

Tracing the first step to speciation: ecological and genetic differentiation of a salamander population in a small forest

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Abstract

Mechanisms and processes of ecologically driven adaptive speciation are best studied in natural situations where the splitting process is still occurring, i.e. before complete reproductive isolation is achieved. Here, we present a case of an early stage of adaptive differentiation under sympatric conditions in the fire salamander, *Salamandra salamandra*, that allows inferring the underlying processes for the split. Larvae of *S. salamandra* normally mature in small streams until metamorphosis, but in an old, continuous forest area near Bonn (the Kottenforst), we found salamander larvae not only in small streams but also in shallow ponds, which are ecologically very different from small streams. Common-environment experiments with larvae from both habitat types reveal specific adaptations to these different ecological conditions. Mitochondrial and microsatellite analyses show that the two ecologically differentiated groups also show signs of genetic differentiation. A parallel analysis of animals from a neighbouring much larger forest area (the Eifel), in which larvae mature only in streams, shows no signs of genetic differentiation, indicating that gene flow between ecologically similar types can occur over large distances. Hence, geographical factors cannot explain the differential larval habitat adaptations in the Kottenforst, in particular since adult life and mating of *S. salamandra* is strictly terrestrial and not associated with larval habitats. We propose therefore that the evolution of these adaptations was coupled with the evolution of cues for assortative mating which would be in line with models of sympatric speciation that suggest a co-evolution of habitat adaptations and associated mating signals.

Keywords: adaptive speciation, amphibians, pond reproduction, population differentiation, *Salamandra salamandra*, sympatric speciation

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Introduction

Spatially structured environments should provide an opportunity for rapid differential adaptations in local populations (Schluter 2001). However, such adaptations would not be expected to develop under conditions of residual gene flow, unless some form of restricted mating among differentially adapted genotypes takes place

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(Coyne & Orr 2004). This notion is of particular interest in the context of models of adaptive speciation (Dieckmann *et al.* 2004). Dieckman & Doebeli (1999) have shown that intraspecific competition can lead to disruptive selection and adaptation to alternative local environmental resources, if these are available. In sexually reproducing populations, their model requires that assortative mating evolves in parallel, to genetically stabilize the differential adaptation. Under appropriate conditions, this adaptive and genetic divergence of sympatric populations can occur relatively quickly, in the order of hundreds to thousands of generations (Doebeli 1996). However, this depends on somewhat restrictive parameters. If these parameters are not met, one expects a fluctuating situation in which partially adapted genotypes may merge repeatedly which can result in long time periods until differential adaptation and speciation is

complete (Bolnick 2004). On the other hand, environmental gradients and spatial structure could facilitate adaptation and genetic splitting (Doebeli & Dieckmann 2003).

Under a parapatric mode of speciation, spatial structuring leads passively to a restricted gene flow between subpopulations. Under this model, genetic drift and an environmental gradient will enhance the geographical variation and can lead to reproductive isolation between geographically distant subpopulations (Gavrilets *et al.* 2000). Accordingly, the parapatric mode of speciation requires neither disruptive selection nor assortative mating to explain species divergence. Parapatric models can also lead to rapid speciation within a few hundred to a few thousand generations, depending on model parameters such as size of the subpopulations, migration rate between subpopulations and the mutation rate (Gavrilets 2003).

Thus, both the adaptive mode of speciation under sympatry and the parapatric mode of speciation could potentially explain recently established diversification in structured environments. Although it is accepted that situations of postglacial re-invasion provide ideal cases to test for the spatial pattern of speciation (e.g. sticklebacks in postglacial lakes in Canada; Schluter 1995), it remains a challenge to identify an area in which such model predictions can be assessed in a natural context (Tautz 2004). Here, we analyse data from mitochondrial D-loop sequences, microsatellite genotypes and common-environment experiments to address the spatial pattern and history of a natural population of fire salamanders where differential adaptations to alternative larval habitats have recently evolved.

Previous studies showed that populations of *Salamandra salamandra* in West Germany are derived from a single recolonization wave after the last glaciation approximately 6000–8000 years ago (Steinfartz *et al.* 2000; Weitere *et al.* 2004). Adult *S. salamandra* are typically found in high frequencies in old broadleaf forests that provide shelter and small streams that serve as larval habitats until metamorphosis is completed. After metamorphosis, the adult life stages, including mating, are completely terrestrial and are not attached to the aquatic habitat in which larval development occurred. The animals reach sexual maturity in their third to fifth year on land (Seifert 1991; Thiesmeier & Grossenbacher 2004). Mating occurs in spring to early summer and eggs are internally fertilized in the mother's oviduct. The zygotes develop into complete larvae within the mother, and in spring of the following year, these are deposited into suitable aquatic habitats.

Our study sites include two old forest areas (the Eifel and the Kottenforst) and some smaller forest patches surrounding them (Fig. 1). The Eifel is an old volcanic area with many steep slopes and small streams. The Kottenforst, on the other hand, lies on an uplifted plateau that is part of the old Pleistocene Rhine terrace and harbours many small temporary ponds. We found that in the Kottenforst, both streams and

ponds are used as larval habitats by salamanders. However, small temporary ponds represent an atypical larval habitat for *S. salamandra* in Central Europe (Thiesmeier & Grossenbacher 2004) and a consistent occurrence of salamander larvae in such ponds is rather unique to this area.

We previously studied such pond larvae from a habitat near Königsdorf, which is located north of, but was in former times connected to, the Kottenforst (Fig. 1a). We found that these larvae display several adaptations, including higher weight at larval deposition, capacity to cope with lower quality food and an early metamorphosis under limited food conditions. All these are adaptations that allow them to survive in this ecologically very different environment (see Weitere *et al.* 2004 for further details of the associated ecological conditions).

The finding of such a new habitat adaptation in a locally confined region suggests that this is a case where an adaptive population splitting process is underway. We have therefore focused our current study on this area in order to explore under which spatial scenario the splitting process occurred, and infer from this the mechanisms involved during the early step of adaptive speciation.

Materials and methods

Study sites and samples

The study area is located in North Rhine–Westphalia near Cologne (Fig. 1a). Samples were taken from 33 locations in the Eifel, the Kottenforst and surrounding forest patches (see Appendix for geographical coordinates and sample sizes per location). The Eifel and the Kottenforst each represent continuous forests of different sizes without major spatial barriers that would hamper migration of adult salamanders. In the Eifel, larvae were exclusively found in permanent streams, whereas in the Kottenforst, larvae of *Salamandra salamandra* can be found in small steep streams and in small temporary ponds and pond-like water bodies, which eventually dry up in summer. Close to the Kottenforst, larvae were also found in ponds at location KoO and in Königsdorf (Koe). DNA extraction was carried out as described in Steinfartz *et al.* (2000).

Mitochondrial D-loop analysis

The phylogeographical colonization pattern of *S. salamandra* for the study area was inferred by sequence analysis for a representative subset of 18 localities — including habitat sites within the Kottenforst, the Eifel and additional populations. The whole mitochondrial D-loop was sequenced (758 bp) as described in Steinfartz *et al.* (2000). D-loop haplotypes were obtained for 438 individuals. Classification of haplotypes into postglacial recolonization lineages followed Weitere *et al.* (2004).

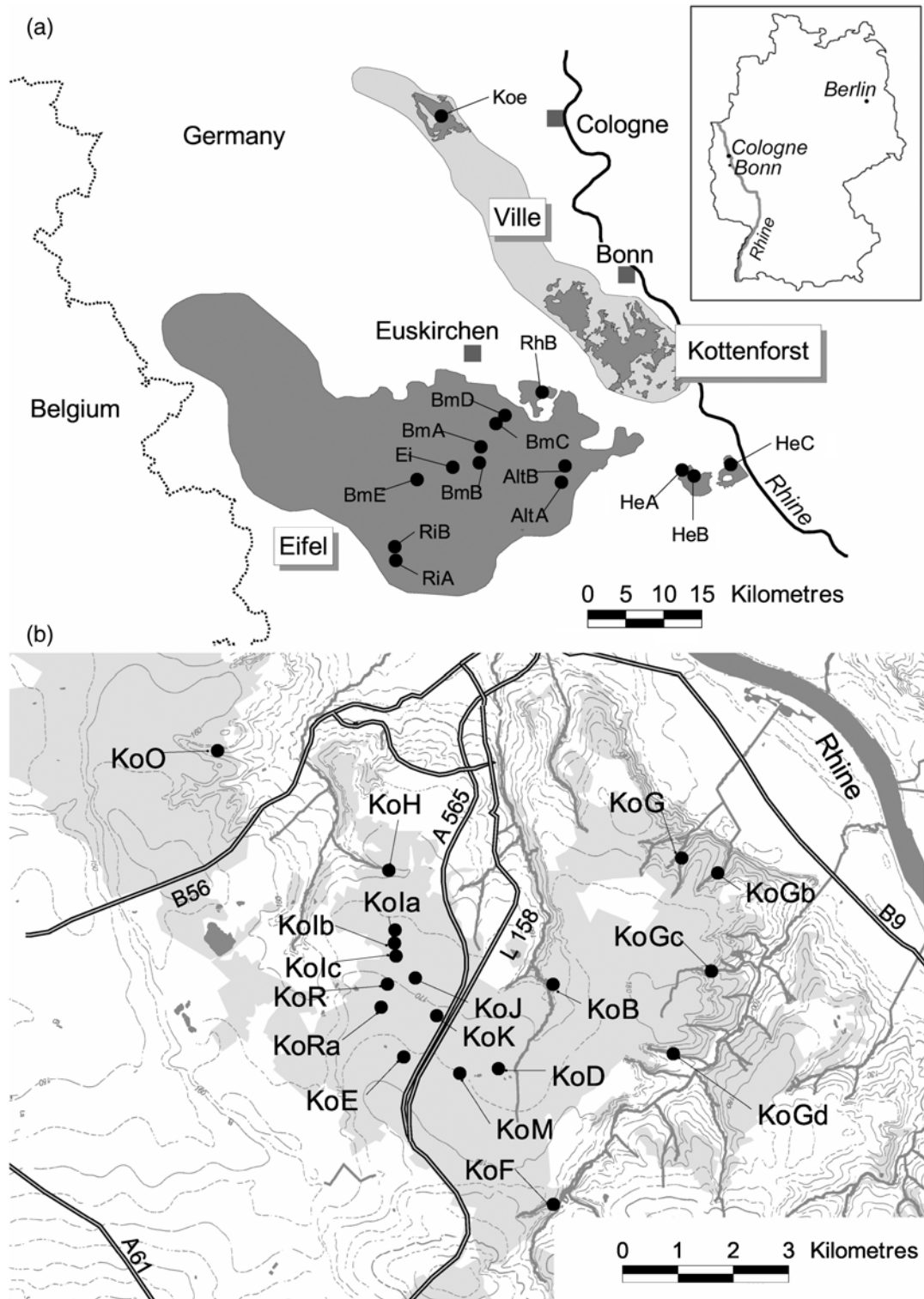


Fig. 1 Sampling locations of *Salamandra salamandra* larvae in the study area; see Appendix for exact coordinates of sampling locations. (a) Sampling locations in the Eifel and surrounding forest patches. The general distribution of possible habitats for adult salamanders in the sampled regions is shown in dark grey, reflecting the forest areas in which they can roam freely. The former extent of old broadleaf forest in the area of the Ville is shown in light grey according to historic maps (Landesvermessungsamt 2005). (b) Sampling localities within the Kottenforst [enlarged section of (a)]. Note that the highway running through the forest had so far no effect on population differentiation, although it is probably an effective migration barrier since about 40 years.

Microsatellite analysis

A total of 1054 individuals (larvae) from 33 sites (ponds or specific stream locations) were genotyped for 11 microsatellite loci. Locus characteristics, amplification of loci and scoring of alleles are described in Steinfartz *et al.* (2004). Chord distances between the 33 sampling sites were calculated with the program MICROSAT 1.5 (Minch *et al.* 1995) and the program PHYLIP 3.65 (Felsenstein 1995) was used to visualize these as a tree.

We inferred the genetic structure on the level of sampling locations with a Bayesian genetic mixture analysis using the implemented option of the program BAPS 3.2 (Corander *et al.* 2003) to cluster groups of individuals, rather than the individuals themselves. In our case, 'groups' represent all individuals from a given sampling location. The optimal number of clusters identified by BAPS 3.2 were selected from a list of 10 best visited partitions according to their log(ml) values (see Corander *et al.* 2003). We conducted also an individual-based cluster analysis with the program STRUCTURE 2.1 (Pritchard *et al.* 2000) using the following run parameters: discrete model of ancestry, correlated allele frequency model, a burn-in period of 100 000 simulations followed by a run length of 2 million Markov chain Monte Carlo simulations and five iterations for each K . Estimation of K was based on the computed posterior probabilities for each K following Bayes' rule (Pritchard *et al.* 2000) for a specific value of K .

Finally, we tested for the differentiation of populations by applying an individual-based self-assignment test using the program GENECLASS 2 (Piry *et al.* 2004) and the implemented Bayesian assignment approach of Rannala & Mountain (1997).

Ecological differentiation

We used the common-environment approach that we developed previously (Weitere *et al.* 2004) for the phenotypic characterization of larvae from pond and stream habitats in the Kottenforst, i.e. larvae from the different habitats were subjected to the same laboratory conditions for their further development. We measured time until and weight at metamorphosis under limited food conditions because larvae experience a lower average food level and a higher desiccation risk in ponds than in streams (Weitere *et al.* 2004). Larvae not older than 15 h (streams and ponds were monitored every 12 h for new larvae at the beginning of the reproduction period of the year 2002) were collected from eight different locations for the common-environment experiments. Most larvae were collected immediately after the deposition in direct proximity to their mother. Thus, larvae were exposed to their typical environment for a brief period. We used this

sampling procedure to ensure that females did in fact deposit their larvae in the particular environment rather than moving on to another location. Five locations (KoH, KoM, KoD, KoIa and KoJ) represent typical ponds that dried up early during the year. Two locations (KoB and KoF) are somewhat atypical ponds connected to shallow, nonpermanent streams that dried up later in the year; we categorize these as intermediate habitat or pond-like habitat. One location (KoG) was a permanent stream with a strong water flow during spring and a constant water flow during the rest of the year. To avoid possible sibling effects, only one larva per gravid female or larvae from different sampling spots within the ponds and streams were taken. Larvae were kept individually in vessels (20 × 9.5 cm) with a 9-cm water level at a constant 20 °C. The vessels with larvae from the different origin were mixed randomly within the climate room in order to avoid spatial effects within the room (such as small possible differences in the temperature or light intensity). The larvae were fed one 12 ± 1 mm long *Chironomus* larva daily, which corresponds to an energy value of approximately 50 J. The size of the salamander larvae at the beginning of the experiments, the size and weight of the young salamanders after completing the metamorphosis (determined by a total reduction of gills) and the time of the total larval period until completion of metamorphosis were recorded.

Results

To assess the population structure of *Salamandra salamandra* in the Eifel and the Kottenforst, we sampled more than a thousand larvae from streams and ponds within these forests. We focused on larval sampling, because larvae are unequivocally associated with habitat types, which is not the case for adult animals. Adult fire salamanders are often considered to display strong site fidelity to a restricted area (Thiesmeier & Grossenbacher 2004). However, our parallel study with transponder-marked animals in a similarly structured neighbouring forest indicates that fire salamanders use much larger home ranges (Schulte *et al.* 2007) and migratory activity is much higher (Schmidt *et al.* 2007) than hitherto thought. Hence, for any given adult salamander caught in the forest, it would not be possible to infer its larval history. Additionally, mating is strictly terrestrial and does not occur in or in close proximity to the larval habitats. Thus, only direct larval sampling can provide clues about habitat-specific adaptations in this setting.

Phylogeography of the study area

Out of 438 individual D-loop sequences of salamander larvae from the Eifel, the Kottenforst and the surrounding

Table 1 Frequencies of three D-loop haplotypes (Type Ia, b and c) found in the study area

Population/site	Type Ia	Type Ib	Type Ic	N
Eifel				
RiB	0.03	0.97	—	33
RiA	0.14	0.86	—	37
BmA	0.38	0.62	—	21
BmB	0.58	0.42	—	24
BmC	0.55	0.45	—	31
BmD	0.42	0.58	—	26
BmE	0.09	0.91	—	11
Ei	0.34	0.66	—	38
Kottenforst				
KoB	0.96	0.04	—	25
KoF	0.7	0.3	—	23
KoG	0.78	0.22	—	37
KoH	0.92	0.08	—	13
KoIa	1	—	—	23
KoIb	1	—	—	13
KoJ	1	—	—	12
KoD	0.9	0.1	—	10
KoK	1	—	—	11
Other locations				
Koe	0.85	—	0.15	33
KoO	1	—	—	17

forest patches only three different haplotypes were detected. Each matched one of the previously identified types Ia–Ic of the western postglacial recolonization lineage (Table 1). These differ only at single nucleotide positions, which is in line with our previous conclusion based on phylogeographical assessment that our study area was colonized by a single postglacial recolonization wave (Steinfartz *et al.* 2000; Weitere *et al.* 2004). This most likely occurred about 6000–8000 years ago, namely at a time when the first pollen records for *Fagus sylvaticus*, the character tree of the natural habitat of *S. salamandra* in Middle Europe, are found in this part of Germany (Frenzel & Gliemer 1995). This would represent up to 2000 generations given an average generation time of 4 years.

Haplotype frequency differences exist between the sampling sites in the Eifel and the Kottenforst. While type Ib is most frequent in the Eifel, type Ia is most frequent in the Kottenforst (Table 1). In Königsdorf (location Koe) type Ib is completely missing and only type Ic is found. Historical maps from the early 18th century (Landesvermessungsamt 2005) show that the Kottenforst and Königsdorf were at that time connected by a continuous deciduous forest, while no forest connection existed to the Eifel (see Fig. 1a). Possible migration routes between the Kottenforst and Königsdorf would have been interrupted about 100 years ago, when strip mining eliminated the forest areas of major parts of the Ville.

Table 2 Average likelihoods for number of genetic clusters based on the program STRUCTURE 2.1 (Pritchard *et al.* 2000) for 508 individuals from the Kottenforst (upper) and 449 individuals from the Eifel (lower) for different values of K from 1 to 5. The probability of K is given as the arithmetic mean plus standard deviation (SD). Following Bayes' Rule the probability (PrK) for $K = 2$ was estimated to be 1 in the Kottenforst and $K = 1$ in the Eifel

K (number of clusters)	$\ln Pr(X K)$ (SD)	PrK
Kottenforst		
1	-12967.38 ± 0.05 SD	0.00
2	-12923.6 ± 6.55 SD	1.00
3	-13027.38 ± 47.17 SD	0.00
4	-12977.88 ± 49.43 SD	0.00
5	-13019.86 ± 46.25 SD	0.00
Eifel		
1	-7661.3 ± 0.0 SD	1.00
2	-7682.6 ± 8.10 SD	0.00
3	-7741.2 ± 19.95 SD	0.00
4	-8090.48 ± 158.26 SD	0.00
5	-8303.45 ± 22.0 SD	0.00

Population genetic structure

In order to assess the patterns of gene flow and population differentiation, we genotyped more than 1000 larvae from the study area for 11 microsatellite loci. We used a Bayesian analysis of genetic differentiation between populations (Corander *et al.* 2003) to assess the genetic relationships between sampling locations. This analysis suggests that there are six genetic clusters in the area, three larger ones covering the Eifel and the Kottenforst and three smaller ones in the surrounding forest patches (Fig. 2a). All sampling locations in the Eifel (represented by 449 larvae from 13 sampling sites) fall into a single genetic cluster whereas sampling sites in the much smaller Kottenforst (represented by 508 larvae from 16 sampling sites) fall into two clusters. Intriguingly, the two clusters in the Kottenforst correlate with larval habitat types, i.e. pond and stream types (Fig. 2a).

When an individual-based cluster analysis (Pritchard *et al.* 2000) is used, we find a single cluster for the Eifel and two clusters for the Kottenforst as optimal partitions (Table 2), thus confirming the results from the sampling location analysis.

A neighbour-joining tree based on Chord distances reveals the same groupings and shows furthermore that the two Kottenforst clusters are most closely related to each other (Fig. 2b).

With individual-based self-assignment tests, we find that 823 out of 1067 individuals (77%) are correctly assigned to their respective clusters (Fig. 3). Within the Kottenforst, 228 out of 331 larvae originating from pond habitats (70%) were assigned to the genetic *Kopond* cluster

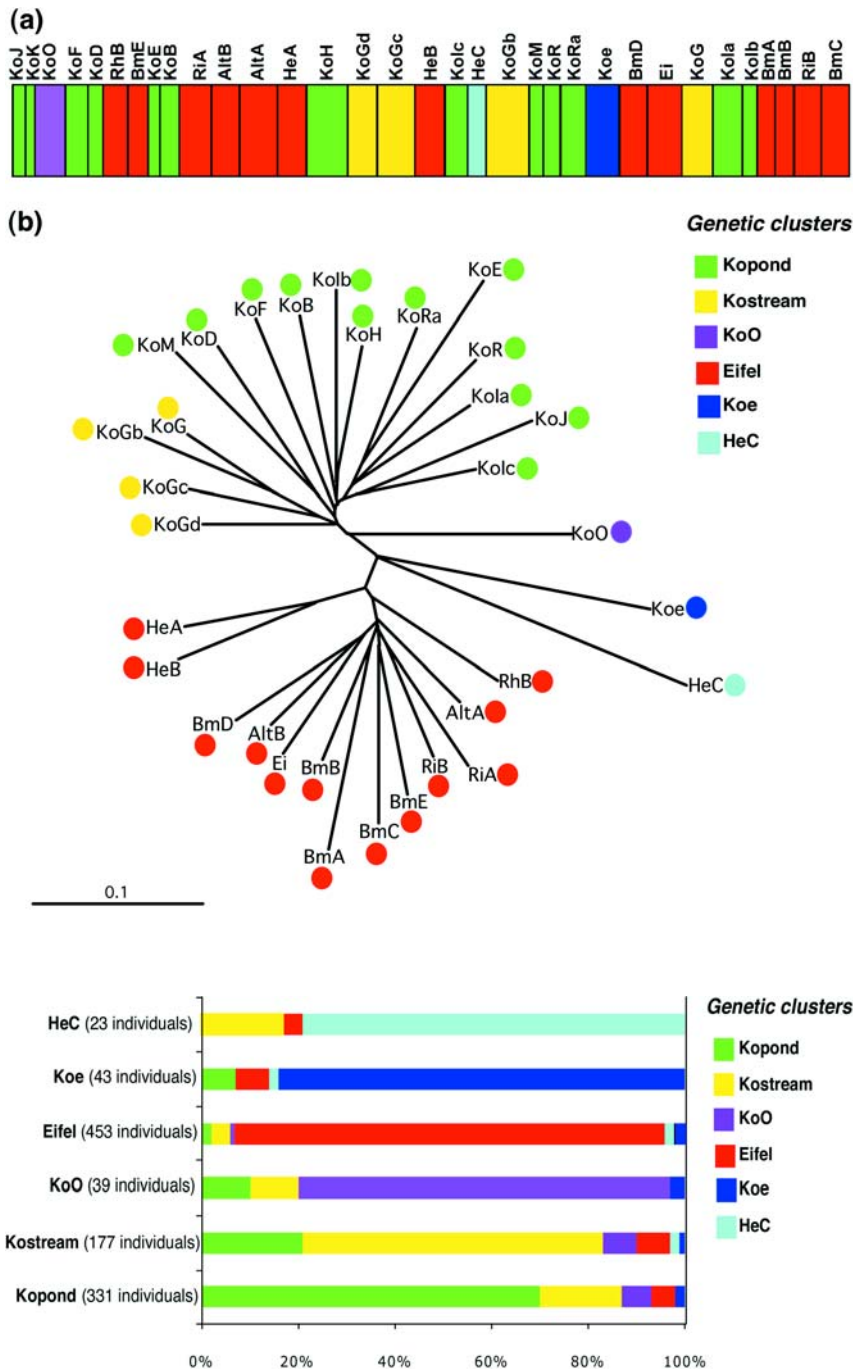


Fig. 2 Cluster analysis and distance analysis of the microsatellite data. (a) Unordered Bayesian cluster analysis of 33 larval sampling locations in the Eifel, the Ville and the Kottenforst using the program BAPS3.2. Six genetic clusters can be distinguished (Kopond, Kostream, KoO, Eifel, Koe and HeC). Sampling locations that belong to the same genetic cluster share the same colour. The respective clustering of pond and stream locations in the Kottenforst is evident (Kopond and Kostream). (b) Population tree inferred from Chord distances for the 33 sampling locations. The groupings of this tree are supported by the results of the Bayesian cluster analysis. The scale on the bottom indicates 0.1 Chord distance units.

Fig. 3 Self-assignment test of 1054 individuals grouped according to the genetic clusters shown in Fig. 2. The proportion of assigned individuals to a respective genetic cluster (Kopond, Kostream, KoO, Eifel, Koe and HeC) is indicated by bars in the same colour.

and 109 out of 177 larvae (62%) from the stream habitats were assigned to the genetic *Kostream* cluster. Interestingly, the majority of the seemingly wrongly assigned larvae were still assigned to the respective other Kottenforst cluster, rather than to an outside cluster (Fig. 3). This confirms the close genetic proximity between the *Kopond* and *Kostream* cluster.

Although the statistical limitations of the approach would only seldom lead to a complete assignment, the

number of incorrectly assigned individuals seems still relatively high. Within the Kottenforst, there is the possibility that the larvae may not always have been deposited in their optimal habitats. Although one might expect that females would deposit their larvae preferentially in the habitat for which they are adapted, some larvae might end up in a habitat that is not optimal for them. This is not unexpected, since it is known that females of *S. salamandra* tend to drop single larvae of their offspring on their

way to their preferred deposition sites (Thiesmeier & Grossenbacher 2004). However, seemingly incorrect assignments also occur between the major forests and the forest patches, although regular migration between them is unlikely. Thus, the pattern of incomplete assignment is more compatible with the assumption that the differentiation has occurred recently and that the coalescent process is not yet complete.

Habitat-specific adaptation

We performed common-environment experiments to obtain a direct estimate of the proportion of larvae with specific adaptations in the respective habitats. In our previous study (Weitere *et al.* 2004), we found that under limited food conditions, salamander larvae from ponds metamorphose much earlier and with a lower weight than larvae from permanent stream habitats. This can be interpreted as an adaptation to unfavourable conditions such as a high risk of desiccation and low food supply in the ponds. The stream larvae, on the other hand, normally experience a continuous supply of food until they reach an optimal size for metamorphosis. They therefore have a prolonged larval period and are larger at metamorphosis (Weitere *et al.* 2004). We used the same type of common-environment experiments as in our previous study, applied to the Kottenforst animals. The larvae were taken directly at the time of deposition (see Methods), to eliminate effects of habitat-specific selection. Among 76 larvae kept under limited food conditions, 51 showed early metamorphosis (time until metamorphosis < 130 days) and 25 late metamorphosis (time until metamorphosis > 200 days) (Fig. 4). All but one individual from the permanent stream (location KoG) showed a late metamorphosis, while most animals from the typical ponds showed an early metamorphosis. Two locations with shallow streams that are ecologically not very different from ponds (locations KoB and KoF) had about equal proportions of early and late metamorphosing larvae (Fig. 4b). This suggests that at least some females are opportunistic with respect to dropping their larvae in the appropriate habitat.

We note that there were no larvae with an intermediate metamorphosis timing. All showed either the early or the late response (see Fig. 4a). If this response represents a complex genetic trait, one might expect that intermediate forms would be found if individuals of the lineages hybridize with each other. In our previous experiments with the animals from Königsdorf, we did indeed find three larvae with an intermediate metamorphosis reaction (Weitere *et al.* 2004), i.e. they appear to exist in principle. However, slight experimental differences between these experiments, which were conducted in different years, might also account for the absence of intermediate types.

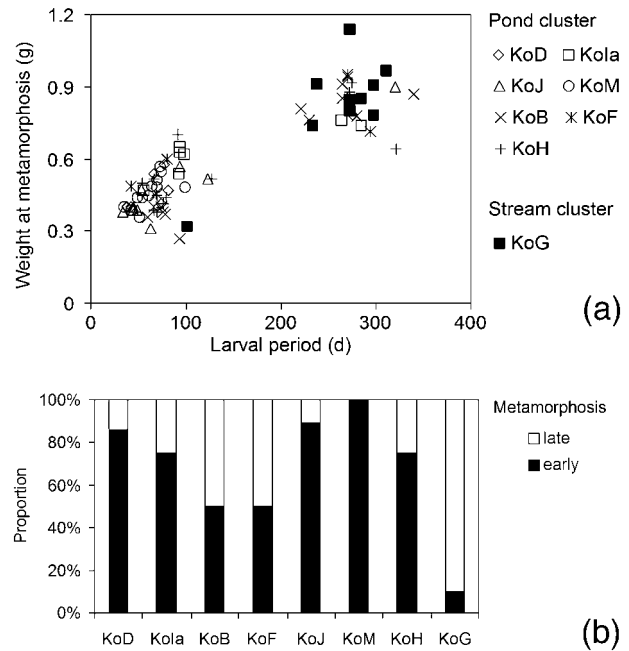


Fig. 4 Results of the common-environment experiments under limited food conditions. Seventy-six individual larvae from eight different locations were used in the experiment. (a) Metamorphosis time and weight were recorded. It is evident that two types exist, namely those that metamorphose early with a low weight and those that metamorphose late with a high weight. Most animals from the permanent stream habitat KoG metamorphose late, while most larvae from the five typical pond locations KoD, Kola, KoJ, KoM and KoH metamorphose early. Only the larvae from locations KoB and KoF, which correspond to the atypical pond locations, show equal proportions of both types. Note that we found no case with an intermediate response. (b) Proportion of early (time until metamorphosis < 130 days) vs. late metamorphosis (time until metamorphosis > 200 days) for the same individuals according to sampling locations.

Discussion

It has long been known that fire salamanders across Europe and the Near East form many local varieties differing in morphology and reproductive characters. This has led to the term 'polytypic species' (Eiselt 1958; Klewen 1991), although genetic analysis has shown that sometimes deep genetic splits exist between the different varieties (Steinfartz *et al.* 2000; Garcia-Paris *et al.* 2003). *Salamandra salamandra* is outstanding among amphibians concerning its intraspecific diversity of reproductive strategies reaching from lecithotrophic viviparity (i.e. females give birth to many small larvae) to being strictly viviparous (i.e. females produce few, large metamorphosed juveniles) (see Grevén 1998 for a review). This intraspecific evolution of different reproductive modes of *S. salamandra* has recently been discussed as a system to study the establishment of evolutionary novelties in a macro-evolutionary context

(Buckley *et al.* 2007). Thus, rather than depicting *S. salamandra* as a 'polytypic' species, it would be more appropriate to view it as a genus with a particularly high propensity for adaptive differentiation and speciation.

The aim of our study was to identify an area where the earliest stages of an adaptive differentiation in a salamander population take place. The results presented here show that the Kottenforst is indeed such an area. The colonization of *S. salamandra* has occurred relatively recently and the specific larval habitat adaptations and genetic differentiation are still in their early stages. In the following, we will first discuss the spatial scenario for the observed adaptive differentiation and, second, the mechanism that may be involved.

The spatial context

Both, the Eifel and the Kottenforst are continuous forest systems in which movement of adult fire salamanders is not significantly hampered by spatial structures. Taking this into consideration, the presence of two genetically and ecologically differentiated groups of salamanders in the Kottenforst is unexpected, given that in the Eifel only one ecological type and a single genetic cluster is found, although sampling sites in the Eifel are much farther apart from each other than in the Kottenforst. Hence, the differentiation in the Kottenforst is likely to be a result of an adaptation to a specific habitat type in this forest, namely shallow ponds that do not occur in the Eifel in large numbers. Although these ponds are spatially somewhat separated from the streams in the Kottenforst (Fig. 1), the situation can be described as fully sympatric, since one has to consider the migration potential of the adults rather than the locations of larval habitats in this context.

Fire salamanders are terrestrial amphibians that spend only their larval period in the aquatic environment and are strictly terrestrial after metamorphosis. They normally start to reproduce after 3–5 years and can live for more than 20 years (Feldmann 1987; Seifert 1991). They are also quite abundant, with population densities reaching 80–400 individuals per hectare (Seifert 1991; Thiesmeier & Grossenbacher 2004). In a parallel study, we found that females mate with multiple males during a given year and that the offspring is sired by up to three males (Steinfartz *et al.* 2006). Mating is not associated with larval habitats and paternity change between the years, suggesting that males compete with each other for access to females (Steinfartz *et al.* 2006). This reproductive behaviour should enhance genetic exchange, rather than reduce it. We further analysed movement patterns of adult *S. salamandra* in a neighbouring but very similar forest, and found that salamanders use rather large home ranges (Schulte *et al.* 2007) with an estimated emigration/immigration rate of nearly 50% per year (Schmidt *et al.* 2007).

Thus, one can conclude that adult salamanders roam freely within these forests, which is not different from free movement of fish in small lakes for which sympatric conditions are usually assumed (Schliewen *et al.* 2001; Schliewen & Klee 2004). Accordingly, the fact that two genetic clusters occur in the Kottenforst can not be ascribed to passive limitations to dispersal, i.e. allopatric or parapatric models are not adequate to explain the differentiation in this case.

The second important finding is that the two genetically differentiated groups in the Kottenforst are the most closely related ones in the area. This is evident from the distance tree (Fig. 2b), which groups all the Kottenforst sampling sites together, and from the self-assignment analysis (Fig. 3) that indicates that the pond and stream clusters in the Kottenforst share the most recent ancestry. One should normally conclude from this that the area was colonized only once and that the habitat-specific adaptations and concomitant genetic differentiation have occurred within the area. However, the situation is more complex, given that we also find the pond adaptation in Königsdorf (site Koe) north of the Kottenforst. It would therefore also seem possible that the pond adaptation evolved there and that these animals then invaded the corresponding habitats in the Kottenforst. The larger genetic distance of the Königsdorf population could be explained by its smaller size that would increase genetic-drift effects. However, the Kottenforst and Königsdorf were connected until about 150 years ago and the distance between these locations is comparable to the distances of sampling sites in the Eifel (cf Fig. 1). Given that no genetic differentiation is found within the Eifel, it seems safe to conclude that Kottenforst and Königsdorf would have been under conditions of free genetic exchange until recently. Independent of the exact origin of the larval pond adaptation, the different lineages have evidently maintained their separate genetic identities within the Kottenforst, which has been a stable forest area for at least several hundred years and possibly even much longer.

The mechanism for differentiation

Since only a small amount of genetic exchange is sufficient to equalize gene pools, it is necessary to invoke additional mechanisms that can explain the genetic differentiation in the Kottenforst. These mechanisms could include habitat choice by females, a balance between migration and selection, or assortative mating.

During the activity period from March to November, which includes the mating period, salamanders are found across the whole Kottenforst, up to a kilometre away from the next possible larval deposition site. However, for some females of *S. salamandra* it has been shown that they have a tendency to return to the same larval deposition site in

successive years (Thiesmeier 1988). On the other hand, studies within the Kottenforst have shown that females stay only very short times close to the larval habitats (an average of 1.3 days) during early spring for the deposition of larvae (Blab 1986). Larvae are already completely developed at this stage, i.e. mating and fertilization occurred much earlier (during late spring and early summer of the previous year). Thus, despite a possible female preference for larval deposition sites, there is no evidence that mating is associated with sites of larval deposition. It seems therefore reasonable to conclude that female preferences for certain larval habitats cannot explain the genetic differentiation seen in the Kottenforst, at least not as the only causative factor.

Maternal imprinting could be another explanation for habitat choice by females. In this case, one would propose that females retain some signal (imprint) of the habitat in which they grew as larvae which would trigger them to use the same habitat for their offspring. This would indeed be a convincing mechanism for explaining the preferential deposition of larvae seen for some females. However, it would not easily explain the genetic differentiation, since the females would have to use the same imprint for identifying males that have grown up in the matching habitat. Although this cannot be excluded, it does not seem very likely either.

The second possibility for explaining the genetic differentiation in the Kottenforst would be a selection against the alternative phenotypes in the respective habitats. Hendry (2004) has shown that selection against migrants can contribute to the rapid evolution of ecologically dependent reproductive isolation. However, the habitat difference is only relevant for the larvae, not for the adults. Hence, there is no selection against migrating animals and the models analysed by Hendry (2004) do not apply in our case, since they assume a direct selection against migrants before reproduction occurs.

Given that we have focused our genetic analysis on larvae shortly after deposition, the results of this analysis are a direct statistical reflection of the mate choice that occurred in the previous year. Since the genetic clusters that we detect in the Kottenforst correlate with the larval habitat types, it seems clear that the adult animals have to use some recognition mechanism for finding a matching mate with the same genetic adaptation. Salamanders show complex mating rituals (Houck & Arnold 2003), which could be subject to modification to find mates of the same type. Alternatively, chemical cues might play a role, as has been shown for other salamander species (Gautier *et al.* 2004; Gautier *et al.* 2006). As discussed above, purely passive modes, such as a restriction of mating to the places where suitable larval habitats occur, seem unlikely. But even that would be a specific behavioural adaptation, since it is not known from the neighbouring Eifel or other study areas.

Speciation models

Specific habitat adaptations and assortative mating are crucial components of sympatric speciation models (e.g. Kondrashov & Shpak 1998; Dieckmann & Doebeli 1999; Bürger & Schneider 2006). Although assortative mating may also evolve as a by-product of adaptation to different environments under allopatric conditions (Vines & Schluter 2006), an allopatric scenario seems unlikely in our case. The fact that the pond and the stream clusters are genetically sister lineages within the Kottenforst suggests an origin within the forest. Comparable small-scale differentiation in similar sized forest areas has also been demonstrated for birds (Garant *et al.* 2005; Postma & van Noordwijk 2005). In these cases, behavioural mechanisms such as differential dispersal and selection were invoked to explain local differences in mean phenotypes. However, it is not yet clear whether such local phenotype fluctuations can have long-term genetic consequences, i.e. would become traceable as genetic differentiation on the basis of neutral molecular markers, as in our case. Modelling has suggested that only a co-evolution between adaptive traits and assortative mating traits can lead to stable genetic splits that lead to speciation (Dieckmann & Doebeli 1999). Assortative mating alone may also lead to splits, or could stabilize them after a split has occurred (Kondrashov & Shpak 1998), but would not explain concomitant differential adaptations.

Many factors are expected to influence the outcome for a given natural situation (Bürger & Schneider 2006). Our data suggest that natural conditions can exist where such splits occur relatively quickly (i.e. within a time frame of less than 2000 generations), which is in line with observations made in other systems (e.g. Hendry *et al.* 2000). Thus, in spite of the ongoing discussions concerning the details of modelling sympatric splits (Polechova & Barton 2005), it is becoming increasingly clear that the process may be frequently found in nature.

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This study represents parts of the PhD project of Sebastian Steinfartz performed at the University of Cologne and parts of his ongoing research on speciation processes in terrestrial salamanders carried out by his group in Molecular and Behavioural Ecology at the University of Bielefeld. Markus Weitere is an Assistant Professor at the Zoological Department at the University of Cologne and generally interested in limnology. Diethard Tautz holds the chair of Evolutionary Genetics at the University of Cologne and is interested in mechanisms of molecular evolution and speciation. He has recently moved to the Max-Planck Institute for Evolutionary Biology in Plön.

Appendix

Sampling localities of *Salamandra salamandra* in the Eifel, the Kottenforst and surrounding forest patches. GPS coordinates (Gauss-Krueger; Potsdam), larval habitat type and number of individuals analysed for 11 microsatellite loci are provided

Locality	GPS coordinates	Habitat type	No. of individuals analysed for 11 microsatellites	Locality	GPS coordinates	Habitat type	No. of individuals analysed for 11 microsatellites
BmA	2556944 GK 5601758	Stream	21	KoG	2579010 GK 5617667	Stream	39
BmB	2556750 GK 5599662	Stream	24	KoGb	2579661 GK 5617398	Stream	54
BmC	2558920 GK 5604825	Stream	35	KoGc	2579548 GK 5615623	Stream	48
BmD	2560111 GK 5605914	Stream	35	KoGd	2578858 GK 5614129	Stream	36
BmE	2548562 GK 5597456	Stream	26	KoH	2573721 GK 5617450	Pond	51
RiA	2545633 GK 5587038	Stream	39	KoD	2576008 GK 5613858	Pond	20
RiB	2545631 GK 5588220	Stream	35	KoK	2574584 GK 5614821	Pond	10
Ei	2553247 GK 5599075	Stream	43	KoO	2570627 GK 5619612	Pond	39
RhB	2565013 GK 5608960	Stream	31	KoIa	2573833 GK 5616371	Pond	37
AltA	2567525 GK 5597094	Stream	49	KoIb	2573824 GK 5616128	Pond	20
AltB	2568014 GK 5599230	Stream	35	KoIc	2573850 GK 5615897	Pond	29
HeA	2583386 GK 5598686	Stream	38	KoR	2573695 GK 5615391	Pond	20
HeB	2584962 GK 5597934	Stream	38	KoRa	2573582 GK 5614970	Pond	32
HeC	2589785 GK 5599435	Stream	23	KoJ	2574200 GK 5615502	Pond	17
Koe	2551744 GK 5645278	Pond	44	KoE	2573990 GK 5614073	Pond	13
KoB	2576687 GK 5615382	Pond-like	26	KoM	2575003 GK 5613776	Pond	18
KoF	2576693 GK 5611408	Pond-like	29				