Molecular phylogeny of the salamandrid genus *Neurergus*: evidence for an intrageneric switch of reproductive biology

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Abstract. A molecular phylogeny of the salamandrid genus *Neurergus* was reconstructed based on two sections of the 12S and 16S mitochondrial ribosomal genes (810 bp), 19 allozyme and three plasma protein loci. When representative species of all closely related salamandrid groups were included, mitochondrial data provided evidence for monophyly of *Neurergus* within the Salamandridae. Mitochondrial and allozyme data showed homogenous intrageneric tree topologies, but different estimates of times of separation. We calibrated the evolutionary rate to 0.46% pairwise sequence divergence per million years. Accordingly *Neurergus* diverged 18 million years ago (mya) from a lineage that comprised *Euproctus asper* and large bodied newts of the genus *Triturus*. A split around 11 mya produced two major clades within *Neurergus*. Further separation within the southern '*N. crocatus*-clade' (comprising *N. crocatus*, *N. microspilotus* and *N. kaiseri*) occurred ca. 5 mya. The northern '*N. strauchii*-clade' separated into *N. s. strauchii* and *N. s. barani* ca. 3 mya. Our molecular phylogeny indicates that stream-reproduction is the ancestral state for *Neurergus,* adaptations to pond-reproduction in *N. kaiseri* are the result of an evolutionary switch of reproductive biology within the '*N. crocatus*-clade'.

Introduction

Within the Salamandridae, newts of the Middle Eastern genus *Neurergus* are basically known as stream inhabiting species (Schmidtler and Schmidtler, 1970, 1975; Schmidtler, 1994). *Neurergus* consists of four species: *N. strauchii* (Steindachner, 1887), (*N. s. strauchii* and *N. s. barani*, Öz, 1994), *N. crocatus* (Cope, 1862), *N. microspilotus* (Nesterov, 1917) and *N. kaiseri* (Schmidt, 1952) (fig. 1). The latter is the only species that breeds in stagnant water. All *Neurergus* species can be easily distinguished on morphological and ecological grounds (Schmidtler and Schmidtler 1970, 1975; Schmidtler, 1994). Phylogenetic studies based on mitochondrial DNA data (using *N. strauchii* as a representative of

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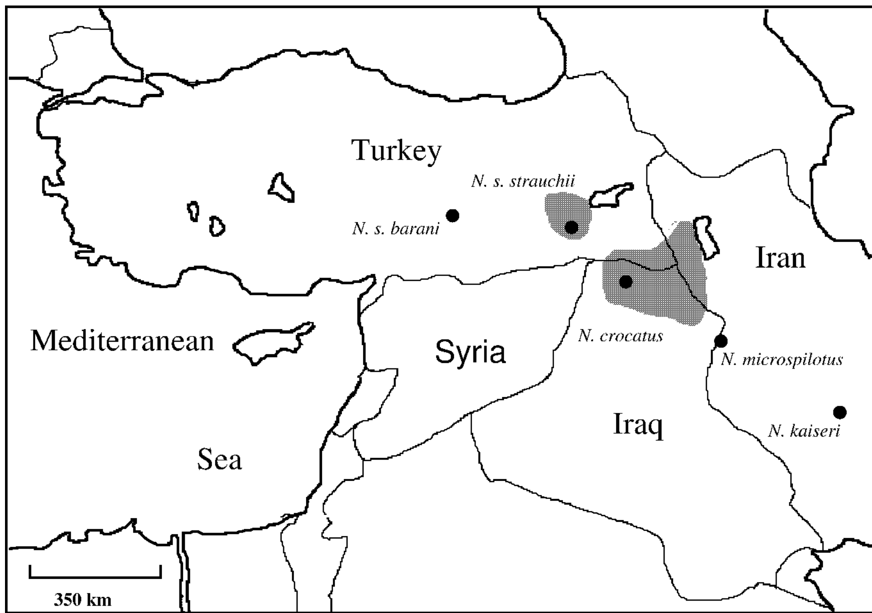

Figure 1. Distribution of *Neurergus* in the Middle East (after Nader, 1970; Schmidtler and Schmidtler, 1970; Öz, 1994). *Neurergus s. barani*, *N. microspilotus* and *N. kaiseri* are only known from their respective type localities whereas *N. s. strauchii* and *N. crocatus* are known from more than one place within a zone (hatched). Sample sites are marked by points: *N. s. barani*: Pütürge SE Malatya, *N. s. strauchii*: Sürüm, near Lake Van, *N. crocatus*: Aqrah, northern Iraq, *N. microspilotus*: Quri-Qaleh SE Paveh, *N. kaiseri*: Sha-Bazan.

the whole genus; Titus and Larson, 1995; Zajc and Arntzen, 1999) place *Neurergus* next to the large bodied newts of *Triturus* and *Euproctus asper*. Skull and vertebrae morphology reveal great differences between *N. strauchii* and *N. kaiseri* — the only *Neurergus* species included in this study — but striking similarities between *N. kaiseri* and *Triturus alpestris* (Haller-Probst and Schleich, 1994). A close relationship of *Neurergus* and *Triturus* had already been assumed on the basis of morphological studies (Bolkay, 1928; Freytag, 1956; Özeti and Wake, 1969). The only comprehensive study on the genus *Neurergus* analysed courtship behaviour, which is very similar among species of *Neurergus* and resembles that of *Triturus* (Sparreboom et al., 2000).

In newts and salamanders, reproductive strategy and larval morphology are strongly correlated to the aquatic breeding habitat (Noble, 1927; Mertens, 1960; Nussbaum, 1985). Pond larvae are characterised by large gills and comparatively high tails and dorsal fins, while stream larvae are adapted to the water current by having low tails, low dorsal fins, and reduced gills. The evolution of body proportions in larvae (long and slender in stream-dwellers, short and squat in pond-dwellers) usually parallels that of gills and fins.

Neurergus is the only aquatic salamandrid genus in which both types of reproduction occur. All species except the pond-breeding *N. kaiseri* are stream breeders (Schmidtler

and Schmidtler, 1970, 1975; Nader, 1970). Three hypotheses may explain the evolution of different reproductive strategies within *Neurergus*: (i) *Neurergus kaiseri* is closely related to *Triturus alpestris* with which it shares numerous morphological and behavioural traits; consequently, species of *Neurergus* would not form a monophylum. The striking osteological differences between *N. stauchii* and *N. kaiseri* (Haller-Probst and Schleich, 1994) support this view. (ii) Pond-breeding is the ancestral state of a monophyletic genus, with stream-breeding being the derived character. (iii) The genus *Neurergus* forms a monophylum with stream-breeding as the ancestral state; pond-breeding in *N. kaiseri* is the result of an intrageneric switch of reproductive biology in *Neurergus* during the evolution of this clade.

A robust phylogenetic analysis is crucial to test these hypotheses. Since the morphological and molecular studies mentioned above used only one or two species of *Neurergus*, monophyly of the genus has never been proven. In order to test for monophyly of *Neurergus* we analysed fragments of two mitochondrial genes for all species and subspecies of *Neurergus* and all presumably closely related salamandrid species (Titus and Larson, 1995; Zajc and Arntzen, 1999). To gain confidence in the intrageneric relationship we analysed 19 allozyme and 3 plasma protein loci for *Neurergus* and *E. asper*.

Material and methods

Samples. We sequenced sections of the 12S and 16S ribosomal mitochondrial genes of *N. strauchii strauchii*, *N. s. barani*, *N. crocatus*, *N. microspilotus* and *N. kaiseri* (GenBank accession numbers AY147242-AY147251). To test for monophyly of *Neurergus* we included *T. marmoratus* (France; GenBank accession numbers AY147252, AY147253), *T. carnifex* (GenBank accession numbers U04702, U04703), *T. vulgaris* (Italy; GenBank accession numbers AY147254, AY147255), *T. alpestris* (Germany; GenBank accession numbers AY147256, AY147257), *E. asper* (France; GenBank accession numbers AY147258, AY147259), *E. platycephalus* (U04698, U04699) and *E. montanus* (U04696, U04697). These taxa are closely related to *Neurergus* based on morphological (Bolkay, 1928; Freytag, 1956; Özeti and Wake, 1969; Haller-Probst and Schleich, 1994; Scholz, 1995), behavioural (Arntzen and Sparreboom, 1989; Schmidtler, 1994), and molecular (Titus and Larson, 1995) data. For outgroup rooting we used the more distantly related *Pleurodeles waltl* (Spain; U04700, U04701). On average we sequenced two individuals per species. Allozyme electrophoresis was performed for 26 specimens of *Neurergus* (see appendix 1). *Euproctus asper* and the salamander *Mertensiella luschani* (Turkey) were used for hierarchical outgroup rooting. One toe was taken for DNA-analysis; blood samples were used for allozyme electrophoresis. All *Neurergus* specimens used in the DNA sequence analysis were also included in the allozyme analysis.

DNA Isolation, sequencing and data analysis. Genomic DNA was extracted from tissue samples using the SDS-proteinase K/Phenol-Chloroform extraction method. We used published primer combinations to amplify fragments of the 12S (L1091 and H1478, Kocher et al., 1989) and 16S gene (16SA and 16SB, Palumbi et al., 1991). Polymerase chain reaction was performed on a 50 μl scale under standard conditions (Kocher et al., 1989). Amplified mtDNA was purified using ultrafree-filters (Millipore) and afterwards processed for symmetric cycle sequencing using either the Ready Reaction kit (Amersham) or the Big-Dye Ready-Reaction kit (Perkin Elmer). Sequencing products were analysed on an ABI™ 377 (Perkin Elmer) using the Sequence Navigator software (Perkin Elmer).

Sequences were aligned using CLUSTAL W (Higgins and Sharp, 1993) and subsequently adjusted by eye. We obtained a final alignment of 810 bp for 12S and 16S after excluding insertions and deletions. Based on the results of Titus and Larson (1995) we defined *Pleurodeles waltl* as the outgroup for phylogenetic reconstruction. Molecular phylogeny was inferred using the maximum likelihood (ML) method. Since we observed strong

among-site variation, phylogenetic reconstruction was done under the HKY-$\Gamma_{\alpha=0.5}$ (Hasegawa et al., 1985; Yang, 1996) substitution model using PUZZLE 4.02 from Strimmer and von Haeseler (1996). Node robustness was tested through 1000 puzzling steps. To resolve the intrageneric phylogeny of *Neurergus* under the same model with the same assumptions as before, we chose *Euproctus asper* as the outgroup, because it came up as the closest relative of *Neurergus*.

DNA sequences should behave in a clock-like manner in order to estimate time of separation between taxa. Therefore we tested for clock-like behaviour using PUZZLE 4.02 with *E. asper* as outgroup. Since strong among-site variation in all sequence comparisons emerged for all species of *Euproctus*, we re-calibrated Caccone's et al. (1994, 1997) estimation of evolutionary rates for *Euproctus* for bp positions homologous to our data set under the HKY-$\Gamma_{\alpha=0.5}$ substitution model.

Allozyme electrophoresis and data analysis. Blood samples were taken from the caudal vein and suspended in citric buffer. Erythrocytes were separated from the plasma via centrifugation. They were washed twice in citric buffer and subsequently lysed in distilled water. Erythrocyte lysate and plasma were used immediately for electrophoresis on cellulose acetate plates from Helena Diagnostics, Texas (Hebert and Beaton, 1993).

Thirteen enzyme systems were stained using standard recipes (Richardson et al., 1986). They provided data on 19 presumptive gene loci: creatine kinase (*ck*), fumarate hydratase (*fum*), glucose-phosphate isomerase (*gpi-1* and *-2*), isocitrate dehydrogenase (*idh-1* and *-2*), lactate dehydrogenase (*ldh-1* and *-2*), NAD-dependent malate dehydrogenase (*mdh-1* and *-2*), NADP-dependent malate dehydrogenase (malic enzyme, *me*), mannose-phosphate isomerase (*mpi*), tripeptidase with phenyl-alanyl-proline as substrate (*pepD*), tripeptidase with glycyl-leucyl-leucine as substrate (*pepB*), 6-phosphogluconate dehydrogenase (*6pgd*), phosphoglucomutase (*pgm-1* and -2), pyruvate kinase (*pk*) and triosephosphate isomerase (*tpi*). Three additional protein loci (praealbumin (*prae*), albumin (*alb*) and transferrin (*tf*) were stained from plasma using coomassie brilliant blue (CBB). We used four different buffer systems for the separation of allozymes and plasma proteins: Phosphate buffer, pH 7.2 (*gpi*, *tpi*); tris-maleic buffer, pH 7.0 (*alb*, *ck*, *idh*, *mdh*, *me*, *mpi*, *pepD*, *6pgd*, *pk*, *prae*, *tf*); tris-citric buffer, pH 8.2 (*fum*, *ldh*, *pepB*); tris-glycine buffer, pH 8.5 (*pgm*). All gels were run at 200 volts for 40 minutes. Allozyme loci and alleles were numbered according to their electrophoretic mobility with the fastest being 1 or a, respectively.

Allele frequencies were calculated using G-STAT (Sigismund, 1997). We calculated Nei's (1972) standard genetic distances since it is the most widely used genetic distance estimate for allozymes. Unweighted pair group method with arithmetic mean (UPGMA), neighbour joining (NJ), and ML trees were calculated from distance or allele frequency data, respectively, with 1000 bootstrap replicates each (PHYLIP; Felsenstein, 1995).

Test for homogeneity between mitochondrial and nuclear data. Mitochondrial and nuclear loci represent two independent data sets. We tested for homogeneity of distance matrices derived from allozymes (Nei's standard distance) and mtDNA data (HKY-85 distance) by applying the Mantel test statistics (NTSYS-PC of Rohlf, 1990). Non-homogeneity would indicate either a sorting out of mitochondrial haplolineages, introgression at nuclear loci, or both.

Results

In a comparison of all taxa, 592 out of 810 bp were constant in the total alignment. Base frequencies were estimated to $\pi_A = 0.356$, $\pi_C = 0.220$, $\pi_G = 0.189$ and $\pi_T = 0.235$, resulting in a clear deviation from equal base frequencies. The transition/transversion ratio was estimated to 2.97, indicating a six-fold bias toward transitions. Corrected intergeneric molecular distances ranged from 14.1% (*Pleurodeles waltl*/*Triturus carnifex*) to 16.9% (*Pleurodeles waltl*/*N. s. barani*). Intrageneric distances for *Neurergus* ranged from 1.4% (*N. s. strauchii*/*N. s. barani*) to 5.8% (*N. kaiseri*/*N. s. barani*) (table 1). Based on 22 presumptive protein loci (allele frequencies are given in appendix 1), Nei's (1972) standard genetic distance ranged from 0.202 between *N. s. strauchii* and *N. s. barani* to 0.981 between *N. s. strauchii* and *N. kaiseri* (table 1).

Table 1. HKY-$\Gamma_{0.5}$ distances (below diagonal) and Nei's (1972) standard genetic distance (above diagonal) between *N. crocatus* (*Nc*), *N. kaiseri* (*Nk*), *N. microspilotus* (*Nm*), *N. s. barani* (*Nsb*), *N. s. strauchii* (*Nss*) and *E. asper* (*Ea*).

	Nc	*Nk*	*Nm*	*Nsb*	*Nss*	*Ea*
			Nei's (1972) standard genetic distance			
Nc	–	0.624	0.725	0.837	0.749	1.820
Nk	0.03154	–	0.836	0.824	0.981	2.234
Nm	0.02612	0.02324	–	0.778	0.735	1.720
Nsb	0.05688	0.05806	0.04739	–	0.202	2.228
Nss	0.05208	0.05067	0.04154	0.01379	–	2.251
Ea	0.08179	0.07451	0.08001	0.09240	0.08681	–
			HKY-$\Gamma_{0.5}$ distance			

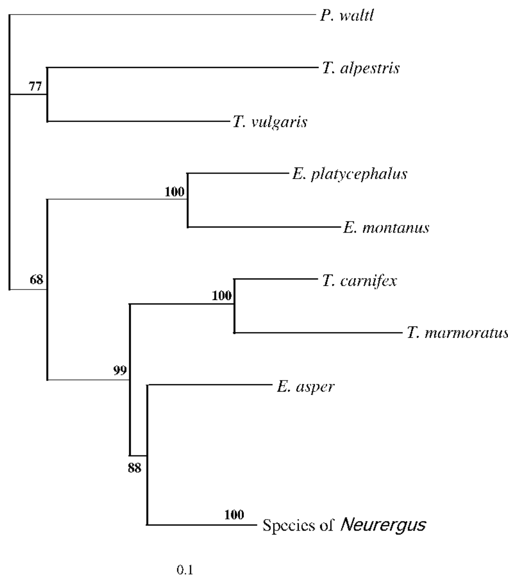

Figure 2. Maximum Likelihood tree (HKY-Γ, $\alpha = 0.5$) of *Neurergus* and closely related salamandrid species based on combined sequences of 12S and 16S; *Pleurodeles waltl* was defined as the outgroup. Percentage support of internal branches (derived from 1000 puzzling steps) is indicated at the nodes. The line at the bottom represents a branch length of 0.1 corrected molecular distance.

Is Neurergus *a monophylum?*

Monophyly of *Neurergus* was strongly supported in the ML tree of DNA sequence data (fig. 2). The sequences also confirmed previous views of non-monophyly of *Triturus* and *Euproctus* (Caccone et al., 1994, 1997; Titus and Larson, 1995; Zajc and Arntzen, 1999). *Euproctus asper* appeared as a sister taxon to *Neurergus* as previously proposed by Titus and Larson (1995). *Triturus marmoratus* and *T. carnifex*, the two representatives of the large-bodied newts of the genus *Triturus*, showed a sister relationship to the *E. asper* and

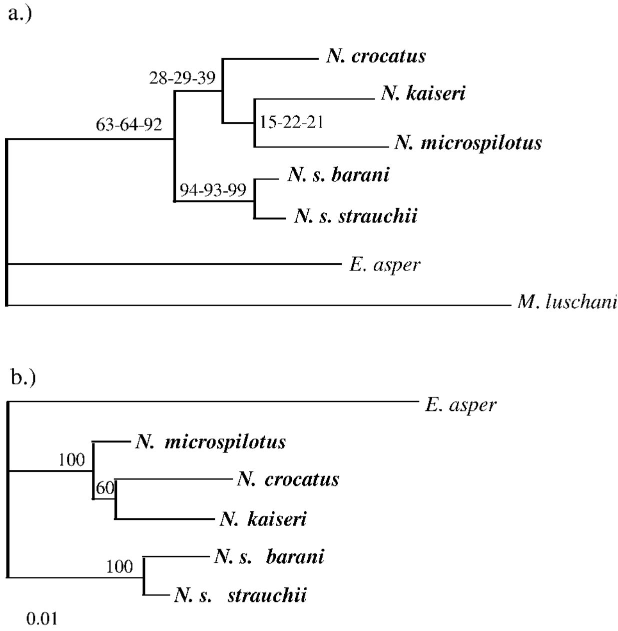

Figure 3. (a) NJ tree of *Neurergus* and two outgroup taxa based on Nei's (1972) standard genetic distances and allele distribution at 19 allozyme and 3 plasma protein loci; numbers at nodes indicate bootstrap support for the respective topology in the UPGMA-NJ-ML approach for 1000 replicates each. (b) Maximum Likelihood tree of all currently known *Neurergus* taxa (*Euproctus asper* was defined as the outgroup) based on mitochondrial DNA sequences (HKY-Γ, $\alpha = 0.5$); numbers refer to percentage support of internal nodes derived from 1000 puzzling steps; line at the bottom represent a branch length of 0.01 corrected molecular distance.

Neurergus clade. In contrast, *T. vulgaris* and *T. alpestris*, members of the small and medium sized *Triturus*, were more distantly related to *Neurergus*.

The Mantel test for homogeneity of mitochondrial and allozyme distance matrices revealed a significant matrix correlation ($t = 1{\cdot}83$, $P_{\text{rand}\cdot\text{Z}<\text{obs}\cdot\text{Z}} = 0{\cdot}9664$).

Phylogenetic relationships within Neurergus

Using *Euproctus asper* as an outgroup we obtained 100% puzzle support for two further clades within *Neurergus*, namely the '*crocatus*-clade', comprising *N. crocatus*, *N. microspilotus* and *N. kaiseri*, and the '*strauchii*-clade', representing *N. s. strauchii* and *N. s. barani* (fig. 3b). This pattern was again supported by the allozyme data (fig. 3a), although bootstrap support was comparatively low. Further resolution of the '*crocatus*-clade' above the 70% puzzling level was not possible. This was also the case when trees were reconstructed with maximum parsimony and neighbour joining (data not shown).

Table 2. A time scale for major splits between *E. asper* and *Neurergus* and within *Neurergus*; mean and standard deviation (s) are given for all (n) pair-wise allozyme (D) and HKY-$\Gamma_{0.5}$ distances among taxa; calibrations given in the text were used to calculate times of separation, including the 95% confidence intervals (*CI*) over all respective pairs. *CI* are based on s of pairwise distances.

		Allozymes			mtDNA		
	n	$D \pm s$	time (my)	95% *CI*	HKY $\pm s$	time (my)	95% *CI*
E. asper from *Neurergus*	5	2.051 ± 0.259	28.7	21.6-35.8	0.0831 ± 0.0068	18.1	15.2-21.0
N. strauchii from '*N. crocatus* clade'	6	0.817 ± 0.090	11.4	8.9-13.9	0.0511 ± 0.0061	11.1	8.5-13.7
Within '*N. crocatus* clade'	3	0.728 ± 0.106	10.2	7.3-13.1	0.0270 ± 0.0042	5.9	4.1-7.7
Within *N. strauchii*	1	0.202	2.8	–	0.0138	3.0	–

A time frame for speciation in Neurergus

The application of a molecular clock for the inference of a paleo-biogeographic scenario requires that the evolution of the respective molecular markers occurred at a more or less constant rate. For the combined 12S and 16S fragment, a clock-like behaviour was not rejected (log $L_{\text{without clock}}$: $-1706{\cdot}99$, log $L_{\text{with clock}}$: $-1710{\cdot}78$, critical significance level: 10.78%). After correcting for homologous bp positions and the underlying substitution model (see above), pair-wise HKY-$\Gamma_{0.5}$ distances of Caccone's et al. (1997) data resulted in a calibration of 0.46 per million years (=0.23 per my and lineage). Consequently, *N. strauchii* would have separated from the remaining *Neurergus* species ca. 10-11 million years ago. Subsequent splitting within the '*crocatus*-clade' and between *N. s. strauchii* and *N. s. barani* would have occurred ca. 3-5 and 3 mya, respectively (see table 2).

Allozyme genetic distances (Nei, 1972) are often used to estimate the time of separation in salamanders (1 $D = 14$ my of separation; Maxson and Maxson, 1979). Consequently, the major radiation within *Neurergus* occurred around 11.5 mya, separating the '*N. crocatus*-clade' from the '*N. strauchii*-clade'. Speciation within the '*N. crocatus*-clade' occurred 10.6 mya while the differentiation of *N. s. strauchii* and *N. s. barani* was the most recent event (ca. 2.8 mya).

Discussion

Monophyly of Neurergus

Mitochondrial DNA markers strongly support monophyly of *Neurergus*. In this context the close osteological similarity between *N. kaiseri* and *T. alpestris* (Haller-Probst and Schleich, 1994) should be due to a convergent morphological evolution. Morphology may

strongly adapt to the environment, as has been shown for other vertebrate groups (e.g., cichlids of the African lakes: Kocher et al., 1993; lizards of the genus *Anolis*: Losos et al., 1998; reviewed in Orr and Smith, 1998). A recent analysis of ranid frogs showed that almost identical ecomorphs with striking morphological similarities have evolved independently in Africa and Asia (Bossuyt and Milinkovitch, 2000). An outstanding example of an environmentally induced osteological homoplasy has been documented for tropical plethodontid salamanders of the genus *Lineatriton* (Parra-Olea and Wake, 2001).

The evolution of reproductive modes within Neurergus

So far *Neurergus s. strauchii*, *N. s. barani*, *N. crocatus* and *N. microspilotus* have been only reported from stream habitats, and their larvae show clear signs of stream adaptation (Schmidtler and Schmidtler, 1970, 1975; Nader 1970). As an example, the aquatic larval phase of *N. s. barani* can last more than one year (Schmidtler, 1994). In contrast, larvae of *N. kaiseri* are characterised by large gills and concentrically-inserting dorsal fins, both are indicators of pond-reproduction (Noble, 1927; Valentine and Dennis, 1964). *Neurergus kaiseri* inhabits stagnant waters such as periodic streams and ponds (Schmidtler and Schmidtler, 1975; Schultschik and Steinfartz, 1996) which do not provide stable environmental conditions, since water availability may drastically fluctuate over time. As a consequence, the breeding period of *N. kaiseri* and subsequent larval development are notably shorter than for the stream-species (Schmidtler and Schmidtler, 1975).

Molecular monophyly of *Neurergus* and the existence of stream- and pond-breeding within the '*crocatus*-clade' indicate that (i) an intrageneric adaptive change of the reproductive mode (stream-breeding to pond-breeding) has occurred for *N. kaiseri*. For such a scenario stream-type reproduction is the ancestral type, since it is shared by *E. asper* and all *Neurergus* except for *N. kaiseri*. To assume the opposite would require the unlikely case of a recurrent parallel evolution of stream-reproduction in the '*crocatus*-' and '*strauchii*-clade' from a pond-reproductive ancestral lineage.

In order to determine the genetical and plastic component of an environmental adaptation elaborate natural enclosure and common environments experiments should be done. For *Neurergus* data from these kinds of experiments are missing and therefore only indirect conclusions on these components can be drawn. We think that stream- and pond-reproduction in *Neurergus* are rather examples of genetical adaptations than of environmental plasticity for the following reasons. (i) Certain characters of the larval (e.g. concentrically inserting dorsal fins) and adult morphology (e.g. the female's cloaca) of *N. kaiseri* are known to be invariable within populations (Schmidtler and Schmidtler, 1975) and should be therefore seen as fixed and heritable. (ii) Throughout the comparatively large distribution areas of *N. strauchii* and *N. crocatus* where the existence of suitable ponds is indicated by the presence of the newt *T. vittatus* (Schmidtler and Schmidtler, 1967; Leviton et al., 1992), *Neurergus* breed solely in streams and brooks. (iii) That adaptations to the desiccation risk are indeed genetically controlled leading to a limited environmental

plasticity even within the same species has been shown for populations of *Rana temporaria* from high desiccation and low desiccation risk environment (Laurila et al., 2002). This example demonstrates that strong environmental selection can eliminate or favour certain adapted genotypes within considerable shorter time frames than we can assume for *N. kaiseri* which separated several millions of years ago from the other species.

Courtship behaviour of Neurergus *in the light of the molecular data*

Courtship behaviour of newts and salamanders has been studied since the 1960s for different families, genera and species (e.g., Salthe, 1967; Salthe and Mecham, 1974; Arnold, 1977; Halliday, 1977; Arntzen and Sparreboom, 1989). In *Triturus*, courtship behaviour is extremely complex (Halliday, 1977; Halliday, 1990; Halliday and Arano, 1991). Several distinct phases, sometimes with feedback mechanisms, have to be completed to assure successful spermatophore transfer from males to females. The basic courtship behaviour of *Neurergus* is almost identical to that of *Triturus*, although a variety of behavioural traits are specific to *Triturus*. Among the Salamandridae, the so-called 'brake', displayed during the final phase of courtship, is only shared by *Neurergus* and *Triturus* and is interpreted as a behavioural synapomorphy (Sparreboom et al., 2000). However, our molecular findings indicate that *E. asper* is the closest relative of *Neurergus*. *Euproctus asper* is a typical stream-breeder (Thiesmeier and Hornberg, 1990). Its courtship includes elements that are typical for stream-breeders, such as close male-female contact, amplexus, and direct sperm transfer, all missing in *Neurergus*. Two explanations could explain these discrepancies. (i) 'brake' is a symplesiomorphy for the *E. asper* + *Triturus* + *Neurergus* lineage and *E. asper* has changed over the time its courtship behaviour mostly. (ii) The mitochondrial data do not reflect the true phylogeny.

In order to illuminate the incongruity between behavioural and mitochondrial molecular markers, nuclear genes should be investigated also.

A paleo-biogeographic scenario for the evolution of Neurergus

The idea of the molecular clock has been a controversial issue (for reviews see Avise, 1994; Rand, 1994; Page and Holmes, 1998) since it was proposed in the mid-1960s by Zuckerkandl and Pauling (1965). However, in the absence of a dated fossil record (which is the case for *Neurergus*) it is still an appropriate way to estimate the time since separation of species.

We compared estimates for mitochondrial genes and protein loci for *Neurergus* and *E. asper* (table 2). Based on the 95% confidence intervals, we achieved congruent estimates only for the separation of the '*N. strauchii*-clade' from the '*N. crocatus*-clade' at 8.5-13.9 mya and the separation of *N. s. barani* and *N. s. strauchii* 3 mya. Other estimates differ greatly between mitochondrial DNA and allozymes. Since we proved clock-like behaviour for mitochondrial DNA sequences we assume that they evolved neutrally in the case of *Neurergus*. We cannot assume neutral evolution for allozymes, since a test for clock-like

behaviour is not at hand and allozyme loci can experience selection (Lemaire et al., 2000). Such an assumption would result in an overestimation of times of separation. In fact, all deviating estimates from allozymes clearly exceed the corresponding estimates obtained from mtDNA sequences (table 2). Accordingly, we will use the mitochondrial estimates to reconstruct a paleo-biogeographic scenario for the evolution of *Neurergus*.

The occupation of a montane niche by the ancestor of *Neurergus* should have taken place between its separation from *E. asper* around 18 mya and the first intrageneric diversification. The rise of the Zagros Mountains roughly 9-10 mya (Buchbinder and Gvirtzman, 1976; Rögl and Steininger, 1983) in association with humid climatic conditions (Lueger, 1978; Thenius, 1982) could have provided ideal conditions for *Neurergus* to spread from the north throughout the Zagros mountain chains (today's Iraq-Iranian mountain chains). The first separation within the *N. crocatus*-clade coincides well with the end of the Messinian salinity crisis, ca. 5 mya. Although this cannot have directly affected mountain species such as *Neurergus*, its indirect effects on the circum-Mediterranean climate must have been dramatic (Azzaroli and Guazzone, 1980; Maldonado, 1985; Oosterbroek and Arntzen, 1992). The subspecific diversification of *N. strauchii* roughly coincides with the onset of a first cycle of oscillating Pleistocene glaciations in the Northern hemisphere about 2.5 mya (Hays et al., 1976; Wilson et al., 1999; Dynesius and Jansson, 2000).

Conclusions

Ecological adaptation of *Neurergus* to high mountain brooks may have trapped them in a restricted area for millions of years. Favourable climatic conditions allowed only for minor range expansions within the Iraq-Iranian mountain chains. Since courtship behaviour seems to be more prone to adaptive changes than previously thought, it remains enigmatic why *Neurergus* evolved or kept a pond-breeding courtship behaviour while living in running water. Only one species, *Neurergus kaiseri*, switched to pond reproduction, since it has colonized an unstable environment. The evolution of ecotypes within *Neurergus* is a nice example of how a habitat switch is paralleled by a dramatic change in larval and adult morphology, while remaining in the original habitat results in morphological stasis.

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Appendix 1. Allele frequencies of *Neurergus crocatus* (*Nc*), *N. kaiseri* (*Nk*), *N. microspilotus* (*Nm*), *N. s. strauchii* (*Nss*), *N. s. barani* (*Nsb*), *Euproctus asper* (*Ea*) and *Mertensiella luschani atifi* (*Mla*).

Locus	*Nc*	*Nk*	*Nm*	*Nsb*	*Nss*	*Ea*	*Mla*
n →	4	7	7	6	2	4	4
alb	(a) 1.00	(c) 1.00	(c) 1.00	(e) 1.00	(e) 1.00	(d) 1.00	(b) 1.00
ck	(c) 1.00	(c) 1.00	(c) 1.00	(c) 1.00	(c) 1.00	(a) 1.00	(b) 1.00
fum	(b) 1.00	(b) 1.00	(b) 1.00	(c) 1.00	(c) 1.00	(a) 1.00	(d) 1.00
gpi-1	(e) 1.00	(b) 0.29 (f) 0.71	(c) 1.00	(c) 0.42 (e) 0.58	(c) 1.00	(a) 1.00	(d) 1.00
gpi-2	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00	(b) 1.00	(c) 1.00
idh-1	(b) 0.25 (c) 0.75	(c) 1.00	(c) 1.00	(e) 1.00	(d) 1.00	(a) 1.00	(f) 1.00
idh-2	(b) 1.00	(b) 1.00	(a) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(b) 1.00
ldh-1	(a) 1.00	(c) 1.00	(e) 1.00	(c) 1.00	(c) 1.00	(d) 1.00	(b) 1.00
ldh-2	(a) 1.00	(c) 1.00	(e) 1.00	(b) 1.00	(b) 1.00	(e) 1.00	(d) 1.00
mdh-1	(b) 1.00	(d) 1.00	(a) 1.00	(e) 1.00	(e) 1.00	(c) 1.00	(f) 1.00
mdh-2	(a) 1.00	(d) 0.86 (e) 0.14	(a) 0.36 (b) 0.64	(f) 0.75 (g) 0.25	(f) 1.00	(a) 1.00	(c) 1.00
me	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00
mpi	(a) 1.00	(a) 1.00	(b) 1.00	(c) 1.00	(c) 1.00	(d) 1.00	(c) 1.00
pepB	(a) 0.88 (c) 0.12	(d) 1.00	(e) 1.00	(b) 1.00	(b) 1.00	(e) 1.00	(f) 1.00
pepD	(c) 1.00	(c) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(a) 1.00	(d) 1.00
6pgd	(b) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(a) 1.00	(c) 1.00
pgm-1	(b) 1.00	(a) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(d) 1.00	(c) 1.00
pgm-2	(b) 0.75 (c) 0.25	(a) 1.00	(b) 1.00	(c) 1.00	(b) 1.00	(e) 1.00	(d) 1.00
pk	(a) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(a) 1.00	(c) 1.00	(d) 1.00
prae	(b) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(b) 1.00	(a) 1.00
tf	(e) 1.00	(a) 1.00	(c) 1.00	(f) 1.00	(f) 1.00	(b) 1.00	(d) 1.00
tpi	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00	(a) 1.00	(b) 1.00	(c) 1.00