in Hartert, 1913), Anura, Ranidae); ocellated skinks (*Chalcides ocellatus* (Forskål, 1775), Squamata, Scincidae); three-toed skinks (*Chalcides chalcides* (Linnaeus, 1758), Squamata, Scincidae); Turkish geckos (*Hemidacylus turcicus* (Linnaeus, 1758), Squamata, Gekkonidae); and Moorish wall geckos (*Tarentola mauritanica* (Linnaeus, 1758), Squamata, Gekkonidae). Samples, were collected from multiple localities in N-Africa, Sicily, the IP and on Mediterranean islands in the Strait of Sicily (Pantelleria, Linosa, Lampedusa and Malta), or obtained from scientific collections (Table S1). Published sequences (GenBank) were used for outgroups (Table S1 for details on specimens examined, and museum vouchers; File S3 for all sequence data and alignments).

2.2. DNA extraction, amplification, cloning and sequencing

Genomic DNA was extracted from ethanol preserved muscle tissue, tail tips or frozen buccal swabs using the DNeasy Blood & Tissue Kit (Qiagen). Two mitochondrial markers were amplified in all species (Table 1). In amphibians, we PCR-amplified most of the mitochondrial cytochrome *b* (from now: cyt-b) using primers MVZ15-L_AmphCytb/Ptacek2-H (94 °C, 7 min, denaturation; cycle [94 °C, 40 s, denaturation; 46 °C, 30 s, annealing; 72 °C, 90 s, extension] 40 times; 72 °C, 10 min, final extension; Moritz et al., 1992). For lizards, cyt-b was amplified with primers Rep_rGlu-1L/Rept_rcytb-1H (94 °C, 1 min, denaturation; cycle [94 °C, 30 s, denaturation; 48 °C, 60 s, annealing; 72 °C, 60 s, extension] 38 times; 72 °C, 5 min, final extension). We further amplified the 16S rDNA, for amphibians using primers 16Sar-L/16Sbr-H (PCR: 95 °C, 3 min, denaturation; cycle [94 °C, 45 s, denaturation; 55 °C, 45 s, annealing; 72 °C, 60 s, extension] 35 times; 72 °C, 5 min, final extension; Kocher et al., 1989); for reptiles with primers L2606/H3056 (PCR: 96 °C, 2 min, denaturation; cycle [94 °C, 30 s, denaturation; 55 °C, 45 s, annealing; 72 °C, 90 s, extension] 38 times; 72 °C, 5 min, final extension; Kocher et al., 1989).

We chose a nuclear marker, which could be commonly amplified in all amphibian and reptile species and that was expected to provide appropriate phylogeographic signals: intron 5–6 of nuclear *alpha*-tropomyosin (Friesen et al., 1999). To amplify this marker, we used primers of Friesen et al. (1999); "frog version" for amphibians, "bird version" for *Chalcides*; we restricted analyses for geckos on mtDNA). PCR conditions for "frogs" were adapted: 95 °C, 1:30 min; cycle [94 °C, 30 s; 59.0°, 30 s; 72 °C, 45 s] 30 times; 72 °C, 5 min (Stöck et al., 2008a); PCR conditions for reptiles were: 94 °C, 1:30 min; cycle [94 °C, 30 s; 55°, 30 s; 72 °C, 45 s] 30 times; 72 °C, 5 min.

Mitochondrial PCR products were sequenced directly. Nuclear amplicons were cloned using the pGEM®easy system (Promega) as described (Stöck et al., 2008a). To identify both alleles in potential heterozygotes prior to sequencing, 1 μl of DNA from 10 positive colonies (clones) was re-amplified (primers M13F/M13R), products

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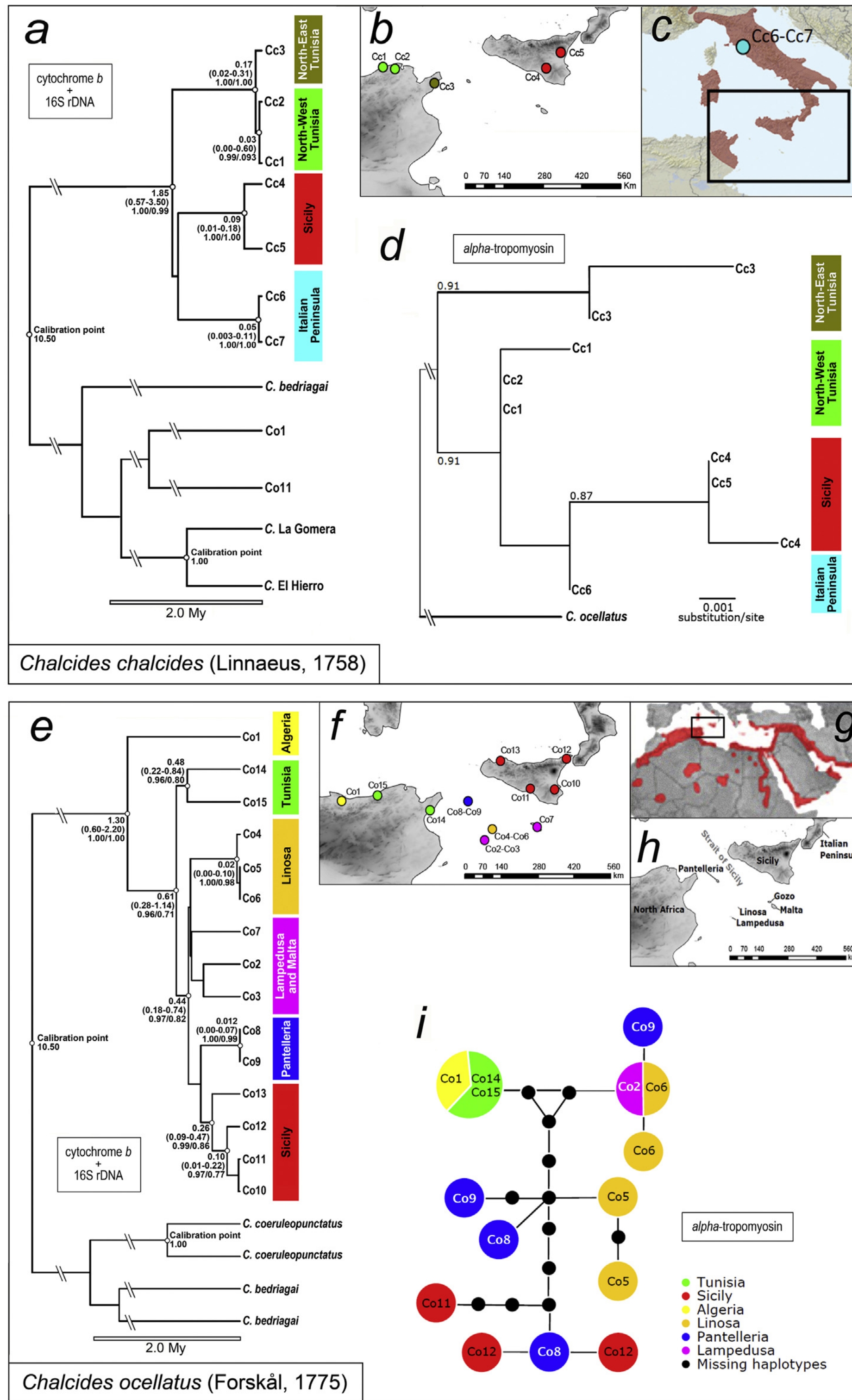

Fig. 1. Phylogeography of three-toed skinks (*Chalcides chalcides, a–d*) and ocellated skinks (*Chalcides ocellatus, e–i*) in Sicily and adjacent regions based on mitochondrial and nuclear DNA sequence data. (a) Bayesian tree from concatenated cytochrome *b* + 16S rDNA (1255 bp) of *C. chalcides* from localities in (b) and (c); *C. ocellatus, C. bedriagai* and *C. coeruleopunctatus* served as outgroup; (c) range of *C. chalcides* (after www.iucnredlist.org) with some sampling localities; (d) ML tree based on 450 bp of a nuclear intron of *alpha*-tropomyosin; (e) Bayesian tree from concatenated cytochrome *b* + 16S rDNA (1280 bp) of *C. ocellatus* from localities in (f), *C. chalcides, C. bedriagai* and *C. coeruleupunctatus* served as outgroup; (g) range of *C. ocellatus* (map from Carranza et al., 2008) with sampling area and (h) names of sampled islands in the Strait of Sicily; (i) network analysis of *C. ocellatus* based on ca. 450 bp of a nuclear intron of *alpha*-tropomyosin (note that heterozygous copies appear twice with the same sample name). Numbers at branch tips and localities refer to specimen numbers in Table S1; numbers at nodes represent divergence time estimates in My (top) followed by confidence intervals (95%; below) and bootstrap probabilities from 1000 resampled datasets and Bayesian posterior probabilities (below). (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

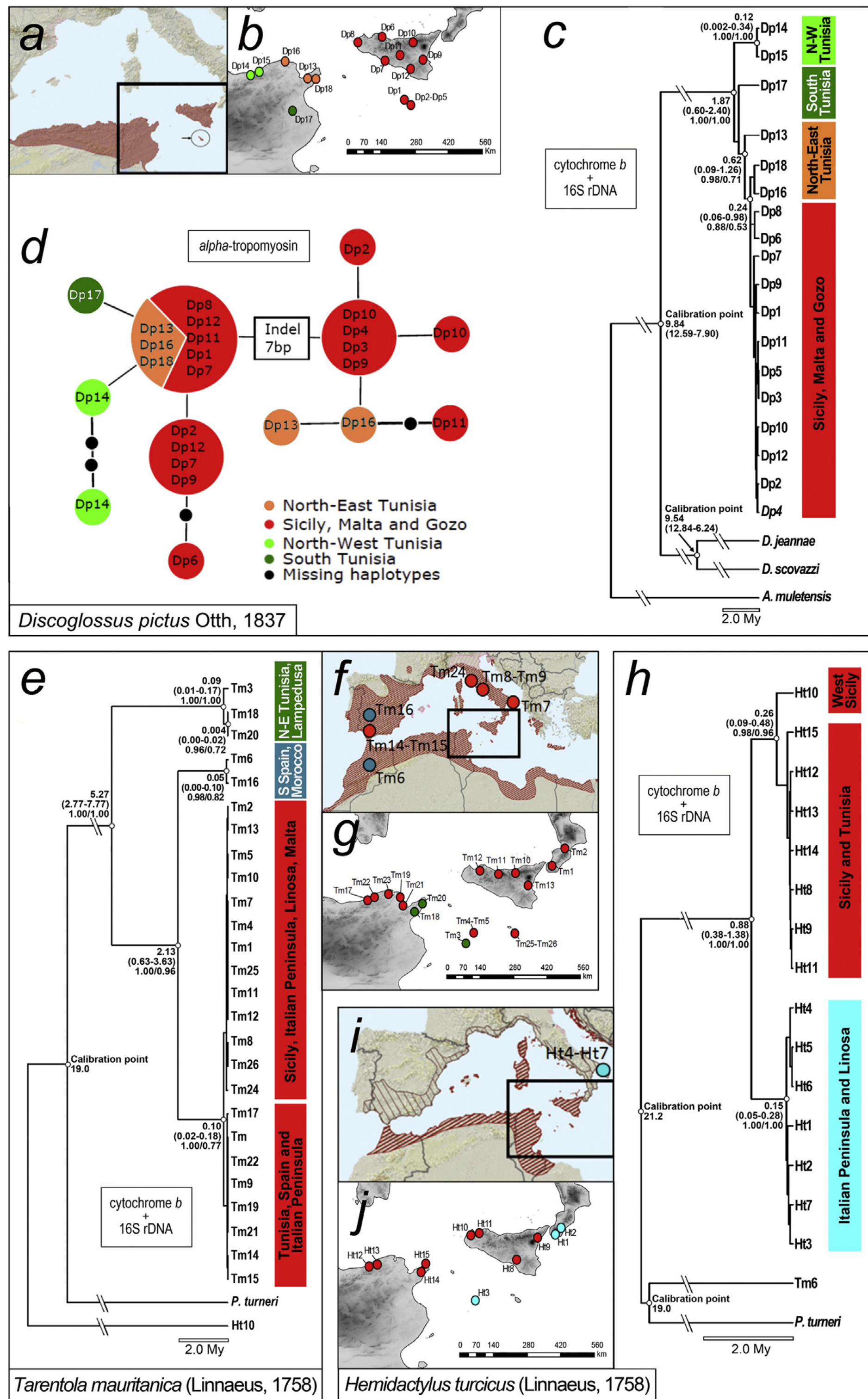

Fig. 2. Phylogeography of painted frogs (*Discoglossus pictus*, a-d) based on mitochondrial and nuclear sequence data, Moorish wall geckos (*Tarentola mauritanica*, e–g) and Turkish geckos (*Hemidactylus turcicus*, h–j) based on mtDNA, in Sicily and adjacent regions. (a) range of *Discoglossus pictus* (after www.iucnredlist.org); (c) Bayesian tree from concatenated cytochrome *b* + 16S rDNA (1460 bp) of *D. pictus* from localities in (b), *D. jeannae*, *D. scovazzi* and *Alytes muletensis* served as outgroup; (d) network analysis of *D. pictus* based on ca. 450 bp of a nuclear intron of *alpha*-tropomyosin (note that heterozygous copies appear twice with the same sample name); (e) Bayesian tree from concatenated cytochrome *b* + 16S rDNA (1235 bp) of *T. mauritanica* from localities in (f) and (g), *Pachydactylus turneri* and *Hemidactylus turcicus* (Ht10) served as outgroup; (f) range of *T. mauritanica* (after www.iucnredlist.org) with some sampling localities; (h) Bayesian tree from concatenated cytochrome *b* + 16S rDNA (1266 bp) of *H. turcicus* from localities in (i) and (j), *Pachydactylus turneri* and *Tarentola mauritanica* (Tm6) served as outgroup; (i) range of *H. turcicus* (after www.iucnredlist.org) with some sampling localities. Numbers at branch tips and localities refer to specimen numbers in Table S1; numbers at nodes represent divergence time estimates in My (top) followed by confidence intervals (95%; below) and bootstrap probabilities from 1000 resampled datasets and Bayesian posterior probabilities (below). (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

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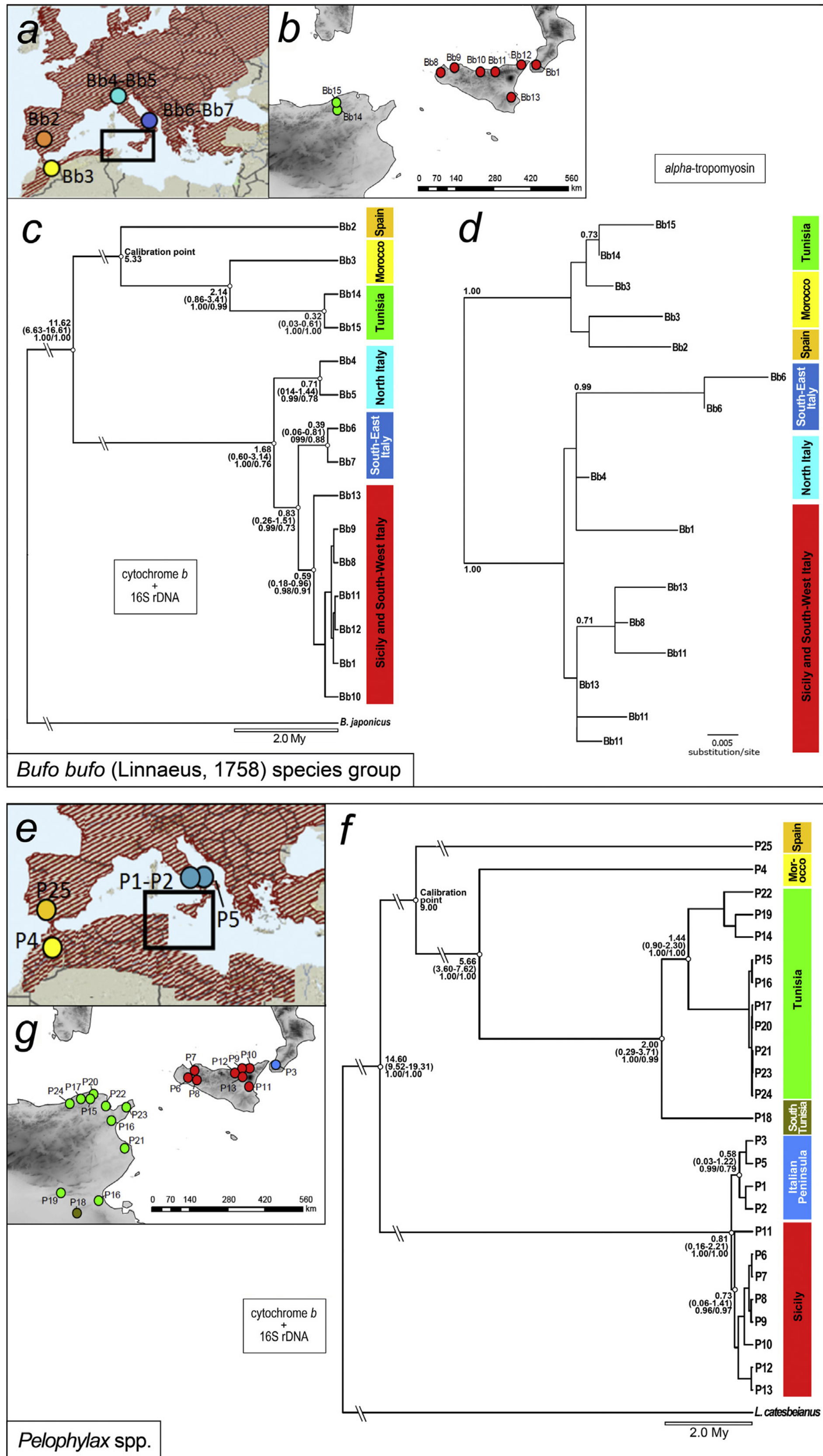

Fig. 3. Phylogeography of common toads (*Bufo bufo* species-group, a–d), based on mitochondrial and nuclear DNA, and of water frogs (*Pelophylax hispanicus, P. bergeri, P. perezi* and *P. saharicus*, e–g), based on mtDNA, in Sicily and adjacent regions. (c) Bayesian tree from concatenated cytochrome *b* + 16S rDNA (1480 bp) of *B. bufo* and *B. spinosus* from localities in (a) and (b), *B. japonicus* was used as outgroup; (a) range of *B. bufo* species-group (after www.iucnredlist.org) with some sampling localities; (d) ML tree for common toads based on 980 bp of a nuclear intron of *alpha*-tropomyosin (note that heterozygous copies appear twice with the same sample name); (f) Bayesian tree obtained from concatenated cytochrome *b* + 16S rDNA (1480 bp) of waterfrogs from localities in (e) and (g), *Lithobates catesbeianus* was used as outgroup; (e) range of *Pelophylax* spp. with some sampling localities (after www.iucnredlist.org). Numbers at branch tips refer to specimen numbers in Table S1. Numbers at nodes represent divergence time estimates in My (top) followed by the confidence intervals (95%; below) and bootstrap probabilities from 1000 resampled datasets and Bayesian posterior probabilities (below). Specimen numbers refer to Table S1. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

Table 1
Details on sequence markers. Primer name, reference, primers sequence, target gene, approximate fragment length per species.

Primer	Source	5′ => 3′ sequence	Target	PCR product sizes
16Sar-L	Kocher et al. (1989)	CGCCTGTTTATCAAAAACAT	16S rDNA	*Bufo* spp. (∼570 bp), *D. pictus* (∼560 bp),
16Sbr-H	Kocher et al. (1989)	CCCGTCTGAACTCAGATCACGT		*Pelophylax* spp. (∼570 bp)
L2606	Kocher et al. (1989)	CTCCGGTCTGAACTCAGATCACGTAGG	16S rDNA	*C. chalcides* (∼415 bp), *C. ocellatus* (∼440 bp), *T.*
H3056	Kocher et al. (1989)	CTGACCGTGCAAAGGTAGCGTAATCACT		*mauritanica* (∼410 bp), *H. turcicus* (∼450 bp)
MVZ15-L	Moritz et al. (1992)	GAACTAATGGCACCCAAWWTCCGNAA	Cytochrome *b*	*Bufo* spp. (∼910 bp), *D. pictus* (∼900 bp),
Ptacek2-H	Busack and Lawson (2008)	TCTTCTACTGGTTGTCCTCCGATTC		*Pelophylax* spp. (∼915 bp)
Rep_rGlu-1L	This paper	GAAAAACCRCCGTTGTWATTCAACTA	Cytochrome *b*	*C. chalcides* (∼840 bp), *C. ocellatus* (∼840 bp), *T.*
Rep_rcytb-1H	This paper	GCGTAGGCRAATAGGAAGTATCA		*mauritanica* (∼835 bp), *H. turcicus* (∼816 bp)
Trop_frog_F	Friesen et al. (1999)	CGGTCAGCCTCCTCCGCAATGTGCTT	*Tropomyosine*	*Bufo* spp. (∼980 bp), *D. pictus* (∼530 bp)
Trop_frog_R	Friesen et al. (1999)	GAGTTGGATCGCGCTCAGGAGCG		
Trop_bird_F	Friesen et al. (1999)	CGGTCAGCCTCCTCCGCAATGTGCTT	*Tropomyosine*	*C. chalcides* (∼530 bp), *C. ocellatus* (∼530 bp)
Trop_bird_R	Friesen et al. (1999)	GAGTTGGATCGCGCTCAGGAGCG		

digested (65 °C, 75 min) with frequently cutting restriction enzyme *Taq*I (Fermentas), and visualized on 2% agarose gels. Two clones per restriction pattern were sequenced (T7/SP6). All PCR-products were Sanger-sequenced in both directions and strands aligned and edited (Dnastar Lasergene 8).

2.3. Phylogenetic analyses

Species group sequences were aligned (Seaview 4.2.4; Gouy et al., 2010) and cut to equal length. None of the cyt-b and only few 16S rDNA sequences exhibited indels (SNPs) but could be unambiguously aligned. Genetic distances were estimated in MEGA (v. 5.2.2, Tamura et al., 2011; Tamura–Nei (TrN) model). Nucleotide diversity was assessed for mtDNA only, and uncorrected p-distances calculated for within N-Africa, within Sicily, between Sicily and Tunisia, as well as between Sicily and the IP.

Phylogenies where obtained from Maximum Likelihood (ML) and Bayesian analyses (BA) for separate and concatenated mtDNA datasets (16S rDNA + cyt *b*). For nuclear DNA, we performed ML analyses. Substitution models were determined (jModeltest 0.1.1; Posada, 2008; Akaike Information Criterion: Table S2), and applied in PhyML (Seaview; Gouy et al., 2010), starting with BioNJ trees, employing NNI & SPR algorithms for tree improvement. Reliability of ML-trees was assessed by bootstrapping with 1000 pseudo-replications.

For BA phylogenies of mtDNAs, we used Beast (v.1.6.1, Drummond and Rambaut, 2007) under partition-specific substitution models (Table S2) and summarized trees in TreeAnnotator (v.1.6.1). Posterior probability cut-offs were set to 0.5, maximum clade credibility trees were the target; median for node heights. To discard unlikely trees, the "burn-in" was selected visualizing log likelihoods of posterior distributions of trees (Tracer v.1.5).

2.4. Molecular clock and estimation of divergence times

Divergence times for the main mitochondrial lineages were estimated using BEAST (MCMC; Drummond et al., 2005, http://beast.bio.ed.ac.uk/). Since intraspecific analyses were performed, a Bayesian Skyline tree prior was chosen (Drummond et al., 2005), started with an UPGMA-tree, assuming an uncorrelated relaxed molecular clock (Drummond et al., 2006). Two independent analyses for 60 million generations were run, sampling every 1000 generations. Convergence and stationarity were checked in Tracer, results combined in LogCombiner (v.1.6.1), "burn-in" determined in Tracer, and unlikely trees discarded. Dated trees with confidence intervals and statistic support were visualized using FigTree (v. 1.3.1).

If available, two or more calibration priors were used to obtain divergence-time estimations in Beast; "2 partition"-models to coding cyt-b and none for 16S rDNA. For cyt-b, the substitution model keeps the 1st and 2nd positions together and the 3rd separately, to account for its faster rate due to the genetic code redundancy. The best suitable outgroups and species-group specific calibration points were chosen from the literature (Table S2 for marker-specific outgroups, calibrations points, priors, substitution models). During tree search, full parameter estimation was performed and posterior probabilities (PP) were calculated as the percentage of samples recovering any particular clade, where PP > 0.95 indicates significant support. A reciprocal 70% bootstrap proportion or a 0.95% PP threshold was used to test topological incongruence among partitions, respectively. We considered topological conflicts significant if two different relationships for the same set of taxa were both supported by bootstrap values (BV) >70% or PP values >0.95. No such conflicts were observed in our analyses.

2.5. Network analyses for nuclear DNA

Haplotype diversity for the nuclear gene tropomyosin was further visualized using haplotype networks. Heterozygous positions were found for all nuclear gene fragments. Minimum spanning tree files between the different nuclear sequences were created with the software Arlequin (v. 3.1, Excoffier et al., 2005). The output files from ARLEQUIN were analyzed with the software HapStar (v. 0.5, Teacher and Griffiths, 2010) resulting in a haplotype network.

3. Results

Phylogenetic analyses using homologous mtDNA and partly nuclear sequences revealed three major patterns among the three amphibian and four reptile species-groups. Bayesian and ML-phylogenies yielded very similar tree topologies of separate and concatenated fragments (analysed in detail), with higher bootstrap support and posterior probabilities for the latter. Calibration points received high statistical support (1.00/1.00).

3.1. Groups compatible with a Plio-Pleistocene out-of-Africa colonization scenario

Two reptile taxa (*C. chalcides*, *C. ocellatus*) show close Plio-Pleistocene relationships across the Strait of Sicily.

3.1.1. Three-toed skinks (*Chalcides chalcides*)

These scincid lizards are distributed in N-Tunisia, on Sardinia, Sicily and the entire IP (Fig. 1c). MtDNA-based trees yielded three major clades (Fig. 1a), which form a polytomy with a well-supported, possible Plio-Pleistocene divergence (ca. 1.8 Mya, HDI: 0.6–3.5 Mya). Samples from Tunisia (Fig. 1a–d: green) appear ancestral in respect to individuals from Sicily (Fig. 1a–d: red) plus the clade representing specimens from the IP (Tuscany, light-blue, Fig. 1a and c). ML and Bayesian *alpha*-tropomyosin trees (Fig. 1d)

show similar topologies as the mtDNA-tree (Fig. 1a). While two major clades (Tunisia: green; Sicily: red) are well-supported, the clade on the IP could not be recovered (possibly due to missing data; Discussion).

3.1.2. *Ocellated skinks (*Chalcides ocellatus*)*

These skinks are distributed throughout N-Africa, the Middle East, on Sardinia and Sicily, as well as on most of the islands in the Strait of Sicily (Fig. 1g). The mtDNA-phylogeny (Fig. 1e) shows four major clades. Although samples from Algeria and Tunisa (Fig. 1e, f, i: yellow, green) vs. Lampedusa, Malta, Linosa, Malta and Sicily (Fig. 1e: deep yellow, pink, blue, red) show slight divergence, clades are not well-supported (Fig. 1e). Individuals from Sicily and N-Africa suggest a Middle Pleistocene (ca. 0.61 Mya, 0.28–1.14) divergence. Samples from Tunisia (Fig. 1e and f: green) appear ancestral with respect to the clade assembling Sicilian samples (Fig. 1e, f: red) plus clades from islands of Pantelleria, Linosa, Lampedusa and Malta (Fig. 1e, f: blue, orange and purple). A ML-tree based on *alpha*-tropomyosin intron data (not shown) did not recover any clade, presumably due to the very recent divergence. As for mtDNA, a nuclear network (Fig. 1i) suggests out-of-Africa colonization of Sicily via Pantelleria.

3.2. *Groups compatible with Pleistocene to Holocene out-of-Africa colonization scenarios*

One anuran (*D. pictus*) and two gekkos (*T. mauritanica, Hemidactylus turcicus*) show close relationships across the Sicily Strait of Pleistocene (ca. 0.24 Mya, *Discoglossus*) to Holocene age (>0.01 Mya, geckos).

3.2.1. *Painted frogs (*Discoglossus pictus*)*

These frogs are distributed in N-Africa (Algeria, Tunisia), on Sicily, Malta and Gozo (Fig. 2a). The MtDNA-tree shows two major clades (Fig. 2c). The only southern individual is strongly separated from the other Tunisian ones (Fig. 2b, c: dark green). Specimens from Sicily and NE-Tunisia (Fig. 2b, c) form a clade, dated at 0.58 Mya (0.05–1.30) using cyt-b only, and then belongs to a well-supported clade on Sicily, Malta and Gozo (0.97/0.78). Samples from Tunisia (Fig. 2b, c: green and dark green) appear ancestral to those on Sicily (Fig. 2b, c: red) plus Malta and Gozo. A ML-tree for *alpha*-tropomyosin did not recover any supported clade (due to recent relationships; Discussion). A nuDNA-network (Fig. 2d) suggests a single colonization event of Sicily, Malta and Gozo from N-Africa.

3.2.2. *Moorish wall geckos (*Tarentola mauritanica*)*

This taxon occurs along all W-Mediterranean coasts, including N-Africa, Iberia, Sardinia, Sicily and the entire IP (Fig. 2f). MtDNA-relationships show three clades (Fig. 2e, g). Sicilian samples clustered (Fig. 2e, g: red) with specimens from NW-Tunisia, Iberia and the IP, Malta and Linosa. Within-clade genetic diversity among Sicilian and Tunisia samples comprises 0.14% only. NE-Tunisian samples form a well-supported clade with those from Lampedusa (Fig. 2e, g: green); the latter and the clade for Morocco and Spain (Fig. 2e, g: bluish) appear ancestral to wall geckos from the entire Sicily and the IP (Fig. 2e, g: red).

3.2.3. *Turkish geckos (*Hemidactylus turcicus*)*

These geckos inhabit costal N-Africa, Iberia, Sardinia, Sicily and the entire IP (Fig. 2i). MtDNA-relationships yielded two major clades (Fig. 2h). All Sicilian samples clustered with Tunisian ones (Fig. 2h, j: red), lacking any genetic diversity (0%, Table 2). However, geckos from the IP and Linosa Island are diverged (Fig. 2h, j: light blue). While dating was impossible for Sicily vs. N-Africa, the major lineages may have diverged ca. 0.88 Mya (0.38–1.38).

3.3. *Groups compatible with a Pleistocene out-of-Italy colonization scenario*

Two anuran groups (*B. bufo*, *Pelophylax* spp.) from Italy, Sicily and N-Africa are distantly related across the Strait of Sicily, while Sicilian representatives exhibit close relationships to the IP.

3.3.1. *Common toads (*Bufo bufo *species-group)*

These toads inhabit SW-Europe and N-Africa (Morocco to W-Tunisia; *Bufo spinosus*), as well as whole Europe including the IP and Sicily (*B. bufo*) (Fig. 3a). Using ML and Bayesian approaches, mtDNA-relationships display four main clades (Fig. 3c) as follows. Single samples from Morocco and Spain were separated (Fig. 3c: yellow, orange), and SW-Italian (Calabria) samples clustered with those from Sicily (Fig. 3a, c: red). Toads from Sicily and N-Africa show a deep split (ca. 11.62 Mya), while Sicilian and SW-Italian ones suggest Pleistocene divergence (0.83 Mya). N-Italian toads (Fig. 3a, c: light blue) appear ancestral to the clade assembling different ones from Sicily (Fig. 3c: red). *Alpha*-tropomyosin (Fig. 3d) yielded a similar tree topology as mtDNA. While two major clades (Tunisia: green; SE-Italy: blue) are well supported, other mtDNA-based ones could not be recovered from nuDNA.

3.3.2. *Palearctic water frogs (*Pelophylax *spp.)*

This frog complex is distributed in N-Africa (*Pelophylax saharicus*), Iberia (*P. perezi* (Seoane, 1885)), continental Europe, the IP and Sicily (*P. bergeri*/*P. hispanicus*) (Fig. 3e). MtDNA-phylogenies yielded the following three clades (Fig. 3f). Samples from Morocco and Spain sit on very well supported branches (Fig. 3e: yellow, light yellow); those from Sicily are deeply diverged from Tunisian ones but closely related to frogs from the IP (ca. 0.81 Mya; 0.16–2.21; Fig. 3f: blue) appearing ancestral to the various Sicilian ones (Fig. 3f: red).

4. Discussion

We acknowledge two general limitations of divergence time estimations to be directly translated into time of island occupancy. In fact, (i) using mtDNA may lead to overestimation of the splitting dates because the most recent common ancestor of haplotypes (their coalescent) does not necessarily correspond to the real temporal split of the populations but may precede their divergence (e.g. Arbogast et al., 2002). (ii) More importantly, the true dispersal events could either pre-date or post-date divergence times. Despite these inherent uncertainties, our analyses suggest three major colonization patterns among the selected amphibians and reptiles from Sicily (Fig. 4): (i) Plio-Pleistocene out-of-Africa colonization of Sicily for the two skinks (*C. chalcides*, *C. ocellatus*), diverged 1.85 Mya (median, CI: 0.57–3.50 Mya) and 0.61 Mya (median, CI: 0.28–1.14). (ii) Different Pleistocene out-of-Italy colonization for Palearctic water frogs (*Pelophylax* spp.), diverged about 0.81 Mya (0.16–2.21), and common toads (*B. bufo*) (>0.59 Mya; 0.18–0.96). (iii) Very close relationships between N-Africa and Sicily are suggested for painted frogs (*D. pictus*), diverged about 0.24 Mya (median, CI: 0.06–0.98), and Holocence connections, most likely due to human introduction, for the two gecko species (*H. turcicus*, *T. mauritanica*).

4.1. *Plio-Pleistocene out-of-Africa colonization of Sicily*

C. chalcides colonized Sicily from Africa at the Plio-Pleistocene boundary, implying possible land connections, and/or very low sea levels. Based on RFLP of fragments of NADH and cyt-b, Giovannotti et al. (2007) estimated a similar divergence (1.8 Mya). Using two

Table 2
Summarized results for single and concatenated mtDNA markers. Mean substitution rate, genetic distances and estimated divergence times with HDP among the analyzed geographic regions for each gene in each species group, and the combined mitochondrial dataset. Note, that divergence time estimates written in *italics* did not receive statistical support.

				Genetic distances (%) between geographic regions				Estimated divergence time Mya (HPD 95%)	
Species	Marker	Length (bp)	Mean substitution rate (% per My)	North Africa/Sicily	Sicily/Italian Peninsula	Within North Africa	Within Sicily	Sicily/North Africa	Sicily/Italian Peninsula
Chalcides chalcides	16S	415	0.55	2.76	1.73	0.87	0.00	2.4 (0.39–4.9)	*1.9 (0.29–3.7)*
	Cyt *b*	840	1.66	4.52	3.56	0.22	0.30	1.8 (0.41–3.71)	*1.13 (0.21–2.26)*
	16S + Cyt *b*	1255	1.49	–	–	–	–	1.85 (0.57–3.50)	*1.26 (0.30–2.41)*
Chalcides ocellatus	16S	440	0.56	0.32	–	0.38	0.20	0.59 (0.23–1.31)	–
	Cyt *b*	840	1.88	1.00	–	2.59	0.40	0.64 (0.24–1.25)	–
	16S + Cyt *b*	1280	1.73	–	–	–	–	0.61 (0.28–1.14)	–
Discoglossus pictus	16S	560	0.39	0.18	–	0.16	0.10	*0.26 (0.00–0.91)*	–
	Cyt *b*	900	1.23	0.76	–	0.81	0.40	0.58 (0.05–1.30)	–
	16S + Cyt *b*	1460	1.11	–	–	–	–	*0.24 (0.06–0.98)*	–
Tarentola mauritanica	16S	410	1.19	2.92	0.25	6.23	0.00	NA	NA
	Cyt *b*	825	2.45	5.45	0.34	10.93	0.00	NA	NA
	16S + Cyt *b*	1235	2.28	–	–	–	–	NA	NA
Hemidactilus turcicus	16S	450	0.72	0.00	0.53	0.00	0.00	NA	0.68 (0.13–1.46)
	Cyt *b*	816	1.51	0.38	2.45	0.24	0.30	NA	0.92 (0.40–1.54)
	16S + Cyt *b*	1266	1.01	–	–	–	–	NA	0.88 (0.38–1.38)
Bufo bufo/B. spinosus	16S	570	0.31	3.93	0.31	1.12	0.30	*9.57 (4.61–14.91)*	NA
	Cyt *b*	910	0.91	10.27	1.25	1.18	0.50	12.28(6.11–19.83)	NA
	16S + Cyt *b*	1480	0.87	–	–	–	–	11.62(6.63–16.61)	NA
Pelophylax hispanicus/P. bergeri and P. saharicus	16S	570	0.39	9.60	0.9	0.40	0.60	14.30(9.53–19.32)	1.14 (0.28-2.15)
	Cyt *b*	915	1.39	24.10	4.5	5.60	2.10	10.10(9.53–13.61)	1.09 (0.15-2.91)
	16S + Cyt *b*	1485	1.08	–	–	–	–	14.60(9.56–19.31)	0.81 (0.16-5.21)

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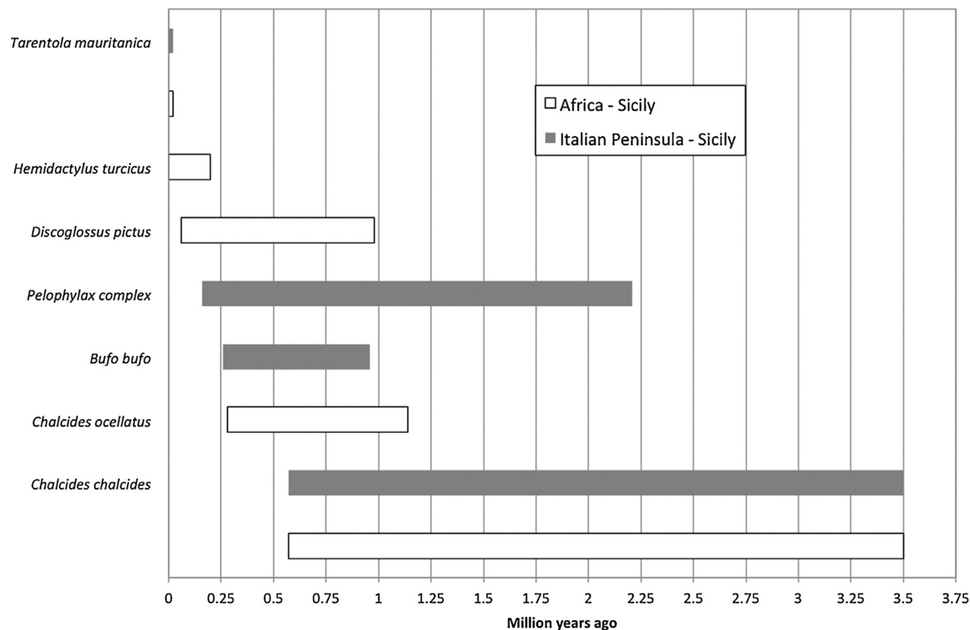

Fig. 4. Summary graph for the three amphibian and four reptile taxa and corresponding time ranges for faunal exchanges (after the end of the Messinian crisis 5.33 Mya), between Africa and Sicily as well as the Italian Peninsula and Sicily, as estimated based on our molecular data.

mtDNA markers (d-loop, 16S rDNA; supported by nuclear markers) Stöck et al. (2008a) calculated a divergence of 1.8 Mya for green toads (*B. boulengeri*/*B. siculus*) across the Strait of Sicily. Carranza et al. (2008) assumed 1.4 Mya divergence of *C. chalcides* between N-Africa and Italy (and, as assumed, also Sicily), but did not include Sicilian samples and used a much shorter cyt-b fragment. Despite identical calibration points, we made three improvements: (i) almost three times longer cyt-b sequences in combination with the mitochondrial 16S rDNA; (ii) an additional nuclear marker; and (iii) we tested samples from Sicily.

Pleistocene sea levels were particularly low at ca. 1.8 Mya (less than −120 m; Dawson, 1992). MtDNA-monophyly of *C. chalcides* in Sicily, supported by nuclear data (Fig. 1a, d), suggests a single colonization from N-Africa and simultaneous dispersal onto the IP.

Following the colonization pattern of *C. chalcides*, during a much younger Pleistocene phase (Fig. 4), *C. ocellatus* experienced vicariance around 0.61 Mya (0.28–1.14) during the lowest Post-Messinian sea level (−200 m; Dawson, 1992). In their range-wide phylogeographic study of *C. ocellatus*, Kornilios et al. (2010); cyt-b + 12S rDNA) speculated about human introduction into Sicily from N-Africa. However, these authors did not focus on the timing but based their interpretation on low genetic distance and the absence of this species from the IP. Kornilios et al. (2010) suggested that the IP was last connected to Sicily 18 kya, during the Last Glacial Maximum (e.g. Shackleton et al., 1984). However, assuming natural dispersal into Sicily, the absence of *C. ocellatus* from the Italian mainland might be due to a more recent arrival of this species in NE-Sicily than *C. chalcides* (<50 kya, i.e. >1 My later), possibly leaving skinks "not enough time" to spread further north across the Strait of Messina. The common ancestor of ocellated skinks on Sicily and islands in the Sicilian Strait lived about 0.44 Mya (0.18–0.74). Pleistocene out-of-Africa colonization using "stepping stone islands" seems well supported by our phylogenetic trees and nuDNA-network (Figs. 1e, i), starting about 0.61 Mya from N-Africa to Pantelleria (and potential intermediate stepping stone islands at that time), followed about 0.44 Mya from Pantelleria to W-Sicily and other islands (Fig. 1e), while lineages from Lampedusa, Linosa and Malta may not have contributed to the colonization of Sicily.

4.2. Pleistocene out-of-Italy colonization of Sicily

Palearctic water frogs (*Pelophylax* spp.) and common toads (*B. bufo* species-group) share similar colonization patterns of Sicily from the IP. In accordance with previous work, we found a very high genetic distance between N-Africa and Sicily, with 11.62 Mya (6.63–16.61) for the *B. bufo* group (Fig. 3c, Table 2; cf. Recuero et al., 2012; Garcia-Porta et al., 2012) and 14.60 Mya (9.52–19.31) for *Pelophylax* spp. (Fig. 3f, Table 2). Divergence between the IP and Sicily is orders of magnitude younger: for Palearctic water frogs ca. 0.81 Mya (0.16–2.21) and thus older than for common toads (<0.30 Mya). *B. bufo* from Sicily and SW-Italy (Calabria) form a clade (Fig. 3c), implying very recent contacts across the Strait of Messina (cf. Garcia-Porta et al., 2012), possibly even including (re-) colonization of Calabria from Sicily, which were episodically connected throughout the Plio-Pleistocene (Bonfiglio et al., 2000). Our data match the recent, but limited mtDNA results by Garcia-Porta et al. (2012), who, however, did not attempt to deduce the timing of colonization of Sicily.

Allozymes (Santucci et al., 1996), and cyt-b (Canestrelli and Nascetti, 2008), yielded Mid-Pleistocene (0.72 ± 0.16 Mya) divergence for water frogs across the Strait of Messina, fitting to ours (0.81 Mya; Fig. 3f, Table 2). Monophyly of water frogs (mtDNA; Fig. 3e) in Sicily suggests a single colonization, without further exchange between Sicily and Calabria, despite later connections. Similar patterns of a single Pleistocene colonization and separation despite low divergence across the Strait of Sicily have been already found for other amphibians like tree frogs (Canestrelli et al., 2007; Stöck et al., 2008b) and green toads (Stöck et al., 2008). In the eastern Mediterranean, Poulakakis et al. (2013) recently recovered analogous Pleistocene relationships in water frogs between Cyprus and mainland.

Another common feature of N-African *Pelophylax* (*P. saharicus*; cf. Amor et al., 2010; Nicolas et al., 2015) and *Bufo* (*B. spinosus*) (Fig. 3) is high genetic distance between NE- and central N-Africa, shared with several other terrestrial species. The divergence was dated 2.14 Mya (0.86–3.41) for common toads, and 5.66 Mya (3.60–7.72) for water frogs, suggesting potential for (sub) species level divergence. This East-West pattern has already been shown

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for other vertebrates including amphibians (Zangari et al., 2006; Stöck et al., 2008b; Vences et al., 2014) and reptiles (cf. Kindler et al., 2013), while recent research highlighted the significance of N-Africa as a refugium for some Palearctic species (Husemann et al., 2013).

4.3. Pleistocene to Holocene out-of-Africa colonization of Sicily

D. pictus clearly colonized Sicily from N-Africa (Fig. 2c), as suggested by previous works (Fromhage et al., 2004; Zangari et al., 2006; Pabijan et al., 2012). Based on combined mtDNA data, we obtained vicariance at 0.24 Mya (0.06-0.98), however with low statistical support (Fig. 2c). The substitution rate for 16S rDNA (560 bp) in *D. pictus* was 0.39% per My and thus potentially insufficient to resolve very close relationships (<1 My). By contrast, cyt-b only (ca. 900 bp) suggested well-supported 0.58 Mya (0.05–1.30; Table 2), in line with cyt-b's higher substitution rate (1.23% per My). Similarly, Zangari et al. (2006) proposed a Pleistocene range expansion ca. 0.40 Mya based on allozymes and mitochondrial 12S rDNA. A probable monophyletic origin of *D. pictus* in Sicily and Malta plus Gozo is also supported by a network (HapStar) of the nuclear tropomyosin-intron (Fig. 2d): two main alleles divided by a 7 bp indel, with a potential origin in NW-Tunisia for both of them (individuals Dp13 and Dp16). Euryhaline painted frogs can even breed at salt concentrations of 8 g/l (Lanza, 1983), which might facilitate transmarine dispersal (e.g. by rafting), as shown for other anurans (Heinicke et al., 2007; Measey et al., 2007). However, even during low Pleistocene sea levels, the Strait of Sicily probably was still 40 km wide, and our vicariance dating for *Discoglossus* corresponds to that for *C. ocellatus* (Fig. 4), suggesting simultaneous, perhaps "stepping stone" dispersals. Nevertheless, the shallow divergence of Sicilian and Tunisian painted frogs, together with their reported introduction from Algeria to SE-France and NE-Spain (cf. www.iucnredlist.org), may not exclude human introduction, as shown for other vertebrates (e.g. porcupines, introduced from Tunisia into Italy: Trucchi and Sbordoni 2009). To rule out this alternative, faster evolving markers like mtDNA D-loop or microsatellites might prove useful.

Representatives from Sicily and N-Africa cluster for both geckos (*T. mauritanica*, *H. turcicus*). Due to extremely low genetic distances (*H. turcicus*: 0%, *T. mauritanica*: 0.01%), their Africa-Sicily divergence (Figs. 2e–j) could not be exactly dated, but seems younger than the last glaciation (ca. 18 kya). Commensalism with people (Harris et al., 2004; Carranza and Arnold, 2006) frequently leads to anthropogenic introductions, including Sicily: Turkish geckos probably from N-Africa and Moorish geckos from N-Africa and/or Southern Europe (cf. Rato et al., 2010). In their comprehensive study on the phylogeography of W-Mediterranean *T. mauritanica*, Rato et al. (2010), characterized a widespread shallow "European clade", comprising geckos from most European and N-African range parts (including NW-Tunisia). Based on our new data, geckos on Sicily, Linosa and Malta belong to this "European clade". We also detected a second well-differentiated mtDNA clade in NE-Tunisia and on Lampedusa, corresponding to the "Italian islands and Libya clade" of Rato et al. (2010). Thorough sampling across the Sicilian Strait and within Tunisia reveals that this clade reached NE- Tunisia but may not occur on the geographically close islands of Linosa and Malta; Fig. 2g. Divergence time of this clade from the "European" one has been dated at about Messinian age, 5.27 Mya (2.77–7.77).

For 38 Mediterranean and American *H. turcicus*, Carranza et al. (2006a) showed mtDNA-based uniformity (partial cyt-b + 12S rDNA; maximum uncorrected divergence <2%) in the Mediterranean region, indicating recent rapid spread. However, no samples from Sicily or the IP were included. Our data provide evidence that Sicilian and N-Africa n gecko populations are comparatively more deeply diverged from those on the IP (and Linosa Island), with a genetic distance of 2.44% on cyt-b (Table. 3), corresponding to a divergence time of 0.88 Mya (0.38–1.38). This implies a recent exchange between Sicily and N-Africa but apparently not with geographically closer geckos on the IP. However, we cannot rule out a sampling bias, although our samples covered geographically proximate NE-Sicily and W-Calabria (Fig. 2j: Ht9, Ht1, Ht2).

4.4. Inferred substitution rates

Our data support the temporal inferences, since comparisons of separately and concatenated analyzed cyt-b + 16S substitution rates (Table 2) agree well with other studies (e.g. for amphibians: Zangari et al., 2006; Busack and Lawson, 2008; for *Chalcides*: Kornilios et al., 2010; Giovannotti et al., 2007; for geckos: Rocha et al., 2010; Yan et al., 2010). We found that amphibian species in general show lower substitution rates compared to reptiles and, as previously suggested (e.g. Mueller, 2006), 16S rDNA exhibits a lower substitution rate than cyt-b.

4.5. Implications for conservation

Our work highlights potential incipient allopatric speciation on some islands in the Sicily Strait and in central (Tunisian) vs. NW-African amphibian and reptile populations (for their distributions see also Sicilia et al., 2009). The degree of endemism on continental islands is expected to reflect the number and duration of ocean level low-stands that allowed exchange with the mainland (Schubart et al., 1998). Founder effects and genetic drift due to small population sizes, plus adaptations under different environmental conditions on the islands, may foster rapid island speciation (e.g. Whittaker and Fernandez-Palacios, 2007; Algar and Losos, 2011). *C. ocellatus* (Fig. 1e–i) exhibits high differentiation as sign of incipient speciation on several islands. *T. mauritanica* possibly shows Messinian divergence within Tunisia and vs. Lampedusa Island (Fig. 2e, g), as reflected in morphometric differentiation (Sarra et al., 2013). For *P. saharicus* and *D. pictus*, we revealed phylogenetic structure within Tunisia. Such isolated populations are potentially important for conservation to maintain high genetic diversity. Conservation management will benefit from future research on a more local scale with larger sample sizes in order to characterize biodiversity, here elucidated in part for the first time.

4.6. Implications for the biogeography of Sicily as a continental island

Focusing on seven species groups of terrestrial amphibians and reptiles, we found phylogeographic signatures on Sicily as typical of continental islands (cf. Williamson, 1981; Cody, 2006; Whittaker and Fernandez-Palacios, 2007; Johnson et al., 2012), with limited diversification and endemism. Widely neglected out-of-Africa and/or out-of-Italy faunal events, facilitated by marine transgressions along with species' ecology and human contributions, shaped the colonization from adjacent continents. Our study helps understanding timing and directions of these colonisations, appearing similarly complex as the faunal exchanges across the Strait of Gibraltar. For the latter, despite its dramatic formation at the end of the Messinian, it has been shown that many species are older or younger than the simple, purely geology-derived hypotheses initially suggested (Busack, 1986; Busack and Lawson, 2008). In a review, Husemann et al. (2013) concluded: "The regions around the sea straits of Gibraltar and Sicily have acted as important biogeographical links between N-Africa and Europe at different times". Although sometimes barely discussed (cf. van der Geer et al., 2010; SIB, 2011), our work backs the recognition of post-Messinian out-of-Africa colonization of Sicily for terrestrial vertebrates, shown for several amphibians (*Bufo siculus*, Stöck et al., 2008a, *D. pictus*) and

reptiles (*C. chalcides, C. ocellatus*), as well as suggested for several invertebrates (e.g. Habel et al., 2010) and plants with low dispersal capacity (e.g. Troia et al., 2012). Our results underline how only multispecies approaches involving ecologically differentiated taxa are able to reveal the complexity of faunal contributions to large continental islands like Sicily (cf. Poulakakis et al., 2013).

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Supplementary data

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