Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular Phylogenetics and Evolution 31 (2004) 910–914
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Genetic variation in an endemic salamander, *Salamandra atra*, using amplified fragment length polymorphism

Alexandre Riberon,[a,b,*] Claude Miaud,[b] R. Guyetant,[b] and P. Taberlet[a]

[a] *Laboratoire de Biologie des populations d'Altitude, UMR CNRS 5553, Université J. Fourier, BP 53, 38041 Grenoble Cedex 09, France*
[b] *Laboratoire de Biologie des populations d'Altitude, UMR CNRS 5553, Université de Savoie, CISM, 73376 Le Bourget du Lac, France*

Received 4 February 2003; revised 14 October 2003

Abstract

The pattern of genetic differentiation of the endemic alpine salamander, *Salamandra atra*, has been studied using amplified fragment length polymorphism (AFLP) from 11 populations throughout the range of the two currently recognized subspecies, *atra* and *aurorae*. Five different primer combinations produced 706 bands and were analyzed by constructing a phylogenetic tree using NJ and principal component analysis. Significant genetic variation was revealed by AFLP between and within populations but, our results show a lack of genetic structure. AFLP markers seems to be unsuitable to investigate complex and recent diversification.

1. Introduction

In the last decade, studies of the genetic structure of many species have been remarkably productive and successful because of the development of DNA markers. The most common genetic marker used to assess the intra- and inter-population relations is the mitochondrial (mt) DNA (Avise, 2000). But, mtDNA has inherent limitations. First, mtDNA represents only a single locus and reflects only the matrilineal history. Second, the effective population size of mtDNA is only a fourth of that of nuclear autosomal marker therefore, genetic diversity can be underestimated by phenomena such as bottleneck or lineage sorting. Fortunately, several markers are available to assay variation across the whole genome. The amplified fragment length polymorphism (AFLP) is a PCR-based technique that involves restriction of genomic DNA, followed by ligation of adapters to the fragments generated and selective PCR amplification of a subset of these fragments (Vos et al., 1995). The AFLP technique, being straightforward (especially with fluorescent labels) and robust, allows the examination of thousands of loci distributed across the whole genome in both sexes. AFLP is a powerful tool for obtaining molecular information at fine intraspecific level (Giannasi et al., 2001; de Knijff et al., 2001; Mock et al., 2002; Ogden and Thorpe, 2002).

The Alpine salamander, *Salamandra atra*, is a very secretive, completely terrestrial, and viviparous amphibian. It is an endemic species to the Alpine arc from Switzerland to Austria with some geographically isolated areas in the Dinaric Alps (Slovenia to Albania). There are two currently recognized subspecies: *S. a. atra* and the yellow spotted *S. a. aurorae* which is restricted to an extremely small area in the Asiago plateau in NE Italy (Grossenbacher, 1997). The species inhabits mainly mixed and coniferous forests often in the vicinity of brooks (Grossenbacher, 1997; Klewen, 1991). Previous studies using mtDNA sequence suggested low-genetic differentiation in *S. atra* population across the current range of this species (Ribéron et al., 2001; Steinfartz et al., 2000).

The objectives of the present study are: (1) to evaluate the AFLP technique to investigate the levels and patterns of genetic diversity in *S. atra* to provide information on population structure and intraspecific variation

* Corresponding author. Present address: CNRS Université Paul Sabatier, EDB UMR, 118, Route de Narbonne, 31062 Toulouse Cedex 4, France. Fax: +33-561-556-096.
E-mail address: riberon@cict.fr (A. Riberon).

1055-7903/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2003.10.016

in *S. atra*, and (2) to compare estimates obtained from nuclear and mitochondrial markers.

2. Materials and methods

2.1. Population sampling and DNA extraction

A total of 26 *S. atra* individuals was sampled from 11 locations across its geographic range (Table 1). Most samples were obtained from live animals captured in the field. A toe of the hind legs was clipped off and stored in 95% ethanol until being processed for DNA extraction. Specimens were released alive in their capture location. Some samples were obtained from museum collection material stored in 70% ethanol. Total genomic DNA was extracted using the Qiagen kit for tissue, following the manufacturer's directions except that one centrifugation step was added after lysis.

2.2. AFLP procedure

The AFLP protocol was carried out following the procedure described in Ajmone-Marsan et al. (1997) with some modifications including the use of non-radioactive fluorescent dye-labelled primers (PE, Biosystems). Genomic DNA (400 ng) was digested with *Taq*I at 65 °C for 1 h; then, *Eco*RI at 37 °C for 1 h. Finally, ligation reaction was incubated at 37 °C for 3 h. Preselective PCR amplifications started with an initial 2-min extension step at 72 °C, and a final extension for 10 min at 72 °C. The initial extension step was necessary for the complete adaptator ligation. Thirty-two primer pairs were initially screened, but five were chosen for the analyses because they gave a clear polymorphism within and among populations (*Eco*RI + ATC/*Taq*I + CCA; *Eco*RI + AGT/*Taq*I + CAG; *Eco*RI + AGA/*Taq*I + CAG; *Eco*RI + ATG/*Taq*I + CAC; and *Eco*RI + AGT/*Taq*I + CCA). The selective PCR products were electrophoresed on an ABI PRISM 377 DNA sequencer (PE) in a 5% Long Ranger gel (FMC) for 5 h.

2.3. Data analysis

Data were collected and analyzed using the GeneScan Analysis software (version 2.1; AB). Only the peaks that ranged from 70 to 400 bp were considered. The AFLP patterns were scored for absence/presence of the bands and coded as 0/1 respectively. For each primer combination, the number of polymorphic and monomorphic bands was determined. Levels of genetic diversity were estimated between and among populations. The binary data matrix was converted to a matrix of similarity values (F) using the formula $F = N_{xy}/(N_x + N_y)$, where N_x and N_y are the numbers of fragments in genotype x and y respectively and N_{xy} is the number of fragments shared by genotypes x and y (Nei and Li, 1979). Relationships among samples was based on similarity matrix using the neighbour-joining method (NJ) and a tree is constructed using PAUP* (Swofford, 1998). Principal component analysis (PCA) was performed on the binary data matrix using Statistica (5.0/W, Statsoft, USA.) to assess the dimensionality of the data and to describe the major patterns of variation within and among populations.

3. Results and discussion

3.1. Genetic diversity

The five AFLP primer combinations used for the analysis in *S. atra* produced a total of 706 clear fragments with an average of 141.2 bands per primer pair for the entire sample set. The percentage of polymorphic bands per primer pair in the data set ranges from 9.7 to 21.9%. The proportion of polymorphic fragments was relatively low (16.3% of fragments for the five primer combinations used) but this polymorphism is sufficient

Table 1
List of populations used in this study and AFLP genotype

Pop. No	Subspecies	Locality (country)	*N*	AFLP genotype	mtDNA haplotype
1	*S. a. aurorae*	Bosco del Dosso (Italy)	3	AUA	Aur
2	*S. a. aurorae*	Val di Nos (Italy)	2	AUB	Aur
3	*S. a. atra*	Sežnick (Slovenia)	2	SLO	Slo
4	*S. a. atra*	Velika Kapela (Croatia)	2	CRO	Cro
5[a]	*S. a. atra*	Sappada (Italy)	1	SAP	Sap
6[a]	*S. a. atra*	Sella Nevea (Italy)	1	SEL	Sel
7	*S. a. atra*	Gerola Alta (Italy)	1	GER	Ger
8	*S. a. atra*	Rüschegg (Switzerland)	4	RUS	Rus
9	*S. a. atra*	Meiringen (Switzerland)	4	MER	Mei
10[b]	*S. a. atra*	Lucerne (Switzerland)	1	LUC	Luc
11	*S. a. atra*	St Anton (Austria)	5	AUS	Aus

[a] Collected in the Natural History Museum of Trieste (No. 1784 and 1830, respectively).
[b] Collected in the Natural History Museum of Bern (No. 1016703).

to distinguish all 26 individuals as separate genotype. A similar level of polymorphism was exhibited in dogs (Kim et al., 2001) but, in general, AFLP studies appear to exhibit a higher level of polymorphism. For instance, Bensch et al. (2002) found that despite a huge variability between closely related bird species, polymorphism varied between 25 and 40%. In amphibians, there are no published data using AFLP comparing populations and it is therefore difficult to make proper comparisons. However, it has been suggested that AFLP and RAPD may give a similar estimation of the genetic diversity (Borowsky, 2001). Thus, the level of polymorphism observed in this study can be compared with amphibian RAPD studies. Our results indicate that the level of genetic diversity found in *S. atra* is similar to that reported for other amphibian species surveyed with the RAPD technique (Gibbs, 1998; Kimberling et al., 1996; Tarkhnishvili et al., 2000). In the present study, five pairs of primers were used out of 32 originally tested. This suggests that the level of polymorphism is probably overestimated. Overall, this confirms the idea that genetic polymorphism is indeed low in amphibians in general and in *S. atra* in particular.

The number of primer pairs used is a typically contentious point. Indeed, the amount of diversity revealed will depend on the number of pairs tested. In most studies, between 4 and 10 primer combinations are typically used. As we used five primer pairs, it seems that our study is well within this range. The number of AFLP fragments depends on both the AFLP procedure (primer combinations and amplification selectivity) and the complexity of the genome (size and repetitive sequence abundance) (Yan et al., 1999). Species with a large genome size have been shown to generate more AFLP amplification products than species with small genome (Vos et al., 1995). As species from the *Salamandra* genus are also known to have very large genome size among Vertebrates (Gregory, 2001), we could expect to observe many bands. However, repetitive sequences occur abundantly in large genomes, and tend to reduce the polymorphism and to make it more difficult to evaluate (Reamon-Büttner et al., 1999; Yan et al., 1999). These two observations may explain why *Salamandra* sp. may produce large numbers of monomorphic bands.

3.2. Genetic structure of S. atra

Given the large number of AFLP loci, we were able to detect higher levels of genetic diversity than in previous studies using mtDNA for both the subspecies (Ribéron et al., 2001). However, the PCA plot (Fig. 1) clearly separated *S. a. aurorae* populations from all others populations. The nominal subspecies, *S. a. atra*, except the Slovenian population, formed a relatively dense cluster (Fig. 1). The high dimensionality of the data demonstrated by the PCA (10 out of 26 axes being required to describe 95% of the variation between populations) indicated complex patterns of variation among populations with no simple trends. While genetic variation within populations, measured by the proportion of polymorphic bands, was relatively low (3.9–6.0%) using AFLP, we found no mitochondrial variation in *aurorae*. The absence (or low level) of mitochondrial diversity in *aurorae* subspecies could be due to different factors such as a population bottleneck or the persistence of low population sizes. Unfortunately, information on population trends in this subspecies is not available and does not allow us to separate the two hypotheses.

The apparent conflict between the limited amount of genetic differentiation and the marked phenotypical differences (e.g., body coloration) can be explained by an early geographical isolation, before the last glacial period in Europe, which took place sufficiently long ago to create phenotypical variability but was too recent to create a sufficient overall genetic differentiation between populations when many recombining loci are analyzed.

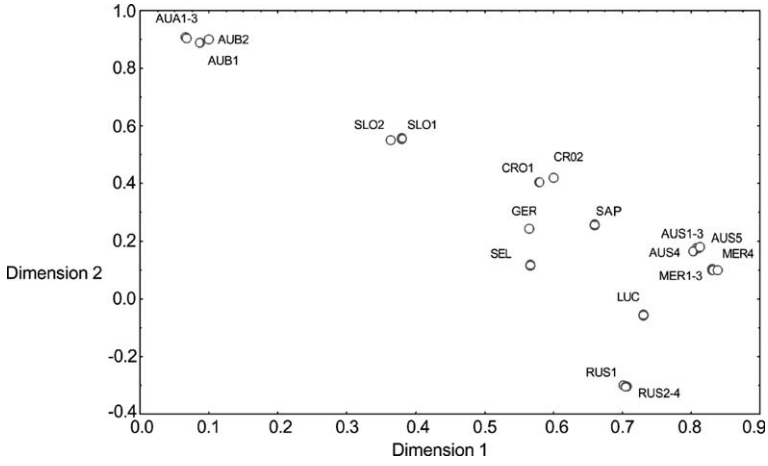

Fig. 1. Plot of first and second principal components of PCA on AFLP characters, together showing 60% of the total variation.

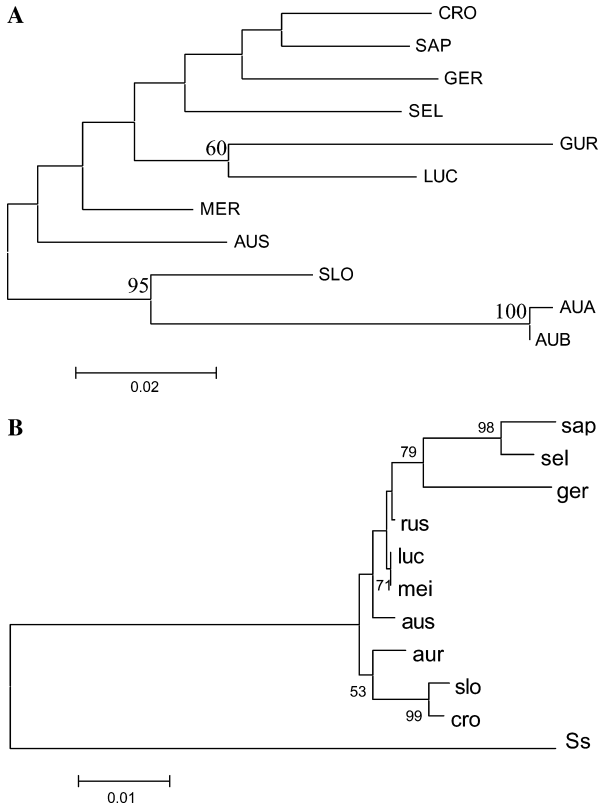

Fig. 2. (A) NJ tree based on Nei and Li (1979) distance calculated from 111 AFLP loci. (B) Relationships for 10 haplotypes of *S. atra* based on NJ analysis of a 1050 bp fragment of cytochrome *b* mitochondrial DNA sequence data (from Ribéron et al., 2001). Bootstrap support values (>50%) from 1000 replicates are shown. *Ss* = *Salamandra salamandra*.

Indeed, patterns of coloration can evolve rapidly (Market et al., 2001). Another explanation is a retention of a recent ancestral polymorphism.

While intra-population diversity was improved with AFLP, the low differentiation and even worse resolution obtained here by the AFLP data (Fig. 2A) contrasts with the previous population structure analyses based on mitochondrial data (Fig. 2B). While one could argue that small sample sizes such as ours could be responsible for our results. In particular, one major consequence of small sample size studies is that low-frequency polymorphism may have gone undetected (Parson and Shaw, 2001). The detection of such polymorphisms may prove quite informative for estimating levels of recent gene flow. However, it is important to note that sampling error associated with use of small sample size for estimating population differentiation is greatly reduced through the use of large numbers of loci (Nei, 1987). Indeed, some studies based on a even more limited sample sizes clearly showed a significant genetic differentiation (Giannasi et al., 2001; for snakes).

Some authors have suggested that AFLP may have little power to resolve complex and recent diversification (Alberston et al., 1999; Kim et al., 2001). Given that the evolutionary rate in the salamander genome is low (Wake et al., 1983) and that equilibrium levels of diversity may not have been reached in short time periods, new markers such as microsatellites may be needed for further understanding of the salamander population history. Microsatellites loci might have the potential to overcome these difficulties owing to their high mutation rates and the potential information content and could at the same time reveal the same genetic variation than AFLP for recent events as hybridization (e.g. Nijman et al., 2003).

Acknowledgments

We are grateful to Drs. N. Bressi (Museum of Trieste), C. Eggert (University of Savoie), K. Grossenbacher (Museum of Bern), and E. Kletecki (Museum of Zagreb) for providing tissue samples and for field assistance. We are indebted to Effimia Sotiriou for help in laboratory. We thank L. Gielly and V. Curry for their technical assistance for the sequencer. We thank Lounes Chikhi and reviewers for providing helpful comments on the manuscript. Financial support was provided by Région Rhône-Alpes, France (Programme AVENIR 1996-98).

References

Alberston, R.C., Markert, J.A., Danley, P.D., Kocher, T.D., 1999. Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. Proc. Natl. Acad. Sci. USA 96, 5107–5110.

Avise, J., 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge.

Ajmone-Marsan, P., Valentini, A., Cassandro, M., et al., 1997. AFLP markers for DNA fingerprinting in cattle. Anim. Genet. 28, 418–426.

Bensch, S., Helbig, A.J., Salomon, M., Seibold, I., 2002. Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. Mol. Ecol. 11, 473–481.

Borowsky, R.L., 2001. Estimating nucleotide diversity from random amplified polymorphic DNA and amplified fragment length polymorphism data. Mol. Phylogenet. Evol. 18, 143–148.

Giannasi, N., Thorpe, R.S., Malhotra, A., 2001. The use of amplified fragment length polymorphism in determining species trees at fine taxonomic levels: analysis of a medically important snake, *Trimeresurus albolabris*. Mol. Ecol. 10, 419–426.

Gibbs, J., 1998. Genetic structure of redback salamander *Plethodon cinereus* populations in continuous and fragmented forests. Biol. Conserv. 86, 77–81.

Gregory, T.R., 2001. Animal Genome Size Database. http//www.genomesize.com.

Grossenbacher, K., 1997. In: Societas Europaea Herpetologica & Muséum National d'Histoire Naturelle (Eds.), *Salamandra atra*. Atlas of Amphibians and Reptiles in Europe, Paris, pp. 68–69.

Kim, K.S., Jeong, H.W., Park, C.K., Ha, J.H., 2001. Suitability of AFLP markers for the study of genetic relationships among Korean native dogs. Genes Genet. Syst. 76, 243–250.

Kimberling, D., Ferreira, A., Shuster, S., Keim, P., 1996. RAPD marker estimation of genetic structure among isolated northern leopard populations in the south-western USA. Mol. Ecol. 5, 521–529.

Klewen, R., 1991. Die Landsalamander Europas. Ziemsen Verlag, Stuttgard.

de Knijff, P., Denkers, F., van Swelm, N.D., Kuiper, M., 2001. Genetic affinities within the herring gull *Larus argentatus* assemblage revealed by AFLP genotyping. J. Mol. Evol. 52, 85–93.

Market, J.A., Danley, P.D., Arnegard, M.E., 2001. New markers for new species: microsatellites loci and the East African cichlids. TREE 16, 100–107.

Mock, K.E., Theimer, T.C., Rhodes, O.E., Greeberg, D.L., Keim, P., 2002. Genetic variation across the historical range of the wild turkey (*Meleagris gallopavo*). Mol. Ecol. 11, 643–657.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269–5273.

Nijman, I.J., Otsen, M., Verkaar, E.C.L., et al., 2003. Hybridization of banteng (*Bos javanicus*) and zebu (*Bos indicus*) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites. Heredity 90, 10–16.

Ogden, R., Thorpe, R.S., 2002. The usefulness of amplified fragment length polymorphism markers in taxon discrimination across graduated fine evolutionary levels in Caribbean Anolis lizards. Mol. Ecol. 11, 437–445.

Parson, Y.M., Shaw, K.L., 2001. Species boundaries and genetic diversity among Hawaiian crickets of the genus *Laupala* identified using amplified fragment length polymorphism. Mol. Ecol. 10, 1765–1772.

Reamon-Büttner, S.M., Schmidt, T., Jung, C., 1999. AFLPs represent highly repetitive sequences in *Asparagus officinalis*. Chromosome Res. 7, 279–304.

Ribéron, A., Miaud, C., Grossenbacher, K., Taberlet, P., 2001. Phylogeography of the Alpine salamander, *Salamandra atra* (Salamandridae), and the influence of the Pleistocene climatic oscillations on population divergence. Mol. Ecol. 10, 2555–2560.

Steinfartz, S., Vieth, M., Tautz, D., 2000. Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial recolonizations of central Europe from distinct source populations of *Salamandra salamandra*. Mol. Ecol. 9, 397–410.

Swofford, D.L., 1998. PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.

Tarkhnishvili, D.N., Thorpe, R.S., Arntzen, J.W., 2000. Pre-pleistocene refugia and differentiation between populations of the caucasian salamander (*Mertensiella caucasica*). Mol. Phylogenet. Evol. 14, 414–422.

Vos, P., Hogers, R., Bleeker, M., et al., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407–4414.

Wake, D.B., Roth, G., Wake, M.H., 1983. On the problem of stasis in organismal evolution. J. Theor. Biol. 101, 211–224.

Yan, G., Romero-Severson, J., Walton, M., Chadee, D.D., Severson, D.W., 1999. Population genetics of the yellow fever mosquito in Trinidad: comparison of amplified fragment length polymorphism (ARLP) and restriction fragment length polymorphism (RFLP) markers. Mol. Ecol. 8, 951–963.