MONOPHYLY OF THE FAMILY PROTEIDAE (AMPHIBIA: CAUDATA) TESTED BY PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL 12S rDNA SEQUENCES

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A phylogenetic analysis of partial 12S rRNA mitochondrial gene sequences from representatives of salamandroid families provided moderate support for the monophyly of the family Proteidae, comprising the North American genus *Necturus* and the European blind cave salamander *Proteus*.

Key words: *Proteus anguinus*, mitochondrial DNA, phylogeny, salamander

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Filogenetska analiza djelomičnih mitohondrijskih genskih sekvenci 12S rRNA predstavnika salamandroidnih porodica umjereno podržava monofiletičnost porodice Proteidae, obuhvaćajući sjevernoamerički rod *Necturus* i europski rod špiljske čovječje ribice *Proteus*.

Ključne riječi: *Proteus anguinus*, mitohondrijalna DNA, filogenija, repaši

INTRODUCTION

The salamander family Proteidae in its current conception comprises two extant genera of permanently aquatic, paedomorphic salamanders: *Proteus* and *Necturus*. The sole recognized species of *Proteus, P. anguinus* Laurenti, 1768, is a specialized and obligate inhabitant of underground waters in the Dinaric Karst of the western Balkan Peninsula. The species is rarely found outside its permanently lightless habitat. Its peculiar morphology, including the reduction of eyes, lack of skin pigmentation, and elongated skull, is partly the result of adaptation to the subterranean environment (PARZEFALL *et al.*, 1999). There are five currently recognized species of *Necturus* (GUTTMAN *et al.*, 1990). They inhabit various types of surface waters throughout eastern

North America. They are active during the night, but are by no means specialized like *Proteus*.

The placement of both genera into the family Proteidae by NOBLE (1931) was based mainly on common paedomorphic characteristics, which can also be found in other neotenic salamanders. HECHT (1957) therefore argued that the perennibranchiate state, caudal fin, and other larval-like characters shared by adult individuals of *Proteus* and *Necturus* are the result of parallel evolution and not an indicator of common ancestry. KEZER *et al.* (1965) demonstrated that both genera have 19 chromosomes in the haploid state, a chromosome number that is unique among urodeles. Since then the monophyly of the Proteidae has become a major dispute in the systematics of urodeles. It has been favored, for example, by BRANDON (1969), LARSEN & GUTHRIE (1974), ESTES (1981) and DUELLMAN & TRUEB (1986), but challenged by HECHT & EDWARDS (1976). Present-day authors remain cautious about the phylogenetic validity of the family (LARSON, 1996; PARZEFALL *et al.*, 1999).

The genus *Proteus* appears to be taxonomically far less diverse than *Necturus* (GRILLITSCH & TIEDEMANN, 1994; SKET & ARNTZEN, 1994). On the other hand, the apparently uniform morphology of geographically remote and isolated populations may have been acquired convergently as a consequence of adaptation to nearly identical subterranean environments. On the basis of allozyme divergence data and biogeographic analyses, SKET (1997) suggested that some populations might actually belong to distinct races or species.

MATERIALS AND METHODS

In an attempt to investigate the degree of divergence and the relationships between *Proteus* populations, we examined the sequences of several mitochondrial genes, among others the 12S rRNA gene. Sequences of this gene have frequently been employed to resolve phylogenetic relationships among salamanders (e. g. TITUS & LARSON, 1995; VEITH *et al.*, 1998; ZAJC & ARNTZEN, 1999). This provided an opportunity to test the hypothesis of proteid monophyly by a phylogenetic analysis of DNA sequences. In this note we present the first molecular-based indication of a sister relationship between *Proteus* and *Necturus*.

Individuals of *Proteus anguinus* were collected in the caves Planinska jama (Postojna, Slovenia) and Kompoljska jama (Grosuplje, Slovenia) in 1998 and in the karst spring of Vedrine (Trilj, Croatia) in 1997. Muscle tissue from the tail was preserved in 96% ethanol at –20°C. Genomic DNA was extracted from muscle tissue using the QIAamp Blood Kit (Qiagen). For PCR amplification of partial 12S rRNA gene sequences, the L-chain primer 5'-AAAACIIGGATTAGATACCC-3' and the H-chain primer 5'-GAGAGTGACGGGCGATGTGT-3' (TRONTELJ & SKET, 2000) were used. PCR was performed by applying 30 cycles of 45 s at 94°C, 45 s at 50°C, and 60 s at 72°C. PCR products were gel-purified and sequenced on an ABI Prism 310 automated sequencer using the Rhodamine Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

12S rRNA gene sequences of other species representing extant salamandroid families were obtained from GenBank under the following accession numbers: X86224

– *Amphiuma tridactylum* (Amphiumidae), AF290183 – *Desmognathus fuscus* and X86248 – *Plethodon yonahlossee* (Plethodontidae), X86251 – *Rhyacotriton olympicus* (Rhyacotritonidae), U04702 – *Triturus carnifex*, U04704 – *Triturus vulgaris* and U04694 – *Euproctus asper* (Salamandridae), X86234 – *Dicamptodon ensatus* (Dicamptodontidae), X86223 – *Ambystoma mexicanum* (Ambystomatidae), X86249 – *Necturus lewisi* (Proteidae). The sequence of *Necturus maculosus* (Proteidae) was taken from TITUS & LARSON (1995).

The multiple sequence alignment for the phylogenetic analysis was produced through a procedure that eliminates regions of unstable and thus ambiguous alignment under a range of alignment parameters. The procedure is implemented in the computer program SOAP (LÖYTYNOJA & MILINKOVITCH, 2001). Gap penalties from 12 to 16 (step 1) and gap extension penalties from 6 to 8 (step 0.5) were applied to produce 25 multiple sequence alignments. Only those positions that were aligned consistently across all 25 alignments were used for further analysis.

Phylogenetic analyses were performed under the parsimony, maximum likelihood (ML) and minimum evolution (ME) optimality criteria employing PAUP*4.0 (SWOFFORD, 1999). The most parsimonious trees were searched for under the heuristic search option with closest stepwise addition and tree-bisection-reconnection branch swapping. Indels were handled as missing data. ML searches under the same heuristic search options incorporated the HKY 85 model of evolution (HASEGAWA *et al.*, 1985), and the model parameters were estimated via ML. The pairwise distances for the ME search were estimated according to the same model of evolution as used in ML. The branch support for the ML tree was assessed using the program TREE-PUZZLE 5.0 (STRIMMER & VON HAESELER, 1996). The most parsimonious tree was compared against the shortest tree that did not include a monophyletic Proteidae member (obtained by reversing the constraint option in PAUP). The difference in tree length was tested for statistical significance using the Kishino-Hasegawa-Templeton test as implemented in Phylip (FELSENSTEIN, 2001).

RESULTS AND DISCUSSION

Three newly obtained 12S rRNA gene sequences (GenBank Accession numbers: AY221119, AY221120, and AY221121) of *Proteus anguinus* from hydrographically isolated populations measured 368 bp in length, excluding amplification primer binding sites. 34 nucleotide sites with unstable alignment were removed from the original sequences and omitted from further analyses, leaving 111 parsimony informative sites. The alignment can be obtained from the first author.

Under all three optimality criteria, the three representatives of *Proteus* clustered together with both *Necturus* species. On a strict consensus tree of all four most parsimonious trees, as well as on the ML and the ME tree, two other families, the Salamandridae and the Plethodontidae, were supported as monophyla. The relationships among families remained unresolved (Fig. 1). However, the goal of this short note was not to study the phylogenetic relationships of salamander families in detail, but rather to present some new evidence for the *Proteus-Necturus* sister re-
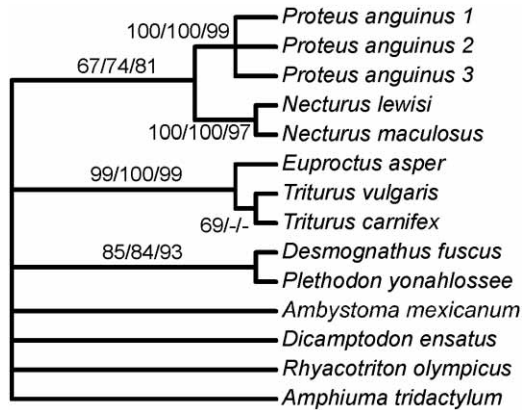
Fig. 1. Strict consensus of four most parsimonious trees from salamander 12S rDNA sequences. Numbers on branches are (in the respective order) per cent bootstrap support values obtained by 500 resamplings applying parsimony and minimum evolution, and branch support values obtained by 1000 puzzling steps applying maximum likelihood. Only values higher than 50% are shown.

lation hypothesis. The bootstrap and branch support values of the proteid clade were slightly above or below 70%. The more rigorous Kishino-Hasegawa-Templeton test showed that the shortest tree not supporting the proteid clade was not significantly worse. However, the support for the monophyly of the proteids increased substantially when certain outgroup taxa were excluded (Tab. 1).

It is known that the choice of the outgroup may affect the topology of the ingroup (GIRIBET & RIBERA, 1998; MESSENGER & McGUIRE, 1998; MILINKOVITCH & LYONS-WEILER, 1998). Therefore, we took the phylogeny estimate of salamander families presented by LARSON & DIMMICK (1993), and successively deleted taxa, starting with the outermost branch (Amphiumidae + Plethodontidae). For each new combination of outgroup taxa we assessed the support of the *Necturus + Proteus* clade. Of course, by reducing the taxonomic inclusiveness one might also exclude taxa whose relationships to *Necturus* or *Proteus* might potentially disrupt the monophyly of proteids. Such exclusion would probably lead to an abrupt increase of proteid support between the last taxa combination including the disruptive taxon, and the first combination without it. However, the bootstrap and branch support changed rather gradually from one tree to another (Tab. 1). When only salamandrids, dicamptodontids and ambystomatids were included, the difference in tree length between the most parsimonious tree and the shortest tree that did not include monophyletic Proteidae became significant. These observations suggest that the relatively low support for proteid monophyly in the complete assortment of salamandroid families results from an increase of phylogenetic noise caused by phylogenetically more distant taxa. SANDERSON & WOJCIECHOWSKI (2000), for example, noted that a decline in bootstrap support with increased taxon sampling appears to be a general tendency of molecular phylogenies.

Tab. 1. Support for the monophyly of the family Proteidae (*Proteus + Necturus*) with different outgroup combinations. Successive, less inclusive outgroups were selected according to the phylogenetic hypothesis of LARSON & DIMMICK (1993). Measures of support are: length of most parsimonious tree vs. length of shortest tree without Proteidae (MP vs. WP), bootstrap values (500 replicates) for the Parsimony and minimum-evolution (ME) optimality criterion, and branch support values (1000 puzzling steps) for the maximum likelihood (ML) optimality criterion.

Outgroup	MP vs. WP (steps)	Parsimony bootstrap (%)	ME bootstrap (%)	ML branch support (%)
Amph, Pleth1, Pleth2, Rhya, Sal1, Sal2, Sal3, Dicam, Amb[a]	333 vs. 337	67	74	81
Amph, Rhya, Sal1, Sal2, Sal3, Dicam, Amb	261 vs. 265	72	82	98
Pleth1, Pleth2, Rhya, Sal1, Sal2, Sal3, Dicam, Amb	306 vs. 309	64	81	79
Pleth2, Rhya, Sal1, Sal2, Sal3, Dicam, Amb	268 vs. 271	65	67	81
Rhya, Sal1, Sal2, Sal3, Dicam, Amb	234 vs. 237	75	94	97
Sal1, Sal2, Sal3, Dicam, Amb	203 vs. 208*	85	94	94
Sal2, Sal3, Dicam, Amb	187 vs. 191*	81	91	93

[a] Amph=*Amphiuma tridactylum* (Amphiumidae), Pleth1=*Desmognathus fuscus* (Plethodontidae), Pleth2=*Plethodon yonahlossee* (Plethodontidae), Rhya=*Rhyacotriton olympicus* (Rhyacotritonidae), Sal1=*Triturus carnifex* (Salamandridae), Sal2=*Triturus vulgaris* (Salamandridae), Sal3=*Euproctus asper* (Salamandridae), Dicam=*Dicamptodon ensatus* (Dicamptodontidae), Amb=*Ambystoma mexicanum* (Ambystomatidae)

* Shortest tree without monophyletic Proteidae is significantly worse (p < 0.05) than most parsimonious tree according to Kishino-Hasegawa-Templeton test as implemented in Phylip (FELSENSTEIN, 2001).

To summarize the results, the phylogenetic information from the analyzed portion of the 12S rRNA gene is consistent with a monophyletic Proteidae family. Nevertheless, the examined sequences are shorter than sequences usually used in contemporary phylogenetic studies. We thus present these results as a first molecular contribution to solve a lasting dispute in the systematics of salamanders. They will have to be corroborated by analyzing additional mitochondrial and nuclear DNA sequences.

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S A Ž E T A K

Monofiletičnost porodice Proteidae (Amphibia: Caudata) testirana filogenetskom analizom sekvenci mitohondrijalne 12s rDNA

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O monofiletičnosti porodice Proteidae raspravlja se gotovo pola stoljeća. Glavni prigovor sestrinskoj vezi europskog roda *Proteus* i sjevernoameričkog roda *Necturus* jest da su njihove pretpostavljene sinapomorfije zapravo pedomorfne osobine i mogu biti rezultat paralelne evolucije. U ovom radu djelomične sekvence mitohondrijskog gena 12S rRNA dobivene su za primjerke roda *Proteus* iz tri populacije, obuhvaćajući pritom većinu njegovog areala. Daljnja filogenetska analiza uključivala je sekvence *Necturus lewisi* i *N. maculosus* kao i sekvence devet drugih predstavnika ostalih porodica repaša. Skupina *Necturus* + *Proteus* je provjerena putem različitih vanjskih grupa i potvrđena sa svim vanjskim grupama i korištenim metodama (parsimon. analiza – *parsimony*, *maximum likelihood*, metoda minimalne evolucije – *minimum evolution*). *Bootstrap* potpora iznosila je od 67% prilikom korištenja svih skupina do 94% pri isključivanju nekih skupina. S dvije vanjske grupe, stablo koje podržava monofiletičnost (= *the most parsimonious tree*) bilo je značajno uvjerljivije nego najkraće stablo bez monofiletičnih Proteidae (p<0.05) prema Kishino-Hasegawa-Templeton testu. Ova opažanja sugeriraju da je relativno slabo podržana monofiletičnost proteida u kompletnom poretku salamandroidnih porodica bila uzrokovana povećanim filogenetičkim šumom čiji izvor su bile filogenetski udaljenije svojte. Tako su filogenetske informacije iz analiziranih dijelova gena 12S rRNA u suglasnosti s monofiletičkom porodicom Proteidae, iako su istraživane sekvence kratke i potrebne su dodatne sekvence kojima bi se procijenila ova trajna rasprava u sistematici repaša.

SUMMARY

Monophyly of the family Proteidae (Amphibia: Caudata) tested by phylogenetic analysis of mitochondrial 12s rDNA sequences

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The monophyly of the salamander family Proteidae has been under debate for nearly half a century. The main objection to a sister relation of the European genus *Proteus* and the North American *Necturus* states that their putative synapomorphies are in fact paedomorphic characteristics and may be the result of parallel evolution. In this study, partial sequences of the mitochondrial 12S rRNA gene were obtained for *Proteus* specimens from three populations spanning most of its geographic range. The phylogenetic analysis further included sequences from *Necturus lewisi* and *N. maculosus* as well as sequences from nine other representatives of extant salamander families. The support for the *Necturus + Proteus* clade was assessed under different outgroup combinations. The clade was supported with all outgroups and methods (parsimony, maximum likelihood, minimum evolution) used, the bootstrap support ranging from 67% using all taxa to 94% when certain taxa were excluded. With two outgroup combinations, the tree supporting proteid monophyly (= the most parsimonious tree) was significantly better than the shortest tree without monophyletic Proteidae ($p < 0.05$) according to Kishino-Hasegawa-Templeton test. These observations suggest that the relatively low support for proteid monophyly in the complete assortment of salamandroid families was caused by an increase of phylogenetic noise from phylogenetically more distant taxa. Thus, the phylogenetic information from the analyzed portion of the 12S rRNA gene is consistent with a monophyletic Proteidae family, although the examined sequences are short and additional sequences are needed to further evaluate this lasting dispute in the systematics of salamanders.