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Morphological, mitochondrial DNA and allozyme evolution in representative amphibians and reptiles inhabiting each side of the Strait of Gibraltar

STEPHEN D. BUSACK[1]* and ROBIN LAWSON[2]

[1]*North Carolina State Museum of Natural Sciences, 11 West Jones Street, Raleigh, NC 27601-1029, USA*
[2]*Center for Comparative Genomics, California Academy of Sciences, Golden Gate Park, San Francisco, CA 94118, USA*

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Patterns of differentiation in morphology, mitochondrial DNA and allozymes in amphibians and reptiles inhabiting northern and southern shores of the Strait of Gibraltar are not concordant, suggesting that each taxon was affected differently by events preceding or following the formation of the Strait of Gibraltar. Mitochondrial DNA and allozyme differentiation between *Discoglossus jeanneae* and *Discoglossus scovazzi* (Anura, Discoglossidae), *Rana perezi* and *Rana saharica* (Anura, Ranidae), and *Blanus cinereus* and *Blanus tingitanus* (Squamata, Amphisbaenia, Amphisbaenidae) is substantial, whereas morphological differentiation is moderate in *Rana* and *Blanus*, but is substantial in *Discoglossus*. Differentiation in mitochondrial DNA and morphology between *Timon* (*Lacerta*) *lepidus* and *Timon* (*Lacerta*) *tangitanus* (Squamata, Lacertoidea, Lacertidae) is considerable, but allozyme differentiation is low. In members of type-I and -II *Podarcis vaucheri* (Squamata, Lacertoidea, Lacertidae), morphology and mitochondrial DNA are moderately differentiated, but allozyme differentiation is low. Spanish and Moroccan populations of *Hyla meridionalis* (Anura, Hylidae), *Mauremys leprosa* (Testudines, Geoemydidae), and *Macroprotodon brevis* (Squamata, Serpentes, Colubridae) demonstrate little allozyme and mitochondrial DNA differentiation, but whereas morphological differentiation between *Mauremys* and *Macroprotodon* populations is moderate, *Hyla* demonstrate substantial morphological differentiation between continental populations. These data suggest that sex-limited mitochondrial markers are reflective of ancient phylogenetic history, whereas biparentally inherited allozyme markers and morphological characteristics reflect more recent population structure and movement.

ADDITIONAL KEYWORDS: cytochrome *b* – discordant character evolution – NADH dehydrogenase subunit 1 – NADH dehydrogenase subunit 2 – NADH dehydrogenase subunit 4.

INTRODUCTION

When a major geographic event is thoroughly investigated and dated, systematic biologists may use that event as a source for information regarding rates and causes of evolutionary change in the organisms believed to be directly influenced by that event. Although we may initiate analysis of such an event by hypothesizing that all representatives of a species assemblage (all amphibian species, all reptile species, etc.) were likely to have been affected similarly by the event, empirical data derived from analyses of biochemical or morphological systems may rapidly falsify this initial hypothesis, and suggest additional avenues of investigation. When Busack (1986a) first published results from an electrophoretic study of genic differentiation in amphibians and reptiles inhabiting adjacent shores of the Strait of Gibraltar, it was apparent that allozyme evolution did not support a parallel effect in all taxa. Several

*Corresponding author. E-mail: sbusack348@aol.com

subsequent studies of individual taxa have been completed since 1986 (see Carranza, Arnold & Pleguezuelos, 2006 for an excellent summary), but our understanding of the comprehensive biological effect of this geographic event on the amphibians and reptiles of this region remains incomplete.

We initiated a broad-based study of mitochondrial DNA (mtDNA) in twelve currently accepted taxa from this region, and compared these data with those resulting from earlier analyses of morphology and allozymes from the same taxa (in many cases from the same individuals). Resulting patterns of concordance and discordance demonstrated by this comparison provide the basis for advising caution in accepting single sources of data regarding differentiation between and among taxa (see also Hillis & Wiens, 2000).

MATERIAL AND METHODS

Taxon sampling

Discoglossus jeanneae (eastern Iberia; García-París, Montori & Herrero, 2004: 15), *Discoglossus scovazzi* (Mediterranean North Africa; Schleich, Kästle & Kabisch, 1996: 110) (Anura, Discoglossidae), *Hyla meridionalis* (south-western Iberia; García-París *et al.*, 2004: 429) (Mediterranean North Africa; Schleich *et al.*, 1996: 134) (Anura, Hylidae), *Rana perezi* (Iberia; García-París *et al.*, 2004: 472), *Rana saharica* (North Africa; Schleich *et al.*, 1996: 140) (Anura, Ranidae), *Mauremys leprosa* (Iberia; Salvador, 1998: 105) (North Africa; Schleich *et al.*, 1996: 147) (Testudines, Geoemydidae), *Blanus cinereus* (Iberia; Salvador, 1998: 336), *Blanus tingitanus* (north-western Morocco; Schleich *et al.*, 1996: 470) (Squamata, Amphisbaenia, Amphisbaenidae), *Podarcis vaucheri* (Type I, southern Iberia and Asilah, Morocco, and Type II, humid mountain regions, Mediterranean Morocco; Busack, Lawson & Arjo, 2005: 240) (Squamata, Lacertoidea, Lacertidae), *Timon (Lacerta) lepidus* (Iberia; Salvador, 1998: 201), *Timon* (*Lacerta*) *tangitanus* (Morocco; Schleich *et al.*, 1996: 414) (Squamata, Lacertoidea, Lacertidae), and *Macroprotodon brevis* (southern Iberia; Salvador, 1998: 433) (north-western Africa; Schleich *et al.*, 1996: 498) (Squamata, Serpentes, Colubridae) were examined. See Appendix 1 for the museum catalogue numbers of the specimens examined during each phase of this project.

Morphology

Because we were interested in the overall differentiation at the population level, we combined data from males and females. A complete explanation of measurement techniques and listing of morphological characters is provided in Appendix 2. We used SYSTAT 11.00.0l (Systat Software, Inc., 2004) for performing classical discriminant function analysis and for calculating Mahalanobis distances (D^2). Populations of origin (Spain or Morocco; localities presumed to be affected by the formation of the Strait of Gibraltar) were considered as grouping variables, untransformed data were considered as predictors, prior probabilities were computed from group sizes, and we used a covariance matrix to calculate Mahalanobis distances to group centroids. The Mahalanobis approach standardizes the data by scaling in terms of standard deviations, and sums pooled within-group variance–covariance, thereby adjusting for intercorrelations among highly correlated variables (Hair, Anderson, Tatham & Black, 1992). Square roots of individual inter- and intracontinental Mahalanobis distances from respective population centroids were then summed and averaged to provide the data displayed in Table 1 and Figure 1. An intracontinental D of 3.3 for Spanish individuals indicates a mean standard deviation of 3.3 between Spanish individuals and the centroid representing the mean value for the Spanish population. An intercontinental D of 11.0 indicates a combined mean standard deviation of 11.0 between Spanish individuals and the centroid for Moroccan individuals, and between Moroccan individuals and the centroid for Spanish individuals.

mtDNA

Our sources of DNA were alcohol-preserved samples derived from cryogenically preserved tissue derived from museum specimens (see Appendix 1 for genes sequenced, voucher specimen numbers, and respective GenBank accession numbers).

We used standard methods for obtaining total genomic DNA (Sambrook, Fritsch & Maniatis, 1989). Template DNA for the polymerase chain reaction (PCR) was prepared by diluting stock DNA with Tris EDTA buffer to give a spectrophotometric absorbance reading of between 0.2 and 0.7 at A260.

The amplification of target DNA was carried out in 100-μl reactions using a hot start method in a thermal cycler, with a 7-min denaturing step at 94 °C, followed by 40 cycles of denaturing for 40 s at 94 °C, primer annealing for 30 s at 46 °C, and elongation for 1 min at 72 °C, with a final 7-min elongation step at 72 °C. The oligonucleotide primers for amplification and sequencing, taken from the literature or designed for this project, are listed in Appendix 3.

PCR products were purified using Promega Wizard® PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions. Cycle sequencing was performed on PCR products using the BigDye® (Perkin-Elmer) reaction premix for 50

Table 1. Morphological (*D*), mitochondrial DNA (*p*), and Nei (D̂) inter- and intracontinental distances for eight congeneric populations of amphibians and reptiles inhabiting northern and southern shores of the Strait of Gibraltar (see text for details)

	Discoglossus jeanneae – Discoglossus scovazzi	***Hyla meridionalis***	***Rana perezi – Rana saharica***	***Mauremys leprosa***	***Blanus cinereus – Blanus tingitanus***	***Podarcis vaucheri****	***Timon lepidus – Timon tangitanus***	***Macroprotodon brevis***
Mahalanobis distance [*D* (SE)]								
Sample correctly classified (%)	100	80	96	88	85	85	99	98
Intercontinental	11.04 (0.15)	9.74 (0.49)	4.21 (0.12)	4.79 (0.28)	3.66 (0.10)	3.81 (0.09)	6.54 (0.09)	4.81 (0.11)
Intracontinental	*D. jeanneae*: 3.29 (0.12)	Spain: 6.58 (0.72)	*R. perezi*: 2.52 (0.11)	Spain: 3.97 (0.24)	*B. cinereus*: 3.03 (0.13)	Type I: 3.13 (0.10)	*T. lepidus*: 2.81 (0.11)	Spain: 3.16 (0.18)
	D. scovazzi: 3.41 (0.13)	Morocco: 7.18 (0.45)	*R. saharica*: 3.69 (0.24)	Morocco: 4.55 (0.52)	*B. tingitanus*: 2.55 (0.15)	Type II: 2.76 (0.16)	*T. tangitanus*: 2.86 (0.10)	Morocco: 3.15 (0.12)
Classification statistics†								
Spanish population‡	14♂♂, 16♀♀, 100%,1.0,1.0-1.0	30♂♂, 29♀♀, 80%,0.7,0.1-1.0	41♂♂, 32♀♀, 100%,1.0,0.7-1.0	13♂♂, 15♀♀, 85%,0.8,0.2-1.0	28♂♂, 29♀♀, 89%,0.8,0.8-1.0	38♂♂, 32♀♀, 87%,0.9,0.8-1.0	43♂♂, 45♀♀, 79%,1.0, 1.0-1.0	25♂♂, 6♀♀, 100%,1.0,1.0-1.0
Moroccan population	5♂♂, 6♀♀, 100%, 1.0,1.0-1.0	30♂♂, 30♀♀, 80%,0.7, 0.0-1.0	18♂♂, 15♀♀, 88%, 0.8, 0.1-1.0	13♂♂, 17♀♀, 90%,0.8,0.2-1.0	13♂♂, 24♀♀, 80%, 0.8, 0.1-1.0	22♂♂, 17♀♀, 82%, 0.8, 0.1-1.0	39♂♂, 20♀♀, 98%, 1.0, 0.0-1.0	40♂♂, 15♀♀, 96%, 1.0, 0.9-1.0
mtDNA distances [*p* (SE, *N*)]								
Intercontinental **Cyt-*b***	13.1 (0.00, 1)	–	16.1 (0.11, 2)	–	14.0 (0.00, 2)	5.3 (0.00, 3)	13.7 (0.07, 8)	0.0 (0.00, 1)
Intercontinental **ND1**	–	0.9 (0.00, 2)	14.3 (0.00, 1)	0.2 (0.11, 2)	–	–	–	0.4 (0.00, 1)
Intercontinental **ND2**	15.4 (0.05, 2)	1.0 (0.06, 4)	15.7 (0.00, 4)	0.2 (0.06, 4)	13.9 (0.02, 3)	6.8 (1.58, 3)	13.3 (0.21, 10)	0.5 (0.00, 1)

Table 1. *Continued*

	Discoglossus jeanneae – Discoglossus scovazzi	***Hyla meridionalis***	***Rana perezi – Rana saharica***	***Mauremys leprosa***	***Blanus cinereus – Blanus tingitanus***	***Podarcis vaucheri****	***Timon lepidus – Timon tangitanus***	***Macroprotodon brevis***
Intercontinental **ND4**	11.1 (0.00, 1)	–	–	0.1 (0.04, 4)	15.8(0.29, 2)	–	11.0 (0.06, 8)	0.3 (0.00, 1)
Intracontinental **Cyt-*b***	–	–	*R. perezi*: 0.7 (0.00, 1)	–	*B. cinereus*: 1.0 (0.00,1)	Type I: 0.4 (0.06, 3)	*T. lepidus*: 0.9 (0.34, 6)	–
Intracontinental **ND1**	–	Spain: 0.6 (0.00, 1)	–	Morocco: 0.2 (0.00,1)	–	–	–	–
Intracontinental **ND2**	*D. jeanneae*: 0.3 (0.00, 1)	Spain: 0.1 (0.00, 1)	*R. perezi*: 0.8 (0.00,1)	Spain: 0.0 (0.00, 1)	*B. cinereus*: 0.5 (0.39, 2)	Type I: 4.5 (1.90, 3)	*T. lepidus*: 1.5 (0.27, 10)	–
	–	Morocco: 0.4 (0.00, 1)	*R. saharica*: 0.1 (0.00,1)	Morocco: 0.4 (0.00,1)	–	–	–	–
Intracontinental **ND4**	–	–	–	Spain: 0.0 (0.00, 1)	*B. cinereus*: 0.7 (0.00, 1)	Type I: 0.6 (0.00, 3)	*T. lepidus*: 0.4 (0.12, 6)	–
Nei distances [$\hat{D}$(*loci*, *N*)]								
Intercontinental	0.39 (34, 17)	0.04 (34, 25)	0.55 (31, 23)	0.00 (37, 19)	0.65 (33, 19)	0.13 (33, 10)	0.15 (39, 15)	0.03 (41, 15)
Intracontinental	*D. jeanneae*: 0.10 (34, 7)	Spain: 0.04 (34, 10)	*R. perezi*: 0.12 (31, 10)	Spain: 0.07 (37, 5)	*B. cinereus*: 0.16 (33, 10)	Type I: 0.07 (33,10)	*T. lepidus*: 0.06 (39, 10)	Spain: 0.00 (41, 10)
	D. scovazzi: 0.11 (34, 10)	Morocco: 0.04 (34, 10)	*R. saharica*: 0.13 (31, 10)	Morocco: 0.00 (37, 10)	*B. tingitanus*: 0.17 (33,8)	–	–	–

*Type I versus Type II (see Busack *et al.*, 2005).

†Number of ♂, number of ♀, percentage of sample that is correctly classified, mean posterior probability for correct classification, and range of posterior probabilities for correct classification.

‡Type I versus Type II for *Podarcis vaucheri* (see Busack *et al.*, 2005).

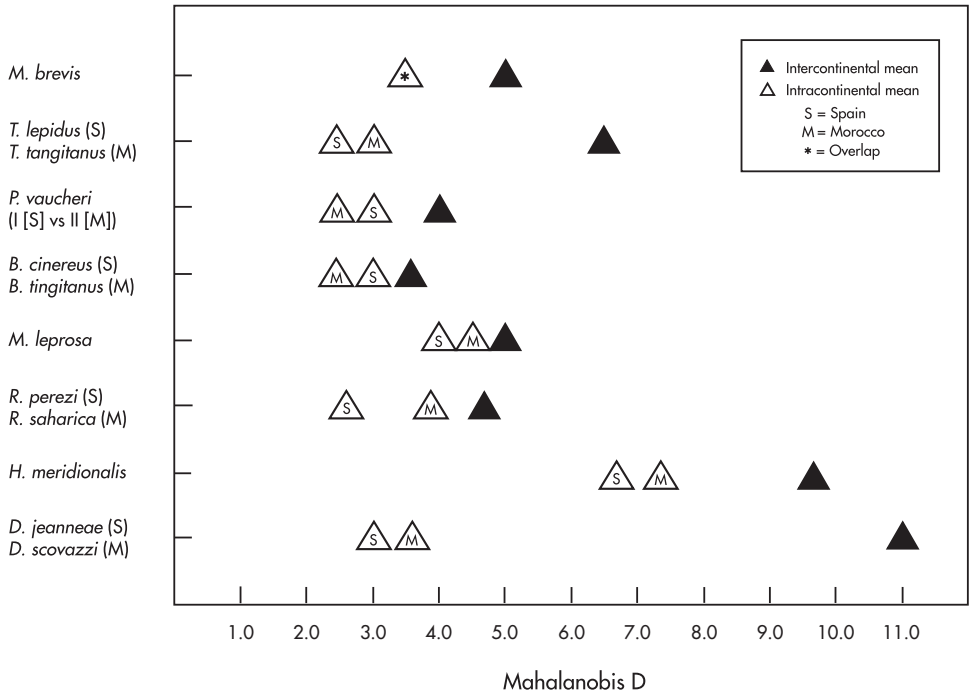

Figure 1. Morphological differences (expressed as Mahalanobis distances, *D*) between inter- and intracontinental populations of congeneric amphibians and reptiles (see Results for details).

cycles of 96 °C for 10 s, 45 °C for 5 s, and 60 °C for 4 min. Nucleotide sequences were determined using an ABI Prism Genetic Analyzer model 3100 (Applied Biosystems), and were aligned with Sequencher™ v4.0 (Gene Codes Corp.); percentage differences (*p*-distances) between sequences were calculated using PAUP* v4.0b4a (Swofford, 2000).

Allozymes

Tissue samples were pooled for each animal. Proteins were separated electrophoretically in horizontal starch gels (11.5% hydrolyzed starch, Sigma Chemical Co.), and were localized by standard histochemical staining procedures (Selander, Smith, Yang, Johnson & Gentry, 1971; Ayala, Powell, Tracey, Mourão & Perez-Salas, 1972; Harris & Hopkinson, 1976).

Allozymic data for each protein system were obtained through 'side-by-side' comparisons of Spanish and Moroccan material, and genetic interpretations were based on criteria developed by Selander *et al.* (1971). The unbiased minimum genetic distance between populations ($\hat{D}$), recommended by Nei (1978) for comparisons utilizing small sample sizes, was computed from allele frequencies using algorithms provided by Nei (1978).

Enzymes and protein systems examined, electrophoretic conditions, and notes regarding the resolution of various systems are provided in Appendix 4. Detailed descriptions of results from the electrophoretic analyses are available elsewhere (see Appendix 4). We report only the $\hat{D}$ values obtained from these analyses here.

RESULTS

Morphology

Confidence limits (95%) for mean Mahalanobis *D* values for *M. leprosa* from Spain (3.5–4.4) and Morocco (3.5–5.6) overlap intercontinental limits (4.2–5.3). Likewise, confidence limits for *R. saharica* (3.2–4.2) overlap those between *R. perezi* and *R. saharica* (4.0–4.4). Confidence limits for mean Mahalanobis *D* values between all other intercontinental comparisons are greater than the limits within either Spain or Morocco, regardless of the degree of perceived taxonomic differentiation between populations (Table 1), but no pattern is apparent (Fig. 1). Although genetically similar populations on each continent (*H. meridionalis*, *M. leprosa*, and *M. brevis*) demonstrate low intercontinental morphological distances (between 1.1 and 1.5 times intracontinental distances), genetically differentiated congeneric populations (*B. cinereus* and *B. tingitanus*, *R. perezi* and *R. saharica*) also demonstrate low levels of intercontinental morphological differentiation (1.3 and 1.5 times that of intracontinental distance, respectively). In contrast, intercontinental distances between *T. (L.)*

lepidus and *T. (L.) tangitanus*, and between *D. jeanneae* and *D. scovazzi*, range between 2.3 and 3.3 times the intracontinental distances, respectively.

MTDNA

We resolved 858 base pairs for cytochrome *b* (cyt-*b*) in *Discoglossus*, 902 in *Rana*, 1143 in *Podarcis* and *Timon*, and 1117 in *Macroprotodon*. Resolution for NADH dehydrogenase subunit 1 (ND1), ND2 and ND4 was as follows: 987 base pairs for ND1 in *Hyla*, 969 in *Rana*, 968–972 in *Mauremys*, and 964 in *Macroprotodon*; 1047 base pairs for ND2 in *Discoglossus*, 1035 in *Hyla*, 1038 in *Rana*, 1044 in *Mauremys*, 1041 in *Blanus*, 1038 in *Podarcis*, 1035 in *Timon*, and 1032 in *Macroprotodon*; 705 base pairs for ND4 in *Mauremys*, 702 in *Blanus*, 709 in *Podarcis* and *Timon*, and 696 in *Macroprotodon*.

Inter- and intracontinental mtDNA differentiation in *M. leprosa* are equivalent ($p \sim 0.2$ vs. $p \sim 0.0$ in Spain and $p \sim 0.3$ in Morocco). With the possible exception of *M. brevis*, for which we have no intracontinental data, all other taxa demonstrate greater differentiation [mean intercontinental p values are between ~2.5 (*Hyla*) and ~154 (*Rana saharica*) times greater (Table 1; Fig. 2) across the Strait of Gibraltar than within populations inhabiting either Spain or Morocco].

ALLOZYMES

Figure 3 provides graphical representation of intra- and intercontinental Nei's $\hat{D}$ values; the actual values and total number of loci resolved for all taxa are summarized in Table 1. Although there were no fixed allelic differences resolved in *H. meridionalis*, *M. leprosa*, *P. vaucheri*, or *M. brevis*, 13 were identified between *B. cinereus* and *B. tingitanus*, nine between *D. jeanneae* and *D. scovazzi*, six between *R. perezi* and *R. saharica*, and three between *T. (L.) lepidus* and *T.* (*L.*) *tangitanus* (Busack, 1986a: Table 2).

Congeneric species pairs currently separated by the Strait of Gibraltar demonstrate intercontinental Nei's $\hat{D}$ values that are greater than intracontinental values (Table 1; Fig. 3). Intercontinental values range between 1.9 and 4.4 times intracontinental values among *D. jeanneae* and *D. scovazzi*, *R. perezi* and *R. saharica*, *B. cinereus* and *B. tingitanus*, *P. vaucheri* types I and II (see Busack *et al.*, 2005), and *T. (L.) lepidus* and *T. (L.) tangitanus*. Inter- and intracontinental Nei's $\hat{D}$ values are more or less equal between populations considered to represent the same species (*H. meridionalis*, *M. leprosa*, and *M. brevis*) on each side of the Strait of Gibraltar (Table 1; Fig. 3).

DISCUSSION

By the time the Strait of Gibraltar had stabilized to form today's watercourse [5.6–5.3 million years ago (Ma); Hsü, Montandert, Bernoulli, Cita, Erikson, Garrison, Kidd, Mèliéres, Müller & Wright, 1977], several geographical changes had affected the European and African land masses it separates (Benson, Rakic-El Beid & Bonaduce, 1991; de Jong, 1998; Gomez, Beauchamp & Barazangi, 2000). Among those changes was the formation of islands, generally short-lived in geological time, and substantial in surface area, that may have provided temporary shelter for sufficiently mobile amphibians and reptiles capable of surviving extremes in environmental conditions. Amphibians and reptiles that do not tolerate saline environments, or that are incapable of swimming relatively long distances, could not be expected to traverse the Strait, and, unless provided with appropriate environments on those islands, would perish. Without recruitment, surviving mainland populations of these taxa would retain the genetic complement available to them immediately after the formation of the Stait was complete.

Mitochondrial DNA and allozyme differentiation between Spanish and Moroccan populations of *H. meridionalis*, *M. leprosa* and *M. brevis* is very low ($p \leq 0.5$, $\hat{D} \leq 0.04$, respectively, in each taxon), and discriminant function analysis (DFA) of morphology accurately assigned 80, 88, and 98% of Spanish and Moroccan samples, respectively, to the population of origin (Table 1; Figs 1–3). With overlapping intra- and intercontinental 95% confidence limits for mean Mahalanobis D values, *M. leprosa* populations retain levels of morphological variation within Spain and within Morocco equivalent to those between Spain and Morocco, whereas *H. meridionalis* and *M. brevis* populations do not. *Mauremys leprosa* requires pond and stream habitat for survival, is known to demonstrate salt-water tolerance, and may occasionally cross the Strait of Gibraltar (Busack, 1977: 291). Equivalent intra- and intercontinental levels of morphological variability probably reflect the relative microhabitat uniformity of this species' habitat in the region. The level of variation in *H. meridionalis* from Spain is less similar to the intercontinental level than is the level of variation in Moroccan populations, suggesting a founder effect in Spain, or greater survival probability associated with greater morphological plasticity in Morocco, or both. This unusual situation (given that regional climate and geography in northern coastal Africa and southern coastal Europe are quite similar) warrants further examination. *Macroprotodon brevis* is a habitat generalist commonly found in association with human settlements. Although mtDNA and allozymic differentiation between continental populations is low, there is substantial morphological variation

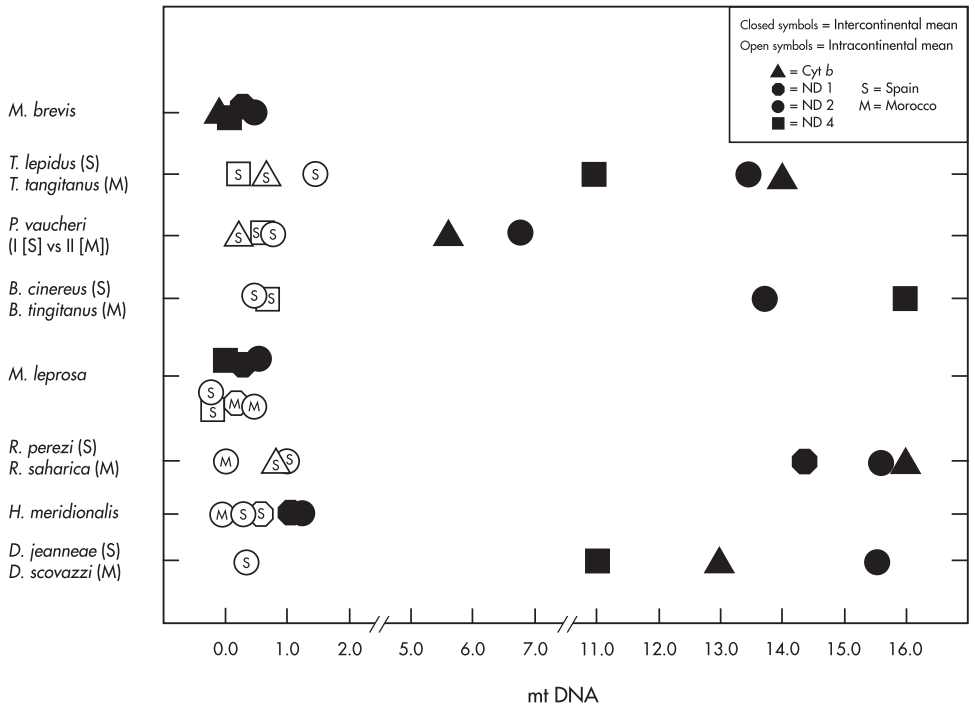

Figure 2. Mitochondrial DNA (mtDNA) sequence differences [expressed as percentages (p)] between inter- and intracontinental populations of congeneric amphibians and reptiles (see Results for details).

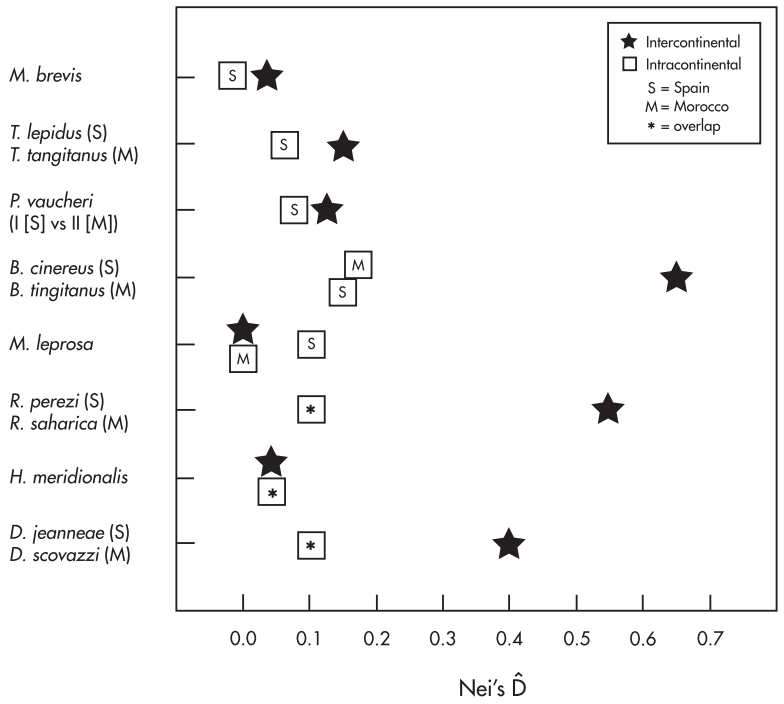

Figure 3. Allozyme differences (expressed as Nei's $\hat{D}$ values) between inter- and intracontinental populations of congeneric amphibians and reptiles (see Results for details).

within and between continents (Fig. 1, Busack & McCoy, 1990; Carranza, Arnold, Wade & Fahd, 2004). Genic similarity is likely to stem from recent colonization of Spain from Morocco: the founder effect, coupled with differing selective pressures within microhabitats on each side of the Strait, may be the driving force for morphological divergence (Carranza *et al.*, 2004).

Morphology and mtDNA (Table 1 and Figs 1, 2, respectively) between *T. (L.) lepidus* (Spain) and *T.* (*L.*) *tangitanus* (Morocco), and between representatives of types-I (both Spain and Morocco) and -II (Morocco only) *P. vaucheri* (*fide* Busack *et al.*, 2005), are substantially differentiated, but allozyme differentiation is relatively low (Table 1; Fig. 3). Paulo (2001) suggests that *T. lepidus* began to diverge as a result of Miocene land-bridge formation and deformation between continents, and Mateo & López-Jurado (1994: Fig. 3) demonstrate an association between bioclimatic region and patterns of coloration in Iberian populations. Geographic and microbioclimatic change associated with the formation of the Strait may have precipitated or accelerated morphological evolution in these taxa, and the moderate level of allozymic differentiation is likely to be reflective of the fact that these wide-ranging terrestrial (*Timon*) and semi-arboreal or saxicolous (*Podarcis*) lizards inhabited the area between Africa and Europe, as changes in sea level variously produced and destroyed emergent land masses. Representatives of *P. vaucheri* Type I continue to inhabit both Spain and Morocco today (Busack *et al.*, 2005: 254).

Mitochondrial DNA and allozyme differentiation between *R. perezi* and *R. saharica*, and between *B. cinereus* and *B. tingitanus*, is substantial (Table 1; Figs 2, 3), suggesting that species formation in these genera may have been precipitated or augmented by the opening of the Strait of Gibraltar. If this were the case, the moderate degree of morphological differentiation found in *Rana* and *Blanus* may simply be reflective of continued survival and reproductive success in relatively stable aquatic (*Rana*) or fossorial (*Blanus*) habitats.

Mitochondrial DNA and allozyme differentiation between *D. jeanneae* and *D. scovazzi* is substantial (*p*-distance ~ 13, $\hat{D}$ ~ 0.39; Table 1), suggesting species evolution preceding or directly related to formation of the Strait of Gibraltar. Considerable morphological differentiation is also apparent in this species pair: DFA correctly assigned 100% of samples to the population of origin (Table 1; Fig. 1). Rapid morphological change following population fragmentation appears probable in this terrestrial genus, as *D. pictus* and *D. sardus* were accorded species status on morphological grounds, yet the cessation of reproductive contact between them is estimated to have been between 4.7 and 1.9 Ma (Zangari, Cimmaruta & Nascetti, 2006): Table 5).

For our summary discussion, we standardized intercontinental data by calculating and plotting, in Figure 4, the percentage of the maximum value obtained for each datum within a data set. The mean value of the Mahalanobis *D* representing morphological differentiation between *D. jeanneae* and *D. scovazzi* was 11.04 (Table 1), and was the maximum value obtained for this datum across all taxa. This result is represented on Figure 4 by the placement of the appropriate symbol at 100% for this taxon pair. Corresponding values of Mahalanobis *D* [88% (9.74/11.04 = 0.88) for *H. meridionalis*, 38% for *R. perezi* and *R. saharica*, etc.], *p*-distance, and Nei's $\hat{D}$ were similarly calculated and placed on Figure 4. Although we fully understand there may be significant differences between genetic and selective components affecting these parameters, both among taxa and within each taxon, we think this procedure presents a reasonably accurate means of presenting differentiation among taxa in this region.

Close examination of these data (see Fig. 4) suggests that sex-limited mitochondrial markers (mtDNA) are likely to be reflective of the ancient phylogenetic history of each taxon, whereas biparentally inherited allozyme and morphological markers are likely to be reflective of more recent movement and assembly of populations. If this is in fact the case, morphological evolution in these taxa apparently may precede (as in the case of *H. meridionalis*, *M. brevis*, and *M. leprosa*), follow (as in *R. perezi*, *R. saharica*, *B. cinereus*, and *B. tingitanus*), or occur almost in concert with (as in *D. jeanneae* and *D. scovazzi*) mtDNA and allozymic differentiation. The pattern (reduced allozymic differentiation coupled with relatively higher levels of morphological and mtDNA differentiation) demonstrated by *Podarcis* and *Timon* suggests that, under certain conditions, allozymic differentiation may also trail behind mtDNA and morphological differentiation.

CONCLUSIONS

Patterns of differentiation in morphology, mtDNA and allozymes in amphibians and reptiles inhabiting the northern and southern shores of the Strait of Gibraltar are not concordant. Whether this discordance was initiated prior to, was exacerbated by, or is completely random, with respect to the formation of the Strait of Gibraltar, the variable degree of 'differentiation among these taxa suggests that the population structure of each was affected differently by the environmental changes the whole region must have undergone' (Busack, 1986a: 33). Our results further suggest that formulating biogeographic scenarios requires multidisciplinary exami-

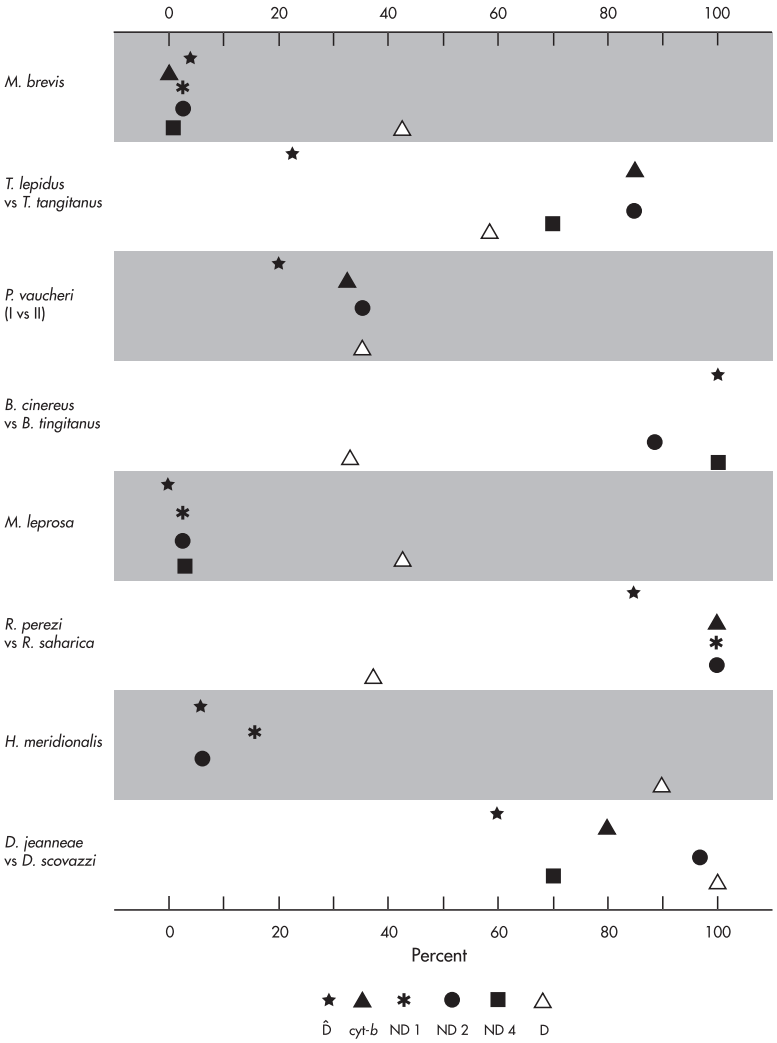

Figure 4. Comparison of relative intercontinental differences in morphology, mitochondrial DNA (mtDNA), and allozymes (expressed as percentages of the maximum values recorded for each characteristic within the taxa sampled) within and among congeneric populations of amphibians and reptiles (see Discussion for details).

nation of the biota involved, and that taxon assignment, which historically has been morphology-based, should proceed with extreme caution in the absence of genetic data.

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APPENDIX 1

SPECIMENS EXAMINED (UNLESS OTHERWISE DESIGNATED, MUSEUM ACRONYMS FOLLOW LEVITON *ET AL.*, 1985)

Mahalanobis distances

Discoglossus jeanneae (♂♂): CM 52127, 52475–52477, 52537, 53087–53088, 53119–53120, 53324, 54582, 54704, and 55742–55743.

Discoglossus jeanneae (♀♀): CM 52125–52126, 52128–52129, 52626, 53884 (a, b, e, and g), 54244, 54581, 54608–54610, 54657, and 55744.

Discoglossus scovazzi (♂♂): MVZ 186124–186126, 186129, and 186133.

Discoglossus scovazzi (♀♀): MVZ 186127–186128, 186130–186132, and 186134.

Hyla meridionalis, Morocco (♂♂): CM 52044, 52123, 52471, 52473–52474, 52485, 52624–52625, 52633, 53118, 53194, 54242, 54271, 54577–54578, 54611, 54611 (b–g), 54621, 54651, 54664–54666, 54700–54701, and 54814.

Hyla meridionalis, Morocco (♀♀): MVZ 162476, 162482, 162484, 177944–177947, 177952, 177954–177956, 177958–177960, 177963, 177965, 177967–177968, 177970, 177972–177975, 177987–177988, 177991, 177993, 186158, and 186160–186161.

Hyla meridionalis, Spain (♂♂): CM 50927–50928, 50930, 50934–50935, 51047c, 52122, 52452–52453, 52461–52465, 52472, 52523, 52533, 52617 (b, c, and e), 53079, 53882, 54270, 54579–54580, 54624, 55285–55287, and 55299.

Hyla meridionalis, Spain (♀♀): MVZ 177930–177932, 177934–177937, 177939–177940, 177948–177949, 177957, 177961–177962, 177971, 177979, 177981–177982, 177984–177986, 177990, 177994, 186142–186145, 186157, and 186162.

Rana perezi (♂♂): CM 51034–51035, 51321, 51321 (b and e), 51324, 51324 (c, d, f–h, and k–l), 51981, 52173,

52186, 52514, 52549, 52619, 52623, 52634, 53035, 53152, 53216, 53225, 53271, 53314, 53354 (c–i), 53395 (c–g), 54620, and 54660.

Rana perezi (♀♀): CM 50926, 51321f, 51324 (b and e), 51345b, 51922, 52120–52121, 52157, 52478, 52623, 52623b, 52635–52636, 53054, 53064, 53171, 53240, 53299, 53354, 53354x, 53395, 53395b, 53406, 53423, 53430, 54585, 54627, 54801, 54880, 55295, and 55445.

Rana saharica (♂♂): MVZ 177997, 178000, 178002–178003, 178006–178012, 178018–178019, 178022, and 178040–178043.

Rana saharica (♀♀): MVZ 177998–177999, 178001, 178004, 178013–178017, 178020, 178024, and 178036–178039.

Mauremys leprosa, Morocco (♂♂): FMNH 197888 and 199764; MCZ 29911; USNM 55614–55615, 196488 (AD224)–196490, and 196492 (AD244–AD247 and AD249).

Mauremys leprosa, Morocco (♀♀): CAS 91522–91525; CM 55050; FMNH 197887, 199761–199763, and 199765; USNM 196486–196487, and 196488 (AD220–AD221 and AD225–AD227).

Mauremys leprosa, Spain (♂♂): CAS 55343; CM 51038, 53421, 54682, 55244, 55331, 55447–55448, and 55675 (c and d); CU 9154; LACM 113889; USNM 195459.

Mauremys leprosa, Spain (♀♀): CM 53355–53357, 53367, 53396, 53422, 55501(22627 and 22630), 55675, and 55675 (b & f); CU 9143–9144; LACM 113888; USNM 195457.

Blanus cinereus (♂♂): CM 53300, 53404, 54675, 54681, 54818–54819, 54867, and 54871–54872; MNCN 10897, 10901–10902, 10905, 11061–11062, 11065, 11067, 11883–11884, 11888–11890, and 11894–11895; MVZ 186053, 186064, and 186451; UCM 44965.

Blanus cinereus (♀♀): CM 2622, 54688, 54832, 54850, 55758, and 55760; MNCN 10896, 10898–10900, 10903–10904, 10906, 11060, 11063–11064, 11066, 11885–11886, 11891–11893, 11896–11899, and 11901; UCM 44964; USNM 195465.

Blanus tingitanus (♂♂): MVZ 178095–178096, 178098–178099, 178102, 178106, 178108, 186168, 186172, 186178–186179, 186186, and 186188.

Blanus tingitanus (♀♀): MVZ 178097, 178100, 178103–178105, 178107, 178110, 186166–186167, 186169–186171, 186173–186177, 186180, 186182–186185, 186187, and 186189.

Podarcis vaucheri, Type I (Busack *et al.*, 2005), Spain (♂♂): CM 53213 (18733, 18735–18737, and 18739), 53416, 54569, 54796, 55494 (22600 and 22602), 54563 (a, f, and j–k), 54569 (b–g, i–j, l, and p), and 54796 (b and d); MVZ 178324, 178326–178329, 178333, 178335, 178337, 186226, and 186230–186232.

Podarcis vaucheri, Type I (Busack *et al.*, 2005), Spain (♀♀): CM 53407, 53415, 53417, 54203, 54232, 54563 (b, c, d, g, h, and i), 54569 (h, k, o, q–r, t, and v), 54603, 55494 (22599, 22603, and 22605), and 55665; MVZ 178325, 178330–178332, 178336, 178338, and 186227–186229.

Podarcis vaucheri, Type II (Busack *et al.*, 2005) (♂♂): CM 55224–55225; MVZ 178292–178298, 178300, 178304–178307, 178311, 178314–178318, 178345, and 186233.

Podarcis vaucheri, Type II (Busack *et al.*, 2005) (♀♀): CM 55221–55223; MVZ 178299, 178301–178303, 178308–178310, 178312–178313, 178343–178344, and 178346–178348.

Timon lepidus (♂♂): CM 51043, 51082, 51085, 53060, 53138, 53176–53177, 53201–53206, 53210–53211, 53218, 53224, 53298, 53316, 53366, 53905, 53917, 53919, 54235, 54268, 54689, 54797–54798, 54870, 54877, 55349, 55439, 55493, and 55673; MVZ 186056–186058, 186061, 186064–186065, 186067, and 186069–186070.

Timon lepidus (♀♀): CM 50948, 51044, 51103, 51918, 51946, 52021, 52180–52181, 52621, 53133, 53136–53137, 53185, 53207–53209, 53212, 53360, 53365, 53370, 53418, 53918, 54214, 54570, 54595, 54799, 54865, 54879, 55332, 55440, 55449, 55469, 55497, and 55669–55672; MVZ 186055, 186059–186060, 186062–186063, 186066, 186068, and 186071.

Timon tangitanus (♂♂): CAS 92417, 92419, and 153730; CM 55216–55218; FMNH 199774 and 199804; MCZ 29972–29975, and 29979; MHNG 1361.58; MNHN 1912.470, 1916.48, and 1925.150; MVZ 162584, 178280, 178282, 178284, 178288–178290, and 186204–186205; NMW 10938.14, 10939.1–10939.3, 10940, and 10945; ZFMK 6386, 17837, 18653, 20402, 26234–26235, and 34559.

Timon tangitanus (♀♀): CAS 92418; FMNH 66607, 199803, and 199805–199806; MCZ 29976 and 29978; MHNG 663.87, 915.45–915.46, and 1510.19; MVZ 178279, 178281, 178283, 178285–178287, and 186203; USNM 196435–196436.

Macroprotodon brevis, Morocco (♂♂): AMNH 7725 and 7727; BMNH 1904.11.28.47–48b, 1906.10.31.9, 1931.8.7.21, 1934.12.3.61, and 89.12.16.110–116c; FMNH 4044, 83311, 83314, and 83318–83319; MCZ 8070 and 29924; MNCN 1742–1743, 1748–1749, 1751–1754, 1757, 1759, 1761, 1763–1764, 1766–1768, 1770–1772, 1775, 1780, 1782–1784, and 1791–1792.

Macroprotodon brevis, Morocco (♀♀): AMNH 7728 and 84183; BMNH 1904.11.28.47–48a, 1907.6.22.30-1b, and 89.12.16.110–116 (a-b, d, and f); CM 55254; FMNH 4043; MCZ 29921; MNCN 1765, 1781, 1785, and 1790.

Macroprotodon brevis, Spain (♂♂): BMNH 1920.1.20.1273 and 95.3.1.2–3 (a & b); CM 50950, 51349, 52041, 52078, 53163, 53178, 53879, 53906, 54699, 54809, and 54842; CU 9128–9129, and 9131; Estación Biologica de Doñana, Sevilla, Spain (EBD)

2084, 2086, 2089, 3127, 4437, and 5262; LACM 113885; USNM 195466.
Macroprotodon brevis, Spain (♀♀): CM 53446; CU 9132; EBD 2087 and 4369; MNCN 1800–1801.

Mitochondrial DNA distances [voucher specimen number followed by mitochondrial gene(s) sequenced and respective GenBank accession number(s)]
Discoglossus jeanneae: MNCN 11844; ND2, DQ902267. MNCN 22891; cyt-*b*, DQ902149 & ND2, DQ902266.
Discoglossus scovazzi: MVZ 186132; cyt-*b*, DQ902148 & ND2, DQ902268.
Hyla meridionalis, Morocco: MVZ 177966; ND1, DQ902199 & ND2, DQ902277. MVZ 186160; ND2, DQ902278.
Hyla meridionalis, Spain: MNCN 11362; ND1, DQ902200 & ND2, DQ902275. S. D. Busack Field Series (SDB, 1914) (voucher lost, collected with MNCN 11868–11875, tissue expended); ND1, DQ902198 & ND2, DQ902276.
Rana perezi: MNCN 11881; cyt-*b*, DQ902145 & ND1, DQ902201 & ND2, DQ902259. MNCN 11882; cyt-*b*, DQ902146 & ND2, DQ902260.
Rana saharica: MVZ 178006; ND2, DQ902261. MVZ 178009; cyt-*b*, DQ902147 & ND1, DQ902202 & ND2, DQ902262.
Mauremys leprosa, Morocco: MVZ 178055; ND1, DQ902196 & ND2, DQ902274 & ND4, DQ902329. MVZ 178060; ND1, DQ902197 & ND2, DQ902272 & ND4, DQ902330.
Mauremys leprosa, Spain: MNCN 11528; ND2, DQ902271 & ND4, DQ902327. MNCN 23659; ND1, DQ902195 & ND2, DQ902273 & ND4, DQ902328.
Blanus cinereus: MNCN 11894; ND2, DQ902263. MNCN 11897; ND2, DQ902264. MNCN 11900; ND2, DQ902265 & ND4, DQ902332.
Blanus tingitanus: MVZ 186189; ND2, DQ902269 & ND4, DQ902331.
Podarcis vaucheri, Type I (Busack *et al.*, 2005): MNCN 11088; cyt-*b*, AY234162 & ND4, AY234173. MVZ 186228; cyt-*b*, AY234161 & ND2, AY234150 & ND4, AY234172. SDB 1531 (voucher lost, collected with MNCN 11078–11082, tissue expended); cyt-*b*, AY234159 & ND2, AY234149 & ND4, AY234170.
Podarcis vaucheri, Type II (Busack *et al.*, 2005): MVZ 186233; cyt-*b*, AY234164 & ND2, AY234153 & ND4, DQ902335.
Timon lepidus: MNCN 11333; cyt-*b*, DQ902140 & ND2, DQ902256 & ND4, DQ902324. MNCN 11977; cyt-*b*, DQ902141 & ND2, DQ902254 & ND4, DQ902321. MNCN 11983; ND2, DQ902253. MVZ 186062; cyt-*b*, DQ902139 & ND2, DQ902252 & ND4, DQ902323. SDB 1672 (voucher lost, tissue catalogued as MVZ 232013); cyt-*b*, DQ902142 & ND2, DQ902255 & ND4, DQ902322.
Timon tangitanus: MVZ 186203; cyt-*b*, DQ902143 & ND2, DQ902258 & ND4, DQ902326. MVZ 186204; cyt-*b*, DQ902144 & ND2, DQ902257 & ND4, DQ902325.
Macroprotodon brevis, Morocco: MVZ 186239; cyt-*b*, DQ907242 & ND1, DQ902203 & ND2, DQ902270 & ND4, DQ902333.
Macroprotodon brevis, Spain: MVZ 186073; cyt-*b*, DQ902150 & ND1, AY486986 & ND2, AY487025 & ND4, AY487064.

Nei distances
Discoglossus jeanneae: MNCN 11363, 11843, 11849–11850, and 22892–22894.
Discoglossus scovazzi: MVZ 18124–18125, and 186132–186134; S. D. Busack Field Series (SDB) 1771–1775 (no vouchers, collected with MVZ 186137–186140, tissue expended).
Hyla meridionalis, Morocco: MVZ 177964–177965, 177970–177972, 186143–186144, 186152–186153, and 186156.
Hyla meridionalis, Spain: MNCN 11361, 11869, 11872–11874, and 11876–11877; MVZ 186013–186014; SDB 1912 (voucher lost, collected with MNCN 11868–11875, tissue expended).
Rana perezi: MNCN 11882 and 11172–11175; MVZ 148895, 148899–148901, and 164861.
Rana saharica: MVZ 178005–178009, and 178032–178039.
Mauremys leprosa, Morocco: MVZ 162520, 178055–178063, 178065–178066, and 231988 (no voucher, tissue only).
Mauremys leprosa, Spain: MNCN 11098–11099, 11527–11529, and 23659.
Blanus cinereus: MNCN 11883–11887, and 11892–11896.
Blanus tingitanus: MVZ 186179–186183, and 186186–186189.
Podarcis vaucheri, Type I (Busack *et al.*, 2005): MNCN 11078–11082; MVZ 178334–178338.
Podarcis vaucheri, Type II (Busack *et al.*, 2005): MVZ 178346–178350.
Timon lepidus: MNCN 11333 and 11983; MVZ 186055–186056, 186061–186064, and 186070; SDB 1672 (voucher lost, tissue catalogued as MVZ 232013).
Timon tangitanus: MVZ 178290 and 186203–186205.
Macroprotodon brevis, Morocco: MVZ 178078 and 186237–186240.
Macroprotodon brevis, Spain: MNCN 11996–11999, and 12004–12005; MVZ 186073–186076.

APPENDIX 2

MORPHOLOGICAL CHARACTERS USED IN ANALYSES

Discoglossus: Straight-line measurements of snout–urostyle; snout (anterior corner of the eye to the tip of the snout); head (posterior angle of the jaw to the tip of the snout); eye (horizontal diameter from posterior corner to anterior corner); eye to naris; tibiofibula (Peters, 1964: Fig. 30, b–1); femur (anus to knee); hand (proximal aspect of central metacarpal tubercle to tip of third digit) and foot (proximal aspect of inner metatarsal tubercle to tip of third digit; Peters, 1964: Fig. 23) lengths, taken to an accuracy of 0.1 mm with dial calipers, were recorded. Head width (angle of jaws), interorbital (between anterior corners of the eyes), and internarial (center to center) distances were also included. Allometric data regarding these characters are summarized in Busack (1986b: Table 4).

Hyla: Straight-line length measurements of snout–urostyle; head (center of imaginary line connecting posterior margins of tympana to tip of snout); eye (horizontal diameter from posterior corner to anterior corner); tympanum (horizontal diameter from anterior margin to posterior margin); tibiofibula (Peters, 1964: Fig. 30, b–1) and foot (proximal aspect of inner metatarsal tubercle to tip of second digit; Peters, 1964: Fig. 23), taken to an accuracy of 0.1 mm with dial calipers, were recorded. Head width (angle of jaws) was also included.

Rana: Straight-line length measurements of snout–urostyle; head (to tip of snout from center of imaginary line connecting posterior margins of tympana); anterior margin of eye to posterior margin of nostril; tibiofibula (Peters, 1964: Fig. 30, b–1); femur (anus to knee); anterior digits III and IV, and posterior digits IV and V, taken to an accuracy of 0.1 mm with dial calipers, were employed. Head width (angle of jaws) was also included.

Mauremys: Straight-line measurements of greatest carapace length; carapace width; plastron length; anterior and posterior plastron lengths; medial seam lengths of all plastron scutes (P1–P6); bridge length; pre-cloacal length; post-cloacal length; tail length; head width (shortest distance across tympana); anterior and posterior plastron widths; carapace height and carapace width at the level of the seam between vertebrals 2 and 3, taken to an accuracy of 0.1 mm with dial calipers, or to 1.0 mm with metal dividers, were recorded. The marginal width was determined by subtracting the width across the pleurals (between marginal–pleural seams at the level of vertebrals 2 and 3) from the carapace width. A partial summary of these data is available in Busack & Ernst (1980: Table 1).

Blanus: Straight-line measurements of prefrontal width, head (tip of snout to back of parietal) and prefrontal lengths were taken to an accuracy of 0.1 mm with dial calipers; snout-vent and tail lengths were measured to an accuracy of 1.0 mm with a steel ruler. Numbers of body and caudal annuli, and dorsal and ventral body segments (Alexander, 1966), were tabulated. Busack (1988) published summaries and interpretations of these data.

Podarcis: Snout-vent length; head length (posterior margin of occipital to anterior edge of rostral); body length (posterior margin of front limb to anterior margin of rear limb); length of fourth digit on front foot and hind leg length were measured to an accuracy of 0.1 mm with dial calipers. Numbers of chin shields (in contact behind the mental); scales comprising the collar; dorsal scales (mid-body); ventral scales (midline, collar to vent), and femoral pores were also recorded. A summary of the data is available in Busack *et al.* (2005: Tables 4 and 5).

Timon: Occipital scale length, fronto-parietal width, occipital width, and frontal width (Busack, 1987: Fig. 1) were measured to an accuracy of 0.1 mm with dial calipers. Snout-vent length (to the nearest mm) was determined with a steel ruler. The numbers of dorsal, ventral, and gular scales, in addition to the number of femoral pores, were also counted. Busack (1987) provides a summary of the data.

Macroprotodon: Snout-vent length and tail length (to the nearest mm) were determined with a steel ruler. The numbers of ventral scales (Dowling, 1951), postocular (left side), caudal, upperlabial, and lowerlabial scales, and dorsal scale rows one head's length posterior to the head, and dorsal scale rows at midbody were tabulated. The labial parietal contact and head pattern (Busack & McCoy, 1990: Fig. 2) were also recorded. Busack & McCoy (1990) present additional details regarding the distribution of these data.

APPENDIX 3

Primers from the current study, listed below, are named to indicate position of the 3′ nucleotide in the mitochondrial genome of *Eumeces egregious* (Kumazawa & Nishida, 1999). Primers for *Podarcis vaucheri* DNA amplification and sequencing are listed in Busack *et al.* (2005: Table 1).

Primer	Primer sequence	Use	Location	Taxa	Target	Reference
16Sb	5′-ACGTGATCTGAGTTCAGACCGG-3′	Amp/Seq.	16sRNA, forward	*Macroprotodon*	ND1	Palumbi, 1996
Busf-ND1f	5′-CGAYATRCAACAYCGMAAAGG-3′	Seq.	ND1, internal, forward	*Hyla & Rana*	ND1	This study
BusGlu-f2	5′-TGAGGWCAAATATCMTTYTGAGG-3′	Seq.	Cyt-*b*, internal, forward	*Timon*	Cyt-*b*	This study
Bus-ND1	5′-TCGTATGARATRATTTGTGCTAC-3′	Seq.	ND1, forward.	*Timon*	ND1	This study
BusThr-r	5′-GGGTTGTTWGADCCBSTTTCRTG-3′	Amp.	tRNA-Thr, reverse	*Blanus & Timon*	ND2	This study
CB6Thr-h	5′-CCTCAATCTTCGRCTTACAGG-3′	Amp.	tRNA-Thr, reverse	*Discoglossus & Rana*	ND2	Palumbi *et al.*, 1991
Frog-16S-f	5′-GGTTTACGACCTCGATGTTGGA-3′	Amp.	16sRNA, forward	*Hyla & Rana*	ND1	This study
Frog-Ile-r	5′-CTCCCTATCAAGGAGGTCCTTA-3′	Amp.	tRNA-Ile, reverse	*Hyla & Rana*	ND1	This study
H12161	5′-AATTTGCCTGTGAAGTTTAT-3′	Seq.	ND4, reverse	*Macroprotodon*	ND4	de Queiroz *et al.*, 2002
H12405	5′-CACAGCTTGAYATTTTWTTTAAATTAC-3′	Seq.	tRNA-his, reverse	*Macroprotodon*	ND4	This study
H15149	5′-CCCTCAGAATGATATTIGTCCTCA-3′	Seq.	Cyt-*b*, internal, reverse	Universal vertebrate	Cyt-*b*	Kocher *et al.* 1989
H15502	5′-GGATTAGCTGGTGTGAAATTGTCTGGG-3′	Amp/Seq.	Cyt-*b*, reverse	*Rana*	Cyt-*b*	Tanaka-Ueno *et al.*, 1998
H15716	5′-TCTGGTTTAATGTGTTG-3′	Seq.	Cyt-*b*	*Macroprotodon*	Cyt-*b*	Slowinski & Lawson, 2002
H16064	5′-CTTTGGTTTACAAGAACAATGCTTTA-3′	Amp/Seq.	tRNA-Thr, reverse	*Mauremys, Timon & Macroprotodon*	ND2	Burbrink, Lawson & Slowinski, 2000
H3056	5′-AGTCADADGTGTTCTTGT-3′	Seq.	ND1, internal, reverse	*Macroprotodon*	ND1	de Queiroz *et al.*, 2002
H3518	5′-CCGTGTCTACTCTATCAAGGTAGTCC-3′	Amp/Seq.	tRNA-Ile, reverse	*Macroprotodon*	ND1	de Queiroz *et al.*, 2002
H5382	5′-GTGTGGGCRATTGATGA-3′	Seq.	ND2	*Macroprotodon*	ND2	de Queiroz *et al.*, 2002
H5877	5′-AAACTAGKAGCCTTGAAGCC-3′	Amp/Seq.	tRNA-trp, reverse	*Macroprotodon*	ND2	de Queiroz *et al.*, 2002
L12140	5′-AACCTAATAAAYATTGCCAC-3′	Seq.	ND4, forward	*Macroprotodon*	ND4	de Queiroz *et al.*, 2002
L14850	5′-TCTCATCCTGATGAAACTTTGGCTC-3′	Amp.	Cyt-*b*, forward	*Rana*	Cyt-*b*	Tanaka-Ueno *et al.*, 1998

APPENDIX 3 *Continued*

Primer	Primer sequence	Use	Location	Taxa	Target	Reference
L14910	5′-GACCTGTGATMTGAAAAACCAYCGTTGT-3′	Amp.	tRNA-glu	*Macroprotodon*	Cyt-*b*	de Queiroz *et al.*, 2002
L14919	5′-AACCACCGTTGTTATTCAACT-3′	Amp/Seq.	tRNA-glu	*Timon & Macroprotodon*	Cyt-*b*	Burbrink *et al.*, 2000
L15584	5′-TCCCATTYCACCCATACCA-3′	Seq.	Cyt-*b*, internal, forward	*Timon & Macroprotodon*	Cyt-*b*	de Queiroz *et al.*, 2002
L2894	5′-ATATCTGGAATATTCACCTA-3′	Seq.	ND1, internal, forward	*Macroprotodon*	ND1	de Queiroz *et al.*, 2002
L4437b	5′-CAGCTAAAAAAGCTATCGGGCCCATACC-3′	Amp/Seq.	tRNA-Met	*Blanus & Macroprotodon*	ND2	Kumazawa *et al.* 1996
L5238	5′-ACMTGACAAAAAATYGC-3′	Seq.	ND2	*Macroprotodon*	ND2	de Queiroz *et al.*, 2002
leu	5′-CATTACTTTTACTIGGATTTGCACCA-3′	Amp/Seq.	tRNA-leu	*Mauremys, Blanus & Macroprotodon*	ND4	Arávalo *et al.* 1994
Llep-2	5′-ATGATCAGTTAAATAGGGCA-3′	Seq.	ND4, internal, reverse	*Timon*	ND4	This study
MVZ15L	5′-GAACTAATGGCACCCAAWWTCCGNAA-3′	Amp.	Cyt-*b*-tRNA-glu, forward	*Discoglossus & Rana*	Cyt-*b*	Moritz, Schneider & Wake, 1992
ND4	5′-CACCTATGACTACCAAAAGCTCATGTAGAAGC-3′	Amp/Seq.	ND4, forward	*Discoglossus, Mauremys, Blanus, Timon, & Macroprotodon*	ND4	Arávalo *et al.* 1994
PD-ND2-f	5′-TCTACYTGRCAAAAACTTGC-3′	Seq.	ND2, internal, forward	*Discoglossus, Hyla & Rana*	ND2	This study
PD-ND2-r	5′-AGGCTATRATTTTTCGAA-3′	Seq.	ND2, internal, reverse	*Discoglossus, Hyla & Rana*	ND2	This study
Ptacek2-h	5′-TCTTCTACTGGTTGTCCTCCGATTCA-3′	Amp.	Cyt-*b*, internal, reverse	*Discoglossus & Rana*	Cyt-*b*	Ptacek, Gerhardt & Sage, 1994
tur-16S-f	5′-AATAAAGACTAGAATGAATGG-3′	Amp.	16sRNA, forward	*Mauremys*	ND1	This study
tur-457f	5′-TYATYACHAAYYTMCTCTCAG-3′	Amp/Seq.	Cyt-*b*, internal, forward	*Mauremys*	Cyt-*b*	This study
tur-511r	5′-GTTARGGTDGCRTTGTCTAC-3′	Amp/Seq.	Cyt-*b*, internal, reverse	*Mauremys*	Cyt-*b*	This study
tur-gln-f	5′-TGATCTCTCTGGTGTAGGTTCGAG-3′	Amp.	tRNA-gln, forward	*Mauremys*	ND2	This study
tur-ile-f	5′-ACCTTGATAGGGTGAATAATAGAG-3′	Amp.	ND1, forward.	*Mauremys*	ND1	This study
tur-ile-r	5′-CTCTATTATTCACCYYATCAAGG-3′	Amp.	tRNA-ile, reverse	*Mauremys*	ND1	This study
tur-trp-r	5′-ATCTCTTGTTTGGGGCTTTGAAGG-3′	Amp.	tRNA-trp, reverse	*Mauremys*	ND2	This study

APPENDIX 4

ENZYMES AND PROTEIN SYSTEMS EXAMINED BY ELECTROPHORESIS

Enzymes and protein systems examined by electrophoresis are arranged first, listed in order by International Union of Biochemistry (1984), Enzyme Commission number in the following format: Name [abbreviation, Enzyme Commission (EC) number, electrophoretic conditions, (notes)]; a listing of electrophoretic conditions follows.

General proteins [Gp, —, B]; haemoglobin [Hb, —, B (resolved for *Timon* only)]; alcohol dehydrogenase [Adh, 1.1.1.1, A (J for *Mauremys*)]; glycerol-3-phosphate dehydrogenase (G3pdh, 1.1.1.8, D); L-iditol dehydrogenase [Iddh, 1.1.1.14, D (not resolved for *Discoglossus*, *Blanus*, or *Podarcis*)]; L-lactate dehydrogenase [Ldh, 1.1.1.27, F (C for *Mauremys*, D for *Hyla*)]; malate dehydrogenase [Mdh, 1.1.1.37, F (D for *Hyla*)]; malate dehydrogenase (Nadp+) [Mdhp, 1.1.1.40, F]; isocitrate dehydrogenase [Idh, 1.1.1.42, E (D for *Mauremys*, G for *Timon*)]; phosphogluconate dehydrogenase [Pgdh, 1.1.1.44, E (J for *Mauremys*)]; glucose-6-phosphate dehydrogenase [G6pdh, 1.1.1.49, D (not resolved for *Discoglossus*, *Hyla*, *Rana*, or *Timon*)]; aldehyde dehydrogenase [Aldh, 1.2.1.3, F (not resolved for *Discoglossus* or *Rana*)]; glyceraldehyde-3-phosphate dehydrogenase [Gapdh, 1.2.1.12, H (not resolved for *Discoglossus*, *Hyla*, *Mauremys* or *Podarcis*)]; glutamate dehydrogenase (Gtdh, 1.4.1.2, D); superoxide dismutase [Sod, 1.15.1.1, D (A for *Timon*, H for *Blanus*)]; aspartate aminotransferase [Aat, 2.6.1.1, D (F for *Rana*)]; hexokinase (Hk, 2.7.1.1, G); pyruvate kinase [Pk, 2.7.1.40, I (resolved for *Mauremys* only)]; creatine kinase (Ck, 2.7.3.2, G); adenylate kinase [Ak, 2.7.4.3, G (not resolved for *Discoglossus*, *Hyla*, *Rana*, or *Mauremys*)]; esterase (non specific) [Est, 3.1.1.–, B]; esterase (non specific) [Est-D, 3.1.1.–, B (not resolved for *Rana* or *Podarcis*)]; acid phosphatase (Acp, 3.1.3.2, G); fructose-bisphosphatase [Fbp, 3.1.3.11, D (H for *Mauremys*, *Blanus*, *Timon*, and *Macroprotodon*)]; *N*-acetyl-β-glucosaminidase [βga, 3.2.1.30, G (J for *Hyla*, C for *Mauremys*)]; peptidase (PEP) L-leucylglycylglycine [Pep-B, 3.4.–.–, C]; dipeptidase I, L-leucyl-L-alanine [La, 3.4.13.11, B (C for *Rana*)]; proline dipeptidase [Pep-D, 3.4.13.9, B]; adenosine deaminase [Ada, 3.5.4.4, A (F for *Mauremys*)]; fructose-bisphosphate aldolase [Fba, 4.1.2.13, H (not resolved for *Hyla, Rana*, or *Podarcis*)]; aconitase hydratase (Acoh, 4.2.1.3, E); mannose-6-phosphate isomerase [Mpi, 5.3.1.8, E (F for *Rana*, D for *Mauremys*)]; glucose-6-phosphate isomerase [Gpi, 5.3.1.9, F], and phosphoglucomutase [Pgm, 5.4.2.2, E (D for *Mauremys*)].

Electrophoretic conditions were as follows: A, Histidine, pH 7.8 gel & electrode buffer (Harris & Hopkinson, 1976), 150 V/3 h; B, LiOH A + B, pH 8.2 gel & LiOH A, pH 8.1 electrode buffer (Selander *et al.*, 1971), 300 V/3 h; C, Poulik, pH 8.7 gel & borate, pH 8.2 electrode buffer (Selander *et al.*, 1971), 250 V/3 h; D, Tris citrate II, pH 8.0 gel & electrode buffer (Selander *et al.*, 1971), 130 V/4 h; E, Tris citrate II, pH 8.0 + NADP gel & Tris citrate II, pH 8.0 electrode buffer (Selander *et al.*, 1971), 130 V/4 h; F, Tris citrate III, pH 7.0 gel & electrode buffer (Ayala *et al.*, 1972), 180 V/3 h; G, Tris citrate III, pH 7.0 + 15% glycerine gel & tris citrate III, pH 7.0 electrode buffer (Ayala *et al.*, 1972), 180 V/3 h; H, Tris citrate III, pH 7.0 + NAD + 2-mercaptoethanol gel & Tris citrate III, pH 7.0 electrode buffer (Ayala *et al.*, 1972), 180 V/3 h; I, Tris EDTA borate II, pH 9.0 gel & electrode buffer (Ayala *et al.*, 1972), 400 V/4 h; and J, Succinate, pH 5.0 + 15% glycerine gel & sodium citrate, pH 5.0 electrode buffer (Culliford, 1971), 100 V/3 h.

Detailed analyses of alleles and allele frequencies for *Discoglossus* (Busack, 1986b: Table 2; *D. scovazzi* reported as *D. pictus*), *Blanus* (Busack, 1988: Table 2), *Podarcis* (Busack *et al.*, 2005: Table 3), *Timon* (Busack, 1987: Table 2; reported as *Lacerta*, *L. tangitanus* reported as *L. pater*):, and *Macroprotodon* (Busack & McCoy, 1990: Table 2; *M. brevis* reported as *M. cucullatus brevis* & *M. c. ibericus*) are already published. See Busack (1985) for allele and allele frequency data regarding *Rana* (Appendix I: 103–107), *Hyla* (Appendix I: 112–115) and *Mauremys* (Appendix I: 116–119).