Determination of genetic diversity within the insular lizard *Podarcis tiliguerta* using mtDNA sequence data, with a reassessment of the phylogeny of *Podarcis*

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Introduction

Despite being the predominant reptile group in Southern Europe, the taxonomy of *Podarcis* Wall lizards is both complex and unstable. Recent attempts to estimate a phylogeny for the genus using molecular methods have been largely unsuccessful, with many poorly resolved nodes and widely different estimates from different studies (Harris and Arnold, 1999; Oliverio et al., 2000; Poulakakis et al., 2003). One possible reason for this is that presently accepted forms may well be species complexes — *Podarcis hispanica** (Steindachner, 1879) contains several highly genetically distinct lineages all of which may deserve species status based on mitochondrial (Harris and Sa-Sousa, 2002; Harris et al., 2002) and protein electrophoretic data (Pinho et al., 2003). *Podarcis erhardii* (Bedriaga, 1882) is also probably a species complex (Poulakakis et al., 2003), *Podarcis sicula* and *Podarcis melisellensis* contain considerable genetic diversity (Podnar et al., 2004, 2005) although *Podarcis lilfordi* (Günter, 1874) and *Podarcis pityusensis* (Boscá, 1883) show much lower levels of intraspecific variation (Terrassa et al., 2004). Other problems in estimating the phylogeny have included laboratory errors (Oliverio et al., 2000). These problems can be overcome by examining variation within species, and by combining previously published data with new sequences. We have therefore collected sequence data from several localities of *Podarcis tiliguerta* (Gmelin, 1789), endemic to Corsica, Sardinia and neighbouring small islands. Our aim was to determine if this form, like *P. erhardii* and *P. hispanica*, might also represent a species complex. Such a scenario seems possible given its fragmented distribution, and given that high genetic diversity was observed within the species using allozyme data (Capula, 1996). At the same time we aimed to combine our data with that previously published to reassess relationships within *Podarcis* and to identify the problematic sequences from previous studies.

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*The last author maintains that *Podarcis* is masculine (Böhme and Kohler, in press). However the first author continues to refer to *Podarcis* as feminine.

Methods

Specimens were identified in the field from both Corsica and Sardinia, and then individuals were released after tail tips were collected (Table 1). Total genomic DNA was extracted from these small pieces of tail using standard methods, following Harris et al. (1998). Polymerase Chain Reaction primers used in both amplification and sequencing were 12Sa and 12Sb and cytochrome *b*1 and cytochrome *b*2 from Kocher et al. (1989). These were the same regions sequenced by Harris and Arnold (1999), so that the data from this earlier study could be combined with the new sequences. Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus.

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Table 1. Specimens sequenced for this analysis with locality and voucher number. * Indicates specimens also sequenced for Cytochrome *b*.

Species	Locality	Island	Map locality and Code
Podarcis tiliguerta *	Porticciolo	Corsica	1 C1.1
Podarcis tiliguerta	Porticciolo	Corsica	1 C1.2
Podarcis tiliguerta *	Rogliano	Corsica	2 C2.1
Podarcis tiliguerta	Rogliano	Corsica	2 C2.2
Podarcis tiliguerta	Centuri	Corsica	3 C3.1
Podarcis tiliguerta	Centuri	Corsica	3 C3.2
Podarcis tiliguerta	Col de Lavezzo	Corsica	4 C4.1
Podarcis tiliguerta	S. Quilico	Corsica	5 C5.1
Podarcis tiliguerta	Palmarella	Corsica	6 C6.1
Podarcis tiliguerta	Corte	Corsica	7 C7.1
Podarcis tiliguerta	Altiani	Corsica	8 C8.1
Podarcis tiliguerta	Solenzara	Corsica	9 C9.1
Podarcis tiliguerta *	Capo Testa	Sardinia	10 S10.1
Podarcis tiliguerta	Trinita d'Agulto	Sardinia	11 S11.1
Podarcis tiliguerta	Asinara Island	Sardinia	12 S12.1
Podarcis tiliguerta	Asinara Island	Sardinia	12 S12.2
Podarcis tiliguerta	Oristano	Sardinia	13 S13.1
Podarcis tiliguerta *	Oristano	Sardinia	13 S13.2
Podarcis raffonei *	Strombolicchio	Aeolian Is.	
Podarcis wagleriana *		Sicily	

Mitochondrial DNA sequences were aligned using Clustal W (Thompson et al., 1994). Previously published sequences included all those available for both genes from Harris and Arnold (1999), representatives of all the major lineages from the Iberian Peninsula and North Africa (Harris et al., 2002), *Podarcis sicula* (Rafinesque, 1810) and *Podarcis muralis* (Laurenti, 1768) (Fu, 2000) and an additional *P. muralis* from GenBank (BA10). The four outgroups, *Lacerta chlorogaster* (Boulenger, 1908), *Lacerta horvathi* Méhely, 1904, *Lacerta dugesii* Milne-Edwards, 1829 and *Lacerta perspicillata* Duméril & Bibron, 1839, were from Harris and Arnold (1999). New sequences for both genes were generated for this study for four *P. tiliguerta*, *Podarcis wagleriana* Gistel, 1868 and *Podarcis raffonei* (Mertens, 1952) Aligned sequences were 873 base pairs long. Additionally, to further examine variation within *P. tiliguerta* we sequenced 18 individuals from 13 populations for the 12S rRNA gene region only. New sequences have been submitted to GenBank. The sequences from *P. sicula* from Harris and Arnold (1999) were not included in this analysis. Since publication of this article it has been noted that the cytochrome *b* sequence published appears to be more similar to haplotypes typical of *P. muralis* (Poulakakis et al., 2003). This appears to be due to a nuclear copy of part of the mtDNA that is preferentially amplified in some populations of *P. sicula* using the standard cytochrome *b* primers (W. Mayer, pers. comm.).

The data were imported into PAUP* 4.0b10 (Swofford, 2002) for phylogenetic analysis. For the phylogenetic analysis of the mtDNA data we used maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference. We followed the approach outlined by Huelsenbeck and Crandall (1997) to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (Posada and Crandall, 1998). Once a model of evolution was chosen, it was used to estimate a tree using ML, and support for nodes estimated by bootstrapping with 500 replicates (Felsenstein, 1985) using the "fast" option. A MP analysis was carried out (100 replicate heuristic search, TBR branch-swapping) with gaps treated as missing data, and support for nodes estimated by bootstrapping with 1000 replicates. The Bayesian analysis was implemented using MrBayes (Huelsenbeck and Ronquist, 2001) which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach. Bayesian analyses were conducted with random starting trees, run 0.5×10^6 generations, and sampled every 1000 generations using a general-time-reversible model of evolution with a gamma model of among site rate variation. In both searches stationarity of the Markov Chain was determined as the point when sampled log likelihood values plotted against generation time reached a stable mean equilibrium value; "burn-in" data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny. Two independent replicates were conducted and inspected for consistency to check for local optima (Huelsenback and Bollback, 2001). To examine variation within *P. tiliguerta* the aligned 12S sequences were examined separately. Because variation is low, the sequences were joined using median networks (Bandelt et al., 2000).

Results

Including the outgroups 30 combined mtDNA sequences were analyzed. We concluded that

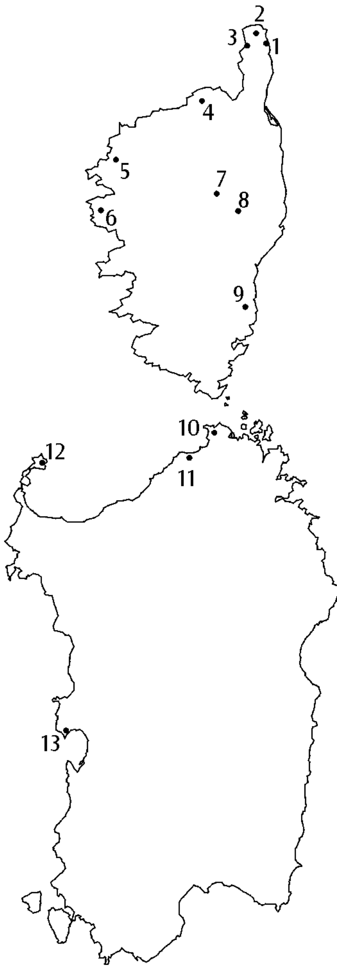

Figure 1. Map showing sampling localities of *Podarcis tiliguerta* sequenced in this study. Codes for samples are given in table 1.

the TrN model (base frequencies A 0.33, C 0.27, G 0.11, T 0.29, relative rates of transitions A/T = 7.84 and C/G = 13.36) with a gamma distributed rate heterogeneity model (4 rate categories, G = 0.619) and an estimated proportion of invariable sites (0.493) was the most appropriate model of evolution for these data. A ten replicate heuristic search incorporating this model found two trees of $-\ln 5174$. Maximum parsimony analysis found two trees of 1030 steps, the 50% bootstrap consensus of which was identical to the ML analysis, but less well resolved (fig. 2). One hundred and ninety-eight characters were parsimony informative. The estimate of phylogeny obtained using Bayesian analyses was similar to the ML tree, although with much higher levels of support (fig. 2).

For the assessment of variation within *Podarcis tiliguerta* a total of 8 haplotypes were found, 5 in Corsica and 3 in Sardinia (fig. 3). Maximum sequence divergence between haplotypes within Corsica was approximately 2.2%.

Discussion

Like earlier estimates of phylogeny within *Podarcis*, many of the nodes between species are poorly resolved. However our results have several significant and well-supported differences from previous studies.

Is Podarcis tiliguerta *a species complex?*

Our results reveal an extremely high degree of genetic diversity within *P. tiliguerta* — maximum uncorrected divergence of 15.3% within the region of cytochrome *b* sequenced. This is higher than typically found between reptile species (Harris, 2002). Thus our results indicate that *P. tiliguerta* may be a species complex. Using nuclear markers Capula (1994) similarly reported high genetic differentiation between populations from Corsica and Sardinian populations but also reported substantial variation between the Cerbicale islands (SE of Corsica) and the main island. Unfortunately, we lack samples of Cerbicale islands to confirm this with mtDNA. Our results based on 12S rRNA sequence data indicate extensive variation within both of the main islands. The insular population of Asinara (NW Sardinia) falls within the genetic variation of Sardinia.

Clearly large scale morphological and molecular studies, such as those carried out on the *P. hispanica* species complex, are needed to determine the exact range and number of forms found within *P. tiliguerta*, and to evaluate their conservation status.

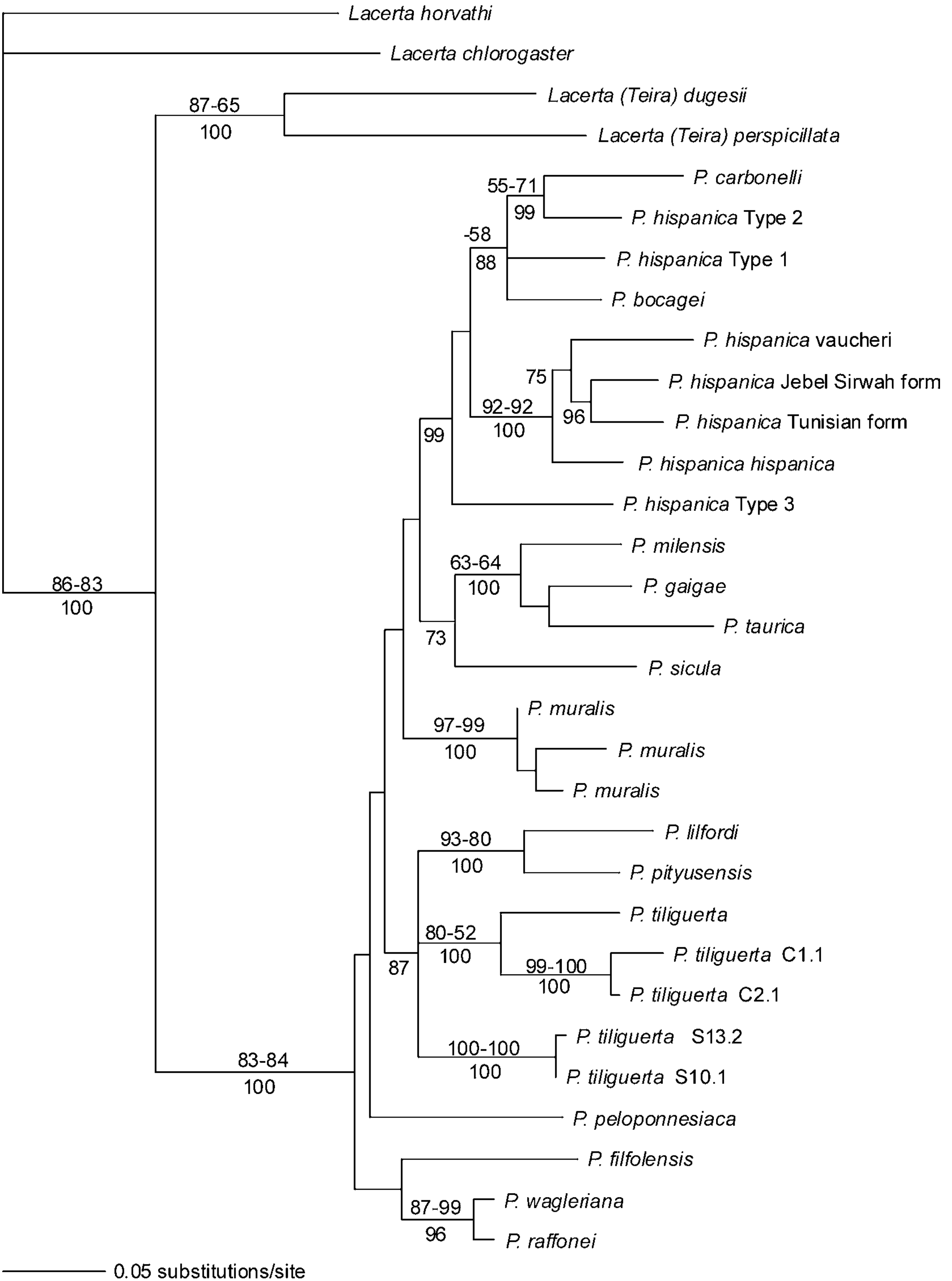

Figure 2. One of two trees derived from a ML analysis using the model described in the text. All analyses produced similar estimates of relationships to the one shown, except for rearrangements of branches with bootstrap support <50%. Posterior node probabilities from the Bayesian analyses are indicated below nodes and bootstrap values for ML-MP are given above nodes.

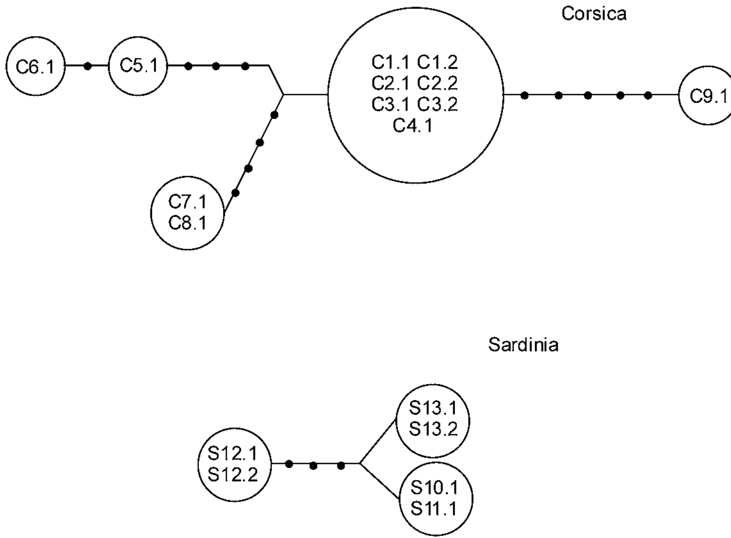

Figure 3. Median-joining network of the 12S rRNA sequences for *Podarcis tiliguerta*. Filled circles indicate presumed missing haplotypes.

Taxonomic implications for Podarcis tiliguerta

The name *Lacerta tiliguerta* Gmelin, 1789 (currently *Podarcis tiliguerta*) was based on Sardinian material so that it is the Sardinian clade which would have to keep the current species name. The next available names (according to the last comprehensive synonymy by Schneider (1986)), viz. *quadrilineata* Gray, 1838, *genei* Cara, 1832, *toro* Mertens, 1932, *oristanensis* Taddei, 1949, and *ranzii* Lanza, 1966, all refer to Sardinian (include offshore islands) type material.

The oldest available names referring to Corsica and its satellite islands are *eiselti, grandisonae* and *maresi*, all described simultaneously in the same paper by Lanza (1972), from the Cerbicale Islands, Isola Pietricaggiosa, Isola Piana and Isolotto Maestro Maria (*eiselti*), Isola Vacca (*grandisonae*) and Isole Toro Piccolo and Toro Grande (*maresi*). However, none of these three names can be assigned to the Corsican clade because of the significant genetic separation in allozyme variation between such populations and those in Corsica (Capula, 1996). Thus, although the taxonomy of *P. tiliguerta* clearly needs to be re-evaluated, in the present state of knowledge any nomenclatural changes would be premature.

Close genetic relationship between Podarcis muralis *and* Podarcis sicula*?*

A significant difference between the estimates of relationships within *Podarcis* between Harris and Arnold (1999) and later studies (Oliverio et al., 2000; Poulakakis et al., 2003) was the close relationship between *P. muralis* and *P. sicula* suggested by the former study. Comparing the two genes (12S rRNA and cytochrome *b*) analysed by Harris and Arnold (1999) separately against sequences published later (Fu, 2000) it is clear that it is only the cytochrome *b* sequences of Harris and Arnold (1999) that support this relationship (analyses not shown). The problem appears to be the existence of a copy of part of the mitochondria of *P. muralis* ancestry found in the nuclear DNA of some *P. sicula* populations (Podnar et al., 2005). This was apparently amplified in the sample used by Harris and Arnold (1999) rather than true mitochondrial DNA. Although a close relationship between *P. sicula* and *P. muralis* is thus not supported by the mtDNA sequence data, exact relationships of these species remain poorly resolved.

Genetic relationship of Podarcis raffonei *with other* Podarcis

Podarcis raffonei is endemic to some Aeolian islands where it is partially sympatric with *P. sicula*. Biochemical data strongly support a sister-taxa relationship with *P. wagleriana* (Capula, 1994) and this is also supported by our data — indeed genetic differentiation between *P. raffonei* and *P. wagleriana* is minimal in our analysis (3.3% cytochrome *b* divergence). Given this, it is surprising that Oliverio et al. (2000) did not recover this estimate of relationships. Their results suggested *P. wagleriana* was a lineage nested within a paraphyletic *P. filfolensis* from the Maltese archipelago, with an extremely low genetic divergence ($p = 0.002$). Examination of the alignment used by these authors shows that an alternative, preferable, alignment would reduce the genetic distance even further (details available from DJH on request). It seems likely therefore that this is erroneous, and the *P. raffonei* is indeed more closely related to *P. wagleriana.* However, further analyses of all three species are clearly warranted.

Relationships between major clades

All earlier estimates of relationships between *Podarcis* species have been weakly supported, suggesting that most major lineages separated during a brief evolutionary time period. However some groups can clearly be discerned. All species belonging to the "Iberian and North African clade" — *P. hispanica* (including all known lineages), *Podarcis bocagei* (Seoane, 1884) and *P. carbonelli* Pérez-Mellado, 1981 — are supported as a monophyletic clade. *Podarcis wagleriana* and *P. raffonei* are sister taxa, as are *P. lilfordi* and *P. pityusensis* from the Balearic islands. *Podarcis milensis* (Bedriaga, 1882), *Podarcis gaigae* (Werner, 1930) and *Podarcis taurica* (Pallas, 1814) form a clade in our analysis, although this was not the result obtained using only cytochrome *b* data (Poulakakis et al. 2003). Again considerable sampling within these species will be needed to determine the cause of this discrepancy. No other relationships are well supported.

Conclusions

Podarcis tiliguerta appears to be a species complex. Further morphological and molecular analyses will be needed to confirm this. *Podarcis sicula* is not sister taxon to *P. muralis*, nor is *P. wagleriana* a genetic lineage within *P. filfolensis* as has been previously suggested. However relationships between most *Podarcis* species remain unresolved.

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