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Combining immunological and molecular data to assess phylogenetic relations of some Greek *Podarcis* species

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Abstract

Most recent molecular studies revealed the phylogeny of Greek *Podarcis* species, which for years remained elusive, due to discordant data produced from various chromosomal, complement fixation and protein studies. In this report, we analyzed cellular immune responses of spleen-derived lymphocytes from six allopatric *Podarcis* species encountered in Greece, by assessing two-way mixed lymphocyte reaction (MLR)-induced proliferation. On the basis of stimulation indices (S.I.) as determined from cultures set up from xenogeneic splenocytes coincubated in pairs, we generated a phylogenetic tree, fully consistent with the phylogenetic relationships of *Podarcis* as determined by parallel analyses based on partial mitochondrial (mt) DNA sequences. Although the exact mechanisms triggering lymphocyte responses in lizard two-way xenogeneic MLR are not fully understood, our results show the potential use of cell-mediated immune responses as an additional approach to mtDNA analysis, for species delimitation within specific lizard taxa.

Keywords: Lacertidae; Xenogeneic mixed lymphocyte reaction; mtDNA analysis; *Podarcis* sp.; Reptile phylogeny

1. Introduction

Reptiles occupy a central phylogenetic position for understanding the evolution of terrestrial vertebrate immune system. However, they are among the less studied exothermic vertebrate classes with respect to the nature of their immunological responses. Several reports have shown that reptiles possess a well-organized lymphoid system and, similar to mammals, their leukocytes are categorized into lymphocytes, monocytes and granulocytes (El Ridi et al., 1988). As in amphibians, birds and mammals, reptilian T cells mature in thymus, whereas spleen serves as a reservoir both, of T and B cells (Manickasundari et al., 1984). Moreover, reptilian peripheral blood, thymic and spleen lymphocytes have been reported to respond to antigenic stimuli *in vitro* and share several common membrane characteristics with mammalian lymphocytes (Cushens and Clem, 1979a; Saad et al., 1984; Saad, 1989; Work et al. 2000; Munoz and De la Fuente, 2001; Burnham et al., 2005).

Within the reptilian lineage of Lepidosauria, lizard and snake primary and secondary lymphoid organs, such as thymus, spleen, bone marrow and gut-associated lymphoid tissue, are well developed and lymphocytes can be distinguished into antibody-producing B cells (Saad et al., 1984) and mitogen-responding T cells (Belliure et al., 2004). Lizard and snake T cells exhibit immunoreactivities similar to those of mammalian T cells, among which is the *in vivo* T cell-mediated

Abbreviations: MLR, mixed lymphocyte reaction; S.I., stimulation index; mtDNA, mitochondrial DNA; FCS, fetal calf serum; CM, complete medium; cpm, counts per minute; cyt *b*, cytochrome *b*; ML, maximum likelihood; BI, Bayesian inference.

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induction of a delayed-type cutaneous hypersensitivity response (Belliure et al., 2004), the *in vitro* fast and vigorous T cell proliferation upon mitogenic challenge (*e.g.* by concanavalin A or phytohemagglutinin) (Farag and El Ridi, 1986; Saad, 1989; Saad and El Deeb, 1990; Burnham et al., 2005) and the *ex vivo* allogeneic stimulation in mixed lymphocyte reaction (MLR) assays (Saad and El Ridi, 1984; Farag and El Ridi, 1985; Burnham et al., 2003, 2005).

As in mammals, birds and amphibians, the principal responder lymphocyte population in lizard and snake MLR are T cells (Farag and El Ridi, 1985; Burnham et al., 2003, 2005). Although in Lepidosauria the identity either of the stimulator cell or the T cell recognition target remains elusive, MLR responses are most probably triggered by alloantigens and/or foreign MHC molecules, and mediated by phagocytes and MHC gene products. In this respect, MHC class I and class II molecules and their encoding genes have been isolated and cloned from the lizard *Amieva amieva*, the snake *Nerodia sipedon* and most recently from *Sphenodon punctatus* (Grossberger and Parham, 1992; Miller et al., 2005) and these MHC gene sequences have been used to generate phylogenetic trees and assess vertebrate species MHC evolutionary relationships (Kasahara et al., 1995). Compared to mammals however, the degree of MHC polymorphism in reptiles, particularly within a specific species, is speculated to be significantly less (Wittzell et al., 1999), as a result of inbreeding depression (Ujvari et al., 2002), or for facilitating animals to acquire superior parasite defence mechanisms (Olsson et al., 2005), or for still unexplored evolution-related reasons. Therefore, allogeneic MLR responses among animals belonging to the same, quasi inbred, reptilian species might not produce maximal T cell stimulation and reveal any potentially existing MHC genetic diversity to infer phylogenesis.

In contrary to mammals, where xenotransplantation models and xenogeneic immune responses have been studied for therapeutic purposes (Hammer, 2004; Andres et al., 2005), xenogeneic MLR among non-mammalian species, including reptiles, has not been studied. Herein, using two-way xenogeneic MLR, we investigated the proliferative responses of spleen-derived lymphocytes isolated from six allopatric species of the genus *Podarcis*. Splenocytes were coincubated in pairs at all possible combinations. Significant variability in the degree of lymphocyte proliferation among paired species was recorded which was further analyzed to infer the phylogenetic relationships of the six *Podarcis* species included in the study. The phylogenetic tree constructed on the basis of cellular immune responses was found to coincide with the recently reported tree constructed on the basis of mitochondrial DNA (mtDNA) analysis of the same species (Poulakakis et al., 2003, 2005a,b). Our results support the existence of a correlation between the degree of proliferative spleen-derived T cell responses and the genetic distance between some *Podarcis* species and further suggest that xenogeneic two-way MLR could be used in conjunction with mtDNA analysis, to reveal ambiguous phylogenetic relations among species of the genus *Podarcis*.

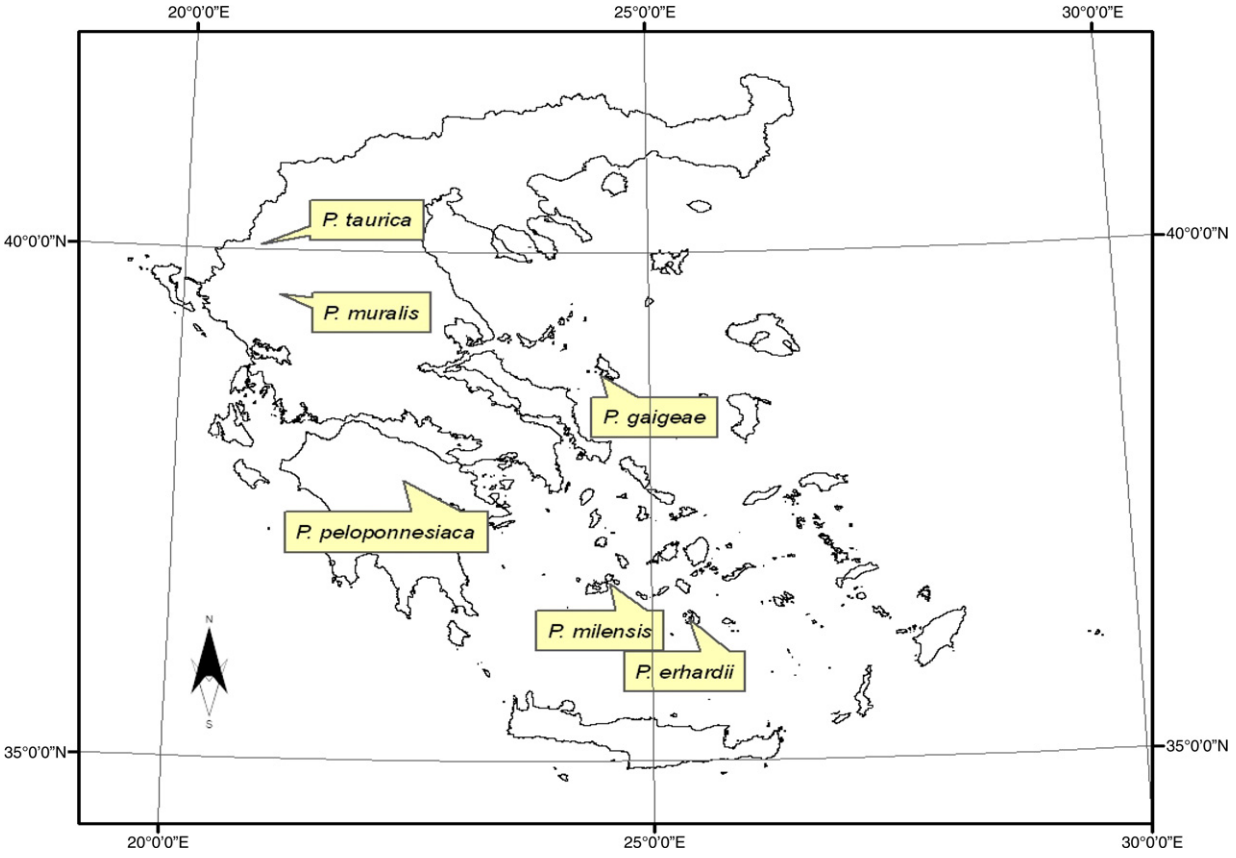

Fig. 1. Map of Greece showing the sampling localities of the six allopatric *Podarcis* species used in MLR assays and for DNA analysis.

2. Materials and methods

2.1. Experimental animals

Male, sexually mature lacertid lizards of the genus *Podarcis*, weighing 4–6 g and of length (snout to vent) 5.5–7.5 cm, were captured in the wild from representative areas of their typical environment. Six allopatric species, *Podarcis erhardii* (Santorini Island), *P. peloponnesiaca* (Stymfalia Lake, Peloponnese), *P. muralis* (Mount Tzoumerka), *P. milensis* (Kimolos Island), *P. gaigeae* (Valaxa Islet) and *P. taurica* (Epirus) were used in the present study (Fig. 1). In accordance with the Hellenic National Law (Presidential Decree 67/81), all animals were collected during the non-reproductive period (October–November). To allow adjustment to conditions in captivity, lizards were individually housed in vitreous terraria (20×25×15 cm) on a sand substrate, where bricks and stones were provided as hiding places. Animals were acclimatized at 26 °C, under a controlled photoperiod (12 h light, 12 h dark) using fluorescent lights for at least 4 weeks prior to sacrifice. Additional incandescent lamps (60 W) allowed animals to thermoregulate behaviorally for 8 h per day. All animals had access to water *ad libitum* and were fed every other day with *Tenebrio molitor* larvae.

2.2. Isolation of spleen cells

Spleens were aseptically excised from each animal and homogenized individually in ice-cold RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 0.1% fetal calf serum (FCS; Gibco) as a source of protein, 2 mM L-glutamine (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), 10 mM Hepes (Gibco), 5×10^{-5} M 2-mercaptoethanol (Gibco) and 1% penicillin–streptomycin (Gibco) (referred to thereafter as complete medium, CM). Spleen cell suspensions were filtered through cotton wool to remove tissue debris and washed twice in CM by centrifugation at 300 ×*g* for 5 min. The number of leukocytes was counted in a Neubauer chamber and varied between 2.0 and 9.3×10^{6} cells/spleen. Viability was assessed using Trypan blue dye exclusion assay and in all cases was >95%.

2.3. Two-way mixed lymphocyte reaction (MLR)

Splenocytes from each animal were adjusted to a concentration of 2×10^{6} cells/mL in CM. 2×10^{5} splenocytes in 100 μL aliquots were mixed with an equal number of allogeneic or xenogeneic spleen lymphocytes in a final volume of 200 μL/well in U-bottomed, 96-well tissue culture plates (Costar, Cambridge, MA, USA). Unmixed splenocytes (2×10^{5} cells/well) from each animal were used as for controls. Plates were incubated for 5 days in a 37 °C humidified 5% CO_2 incubator. During the last 18 h, 1 μCi [^{3}H]-thymidine (The Radiochemical Center, Amersham, UK) was added per well. Cultures were harvested in a semi-automated cell harvester (Skatron Inc., Tranby, Norway) and the radioactivity incorporated into cellular DNA was determined by liquid scintillation counting. These conditions were determined as optimal for MLR using *Podarcis* lizard spleen cells, after a series of pilot experiments performed with splenocytes from two species (*P. erhardii* and *P. taurica*), where various concentrations of FCS (0.1–10%), two incubation temperatures (35 °C and 37 °C) and different incubation periods (1–6 days) were assayed. All cultures were set up in triplicates or quadruplicates depending on the number of cells available per animal. Data are expressed as counts per minute (cpm) and stimulation indices (S.I.) are calculated according to the formula: cpm of MLR culture/sum of cpm of unmixed splenocytes.

2.4. DNA sequence retrieval

Cytochrome (cyt) *b* and 16S rRNA sequences of the six allopatric species were retrieved from GenBank database: *P. erhardii* (Santorini Island): cyt *b* AF486226 (Poulakakis et al., 2003) and 16S AY896225 (Poulakakis et al., 2005b); *P. peloponnesiaca* (Stymfalia Lake): cyt *b* AF486231 (Poulakakis et al., 2003) and 16S AY896172 (Poulakakis et al., 2005b); *P. muralis* (Mount Tzoumerka): cyt *b* AY896126 and 16S AY896183 (Poulakakis et al., 2005b); *P. milensis* (Kimolos Island): cyt *b* AY768776 and 16S AY768740 (Poulakakis et al., 2005a); *P. gaigeae* (Valaxa Islet): cyt *b* AY768775 and 16S AY768739 (Poulakakis et al., 2005a); *P. taurica* (Epirus): cyt *b* AY768772 and 16S AY768726 (Poulakakis et al., 2005a). Alignment of the concatenated cyt *b* and 16S sequences was performed with Clustal X (Thompson et al., 1997) and corrected by eye.

2.5. Phylogenetic analysis and construction of trees

S.I. values from four individually performed experiments using animals from the same species were used as genetic distances among specimens under study and a neighbour-joining analysis was performed using MEGA (v.3.1, Kumar et al., 2001).

To examine whether the sequences from the two genes should be combined in a single analysis, a partition homogeneity test was run in PAUP (v.4.0b10, Swofford, 2002), and significance was estimated by 1000 repartitions. This test, which was described as the incongruence-length difference test by Farris et al. (1995), indicated no conflicting phylogenetic signals between the datasets ($p=0.971$) and, given that the mtDNA genes are linked, datasets from both genes were analyzed together. Analyses for phylogenetic inference were conducted using three methods: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI).

MP analysis (Swofford et al., 1996) was performed with PAUP* (v.4.0b10), using exhaustive search for most parsimonious tree. Our dataset is sufficiently small that an exhaustive search of all possible trees can be performed. Confidence in the nodes was assessed by 1000 bootstrap replicates (Felsenstein, 1985), using the same algorithm.

For ML analysis (Felsenstein, 1981), the best-fit model of DNA substitution and the parameter estimates used for tree construction were chosen by performing hierarchical likelihood-

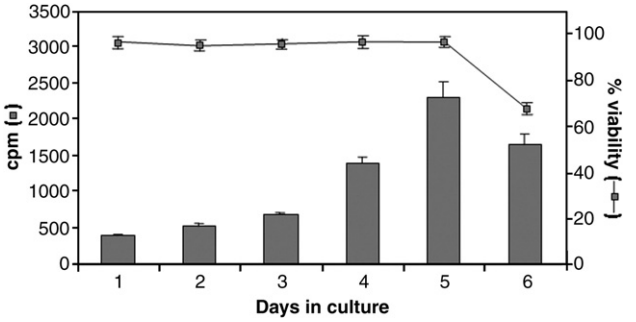

Fig. 2. Time response of *P. erhardii* and *P. taurica* spleen cells in xenogeneic MLR. Splenocytes (2×10^5/animal/well) were co-cultured in CM at 37 °C for 1–6 days. Cultures were pulsed for the last 18 h with 1 μCi/well [^{3}H]-thymidine and isotope incorporation is expressed as counts per minute (cpm)±standard deviation (SD). Viability was assessed by Trypan blue dye exclusion and is expressed as % of viable cells±SD. Pooled data from 3 individual experiments set up with spleen cells from 3 *P. erhardii* and 4 *P. taurica* lizards are shown. Bars (■) represent cpm; solid line (-■-) represents % viability.

ratio tests (Huelsenbeck and Crandall, 1997) in Modeltest (v.3.06; (Posada and Crandall, 1998)). Likelihood-ratio tests indicated that the Tamura and Nei (TrN) (1993) model+G showed a significantly better fit than the other less complicated models for the combined dataset (cyt *b* and 16S rRNA), because in PAUP is not possible to specify a separate model of evolution for each gene partition (model parameters: $a=0.2052$; base frequencies A=0.31, C=0.25, G=0.15, T=0.29; and rate matrix A/G=2.18, C/T=9.76). Exhaustive ML searches were performed in PAUP* (v.4.0b10). Bootstrapping with 100 pseudo-replicates and heuristic search were used to examine the robustness of clades in resulting trees (Felsenstein, 1985).

For the BI, the best-fit model of DNA substitution and the parameter estimates used for tree construction were chosen by performing hierarchical likelihood-ratio tests (Huelsenbeck and Crandall, 1997) in Modeltest (v.3.7; Posada and Crandall, 1998). Likelihood-ratio tests indicated that Tamura and Nei (TrN) (Tamura and Nei, 1993) model+G (model parameters: $a=0.28934$; base frequencies A=27.1, C=27.7, G=12.6, T=32.6; rate matrix A/G=3.0452, C/T=11.2325) and Tamura and Nei (TrN) model+G (model parameters: $a=0.1445$; base frequencies A=34.3, C=21.9, G=18.5, T=25.3; rate matrix A/G=1.8335, C/T=5.8878) showed a significantly better fit than the other less complicated models for the cyt *b* and the 16S rRNA respectively. Bayesian inference (BI) analysis was performed with the software MrBayes (v3.1; Ronquist and Huelsenbeck, 2003) using the ability of MrBayes to handle a wide variety of data types and models, as well as any mix of these models, based on the procedure described in MrBayes (3.1) manual. The analysis was run with four chains for 10^7 generations and the current tree was saved to file every 100 generations. This generated an output of 10^5 trees. The −lnL stabilized after approximately 10^4 generations and the first 10^3 trees (10% "burn-in" in Bayesian terms, chain had not become stationary) were discarded as a conservative measure to avoid the possibility of including random, sub-optimal trees. The percentage of samples recovering any particular clade in a BI analysis represents that clade's posterior probability (Huelsenbeck and Ronquist, 2001). We used one of the methods of Leaché and Reeder (2002) to assure that our analyses were not trapped on local optima. In particular, the posterior probabilities for individual clades obtained from separate analyses (4 runs) were compared for congruence (Huelsenbeck and Imennov, 2002), given the possibility that the analyses could appear to converge on the same ln-likelihood value while actually supporting incongruent phylogenetic trees.

3. Results

3.1. Establishment of optimal culture conditions

Although lymphocyte culture conditions are well established for humans and other mammals, optimal reptilian lymphocyte culture techniques vary greatly among species. Thus, we performed a series of pilot studies using splenocytes from two *Podarcis* species, *P. erhardii* and *P. taurica*, since a sufficient number of animals of the above populations were available in our laboratory. As shown in Fig. 2, when xenogeneic splenocytes were co-cultured at a ratio of 1:1 for 1 to 6 days in a 37 °C, 5% CO_2 incubator, two-way MLR responses gradually increased and optimal MLR values were obtained at 5 days upon culture initiation. Proliferative responses of the same cultures were reduced on day 6 and cell viability, as concomitantly determined by Trypan blue dye exclusion test, revealed that less than 70% of lymphocytes were viable at that time (Fig. 2). No differences in MLR responses in cultures set at 35 °C for 5 days were noticed (data not shown). Thus, culture temperature of 37 °C was constantly used thereafter, since it is in the range of the ambient temperature of free-living individuals belonging to the particular species (Pafilis, 2003; Adamopoulou and Valakos, 2005) and allows comparisons with the majority of available relevant studies performed with reptilian immune cells (Saad et al., 1984; Saad and El Deeb, 1990; Munoz et al., 2000; Munoz and De la Fuente, 2001; Burnham et al., 2003).

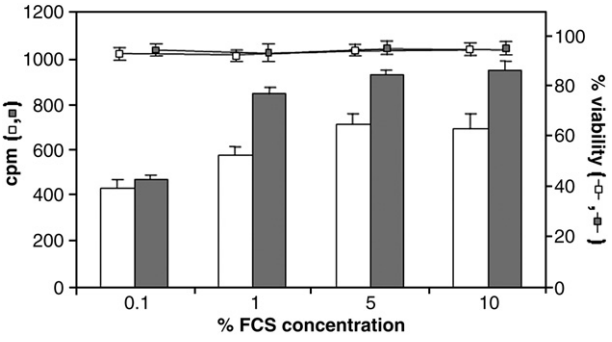

Fig. 3. Effect of serum concentration on *P. erhardii* and *P. taurica* spleen cell proliferation and viability. Splenocytes from individual animals (2×10^5/well) were cultured in RPMI-1640 medium supplemented with 0.1, 1, 5 or 10% FCS at 37 °C for 5 days. Cultures were pulsed for the last 18 h with 1 μCi/well [^{3}H]-thymidine and isotope incorporation is expressed as cpm±SD. Viability was assessed by Trypan blue dye exclusion and is expressed as % of viable cells±SD. Data shown are from 3 individual experiments performed with 3 *P. erhardii* and 3 *P. taurica* lizards. Bars represent cpm for *P. erhardii* (□) and *P. taurica* (■); solid lines represent % viability for *P. erhardii* (-□-) and *P. taurica* (-■-).

Table 1
Xenogeneic and allogeneic MLR-induced proliferation of *Podarcis* spleen cells

	P. erhardii	*P. peloponnesiaca*	*P. taurica*	*P. gaigeae*	*P. milensis*	*P. muralis*
P. erhardii ($n=5$)[a]	0.95±0.03[b]					
P. peloponnesiaca ($n=4$)	1.38±0.13	0.67±0.18				
P. taurica ($n=6$)	2.87±0.13	4.56±0.27	0.77±0.08			
P. gaigeae ($n=2$)	3.96±0.08	2.60±0.20	2.40±0.20	0.93±0.04		
P. milensis ($n=3$)	4.42±0.37	2.54±0.11	1.87±0.32	1.10±0.08	0.80±0.14	
P. muralis ($n=3$)	4.07±0.10	3.42±0.14	3.20±0.08	3.11±0.06	3.08±0.03	0.81±0.12

[a] *n*, total number of animals sacrificed.
[b] Mean S.I. values±SD from 4 individual experiments.

Optimal FCS concentration to supplement RPMI-1640 medium *in vitro* was determined by setting up 5-day cultures of each species' spleen cells individually. The FCS concentrations tested varied from 0.1% to 10%. Differences in the autochthonous proliferative ability of *P. erhardii* or *P. taurica* splenocytes were noticed with the increase of FCS concentration (Fig. 3). Although overall [^{3}H]-thymidine incorporation was lower in 0.1% FCS-supplemented cultures (*ca.* by 30–50% compared to 10% FCS), we noticed, using Trypan blue dye exclusion assay, that after 5 days in 0.1% FCS-supplemented medium *Podarcis* spleen cells presented high viability (over 95%). Thus, to eliminate any possible additional recognition of xenogeneic bovine proteins or peptides in two-way MLR, we selected 0.1% FCS as optimal supplement concentration for our experiments.

3.2. Lymphoproliferative response in two-way MLR

The number of splenocytes that could be obtained from a single spleen varied between 2.0 and 9.3×10^6 cells. In the former case, large-scale experiments could not be designed, and this resulted in an increase of the total number of lizards sacrificed. In total, splenocytes from 23 male adult lizards were tested, which were morphologically defined as *P. erhardii* (5 animals), *P. peloponnesiaca* (4 animals), *P. muralis* (3 animals), *P. milensis* (3 animals), *P. gaigeae* (2 animals) and *P. taurica* (6 animals).

As shown in Table 1 and Fig. 4, spleen lymphocytes from analyzed species, showed variable stimulation rates in response to xenogeneic splenocytes. MLR values were consistently found to decrease when lymphocytes from individual lizards, but of the same species (allogeneic MLR), were coincubated. In all cases, S.I. values determined from such combinations did not exceed 0.95. On the contrary, two-way MLR responses were higher when splenocytes from allopatric species were co-cultured (xenogeneic MLR). Moreover, some combinations showed significantly increased proliferative rates (*e.g. P. taurica–P. peloponnesiaca*; S.I. 4.56), whereas others showed marginal levels of stimulation (*e.g. P. gaigeae–P.*

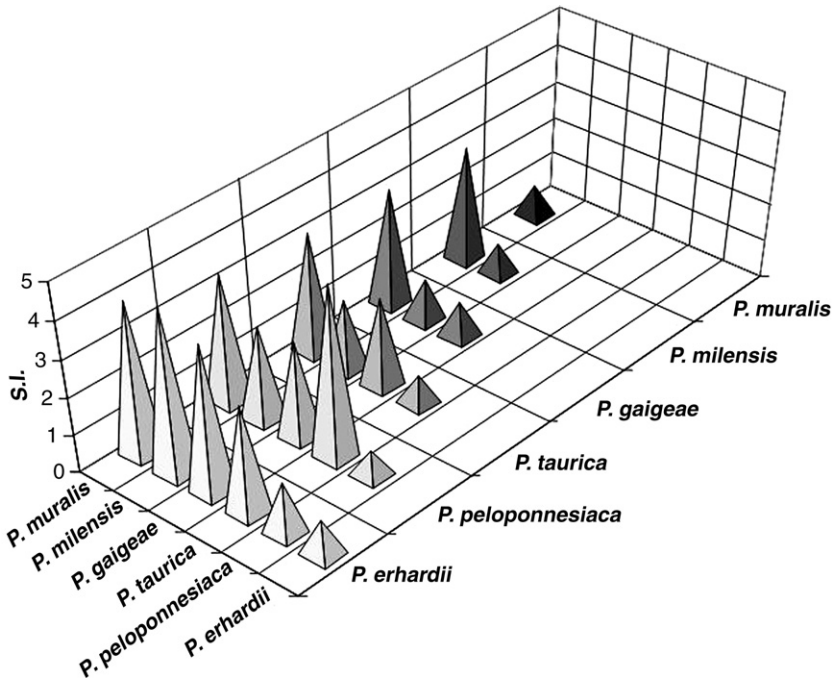

Fig. 4. Three-dimensional schematic representation of S.I. values of Table 1 showing the differences in xenogeneic MLR values among the *Podarcis* species tested. Cultures were set up by coincubating splenocytes from paired species at all combinations and results are presented as mean S.I. values from 4 individual experiments performed.

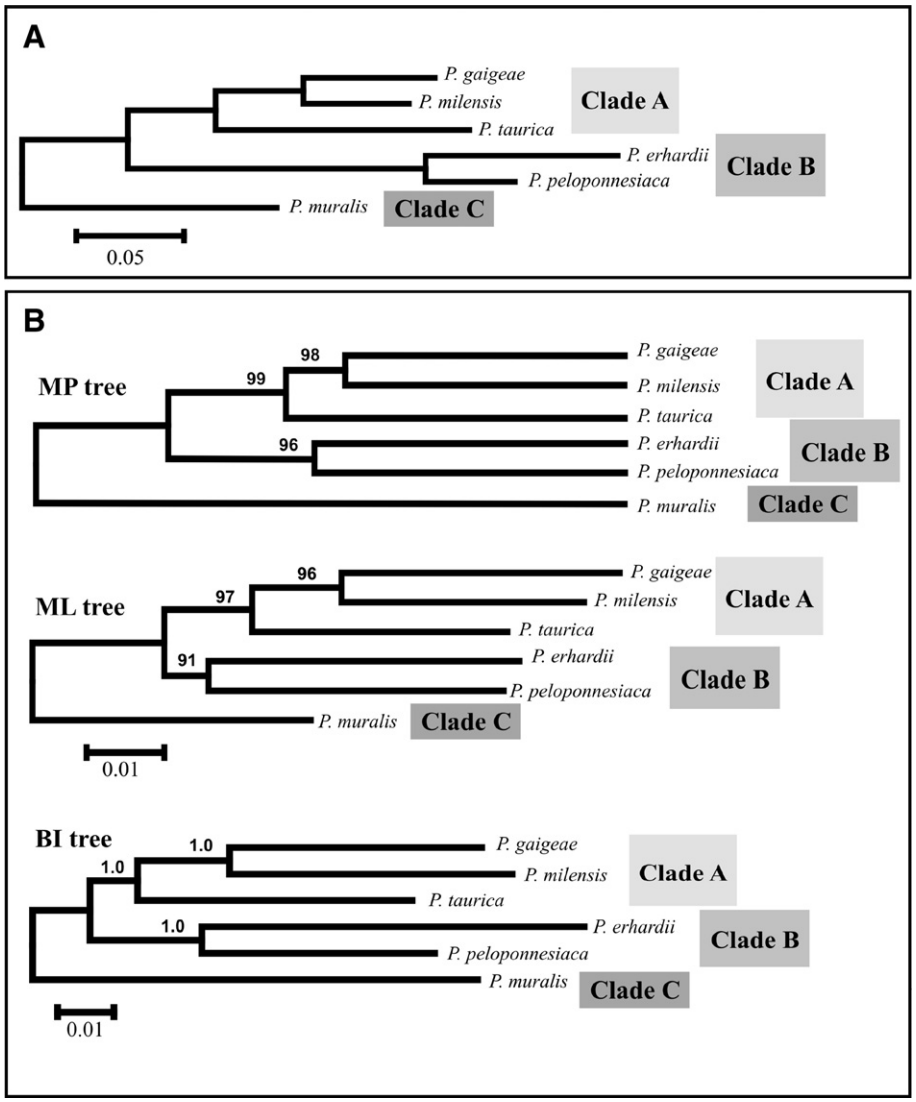

Fig. 5. A, Neighbour-joining tree of the six *Podarcis* species encountered in Greece, using the S.I. values as genetic distances among the species. B, Cyt *b* and 16S phylogeny of the same Greek *Podarcis* species. Phylogenetic analyses of maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) produced trees with the same topology. Numbers above branches are bootstrap values on MP and ML and posterior probabilities values of BI above 95%.

milensis; S.I. 1.10). Neighbour-joining analysis of all S.I. values calculated, generated tree A (Fig. 5A), on the basis of which three clades of *Podarcis* are identified. Clade A comprises *P. taurica*, *P. milensis*, and *P. gaigeae*, in which *P. gaigeae* and *P. milensis* form a closely related pair. Clade B consists of *P. erhardii* and *P. peloponnesiaca*, and clade C comprises *P. muralis*, and appears as a sister group of clades A and B.

3.3. Phylogenetic analysis based on partial mtDNA sequences

To infer the value of xenogeneic MLR responses in the phylogeny of the genus *Podarcis*, we further performed phylogenetic analysis based on partial mtDNA sequences. Of the 927 sites examined, there were 59 variable cyt *b* sites, of which 55 were parsimony informative and 41 variable 16S sites, 20 of which were parsimony informative. For cyt *b*, sequence divergence (Tamura and Nei+G model) ranged from 11.3 to 18.9%, while for 16S sequence divergence ranged between 3.2 and 7.1%. Tree length distribution, determined from random sampling of 10^6 unweighted trees, was significantly skewed to the left ($g_1 = -0.479$), suggesting a strong phylogenetic signal in the data ($p < 0.01$; Hillis and Huelsenbeck, 1992).

All analyses (MP, ML, and BI) were congruent regarding the branching order of the deeper clades. Equally weighted parsimony analysis of the 75 parsimony-informative characters produced one most parsimonious tree with a length of 248 steps (consistency index, CI = 0.79 and retention index, RI = 0.435). ML analysis under the TrN+G model resulted in a topology with lnL = −2402.267 (Fig. 5B). For the BI method, which is consistent with the likelihood one, identical topologies were recovered for each of the 4 runs with the full dataset, although posterior probabilities for some of the nodes differed slightly between each of the Bayesian runs. The mean −ln-likelihood of these trees was −2505.47. In all phylogenetic analyses of the partial mtDNA sequence, three very well supported clades of

Podarcis were identified (Fig. 5B) and these clades coincided well to the clades predicted on the basis of immunological (two-way MLR) data, as shown in the respective tree of Fig. 5A.

4. Discussion

Over the years various methodological approaches have been used to resolve Greek *Podarcis* phylogeny; however, reported results were conflicting and in some cases contradictory (Lanza and Cei, 1977; Mayer and Tiedemann, 1980; Tiedemann and Mayer, 1980; Mayer and Tiedemann, 1981; Olmo et al., 1986, 1987; Capula, 1994, 1996, 1997; Chondropoulos et al., 2000). Recent studies based on mtDNA sequences were able to resolve Greek *Podarcis* species phylogenetic relationships (Harris et al., 1998; Harris and Arnold, 1999; Poulakakis et al., 2005a,b). However, since mtDNA is maternally inherited and thus might reflect only female gene flow, any additional method to confirm the obtained results would be of importance (Wiens and Penkrot, 2002). Herein, data acquired from the analysis of cell-mediated immune responses of spleen-derived lizard lymphocytes from some *Podarcis* species were used to construct a phylogenetic tree, which is shown to fully corroborate with the tree, generated according to results inferred from mtDNA analysis.

For our study, six allopatric lizard species were selected on the basis of recent molecular studies (Harris and Arnold, 1999; Poulakakis et al., 2003, 2005a,b) showing that Greek species of *Podarcis* are divided into three subgroups, one containing *P. taurica*, *P. milensis*, and *P. gaigeae*, the second containing *P. erhardii* and *P. peloponnesiaca* and the third with *P. muralis*. Furthermore, the members of these groups are highly diversified, present great morphological and ecological plasticity, inhabiting many different ecotypes and thus were ideal for assessing the predictive validity of our immunologically-based phylogenetic approach.

In our experimental setting, we used *Podarcis* spleen cells, since, due to the limited size and number of the animals, withdrawal of sufficient amount of peripheral blood was not possible. Reptilian splenocytes, which consist of both, T and B cells as well as of phagocytic cells, have been widely used in assays aiming to the study of cell-mediated and humoral immune responses and, similarly to peripheral blood lymphocytes (PBL), can be satisfactorily activated *in vitro* under optimized culture conditions (Saad et al., 1984; Farag and El Ridi, 1985, 1986; Saad and El Deeb, 1990; Burnham et al., 2003; Munoz and De la Fuente, 2004). Moreover, spleen cells are speculated to be less influenced than PBL by serum-circulating hormonal factors (Saad et al., 1984; Belliure et al., 2004; Munoz et al., 2000) and to exhibit lower variability both, in number and activity according to seasonal changes (Burnham et al., 2003; Munoz and De la Fuente, 2004). To eliminate further hormonal influence in the detected immune responses, we selected to use only male animals, and therefore the differences observed in our study would not reflect any possibly occurring sex hormonal imbalance (Saad, 1989; Saad and El Deeb, 1990; Smith and John-Adler, 1999).

Prior to sacrifice and spleen cell isolation, lizards were acclimatized to laboratory conditions for at least 4 weeks. Since animals were collected in autumn, *i.e.*, during the non-reproductive period, this prolonged adaptation was important to eliminate seasonal changes, reported to significantly affect lymphocyte number and proliferation rate in lizards (Saad and El Ridi, 1984; El Masri et al., 1995; Smith and John-Adler, 1999), as well as in other reptilian taxa, as snakes (Farag and El Ridi, 1986), tuatara (Burnham et al., 2005) and turtles (Munoz et al., 2000).

Culture conditions selected for *in vitro* MLR studies included the determination of optimal (a) incubation temperature to sustain lymphocyte viability, (b) incubation period to allow maximal proliferation and (c) FCS concentration used for supplementing the culture medium. After a series of pilot experiments, maximal MLR responses were recorded when *Podarcis* splenocytes were maintained in *in vitro* culture at 37 °C in a 5% CO_2 incubator over an overall period of 5 days. These empirically established conditions for *Podarcis* cells are in agreement with previous reports showing that lizard *Chalcides ocellatus* or *Sceloporus occidentalis* spleen cells proliferate in MLR or in response to mitogenic stimuli in the range of 4–5 day incubation at 37 °C (Saad et al., 1984; Saad and El Ridi, 1984; Saad and El Deeb, 1990; Burnham et al., 2003).

The most appropriate culture supplement for reptilian lymphocytes has not yet been established. Although 1–10% autologous serum, whenever available, has successfully supplemented alligator (Cushens and Clem, 1979b), snake (Farag and El Ridi, 1985) and lizard (Saad and El Deeb, 1990) cell cultures, in the majority of previously performed reptilian lymphocyte proliferation assays, 5–10% FCS has been consistently used (Munoz and De la Fuente, 2001; Munoz et al., 2000; Burnham et al., 2003, 2005). In a unique report using blood-derived lymphocytes from turtles, the lipid-enriched bovine serum albumin replacement, Albumax-I, was also tested; however, compared to previous studies, an unexplained increase in S.I. values was recorded (Work et al., 2000). Herein, FCS was used as supplementary protein source in the culture medium, since sufficient quantities of homologous *Podarcis* serum were not available. Surprisingly, only 0.1% FCS medium supplementation was sufficient to sustain high *Podarcis* splenocyte viability (Fig. 3). In our system, this very low FCS content was considered advantageous and was further routinely used in our assays, since it eliminates batch-to-batch FCS variation that has often been reported to complicate cell proliferation assays in mammals (Swift et al., 1996) and, most importantly, minimizes the exposure of *Podarcis* splenocytes to additional xenogeneic protein or peptide molecules. Such stimuli, massively provided by higher than 0.1% FCS concentrations, could eventually mask or false positively modulate any immunoreactivity naturally occurring *via* T lymphocyte xenorecognition (xenogeneic antigenic or MHC determinants) among the *Podarcis* splenocyte populations tested. With respect to the latter, doubling the FCS concentration in the culture medium of turtle lymphocytes resulted in duplication of their mitotic index values (Ulsh et al., 2001). To our knowledge, this is the first report where minimum bovine serum supplementation is required for the potentiation of reptilian T cell proliferative responses *in vitro* and suggests that empirically established reptilian lymphocyte culture conditions should be carefully considered.

S.I. values acquired in our two-way MLR assays greatly varied between the paired *Podarcis* species tested. Surprisingly, in all separate experiments performed, allogeneic MLR S.I. values were below 1, whereas xenogeneic MLR S.I. values ranged from 1.10 to 4.56. Significant variability in the degree of MLR responses between reptiles belonging to the same species has been previously reported. In particular, two-way allogeneic MLR S.I. values of alligator blood lymphocytes ranged between 1 and 12.5 (Cushens and Clem, 1979a), whereas snake splenocytes yielded S.I. values from 1 to 20 (Farag and El Ridi, 1985) and the degree of lymphocyte responsiveness was highly dependent on seasonal conditions. To further explain this discrepancy in lymphocyte responses among members of the same snake species, Farag and El Ridi (1985) proposed that some individuals share most, if not all, MLR-stimulating determinants and thus responded weakly in MLR, whereas others possessed strong and diverse lymphocyte activating determinants that resulted in enhanced alloreactive T cell stimulation. However, since the sex of the animals in both studies was not reported, the possibility of sex-related determinants triggering allorecognition between male versus female alligator or snake T cells cannot be ruled out. This is further supported by the recent report of Burnham et al. (2005), who detected low levels of blood lymphocyte responsiveness in two-way MLR of two male tuatara, attributed to the activation of low frequency of T cells due to the possession of minor, if any, genetic differences in histocompatibility loci in members of the same reptilian species.

Due to (a) the use of solely male *Podarc*is lizards throughout the study, (b) the prolonged adaptation period of the animals under laboratory conditions and (c) the use of low FCS concentration minimizing artificial stimulation, the differences in MLR values should not be attributed to sex-, seasonal- or non-specific-related stimulation of responsive T cells. On the contrary, the S.I. differences we detected more likely represent differences in xenogeneic stimuli provided by each species and recognized by xenoreactive T cells or T cell-like populations present among *Podarcis* splenocytes.

To further investigate the use of the observed diversity in xenogeneic MLR S.I. values between lizards belonging to allopatric *Podarcis* species, we referred to available data on *Podarcis* phylogeny (Harris and Arnold 1999; Poulakakis et al., 2003, 2005a,b). Our comparisons revealed the existence of a correlation between the degree of spleen cell proliferation and the genetic distance between the *Podarcis* species tested, *i.e.*, phylogenetically related species (*e.g. P. gaigeae* and *P. milensis*) exhibited lower MLR responses than phylogenetically distant groups (*P. taurica* and *P. peloponnesiaca*). Based on these immune responses we constructed a phylogenetic tree, which was compared with the analogous tree generated on the basis of mtDNA analysis of the same *Podarcis* species. As shown in Fig. 5, the two trees are identical with respect to their predictive validity on *Podarcis* phylogeny.

The mechanisms underlying this correlation are not understood and are currently under investigation in our laboratory. However, if we assume that a mechanism similar to mammalian xenoreactive T cell stimulation occurs in *Podarcis* splenocyte MLR, we suggest that the differences in MLR responses between phylogenetically distant species are due to differences in xeno-determinants presented by phagocytic cells to T cells, originating from animals inhabiting different ecotypes and belonging to distinct, non-inbred populations. These determinants could be either antigenic peptides recognized by T cell receptors in conjunction with MHC molecules or xenogeneic MHC or MHC-like molecules. In the former case, since the six *Podarcis* species are allopatric, any variability in the microbial/parasitic load of their environment could result in the presentation of peptide epitopes recognized as foreign by T cells in the context of self- or xeno-MHC molecules.

The latter possibility however, seems more likely to occur. Taking into consideration that in mammals MLR reactivity is poorer in MHC homozygous animals than in animals that are heterozygous, we can suggest that phylogenetically related *Podarcis* species may share common MHC alleles, preferentially selected under the pressure of evolution and/or inherited in the siblings as a consequence of inbreeding, whereas phylogenetically distant species exhibit a genetically diverse MHC phenotype. Indeed, Olsson et al. (2005) recently reported that a species-specific MHC genotype correlated with resistance to parasites and success in mate acquisition is preferentially selected in male *Lacerta agilis* lizards.

The existence of some degree of polymorphism in the genetic loci encoding for MHC or MHC-like molecules was most recently reported for an evolutionary lower taxa of reptiles, the tuatara (Miller et al., 2005). Whether, similar to mammals, genetically diverse MHC molecules are presented by specific stimulator lizard cells, needs further investigation. Antigen-presenting cells, as monocytes, have been identified in blood cells of the lizard *Gallotia simonyi* (Martinez-Silvestre et al., 2005) and an adherent lymphocyte subpopulation among alligator blood lymphocytes and snake spleen cells has been isolated (Farag and El Ridi, 1985, 1986; Cushens and Clem, 1979a,b). However, it seems that the strength of MLR response does not solely depend on the presence of adherent cells since cell fractions deprived of adherent cells can also stimulate sufficient MLR responses (Farag and El Ridi, 1985, 1986; Cushens and Clem, 1979a,b). Thus, the exact participation of reptilian stimulator cells in triggering MLR responses is confusing. We assume that this vagueness will be clarified soon and the general lack of corresponding literature will be compensated.

Altogether, this is a strong indication that cell-mediated immune responses induced by two-way MLR in lizards can be used as an additive research tool for the elucidation of the phylogeny within *Podarcis* species. Furthermore, the combination of both, molecular and immunological characteristics in phylogenetic studies can shape a more accurate perception for the evolution pattern of specific, if not all, lizard families. Complementary research pathways could reveal, when integrated, a solid basis for understanding the interdisciplinary character of phylogenetic history.

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