CARYOLOGIA Vol. 54, no. 1:97-101,2001

Molecular analysis of Salamander family: the new alpine salamander *Salamandra atra aurorae*

AURORA PEDERZOLI*, PAOLA AZZONI and MAURO MANDRIOLI

Dipartimento di Biologia Animale, Universita degli Studi di Modena e Reggio Emilia, via Campi 213/d, 41100 Modena, Italy.

Abstract — A portion of the mitochondrial DNA from the 3' terminal of gene 12S rRNA to the 5' extremity of 16S rRNA gene has been amplified by PCR from *Salamandra atra aurorae* genomic DNA in order to define the taxonomic position of *S. atra aurorae* within the *Salamandra* species. Analysis of the molecular data indicated that *S. a. aurorae* is quite different from *S. salamandra,* whereas *S. a. aurorae* and *S. a. atra* are more similar, although their mitochondrial DNA present several mutations. Our analysis suggested therefore that *S. a. aurorae* could really represent a new subspecies of alpine salamander. Finally, on the basis of the rate of sequence divergence, we could suggest that *S. a. atra* and *S. a. aurorae* diverged about three million years ago.

Key words: mitochondrial DNA, phylogeny, Salamandra, taxonomy.

INTRODUCTION

Until 1969, *Salamandra atra* was considered to be a monotypic, melanic, urodelan species occurring between 450 and 3000m in the alpine regions of central Europe and into the western Balkans. In 1969, Miksic described the Dynaric form as a new subspecies, *Salamandra atra prenjensis,* characterized by a brownish colour, but a review of morphological characters has suggested that this distinction may not be valid (KLEWEN 1986).

In 1981, TREVISAN *et al.* described a new form of alpine salamander, collected in north east Italy near Asiago. It was characterized by straw-yellow markings on the head extending down the body in a broad fairly continuous dorsal band. Spots are always present on the proximal limbs and often on the tail.

From the morphological and embryological characteristics and in view of the electrophoretic patterns of haemoglobins and LDH isoenzymes, this taxon was interpreted as a new subspecies of *Salamandra atra* and named *Salamandra atra aurorae* (TREVISAN 1982). The Author hypothesized that *S. a. aurorae* originated from a mutation that had arisen and become established somewhere on the Asiago plateau after the last glacial period (TREVISAN 1982).

TREVISAN *et al.* (1984) analyzed the electrophoretic patterns of serum proteins of *S. a. atra* and *S. a. aurorae* on cellulose acetate strips observing some differences in albumins and a-and (3-globulins bands. JOGER (1986) applied serum protein PAG electrophoresis to *S. a. atra* and *S. a. aurorae* and observed that *S. a. aurorae* differs from *S. a. atra* "in such significant electrophoretic characters that it may represent a third species within the genus *Salamandra".*

In order to provide a more definitive study of the taxonomic status of *S. a. aurorae,* a molecular analysis on *S. atra aurorae* mitochondrial DNA should be performed.

Phylogenetic analysis of DNA has become in fact an important tool for studying the evolutionary history of organisms at all the taxonomic levels. Such an approach could be particularly useful in urodels, where the extensive homoplasy in morphological characters and the limited number of morphological markers available make difficult the reconstruction of phylogenetic tree (LARSON 1991; WAKE 1991; LAR-

* Corresponding author: fax ++39-(0)59-2055548 -E-mail: pederzoli.aurora@unimo.it.

```
CGTCAGGTCAAGGTGTAGCAGATAAGGTGGTAAGAAATGGGCTACATTTTCTAACTTAGA
AAACACGGATGATTCTATGAAATAGTTTCTGAAGGTGGATTTAGCAGTAAAAAGAAACAA
GAATGTTCTTTTTAAGACGGCAATTAAGCGCGCACACACCGCCCGTCTCCGTCTTCAAAT
AATTTAATCCTTAAATAAATACAACCAACAAGTAAGAAGAGGCAAGTCGTAACATGGTAA
GTCTACCGGCAGGTGAACTTGGAATATCAACTCGTAGCTTAATTAAAGCATCTTGCTTAC
ACCAAGAAAATACCCGTTAAACCCGACTCAAGTAGAGTTATAATTCTAGCCACCCCACAA
CAACAATTCTATTAACCATATAAATAAATCGTAAGGGCGACAGAAAACCT
```

Figure 1 — Nucleotide sequence of the portion of the mitochondrial DNA from the 3' terminal of gene encoding for 12S rRNA to the 5' extremity of 16S rDNA gene from *S. a. aurome.*

SON and CHIPPINDALE 1993; TITUS and LARSON 1995; VEITH *etal.* 1998). The combined analysis of molecular and morphological data can be therefore very useful in investigating the inter-generic relationship in salamandrids (LARSON 1994; VEITH *et al.* 1998).

In order to evaluate the monophyly of the Salamandridae and to develop a phylogenetic hypothesis for the major salamandrid lineages, TITUS and LARSON (1995) begun a molecular analysis of salamandrids on the basis of mitochondrial DNA sequences.

However, the wide amount of data obtained by TITUS and LARSON (1995) leave open the question regarding the relationship between the different *Salamandra* species. To solve this question, VEITH *et al.* (1998) provided several data coming both from morphological and molecular analysis but they left unresolved the taxonomic status of *S. a. aurorae.*

In this paper, we study the sequence variation in a mitochondrial DNA fragment from the 3' terminal end of 12S rRNA to the 5' extremity of 16S rRNA gene. Sequence data obtained allowed us to evaluate the taxonomic position of *S. atra aurorae* within the *Salamandra* species.

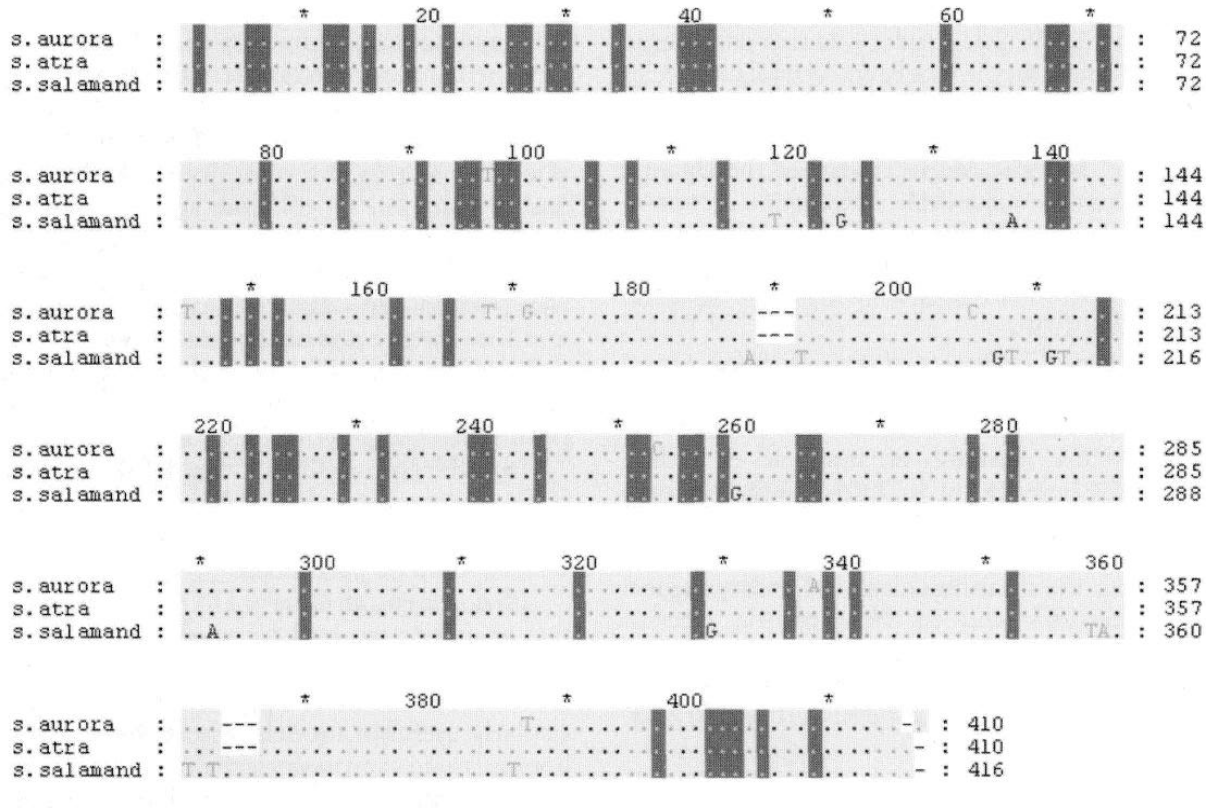

Figure 2 — Schematic representation of the alignment between the three Salamander sequences evidencing the presence of several mutations.

Figura 3 — The mitochondrial sequence amplified by PCR in *S. a. aurorae* has been aligned with the same sequences from *S. s. salamandra* and *S. a. atra*. Sequence alignment shows that, despite that this mitochondrial region is highly conserved, sequence differences can be evidenced, including two trinucleotide deletions.

MATERIALS AND METHODS

Three different salamanders were used in our study. Three adult specimens of *S. a. aurorae* were caught in "terra typica" near Asiago (Vicenza) at heights between 1440-1600m.

DNA extraction was performed from tail tissues as described by HILLIS *et al.* (1990). PCR amplification was carried out using two primers F (5'-GTCAGGTCAAGGTGTAGCAAT) and R (5'-AGGTTTTCTGTCGCCCTTAC) selected according to the primers described by TITUS and LARSON (1995). The amplification mix contained 100 ng genomic, 1 μM of each primer, 200 μM dNTPs and 2U of DyNAZyme II polymerase (Finnzymes Oy, Finland). Amplification was performed with a thermo-cycler Hybaid OMN-E at an annealing temperature of 60°C for 1 min and making extension at 72°C for 1 min. The amplified fragments were cloned using the "PCR Blunt Cloning Kit" (Roche) according to Roche protocols and sequenced with an automatic ABI sequencer (Perkin-Helmer) at "Servizio di Sequenziamento ENEA-Biogen (Casaccia, Italia). Sequence analysis and alignments were performed using the GCG package (Genetics Computer Group, Winsconsin, USA).

RESULTS AND DISCUSSION

In order to investigate the taxonomic status of *S. a. aurorae* we coupled a molecular analysis of the mitochondrial DNA sequences by PCR

to the morphological analysis previously performed (TREVISAN *et al.* 1981; TREVISAN 1982).

PCR amplification of the portion of the mitochondrial DNA from the 3' terminal of gene encoding for 12S rRNA to the 5' extremity of 16S rDNA gene from *S. a. aurorae* genome provided a 416 bp DNA fragment. This result agrees with the sequence data available in other urodel species (TITUS and LARSON 1995). The amplified fragments have been cloned and sequenced in both directions to check putative mutations (Figure 1).

S. a. aurorae sequence was aligned with sequences from *S. s. salamandra* and *S, a. atra* available in literature. Sequence alignment showed that, despite the fact that mitochondrial DNA is highly conserved, differences between the taxa can be assessed (Figure 2). In particular pair-wise divergence between *S. a. aurorae* and *S. a. atra* is 2.2%, whereas the divergence between *S. a, aurorae* sequence and *S. s. salamandra* is 5.6%. Moreover the alignment showed two deletions of a trinucleotide in *S. a. aurorae* and *S. a. atra* sequence in comparison to *S. s. salamandra* (Figure 3).

Our results showed therefore the presence of clear sequence differences not only between the *atra* group and *S. s, salamandra* but also within the *atra* group.

The analysis of the molecular data strongly confirmed that *S. a. aurorae* is quite different from *S. salamandra,* whereas *S. a. aurorae* and *S. a. atra* are more similar, although they present several mutations in the sequenced portion of the mithocondrial DNA. Our analysis, therefore, reinforces the data previously reported (TREVISAN *et al.* 1981; TREVISAN 1982) and suggests that *S. a. aurorae* could really represent a new subspecies of alpine salamander, as previously suggested (TREVISAN 1982), and not merely an aberrant population of the species *S. atra.*

On the basis of the sequence analysis on 16S mitochondrial DNA, it has been estimated a rate of sequence divergence of 0.7 % per million years when counting both transition and trans-version (VEITH *et al.* 1998). On the basis of such molecular clock we could estimate that the first divergence between *S. salamandra* and the S. a*tra* species took place about five million years ago. These data agree with results reported by VEITH *et al.* (1998) suggesting that the major divergence within the genus *Salamandra* took place between five and ten million years ago. On the basis of this hypothesis it could be suggested that a first split within such genus could have separated the species more or less simultaneously into Iberian, Central and Eastern Mediterranean population about 10 million years ago, during the final structuring of the Alps and the Neo-Pyrenees (DECOURT *et al.* 1986; VEITH *et al* 1998).

On the basis of the rate of sequence divergence reported by VEITH *et al.* (1998) we could suggest that *S. a. atra* and *S. a. aurorae* diverged about three millions years ago as already suggested (TREVISAN 1982).

In order to go in depth into the taxonomic status of *S. a. aurorae* and to confirm the preliminary data here reported, we are planning to extend the molecular analysis using nuclear genes such as genes coding for 18S and 28S rRNA.

REFERENCES

DECOURT J., ZONENSHAIN L.P., Ricou L.-E., ZAKMIN V.G., LE PICHON X., KNIPPER **A.L.**, **GRAND**-JACQUET C., SBORTSHOKOV I.M., GEYSSANT L., LEPVRIER C., PECHERSKY D.H., BOULIN J., SIBUET J.-C., SAVOSTIN L.A., SOROKHTIN O., WESTPHAL M., BAZHENOV M.L., LAUER J.P. and Biju-DuvAL B., 1986 — *Geological evolution of the Tethys belt from the Atlantic to the Pamirs since the Lias.* Tectonophysics, 123:241-315.

JOGER U., 1986 — *Serumproteinelektrophoretische Daten zur Frage der Validitat der Unterarten des Alpensalamanders Salamandra atra Laurenti, 1768.* Salamandra, 22:218-220.

HILLIS D.M., LARSON A., DAVIS S.K., and ZIMMER E.A., 1990 — *Nucleic acid sequencing.* In: D.M. Hillis and C. Moritz (Eds) "Molecular systematics", pp. 318-370. Sinauer, Sunderland, Massachusetts.

KLEWEN R., 1986 — *Untersuchungen zur Verbreitung, Oeko-Ethologie und innerartlichen Gliederung von Salamandra altra* Laurenti 1768. Diss. Mathemat.-Naturwissensch. Fakultat Univ. Koln, 185 p.

LARSON A., 1991 — *A molecular perspective on the evolutionary relationships of the salamander families.EvoLKiol.,25:211-211.*

—, 1994 — *The comparison of morphological and molecular data in phylogenetic systematics.* In: B. Schierwater, B. Streit, G.P. Wagner, and R. DeSalle (Eds) "Molecular ecology and evolution.

Approaches and applications", pp. 371-390. Birkhauser Verlag, Basel, Switzerland.

LARSON A. and CHIPPINDALE P., 1993 — *Molecular approaches to the evolutionary biology of plethodonidsalamanders.* Herpetologica, 49:204-215.

MiKSic S., 1969 — *A new subspecies of the Alpine Salamander (Salamandra atra prenjensis* nov.). Glasnik Zemaljskog muzeja, 8:93-96.

TITUS T. A. and LARSON A., 1995 — *A molecular phylogenetic perspective on the evolutionary radiation of the salamander family Salamandridae.* Syst. Biol., 44:125-151.

TREVISAN P., 1982. — *A new subspecies of alpine salamander.* Boll. Zool., 49:235-39.

TREVISAN P., PEDERZOLI A. and AGRUSTA M.A., 1984 — *Primi risultati di uno studio elettroforetico sulle sieroproteine in Salamandra atra.* Boll. Zool., 51 sup.: 107.

TREVISAN P., PEDERZOLI A. and CALLEGARINI C., 1981 — *A new form of alpine salamander.* Boll. Zool., 48:77-82.

VEITH M., STEINFARTZ S., ZARDOYA R., SEITZ A. and MEYER A., 1998 — *A molecular phylogeny of 'true' salamanders (family Salamandridae) and the evolution of terrestrially of reproductive modes. J.* Zool. Syst. Evol. Res., 36:7-16.

WAKE D. B., 1991 — *Homoplasy: The result of natural selection, or evidence of design limitations?* Am. Nat., 138:543-567.

Received 17 January 2001; accepted 27 February 2001