

# 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*

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## Abstract

Representatives of the genus *Salamandra* occur in Europe, Northern Africa and the Near East. Many local variants are known but species and subspecies status of these is still a matter of dispute. We have analysed samples from locations covering the whole expansion range of *Salamandra* by sequence analysis of mitochondrial D-loop regions. In addition, we have calibrated the rate of divergence of the D-loop on the basis of geologically dated splits of the closely related genus *Euproctus*. Phylogenetic analysis of the sequences suggests that six major monophyletic groups exist (*S. salamandra*, *S. algira*, *S. inframaculata*, *S. corsica*, *S. atra* and *S. lanzai*) which have split between 5 and 13 million years ago (Ma). We find that each of the *Salamandra* species occupies a distinct geographical area, with the exception of *S. salamandra*. This species occurs all over Europe from Spain to Greece, suggesting that it was the only species that has recolonized Central Europe after the last glaciation. The occurrence of specific east and west European haplotypes, as well as allozyme alleles in the *S. salamandra* populations suggests that this recolonization has started from at least two source populations, possibly originating in the Iberian peninsula and the Balkans. Two subpopulations of *S. salamandra* were found that are genetically very distinct from the other populations. One lives in northern Spain (*S. s. bernardezi*) and one in southern Italy (*S. s. giglioli*). Surprisingly, the mitochondrial lineages of these subpopulations group closer together than the remainder *S. salamandra* lineages. We suggest that these populations are remnants of a large homogeneous population that had colonized Central Europe in a previous interglacial period, approximately 500 000 years ago. Animals from these populations were apparently not successful in later recolonizations. Still, they have maintained their separate genetic identity in their areas, although they are not separated by geographical barriers from very closely related neighbouring populations.

*Keywords:* biogeography, D-loop, phylogeny, population differentiation

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## Introduction

The biogeography of many species in Europe suggests that their population structure was heavily influenced by the quaternary climatic oscillations that have led to repeated glaciations of Central Europe. Most species

probably went through range contractions when the temperatures decreased and range expansions from different refugia in the following warm periods (Hewitt 1996). Such repeated cycles of separation and recolonization could act as a speciation motor, either through allopatric adaptations to new environments or by specializations into new niches after recolonization of deserted areas under sympatric conditions (Hewitt 1989; Hewitt 1993; Bush & Smith 1998; Orr & Smith 1998). However, a recent survey of the current data available on 10 European animal and plant genera

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suggests that most of the respective species may have originated before the quaternary glaciation cycles began (Hewitt 1996; Taberlet *et al.* 1998; Hewitt 1999). Thus, to understand the influence of the glaciation cycles on the speciation process, it seems necessary to focus not so much on well differentiated species, but rather on subspecies and subpopulations of a given species. These are more likely to reflect the influence of the separation-recolonization cycles and thus would give insights into the processes that have led to the differentiation of populations and eventually to speciation.

We have analysed here the phylogeographic distribution patterns of fire salamander and alpine salamander populations, which present a particularly interesting model system in this respect. Salamanders have received much attention by naturalists and evolutionary biologists because of their large variations in colour patterns, life history and ecology (e.g. Degani & Warburg 1978; Grossenbacher 1994; Alcobendas *et al.* 1996; Dopazo *et al.* 1998; Greven 1998). The genus *Salamandra* consists of the two well recognized Alpine salamander species, *S. atra* and *S. lanzai*, and the fire salamander complex, which covers the main part of the distribution area. Fire salamanders have been considered to constitute polytypic species assemblages with many subspecies and subpopulations (Eiselt 1958; Fachbach 1976a,b; Klewen 1991). However, the morphological and molecular data obtained so far could not fully clarify whether the fire salamander complex reflects a single polytypic species, *S. salamandra*, with up to 16 subspecies distributed over three continents (Eiselt 1958; Klewen 1991), or whether it has to be split into at least four distinct species, *S. salamandra* (Europe), *S. corsica* (Corsica), *S. algira* (northern Africa) and *S. infraimmaculata* (Near East), each with its associated subspecies. The latter hypothesis is based on plasma protein electrophoresis data (Gasser 1978; Joger & Steinfartz 1994a,b) and on allozyme data (Veith 1994), but a formal revision of the species complex is still pending. Accordingly, because it is unclear which taxa should be treated as species, it is even less clear which ones should be called subspecies or subpopulations. However, for the purpose of this paper, we are going to retain the taxonomic assignments that can currently be found in the literature. This then provides a basis for suggestions of future revisions.

To analyse the genetic differentiation between the *Salamandra* populations, we have employed comparisons of mitochondrial D-loop sequences. In previous studies, mitochondrial restriction fragment length polymorphisms were used to analyse *Salamandra* populations in northern Spain (Dopazo *et al.* 1998). However, the mitochondrial control region or D-loop is usually considered to be the most rapidly evolving part of the mitochondrial genome (Upholt & David 1977; Wilson *et al.* 1985; Saccone & Sbisà 1994) and should thus yield the highest degree of

resolution among closely related populations. One study in anurans (*Rhacophorus taipeianus*, Yang *et al.* 1994) and one in salamanders (*Ambystoma tigrinum* complex, Shaffer & McKnight 1996) employing D-loop sequences showed good levels of differentiation between populations, although the deeper phylogenetic relationships could not be resolved. However, in both cases it remained unclear whether this lack of resolution at deep nodes is caused by short separation times (i.e. radiations) or by inherent evolutionary constraints of the D-loop itself. We have, therefore, attempted to calibrate the evolutionary substitution rate in the D-loop, using the splitting of the salamandrid genus *Euproctus*. The well documented disjunctions of the Corsican–Sardinian microplate from the European mainland at the Oligocene nearly 29 Ma (paralleled by the separation of *E. asper* from the *E. platycephalus/montanus* lineage), and the subsequent disjunction of Sardinia and Corsica around 13–15 Ma (Alvarez 1972, 1974; Alvarez *et al.* 1973) resulting in the splitting of *E. platycephalus* (Sardinia) and *E. montanus* (Corsica) constitute two independent events for estimating substitution rates. This system of rotating islands has also been used by Caccone *et al.* (1994, 1997) to estimate evolutionary rates for mitochondrial ribosomal genes and also cytochrome *b* in *Euproctus*.

## Materials and methods

### Samples

To analyse the phylogeography of *Salamandra salamandra* populations we have obtained samples from all European localities where specific populations or subspecies had previously been described. The currently recognized subspecies comprise *S. s. salamandra* and *S. s. beschkovi* (eastern range of Europe), *S. s. terrestris* (western Central Europe), *S. s. giglioli* (Italy), *S. s. fastuosa* (Pyrenees and Cantabric Mountains), *S. s. bejarae* (central parts of Spain), *S. s. almanzoris* (Sierra de Gredos, central Spain), *S. s. bernardezi* (Cantabria), *S. s. morenica* (southern Spain, north of the Rio Guadalquivir), *S. s. longirostris* (southern Spain, south of the Rio Guadalquivir), *S. s. crespoi* (southern Portugal, Algarve), *S. s. gallaica* (rest of Portugal). In addition, populations from all other species of the genus *Salamandra* were also sampled, in particular *S. algira* in northern Africa, *S. corsica* from Corsica, *S. atra* and *S. lanzai* from the Alps and *S. infraimmaculata* from various subpopulations of the Near East (see Fig. 1). The subspecific differentiation for *S. infraimmaculata* populations has yet to be revised. With the exception of *S. i. orientalis* and *S. i. semenovi* for which subspecies status is confirmed (Joger & Steinfartz 1995) we will refer to the remaining *S. infraimmaculata* populations as *S. i. ssp.* (location).

Calibration of the D-loop substitution rate was done

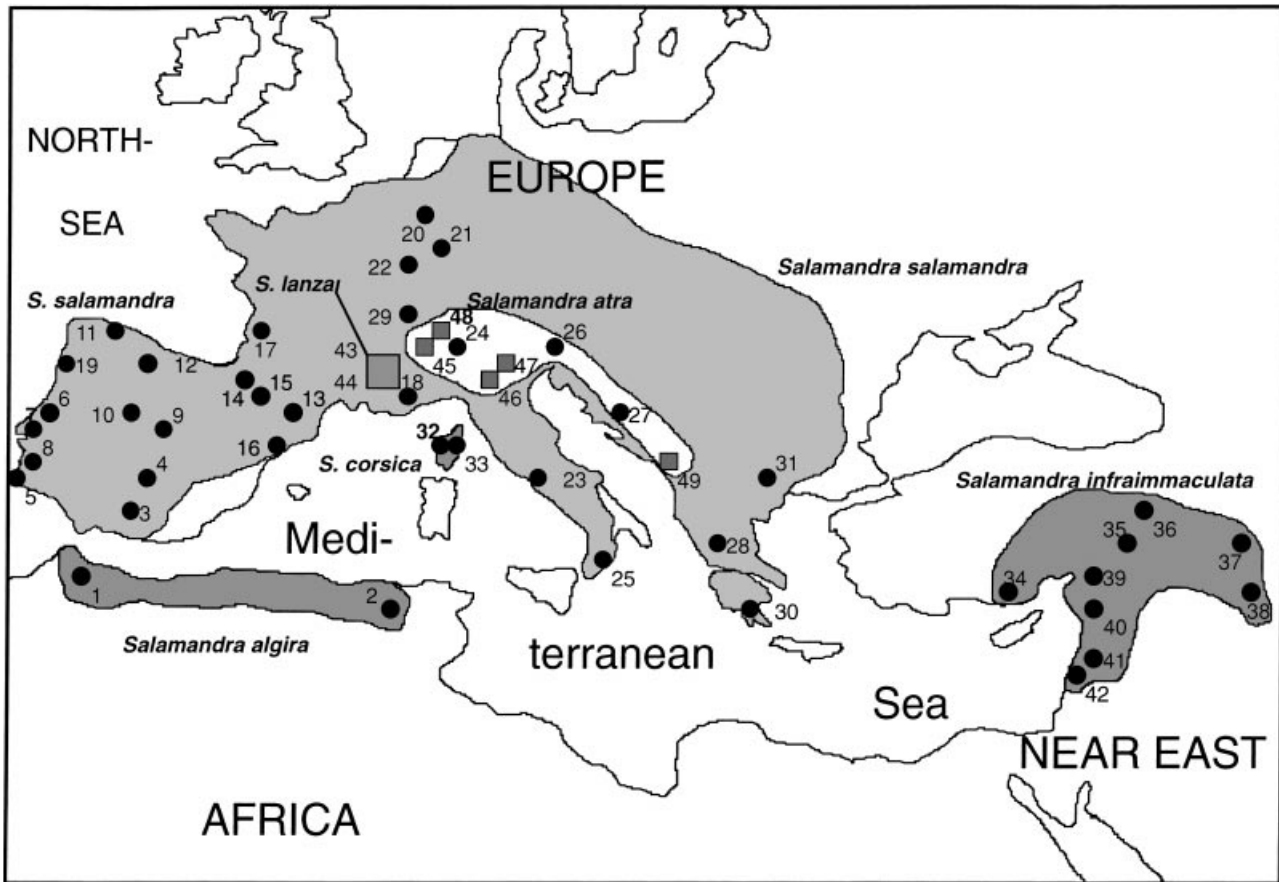


Fig. 1 *Salamandra* species distribution and sampling locations. The approximate distribution ranges are indicated, according to Klewen (1991) and Gasc *et al.* (1997). *S. lanzai* is found at only one location, indicated by a square. The distribution range of *S. atra* refers to altitudes above 1500 m only. The numbers refer to the sampling locations listed in the appendix.

using samples from all three species of the genus *Euproctus* (*E. asper* from the Pyrenees, *E. platycephalus* from Sardinia and *E. montanus* from Corsica).

Blood or tissue samples of salamanders used for this study were taken either from animals registered to local authorities of Germany or field collected with permission of the corresponding country or from museum specimens (see also appendix).

#### DNA isolation and DNA sequencing

Genomic DNA was extracted from clipped toes or blood using the SDS-proteinase K/Phenol-Chloroform extraction method. The primer combination L-Pro-ML (5'-GGCACCCAAGGCCAAAATTCT 3') and H-12S1-ML (5'-CAAGGCCAGGACCAAACCTTTA 3') was used for amplifying the D-loop and sequencing. These primers were initially designed on the basis of sequences from *Mertensiella luschani atifi*. For sequencing the D-loop of *E. platycephalus* we used the primer H-1478 of Kocher *et al.* (1989) and the specific primers E. platyc.-L (5'-

GGCCCATGATCAACAGAACT 3') and E. platyc.-H (5'-GCTGGCACGAGATTTACCAA 3').

PCR was done in a 50- $\mu$ L scale under standard conditions (Kocher *et al.* 1989). Amplified D-loop DNA was purified by using ultrafree-filters (Millipore, Bedford, MA, USA) and was afterwards used for symmetric cycle sequencing using either the Ready Reaction kit (Amersham, Freiburg, Germany) or the Big-Dye Ready-Reaction kit (Perkin Elmer, Weiterstadt, Germany). Sequencing products were analysed on an ABI™ 377 (Perkin Elmer) and the Sequence Navigator software (Perkin Elmer). We sequenced between one and three individuals from 49 *Salamandra* populations and this includes some populations that had formerly been given subspecific rank and that were subsequently synonymized (e.g. *S. s. hispanica*, *S. s. wernerii*, *S. s. bonnali*). Altogether we have sequenced the mitochondrial D-loop from 95 samples.

#### Tree reconstruction

Initial automatic alignments (Clustal W; Thompson *et al.* 1994)

were adjusted by hand. Indels were excluded from the analysis. 783 bp of mt D-loop were included for the final alignment. A sequence from *M. luschani* (subspecies *atifi*) which can be considered as a closely related sister taxon of *Salamandra* (Özeti 1967; Titus & Larson 1995; Veith *et al.* 1998) was used as out-group. Tree reconstruction was done with the neighbour-joining algorithm using the Beta version of PAUP 4.0 (Swofford 1999). Robustness of the branches was estimated by 1000 bootstrap replicates (Felsenstein 1985).

For the rate calibration, the whole D-loop represented by 755 bp for *E. asper*, *E. platycephalus* and *E. montanus* was aligned with homologous positions of *M. l. atifi*. Initially we had also sequenced *Triturus marmoratus* and *T. vulgaris* for use as out-groups. However, it became clear from these data, as well as additional data from other mitochondrial regions, that the *Triturus* taxa would not form a monophyletic group, a notion that was also suggested by Titus & Larson (1995). Thus, until the resolution of this issue, it seems more appropriate to use the slightly more distant *Mertensiella* as an out-group, although the results are not much different when one of the *Triturus* taxa is chosen. All indels were excluded from the alignment before reconstructing the tree by maximum likelihood analysis (PUZZLE 4.0, Strimmer & Haeseler 1996). Estimates of branch lengths were taken from this tree. Because clock-like behaviour is crucial for estimating separation times from molecular data we tested the tree for the *Euproctus* species for clock-like behaviour by using PUZZLE 4.0. Likelihoods were estimated for the tree either under the assumption of clock-like or non clock-like behaviour by using *M. l. atifi* as an out-group.

#### Testing for nuclear insertion

To check whether we might have inadvertently amplified only nuclear copies (Zischler *et al.* 1995; Zhang & Hewitt 1996; Zischler *et al.* 1998) of the D-loop, we have isolated DNA from purified mitochondria (employing the QUIAGEN kit, Hilden, Germany) of the liver of one animal. From this and from the total DNA of this animal, we have amplified and sequenced the D-loop fragment, which yielded identical sequences. Furthermore, the amplified fragment was used for high stringency southern blots on the mitochondrial and genomic DNA fraction, which yielded a strong signal with the mitochondrial DNA-fraction, but not with the genomic one. Finally, sequencing of the population samples did not yield ambiguous results at polymorphic positions within populations, which would have been expected to occur in animals that are heterozygous for the nuclear insert. None of these tests can rule out that there is also a nuclear mitochondrial insert in the *Salamandra* genome, but we can at least be confident that the fragment that we have amplified is indeed derived from the mitochondrial genome.

## Results

In order to analyse the phylogenetic relationships of the various described species, subspecies and subpopulations of the genus *Salamandra*, we have obtained 95 samples from across the whole expansion range, including all described groups and subgroups. D-loop sequences were obtained for all these samples and aligned with *Mertensiella luschani* as an out-group.

Because of the large number of samples involved, tree reconstruction could only be done by neighbour joining. To obtain a realistic model of nucleotide substitution for this, we first estimated the sequence divergence parameters using the program PUZZLE 4.0 (Strimmer & Haeseler 1996). The average transitions:transversions (ti:tv) ratio between all possible taxa combinations was found to be 1.45, which indicates a much lower bias for ti than in mammalian mitochondrial DNA (Horai & Hayasaka 1990; Tamura & Nei 1993). Base frequency parameters were determined as  $\pi_A = 0.306$ ,  $\pi_T = 0.225$ ,  $\pi_C = 0.140$  and  $\pi_G = 0.329$  showing a clear deviation from equal base composition. Finally, we found strong rate heterogeneity across sites which can be approximated by a  $\Gamma$ -distribution with the estimated shape parameter  $\alpha = 0.21$  (Yang 1996). These parameters were used to calculate the distance matrix underlying the tree shown in Fig. 2.

#### Between species relationships

From the tree (Fig. 2), it is evident that all recognized species of *Salamandra* are faithfully resolved into monophyletic assemblages. Less resolution was obtained between some of the major lineages, suggesting that these have separated within a comparatively short time span and that additional sequences will be required for resolving these deeper nodes. The most striking outcome of the present tree is that the two melanic Alpine salamander species, *S. lanzai* and *S. atra*, are not grouped together. Instead *S. lanzai* comes out as a separate unit, while the sister group of *S. atra* is *S. corsica*, which is not a melanic form. Preliminary sequence information from the 12S and 16S mitochondrial genes suggests that *S. lanzai* may even be basal to the remainder of the *Salamandra* taxa (data not shown). The second major outcome is that the sister group to the Central European salamanders (*S. salamandra*) is the North African species *S. algira*, a relationship that receives also slightly higher support from the partial 12S and 16S sequences obtained so far (data not shown).

#### Subspecies resolution

The tree provides also a good resolution at the subspecies and population level. Although bootstrap values are sometimes not much higher than 50, the reconstruction is



that the deeper nodes correspond to differences between localities. For example, the population *S. i.* ssp. (sampling location 34, see Fig. 1) from the Mediterranean coast of Turkey comes out as a separate unit. Interestingly, a population sampled slightly more east (locations 39, 40) groups closer with samples from central Turkey (location 35). Samples from Israel (locations 41, 42) form also a recognizable unit. Finally, samples from the subspecies *S. i. semenovi* in Iran (location 38) group with samples collected in eastern Turkey (locations 36, 37). Thus, a structuring according to geographical origin seems possible for *S. infraimmaculata* populations, although the detailed delimitations of the groups have yet to be determined by further sampling. Some structuring according to origin might also exist for the *S. algira*, the *S. corsica* and *S. atra* populations (see Discussion) although further sampling will be required to verify such subgroups.

In contrast to the more restricted geographical origin of the *Salamandra* species discussed above, the *S. salamandra* subspecies and populations are found all over Europe. Nevertheless they can also be resolved into geographical subgroups to some extent. The most basic one concerns the populations from southern Spain, which are currently classified as three subspecies, namely *S. s. morenica*, *S. s. crespoid* and *S. s. longirostris* (group A). These come from a Pleistocene refugial area in the South of Spain (Garcia-Paris *et al.* 1998) and may not have been affected much by the glaciation cycles. Among these there is a further subdivision between populations living north and south of the Guadalquivir river (*S. s. morenica* and *S. s. crespoid* vs. *S. s. longirostris*), confirming previous similar findings that were derived from serum protein analysis (Joger & Steinfartz 1994a,b) and cytochrome *b* sequences (Garcia-Paris *et al.* 1998).

The second resolved subgroup is the subspecies *S. s. bernardezi* from northern Spain and *S. s. gigliolii* from southern Italy (group B). This grouping seems surprising because it is difficult to see how they could have been connected in previous times. However, analysis of serum proteins from animals of the same populations had also indicated a close phylogenetic relationship (Joger & Steinfartz 1994b). Moreover, we have sequenced other mitochondrial regions for a subset of the samples and found that all these additional sequences support also the close relationship of the *S. s. bernardezi* individuals from Spain and the *S. s. gigliolii* individuals from southern Italy (data not shown). Thus, the close relationship of these disjunctive populations is solidly supported (see Discussion).

Finally, there is a large polytomy of taxa which come from the whole of Central, Western and Eastern Europe, including taxa from the former Yugoslavia, from Bulgaria and from Greece (group C). Intriguingly, none of the described subspecies in this area forms a separate entity in the D-loop tree. Nevertheless, no identical haplotypes

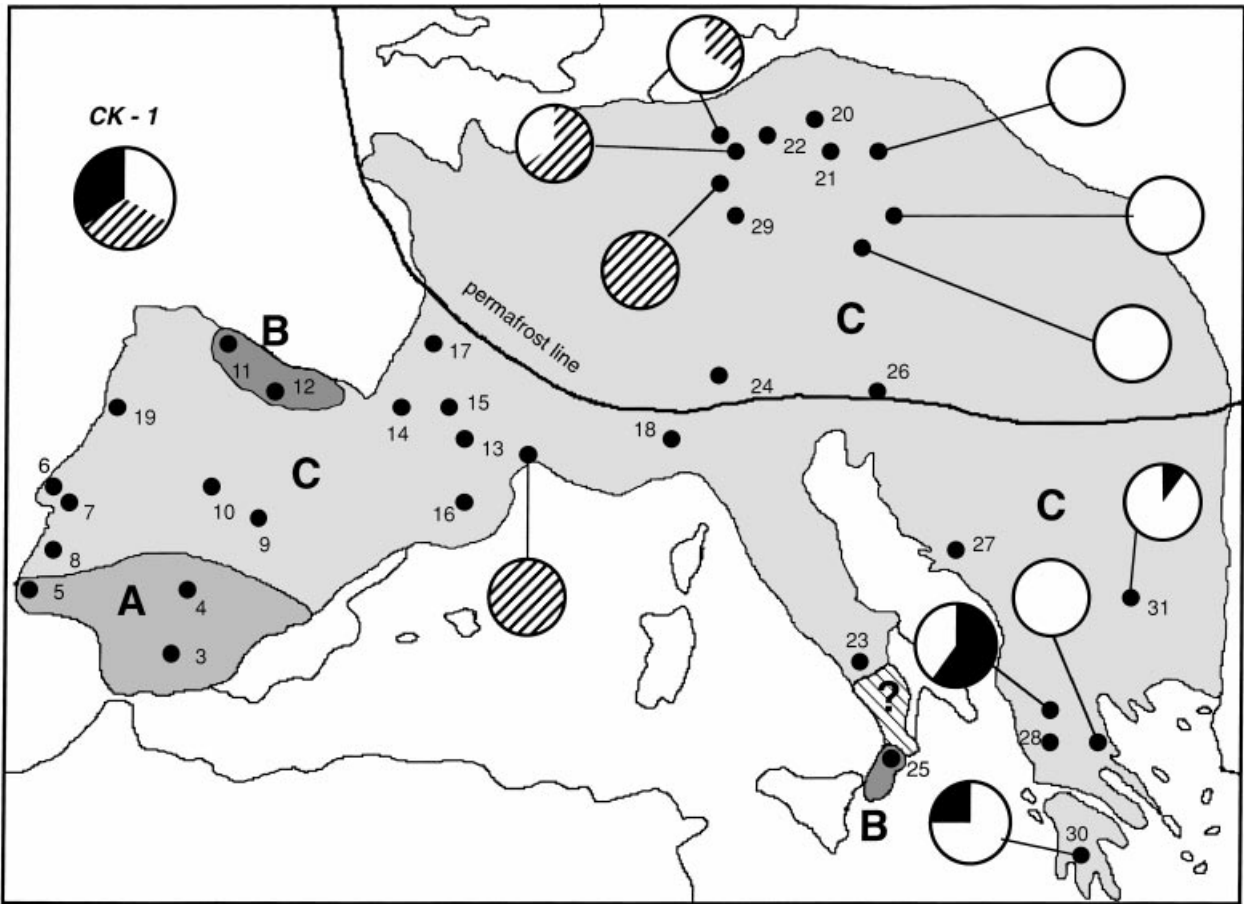
were found between East and West European populations. This indicates that the current distribution is not due to a recolonization from a single source population after the last glaciation and that the original founder population must be older than that.

#### *Allozyme analysis*

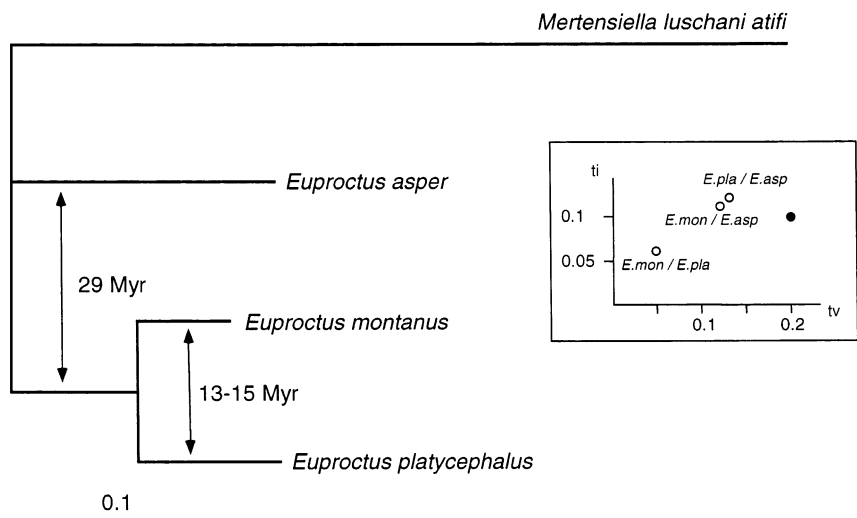
A further resolution can be obtained from allozyme data in this respect. A previous study of Central European samples (mainly from Germany) has shown that only very few polymorphic loci can be detected, supporting the inference of a small ancestral population (Veith 1992). Still, for those loci that showed polymorphism, a clear differentiation into eastern and western alleles was detected (Veith 1992). This suggested that a separate recolonization from western and eastern refugia may have occurred after the last ice age and that these populations now show a zone of overlap in Central Europe. To substantiate this inference, we have analysed additional samples from Southern France, as well as from Greece for the same loci. It is indeed evident that the allele that occurs in Western Germany is the same as in Southern France and that the allele from Eastern Germany occurs also in Greece (Fig. 3).

#### *Calibration of the D-loop substitution rate*

To allow a better assessment of the historical parameters that have led to the distribution patterns of the *Salamandra* species and populations described above, we have sought to calibrate the rate of D-loop divergence. This was done on the basis of well dated geological events that have most likely led to the splitting of the species in the genus *Euproctus* (see also Introduction) which is independent of the evolutionary patterns found in *Salamandra*. To assess the substitution behaviour of these sequences, as well as to assess whether a substitutional saturation may have occurred during this time, we calculated  $t_i$  and  $t_v$  at the substitutional equilibrium for the consensus tree (Fig. 4) using the formulas of Hasegawa *et al.* (1985). The results (Fig. 4) show that  $t_i$  are saturated for the distances of *E. asper* with respect to *E. platycephalus* and *E. montanus* and estimates derived from this split should, therefore, be treated with caution. On the other hand, neither  $t_i$  nor  $t_v$  are saturated for the distance of *E. platycephalus* and *E. montanus*. Furthermore, the divergence is compatible with a clock model, as the likelihood of the clock-like ML tree (log L: -2290.86) is not significantly higher when compared to the nonclock like tree (log L: -2290.17; critical significance level 0.5). Thus, this sequence set is acceptable for calibrating a clock. The estimated distance under the HKY- $\Gamma$  model is 11.12% between *E. montanus* and *E. platycephalus*. Applying the divergence times of 13–15



**Fig. 3** D-loop haplotype and allozyme allele distribution of *Salamandra salamandra* populations. The assignment to groups A, B and C was done according to the groupings revealed in the D-loop tree (Fig. 2). The distribution range of *S. s. gigliolii* is uncertain (hatched area). For CK-1, three alleles were found, two in middle and east European populations and one in middle and west European populations. The pie charts represent the frequencies for these alleles found in the populations of the indicated sampling locations. Note that these locations are not always the same as for the D-loop sequences. A more detailed analysis of the allozyme data will be presented by Veith *et al.* (in preparation). The figure indicates also the approximate line of permafrost during the height of the last glaciation (after Frenzel *et al.* 1992). The refugia of the Salamander populations are likely to have been even further south of this line, as they require a temperate climate for survival.



**Fig. 4** Tree used for the calibration of D-loop divergence rate. The tree was constructed with the three sequences from the *Euproctus* taxa (*E. asper*, *E. montanus* and *E. platycephalus*) with the *Mertensiella* sequence as outgroup (see Methods for choice of out-group). The inset shows the degree of saturation for transitions (ti) and transversions (tv) for the three in-group species pairs (open circles). The filled circle represents the calculated level of saturation for the ti and tv, respectively, under the model of sequence evolution that was inferred from the data. Values higher than this (such as the transition rates for the pairwise comparisons with *E. asper*) must be considered to be partially saturated.

Ma, we obtain a range of 0.74–0.86% divergence per million years (Myr). We will, therefore, apply an average value of 0.8% pairwise divergence per Myr. Although the divergence of *E. asper* shows signs of saturation (see above), the estimate is not so much different (24% divided by 29 Myr is 0.83% per Myr). Thus, two separate geological events would support the same rate. This rate is relatively low for a mitochondrial D-loop region and we have, therefore, considered the possibility that we have amplified a nuclear insert rather than a truly mitochondrial fragment. However, none of the tests (see Methods) suggested that the fragment was not of mitochondrial origin.

## Discussion

The D-loop sequences provide for most populations astonishingly good species level and phylogeographic resolution. The major *Salamandra* lineages are well resolved and most of the local forms and subspecies can be further resolved according to their geographical origin. The only exception are the populations from the Central European regions, which are the ones that have been influenced directly by the glaciations. This is mostly the case for *S. salamandra* populations suggesting that their distribution pattern has to be seen in the light of recolonizations from refugial populations.

In the following, we want to discuss the different levels of resolution in turn, as well as the evolutionary implications. However, to have a time frame for the approximate dating of the separation events at the species and subspecies level, we should like to discuss first the validity of the molecular clock that we have inferred from our data.

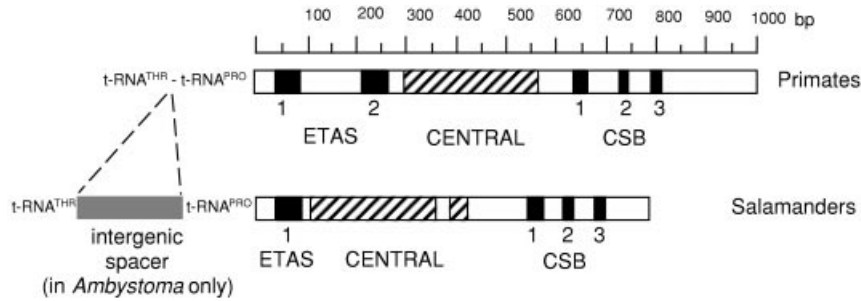
### Clock calibration

Our estimates for the sequence divergence rate in the part of the D-loop that we have analysed here rests on comparisons between *Euproctus* taxa that were separated by island formation at known times. Evidently, there is no formal proof that the *Euproctus* species have separated at the same times, but this is still the most parsimonious scenario especially because two independent geological events appear to have led to the same result. On the basis of this, we estimate a divergence rate of 0.8% pairwise divergence per Myr, which is unusually low for D-loop sequences. A homologous subregion of the D-loop (506 bp) was also sequenced for the North American salamanders of the genus *Ambystoma* (Shaffer & McKnight 1996). If we compare this part with the divergence dates provided in Shaffer & McKnight (1996), we find a rate of 0.82% pairwise divergence per Myr very similar to ours. Wallis & Arntzen (1989) have estimated the general rate of substitution in mitochondria from the

restriction fragment length polymorphism (RFLP) data of the salamandrid species *Triturus cristatus* and *T. marmoratus* ( $d = 8.4%$ , divergence time 10 Myr) suggesting a value of 0.84% per Myr. This would suggest that the substitution rate in the D-loop of salamanders is in the range of the substitution rate for the whole mitochondrion. This is in contrast to substitution rates found in other vertebrates like *Acipenser transmontanus* (Brown *et al.* 1993) or humans (Horai & Hayasaka 1990), where the D-loop rate is 4–5 times higher than in the remainder of the mitochondrion. On the other hand, low D-loop divergence was also found for brown trout (*Salmo trutta*, Bernatchez *et al.* 1992; Apostolidis *et al.* 1997) and for chum salmon (*Oncorhynchus keta*, Park *et al.* 1993) where D-loop variation is unexpectedly low in comparison with other mitochondrial regions. Finally, the analysis of cytochrome *b* sequence divergence in the same *Euproctus* species as the ones analysed by us has also provided evidence for an unusual slow divergence rate (Caccone *et al.* 1997).

In order to better understand the reason for this comparatively low rate of divergence, we have aligned the D-loop for Salamandridae (based on the data for the genera *Salamandra*, *Mertensiella*, *Euproctus* and *Triturus*) and Ambystomatidae (genus *Ambystoma*) with a consensus sequence from mammalian D-loops (Sbisà *et al.* 1997). Such an alignment is possible, as even the D-loop harbours several more highly conserved regions that are likely to be of functional significance (Sbisà *et al.* 1997). From this alignment (Fig. 5) it becomes clear that the whole salamander D-loop (approximately 740 bp) is on average one-third shorter in length when compared to the mammalian D-loop (around 1000 bp on average). The subdomain ETAS2 is totally absent in the investigated salamanders and the region between ETAS1 and the central region, which is known to be particularly polymorphic in mammals is very short in salamanders. Thus, the lack of hypervariable regions in *Salamandra* might explain why the D-loop is so comparatively slow evolving. This inference is also in line with the results from a recent comparison of mammalian D-loop regions (Pesole *et al.* 1999). Interestingly, in *Ambystoma* there is a new hypervariable spacer region next to the D-loop (Fig. 5), which is absent in the salamandridae analysed here and which shows a much higher divergence rate (McKnight & Shaffer 1997). This is also the reason why Shaffer & McKnight (1996) have calculated a rate of 1–1.5% pairwise divergence for the whole region they sequenced, as this includes this hypervariable spacer region.

We conclude that most of the *Salamandra* D-loop appears to be under some sort of constraint and lacks larger regions that are more free to diverge which results in a low overall divergence rate. Accordingly, our estimate of 0.8% divergence per Myr seems credible and forms a solid basis in the following discussion.



**Fig. 5** Structural domains and lengths in bp of primate D-loop regions (after Sbisà *et al.* 1997) and homologous regions of salamander D-loops (Salamandridae and Ambystomatidae), from our data and from Shaffer & McKnight (1996). The intergenic spacer that lies between the *cytb* and the t-RNA<sup>PRO</sup> (shaded) has so far been found only in the Ambystomatidae (McKnight & Shaffer 1997). The dark boxes denote extended termination associated sequences (ETAS), as well as conserved sequence blocks (CSB), as defined by Sbisà *et al.* (1997), which exist in addition to the known conserved central domain (coarsely hatched). The white boxes are the regions that are more free to diverge.

### Species level differentiation

Previous analysis of ribosomal 16S gene sequences with all representatives of 'true' salamanders comprising *Salamandra*, *Mertensiella* and *Chioglossa* has shown that the genus *Salamandra* forms a well supported monophyletic group (Veith *et al.* 1998). However, no significant resolution was obtained for the different *Salamandra* taxa in this analysis. Our more extended analysis here provides evidence that *Salamandra* now has to be split into at least six definite species, namely *S. salamandra*, *S. algira*, *S. infraimmaculata*, *S. corsica*, *S. atra* and *S. lanzai*. These come out as clear monophyletic units supporting previous inferences from protein and allozyme data (Gasser 1978; Joger & Steinfartz 1994a; Veith 1994). A revision of the concept of *Salamandra* being a single polytypic species (Eiselt 1958; Klewen 1991) seems therefore mandatory.

Using our molecular clock estimate we can infer the approximate divergence times of the different taxa. *Salamandra* would have diverged from the sister group *Mertensiella* 25 Ma. The Near East species *S. infraimmaculata* would have separated from the rest 13 Ma. The African species *S. algira* would have separated from the European *S. salamandra* 8 Ma. If this value is correct, it would suggest that they had already been separated before the flooding of the strait of Gibraltar, 5.3 Ma (Riding *et al.* 1998). An alternative geological event that could have been the reason for the separation of these species is the expansion of the northern Betic Sea (Maldonado 1985) which could have resulted in a northern clade *S. salamandra* and a southern clade *S. algira*. This expansion of the sea is also supposed to have caused the first basal split in midwife toads (genus *Alytes*) leading to the separation of *A. cisternasii* and proto-*A. obestricans* (Arntzen & Garcia-Paris 1995).

*S. corsica* and *S. atra* would have split 5 Ma. This fits with the time of rapid desiccation of the Mediterranean

5–6 Ma (Hsü *et al.* 1977; Berggren *et al.* 1995) where colonization of Corsica could have become possible for amphibians due to the dramatic environmental change in the Mediterranean towards freshwater characterized habitats (Hsü *et al.* 1977; Bianco 1990).

In conclusion, even if our clock calibration is wrong by a factor of five (which is very unlikely to be the case), the separation of the major *Salamandra* species predates the pleistocene glaciation cycles, as was similarly inferred for several other species for which molecular data are available (Hewitt 1996; Taberlet *et al.* 1998).

### Subspecies level differentiation

Almost all of the well separated species discussed above can be further subdivided into subspecies on the basis of morphological and coloration characters. These subspecies assignments have sometimes been contentious because they were supported by rather different levels of rigour. Nevertheless, the molecular data support most of these assignments. One of the most notable ones is the clear difference that we see between *S. atra atra* and *S. a. aurorae*. The latter live in a similar montane habitat to the former, but occupy only a small region in the Dolomite mountains located at the edge of the species distribution area, which does not appear to overlap with the *S. a. atra* range (Grossenbacher 1994, personal communication). They differ from the former by a conspicuous yellow mark, but are otherwise morphologically very similar (Trevisan *et al.* 1981). Thus, they might constitute only a very recently, possibly postglacially, derived variety. However, the molecular analysis shows that they are very different from *S. a. atra* and should be considered their sister group. The molecular distance indicates that they have been separated since 0.7–1.1 Ma indicating that they must have recolonized the Alpine area independently after the last glaciation. In contrast, the other *S. a. atra*

lineages, from across the Alps, group more closely together including the former subspecies *S. a. prenjensis* from Bosnia.

The *S. infraimaculata* assemblage shows a rather heterogeneous distribution. Only the subspecies *S. i. orientalis* and the three individuals from Malatya (sampling location 35) come out as well supported monophyletic units. The two Iranian animals which are considered to belong to the subspecies *S. i. semenovi* are very closely related to animals of unassigned status (*S. i. ssp.*) from eastern Turkey. Further clades within this group include an assemblage of animals from Israel and an assemblage of animals from central Turkey to the Mediterranean coast (Fig. 1). If one considers that the molecular clock suggests that these assemblages have been separated between 1.3 and 4.6 Myr, one could propose assigning them a higher taxonomic status. This will be further discussed elsewhere (Steinfartz *et al.* in preparation).

A similar situation might exist for the *S. algira* taxa. The two animals from Morocco are very different from the one from Algeria, suggesting a separation time of at least 6–7 Myr.

In contrast to this, all animals of *S. lanzai* show very similar haplotypes, in spite of sampling in two different areas. This species seems to consist of a single population which may have expanded only relatively recently from a small founder population. As the different haplotypes differ at only three positions by one nucleotide at most, one could even propose that this may have occurred after the last glaciation.

The *S. salamandra* taxa are more diverse. The clade inhabiting a range in the south of the Iberian Peninsula (*S. s. longirostris*, *S. s. morenica*, and *S. s. crespoides*) comes out as a reasonably well separated unit of about 2–4 million years of age from the remaining *S. salamandra* clades. The validity of this southern clade and the basal position of *S. s. longirostris* were also supported when more individuals from different populations were compared on the basis of cytochrome *b* sequences (Garcia-Paris *et al.* 1998).

The second separable unit within *S. salamandra* concerns populations from northern Spain (subspecies *S. s. bernardezi*) together with a population from southern Italy (*S. s. gigliolii*—note that one animal named as *S. s. gigliolii* which came from further north, location 23 (Naples) does not fall into this group). This group is solidly supported (Fig. 2), and it was also suggested on the basis of the analysis of serum proteins (Joger & Steinfartz 1994b). We interpret them as remnants of a former population that had covered the whole of Central Europe in a previous interglacial period (see below).

Although the taxonomic category of a subspecies has sometimes been questioned it appears, nonetheless, that the molecular data support these assignments very well. The sequences of assigned subspecies always fall into

monophyletic groups of the whole species and form often separate units within these groups. Thus, molecular and morphological data conform very well and one might, in the long term, consider assigning species status to the so far discussed subspecies.

On the other hand, the sequences of the subspecies falling into the *S. salamandra* group C (Fig. 2) form a single large, essentially nonresolved polytomy, indicating that they are derived from a single founder population which has expanded only relatively recently. In this case it may be warranted to consider them as subpopulations, although some of them have been assigned subspecies status as well. Nevertheless, as the genetic and morphological differentiation between these is still ongoing, they may in fact constitute the most interesting group for future studies on the mechanisms of speciation. This is particularly so in the context of the ice age cycles, which requires a more detailed discussion.

#### Subpopulation differentiation

Between the group C haplotypes of *S. salamandra* we find on average 2.24 substitutions, most of which are independent. Applying our clock estimate, we should expect no more than one substitution per 175 000 years. Thus, the founder population that has given rise to group C would have existed approximately 400 000 years ago. This would suggest that the current population had already successfully colonized Central Europe in previous interglacial periods. With the beginning of new glaciations, they would have been forced back into refugial areas in Southern Europe as well as the Balkans, from where recolonizations would have started again. This scenario would explain why eastern and western populations harbour different (albeit closely related) haplotypes. Furthermore, it is also in line with the results of the allozyme studies, which indicate the existence of specific eastern and western alleles (Fig. 3).

This finding could imply that only the animals from the C population had been capable of recolonizations after the retreat of the ice. However, the intriguing distribution of the B population suggests that this has not always been so. This pattern can only be explained if one assumes that it was previously the B population that has colonized the whole of Central Europe in one or more interglacial periods. The now existing remnants in Northern Spain and Southern Italy would thus constitute refugial populations that were unable to spread further. The reason for this is unclear, but it seems likely that it was a new competitive advantage acquired by the C population that has caused this dramatic shift in colonizing ability.

A more detailed study of populations in northern Spain using allozymes (Alcobendas *et al.* 1996) and mitochondrial RFLP patterns (Dopazo *et al.* 1998) supports the

notion of a special status of *S. s. bernardezi* populations (equivalent to part of the B population in our study) vs. the *S. s. gallaica*, *S. s. fastuosa* and *S. s. terrestris* populations (equivalent to part of the C population in our study). These previous results also show that there is practically no mixing of animals between the C and B populations and that possible hybrids can only be found at the borders of the distribution range. Intriguingly, the area occupied by the *S. s. bernardezi* animals is not geographically or ecologically distinct from the areas occupied by the other populations. Thus, it is apparently not a geographical isolation that makes these populations different. Therefore, one has to assume behavioural reasons for this differentiation, for example a tendency for assortative mating and/or territory defence. Another potential reason for this, namely very low migration ability, can now be ruled out. Animals of the B population must have been able to spread in previous times and it is not evident that they should not be able to do so nowadays as well.

The patterns found for the populations that were not directly affected by the glaciation periods, i.e. the African and Near East populations support the notion of an active process of maintaining population identity. Each of these populations appears to have remained in its geographical setting for a long time, as the mitochondrial lineages are well resolved. Previously one would have proposed that this separation is due to geographical barriers or very slow dispersal. But as we now see that salamanders must be able to migrate far distances, even under complex geographical settings, it seems much more likely that maintaining population integrity in a particular area is an active process, involving also the displacement of potentially invading animals.

## Conclusion

The detailed study of *Salamandra* populations in Europe has yielded intriguing insights into phylogenetic relationships and interglacial recolonization patterns. The results show that the territories occupied by the different populations are most likely actively maintained and can not be easily invaded by conspecific populations. Accordingly, extensions of expansion ranges can only occur under conditions where new territories can be occupied, i.e. in interglacial periods. Most intriguingly, a specific source population appears to have been most successful at a former time while a different, but closely related, source population has been successful at later times and has largely displaced the first one. These observations are highly relevant to the question of how new species arise and how they maintain their distinct status. A more detailed study in carefully chosen regions is, therefore, likely to provide deep insights into speciation processes of terrestrial vertebrates.

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This work represents the Diploma thesis of Sebastian Steinfartz, who has studied the populations and collected the samples during the past decade and has carried out the molecular analysis in the laboratory of Diethard Tautz in Munich. Michael Veith has designed the primers for amplifying the D-loop region and has contributed the data of the allozyme study. Diethard Tautz has now taken up a chair in Evolutionary Genetics at the University of Cologne and Sebastian Steinfartz is continuing this work as a PhD in Cologne on the question of speciation in salamanders.

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## Appendix I

Names of locations from which individuals were sampled. Synonyms describing former subspecies are given in brackets.

### *Salamandra algira* (Africa)

1. *S. algira* ssp. Chefchauen, Rif-mountains, northern Morocco.
2. *S. algira* ssp. Annaba, northern Algeria.

### *Salamandra salamandra* (Europe)

3. *S. s. longirostris*: Sierra de Ronda, south of the Rio Guadalquivir, Spain.
4. *S. s. morenica*: Sierra Morena, Spain.
5. *S. s. crespoi*: Serra do Monchique, Portugal.
6. *S. s. gallaica*: Setubal, Portugal.
7. *S. s. gallaica*: Serra de Sintra, Portugal.
8. *S. s. gallaica*: Serra de Grandola, Portugal.
9. *S. s. bejarae*: near Madrid, Spain.
10. *S. s. almanzoris*: Sierra de Gredos, Spain.
11. *S. s. bernardezi*: Oviedo, Spain.
12. *S. s. bernardezi*: Pico de los Cuadrazales, Spain.
13. *S. s. fastuosa (bonmalli)*: Cauterets, Pyrenees, France.
14. *S. s. fastuosa*: Pyrenees, France.
15. *S. s. fastuosa*: Bagnères-de-Bigorre, France.
16. *S. s. bejarae (hispanica)*: Monseny, near of Barcelona, Spain.
17. *S. s. terrestris*: Bordeaux, France.
18. *S. s. terrestris*: near of Torino, Italy.
19. *S. s. gallaica*: Serra do Geres, Portugal.
20. *S. s. terrestris*: Edertalsperre, Germany.
21. *S. s. terrestris*: Spessart, Germany.
22. *S. s. terrestris*: Taunus, Germany.
23. *S. s. giglioli*: near Naples, Italy.
24. *S. s. salamandra*: Tecino, Italy.
25. *S. s. giglioli*: Calabria, Italy.
26. *S. s. salamandra*: Loibl-pass, Slovenia.
27. *S. s. salamandra*: Biokovo-mountains, Bosnia.
28. *S. s. salamandra*: Pindos-mountains, Greece.
29. *S. s. terrestris*: Schwarzwald, Germany.
30. *S. s. salamandra (werner)*: Peleponnes, Greece.
31. *S. s. beschkovi*: Bulgaria.

### *Salamandra corsica* (Island of Corsica)

32. *S. corsica*: Foret de Valdo Niello, Corsica (France).
33. *S. corsica*: Col de la Bavella.

### *Salamandra infraimmaculata* (Near East)

34. *S. i. orientalis*: Findikpinar, near Adana, Turkey.
35. *S. i.* ssp. Aslantepe, near Malatya, Turkey.
36. *S. i.* ssp. Kemaliye, near Erzurum, Turkey.
37. *S. i.* ssp. Bitlis, near the Van lake, Turkey.
38. *S. i. semenovi*: Sarvabad, province of Kurdistan, Iran.
39. *S. i.* ssp. Iskenderum, Turkey.
40. *S. i.* ssp. Antakya, Turkey.
41. *S. i.* ssp. Tel.: Dan, Israel.
42. *S. i.* ssp. Kibutz Sasa, Israel.

### *Salamandra lanzai* (south-western (Cottian) Alps, Europe)

43. *S. lanzai*: Mon Viso, Italy.
44. *S. lanzai*: Germanasca valley, Italy.

### *Salamandra atra* (central and eastern Alps, Europe)

45. *S. a. atra*: Berner Oberland.
46. *S. a. atra aurorae*: Bosco del Dosso, near Asiago, Italy.
47. *S. a. atra*: Tambre, near Belluno, Italy.
48. *S. a. atra*: Mittenwald, near Garmisch, Germany.
49. *S. a. prenzensis*: Prenj-mountains, Bosnia.