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The genetic structure of adders (*Vipera berus*) in Fennoscandia: congruence between different kinds of genetic markers

M. CARLSSON, L. SÖDERBERG and H. TEGELSTRÖM*
Department of Conservation Biology and Genetics, EBC, Uppsala University, Norbyv. 18D, SE-752 36 Uppsala, Sweden

Abstract

In order to elucidate the colonization history of Fennoscandian adders (*Vipera berus*), the phylogeographical patterns of two nuclear sets of DNA markers (random amplified polymorphic DNA and microsatellite) are compared with that previously obtained from mitochondrial DNA. An eastern and a western lineage within Fennoscandian adders is readily distinguishable using both sets of nuclear markers, corroborating the hypothesis that the lineages stem from separate glacial refugia. Moreover, the same contact zones as were derived from mitochondrial data are clearly identifiable. Both sets of nuclear markers detect a high level of admixture across one zone in northern Finland, with introgression reaching far west into Sweden.

Keywords: adders, assignment, microsatellites, phylogeography, RAPDs, *Vipera*

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Introduction

The postglacial phylogeography of northern European fauna and flora was first addressed by Nilsson (1847) who took note of morphological differences within and among taxa and suggested different colonization routes into the region. With modern molecular genetic techniques there has been an upsurge of investigations of colonization by morphologically nondifferentiated taxa (Avise 2000). Among terrestrial vertebrates, a common colonization pattern into Fennoscandia has proven to be bidirectional, with historically isolated populations colonizing from the south and the east. For several species studied, the descendants of the separate lineages meet within a 150 km area of northern Sweden (reviewed in Jaarola *et al*. 1999) forming a suture zone (Remington 1968).

The adder, *Vipera berus*, is distributed across most of temperate Eurasia and, being a cold-tolerant species, can be found in Fennoscandia north of the Arctic Circle (Schiemenz 1995). The mitochondrial phylogeography for the entire geographical distribution of the adder has recently been investigated (Carlsson 2003). The mitochondrial DNA (mtDNA) data suggest that the adders of Northern Europe originate from two separate glacial refugia situated north of the Romanian part of the Carpathian Mountains. In Romania and further south several other refugial populations have prevailed (Carlsson 2003). In Fennoscandia, descendants of these two geographically widespread lineages meet in three different areas. Colonization of mainland Fennoscandia has produced a zone of contact in the north of Finland (Carlsson & Tegelström 2002), while dispersal across dozens of kilometres of open water has resulted in mitochondrial contact zones in both southwestern Finland and in the Umeå Archipelago of northern Sweden. The postglacial colonization history of Fennoscandian adders thus appears to differ in some respects to that of other vertebrate species studied to date.

Most phylogeographical studies of animals are based on mtDNA as genetic marker, as the mitochondrial genome holds several advantages compared with the use of nuclear DNA (Wilson *et al*. 1985). However, the mtDNA is effectively transmitted as a single gene, and any singular gene genealogy may not accurately describe the history of populations within a species (Pamilo & Nei 1988; Rosenberg & Nordborg 2002). It is therefore prudent to compare the phylogeographical pattern derived from mtDNA with that of nuclear markers.

Random amplified polymorphic DNA (RAPD) (Williams *et al*. 1990) is a relatively quick and inexpensive approach to

Correspondence: Martin Carlsson. Fax: +46 18 471 6424; E-mail: martin.carlsson@ebc.uu.se
*Håkan Tegelström died on 20 March 2004 after a brave struggle against a long illness. He will be greatly missed.

derive a nuclear dataset for the study of population structure and gene flow (Hadrys *et al*. 1992). The sensitivity of the polymerase chain reaction (PCR) must be taken into account, however, and any RAPD study warrants some kind of reproducibility check (e.g. Gibbs *et al*. 1994; Mockford *et al*. 1999). The most commonly applied method for assessing nuclear DNA variation in population genetic enquiries is analysis of microsatellite loci. Some advantages of studying microsatellite repeats include the codominance of the diploid gene products which facilitates interpretation, and the reproducibility of the PCR — both in contrast to that of RAPD data. Relatively few studies have utilized more than one of the methods mentioned above, although some reports have compared the resolution of different methods (Lougheed *et al*. 2000; Yue *et al*. 2002; Kjølner *et al*. 2004).

In this study, we investigate the population structure and patterns of gene flow of nuclear DNA in Fennoscandian adders and compare that with the phylogeography derived from mitochondrial evidence. We compare data from RAPDs and six microsatellite loci to detect genetic structure on a regional and local scale and gene flow across the three mitochondrially defined contact zones of adders in Fennoscandia (Carlsson & Tegelström 2002).

Materials and methods

In order to investigate overall genetic diversity in northern Europe, while also enabling an assessment of introgression across contact zones, the number of sampled localities was given priority over local sample sizes. In total 122 individuals were collected from 71 localities (Fig. 1). Blood was extracted from the caudal vein and stored in 1× SSC buffer (Höggren & Tegelström 1995) or 96% ethanol. Muscle tissue was taken from previously collected and frozen material. DNA was isolated by salt precipitation (Paxton *et al*. 1996) or using Qiagen DNEasy Tissue Kit and eluted in 0.01 M Tris–HCl (pH 8.0). DNA concentration was measured in a Hoefer DyNA Quant 200 fluorometer and the samples were diluted to a standardized template concentration of 10 ng/μL.

For the RAPD data, PCRs were performed using 25 μL reaction mixtures containing 10 ng of template DNA, 1× PCR buffer (10 mM Tris–HCl, pH 8.8 at 25 °C, 50 mM KCl, 0.8% Nonidet P40), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 mM primer and 0.8 units of *Taq* DNA polymerase (MBI Fermentas). Amplification was performed on a Perkin–Elmer PTC-100 Thermal Cycler (2 min at 94 °C, followed by 45 cycles of 1 min denaturing, 1 min annealing and 2 min extension at 72 °C with a final 8 min extension). PCR products were separated on 1.4% agarose gels (Williams *et al*. 1990) during 2 h of electrophoresis in 1× TBE buffer at 100 V, stained in ethidium bromide and visualized under ultraviolet light.

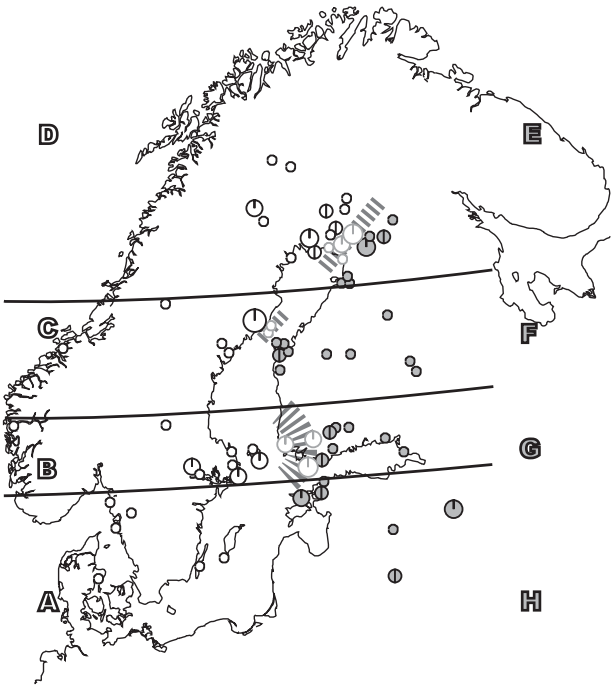

Fig. 1 Map showing localities (circles) for the 122 samples of *Vipera berus* used in this study with the diameter of the circles corresponding to sample size. Circles with black edges denote samples used when testing for genetic structure. Circles with shaded edges represent potential hybrid individuals located at or near the mtDNA contact zones (hatched areas). Lines along the 58th, 61st and 64th parallels define arbitrary subdivisions for population samples (samples A–H) used in analyses of genetic structure. Sample definitions correspond to Tables 2 and 3 in the online supplementary data (http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2315/MEC2315sm.htm).

Polymorphic RAPD primers were selected in a three-step process. Sixty primers (Operon Technologies' Primer Kits H; R and Y, each with 20 oligos of 10 bp length) were initially screened at an annealing temperature of 37 °C on four individual samples, two from each of the two mitochondrially defined populations (Fig. 1). This procedure yielded 18 primers producing seemingly scorable PCR band patterns. The 18 primers were then evaluated on a set of four additional individuals from each population in order to identify polymorphic markers. For some of the 18 primers annealing temperatures were increased from 37 to 38 or 39 °C to achieve a higher specificity. Seven primers were found to yield polymorphic and scorable band patterns (see supplementary data online, http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2315/MEC2315sm.htm). The remaining 110 individuals were examined using these seven primers, and 42 polymorphic loci were defined and scored. We evaluated the reproducibility of the markers used by scoring some individuals repeatedly. One individual was

scored through 13–17 independent PCR for each primer and used as a size reference on each agarose gel. Only loci with consistent patterns for all independent amplifications were included in the final dataset.

Primers and PCR conditions for the six microsatellite loci analysed in this study have been described elsewhere (Carlsson *et al*. 2003).

The two datasets were divided into two separate subsamples, one eastern and one western group, in accordance with the pattern found in mtDNA for adders in Fennoscandia (Carlsson & Tegelström 2002). An AMOVA was computed for each set of data using ARLEQUIN v. 2.0 (Schneider *et al*. 1997) for the dominant RAPD dataset, and GENALEX v. 5 (Peakall & Smouse 2001) for the codominant microsatellite markers. The two groups were then arbitrarily divided along the 58th, 61st and 64th parallel into four population samples each to investigate whether there was any geographical structure within the eastern and western groups (Fig. 1). Some individuals ($n = 24$) sampled at the previously defined contact zones for mtDNA were excluded from these analyses to avoid disturbance of any geographical signal present in the data by including potential hybrids (Fig. 1).

In addition to the AMOVA we tried detecting geographical substructuring of the samples by analysing both datasets using STRUCTURE v. 1.0 (Pritchard *et al*. 2000). Data was divided into $K = 1$ to $K = 20$ populations with three independent runs for all most likely values of K. For each run 100 000 Markov Chain Monte Carlo (MCMC) iterations were performed of which the first 50 000 were discarded as burn-in of the chain.

We also used the assignment calculator DOH (available at: http://www2.biology.ualberta.ca/jbrzusto/Doh.php, courtesy of John Brzustowski 2002), which applies an assignment test where each individual's probability of belonging to a given population is the product of its genotype frequencies for all loci in that population (Paetkau *et al*. 1995). The program allows assignment of individuals with unknown origin, and also implements a method to compensate for unequal sample sizes. We ran DOH once for each dataset by dividing the samples into an eastern and a western population to see how well the assignments based on nuclear markers matched our previous mtDNA results. The 24 individuals that were excluded in the AMOVA analysis were added without a specified locality in order to achieve a better resolution at and around the mitochondrially defined zones of contact (Carlsson & Tegelström 2002) in order to identify potential hybrids. The same eight arbitrary population-groupings as used for the AMOVA were also used for assignment tests in DOH (Fig. 1).

A large difference in sample size can have a dramatic effect on the outcome of the assignment tests. We set the null allele frequency to $p = 1/(\text{population sample size} \times \text{ploidy} + 1)$, which assumes that the next allele sampled would have been the one now missing in the population sample. To compensate for size differences among population samples we performed a jackknife resampling of our data with standardized population sample sizes corresponding to the smallest population sample size. By randomly drawing individuals from each respective subsample without new replacement data matrices ($n = 10\,000$) were generated and assignments performed. The mean assignment values for all replicates were then scored. These values were compared with standard assignment test scores.

Results

The results of the AMOVAs showed an east–west division for both types of data, accounting for 29 and 6% of the total variation in RAPD and microsatellite data, respectively. For both kinds of data there was also a detectable component of variation among populations within groups, representing 4 and 3% of the overall variation of the RAPD and microsatellite data, respectively.

The results of the STRUCTURE analysis revealed a high probability that our data comprised two populations for both types of data ($P(\text{data} \mid K = 2) = 0.96$). The only natural division of the datasets was found to be between east and west, in accordance with our previous results for mtDNA (Carlsson & Tegelström 2002). The assignment outputs from DOH, when data was divided into an eastern and a western group, also corresponded well with previous results based on mtDNA (Carlsson & Tegelström 2002). Seven individuals mis-assigned for both RAPDs and microsatellites and the majority of these individuals were located very close to, but west of the northern mtDNA contact zone, yet they assigned to the eastern lineage (Fig. 2a,b).

Assignment outputs from DOH are in the form of absolute probabilities, i.e. all locus-specific assignment probabilities multiplied (Brzustowski 2002), and naturally become very small, typically in the range 10^{-6} to 10^{-15}. Arguably, with more or less equal probability scores for each of two respective populations, an individual could be considered to be of mixed origin. We plotted individuals with assignment probability scores within a factor 10 for the eastern and western populations in order to discern areas of potential admixture (Fig. 2a,b). As is evident in Fig. 2, the vast majority of these ambiguous individuals are indeed located at or near areas of contact, although the microsatellite data do not display as clear a signal as the RAPD data.

The results from the different DOH assignment runs show some evidence of population substructuring within the eastern and western groups even given the crude and arbitrary population subdivision employed. The jackknife randomization procedure clearly decreases the number of erroneous assignments to the smallest population samples, although there is limited improvement of the overall assignment score (see supplementary data online).

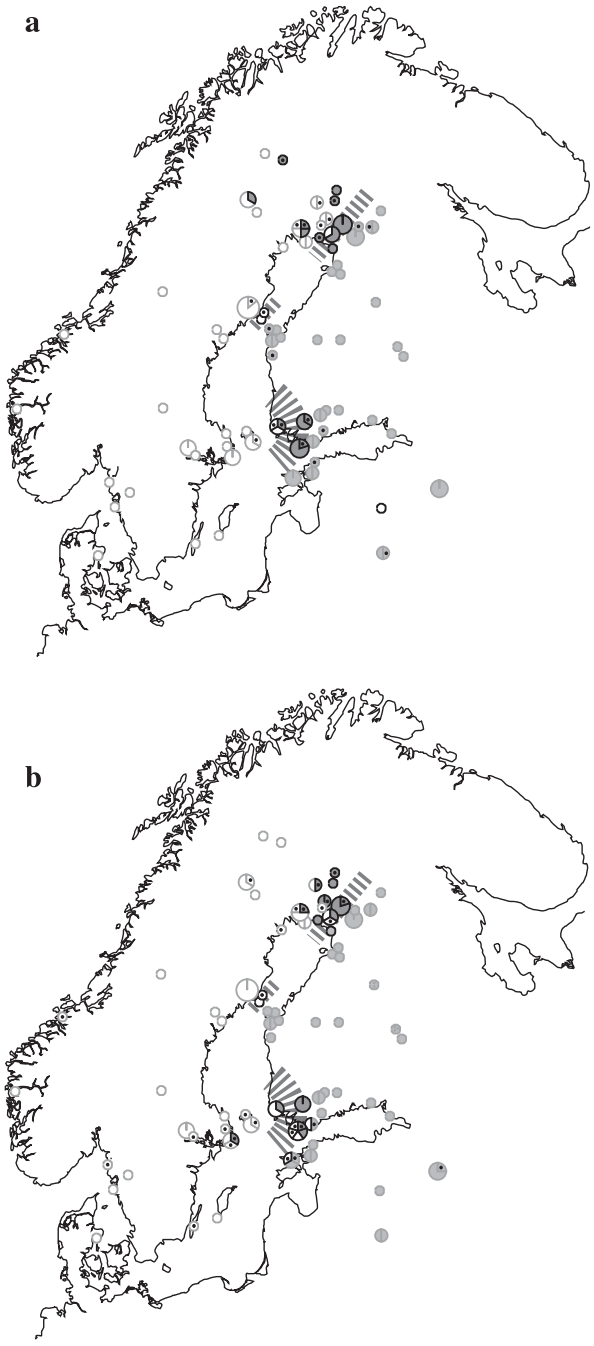

Fig. 2 DOH assignment test results for data divided into two population samples are presented, where grey samples are assigned to the eastern group and noncoloured samples are assigned to the western group. The 24 individuals omitted from the population definitions located close to the mtDNA contact zones (hatched areas) and individuals that assigned contrary to expectations are shown with block circle edges. As a potential indicator of gene flow, individuals with more or less equal assignment probability scores for both population samples are also indicated. When one individual's eastern and western assignment probabilities ranged within a factor 10 this is represented by a dot, respectively. (a) Assignments from RAPD genotypes. (b) Assignments from microsatellite genotypes.

Discussion

This study corroborates the phylogeographical pattern described for Fennoscandian adders based on mtDNA (Carlsson & Tegelström 2002). Both RAPD and microsatellite data indicated an eastern and a western lineage in the region, evident in both the assignment analyses and in the AMOVA analysis. The zones of contact between the two different adder lineages are situated congruently for both the microsatellite and RAPD markers and for the previously reported mtDNA data. The two contact zones, which are the result of across-water dispersal, are similar in appearance for both nuclear datasets (Fig. 2a,b) and for the previous mtDNA data, with a limited amount of gene flow being detected. However, all assignment results indicate a higher extent of gene flow across the northern contact zone than previously detected in mtDNA data (Carlsson & Tegelström 2002). Both for the RAPD and microsatellite data, seven individuals assigned to the wrong population of which six were situated in the northwest, suggestive of nuclear gene flow to the west across that particular contact zone (Fig. 2a,b). Alternatively, the contact zone was originally positioned further to the west, but has since moved eastwards. The pattern observed could reflect the larger population size of nuclear markers compared with mtDNA, in as much as some nuclear DNA of eastern origin may have survived where the mtDNA has become fixed for the invading haplotype.

Several methods have been suggested to infer hybrid origin or to detect migrant individuals through assignment tests (Campton & Utter 1985; Paetkau *et al*. 1995; Rannala & Mountain 1997; Pritchard *et al*. 2000; Anderson & Thompson 2002). Here we present a very simple approach to detect hybrid origin based on direct comparison of absolute probabilities. This approach is reminiscent to that of Campton & Utter (1985) who derived a hybrid index based on relative probabilities of combined genotypes, also in effect an assignment test.

The objective of the jackknife randomization was to reduce or eliminate the assignment bias towards smaller samples, which it succeeded in doing for both datasets and population sample divisions. The percentage of misassignment from any population sample to the smallest population sample was lowered, almost without exception. There was also an apparent improvement in the correct assignment scores evident for the microsatellite dataset (see supplementary data online).

Our findings suggest that the previously identified mtDNA contact zones can be roughly located with both kinds of nuclear DNA markers utilized in this study. The detection of individuals of dubious origin by straight comparison of assignment probabilities appears warranted as the majority of the individuals with equal assignment probabilities to either east or west are situated at or near

contact zones. Overall performance of the two nuclear DNA datasets indicates that our microsatellite markers hold much better promise for correctly assigning individuals on a local scale, using true population samples, than the RAPD markers, although both types of data show sufficient signal for regional comparisons (see supplementary data online). The jackknife method for reducing bias from unequal population samples in assignments appears to work well, although it may be a crude method and our datasets hold too little resolution for strong conclusions.

Gene flow across the contact zones of adders in Fennoscandia is evident using both microsatellite and RAPD markers. Both datasets suggest a far-reaching influx of nuclear DNA from the east to the west in the northern contact zone and to a lesser extent in the southern zone. Thus, the reported pattern of nuclear admixture could be the effect of either a shift in the location of the contact zones or differential dispersal between sexes and phylogroups. Extensive sampling in and around the zones of contact should facilitate a resolution of the unique pattern of introgression found among North European adders.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2315/MEC2315sm.htm

Table S1. PCR annealing temperatures, number of polymorphic loci for each of the seven RAPD primers (Operon Technologiesí nomenclature) and number of alleles for each of the six microsatellite (MS) loci used in the present study

Table S2. Assignment values from 98 RAPD and microsatellite genotypes of adders divided into eight population samples. Sample designations correspond to Fig. 1. Upper values (%) represent Doh single test results for given population sample sizes (n), whereas lower values (% in bold) are means from 10 000 jack-knife randomisations for sample size $n = 5$. Dotted lines separate eastern from western population samples. Values are rounded off to nearest integer

Table S3. Assignment values from 108 RAPD and microsatellite genotypes of adders divided into eleven population samples. Population sample designation letters correspond to Figure 1. Upper values (%) are standard Doh test output for number (n) of individuals per population sample, lower values (%) in bold are means from 10 000 jack-knife randomisations, (where $n = 5$). Dotted lines delineate mtDNA contact zone population samples. Three true population samples only used in these assignment tests (I and J from contact zone samples previously classified as ìunknownî and the third true population sample (C_2) which is a subset of the C sample above ñ C_1 comprising the rest of the C-sample)

References

Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. *Genetics*, **160**, 1217–1229.

Avise JC (2000) *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, MA.

Brzustowski J (2002) Doh assignment test calculator [online]. http://www2.biology.ualberta.ca/jbrzusto/Doh.php.

Campton DE, Utter FM (1985) Natural hybridization between steelhead trout (*Salmo gairdneri*) and coastal cutthroat trout (*Salmo clarki clarki*) in two Puget Sound streams. *Canadian Journal of Fisheries and Aquatic Science*, **42**, 110–119.

Carlsson M (2003) Phylogeography of the Adder, Vipera berus, PhD Thesis, Uppsala University, Sweden.

Carlsson M, Isaksson M, Höggren M, Tegelström H (2003) Characterization of polymorphic microsatellite markers in the adder, *Vipera berus*. *Molecular Ecology Notes*, **3**, 73–75.

Carlsson M, Tegelström H (2002) The phylogeography of adders (*Vipera berus*) from Fennoscandia. In: *Biology of the Vipers* (eds Schuett GW, Höggren MH, Douglas ME, Greene HW), pp. 1–9. Eagle Mountain Publishing, Sandy, UT.

Gibbs HL, Prior KA, Weatherhead PJ (1994) Genetic analysis of populations of threatened snake species using RAPD markers. *Molecular Ecology*, **3**, 329–337.

Hadrys H, Balick M, Schierwater B (1992) Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology*, **1**, 55–63.

Höggren M, Tegelström H (1995) DNA fingerprinting shows within-season multiple paternity in the adder (*Vipera berus*). *Copeia*, **2**, 271–277.

Jaarola M, Tegelström H, Fredga K (1999) Colonization history in Fennoscandian rodents. *Biology Journal of the Linnean Society*, **68**, 113–127.

Kjølner S, Såstad SM, Taberlet P, Brochmann C (2004) Amplified fragment length polymorphism versus random amplified polymorphic DNA markers: clonal diversity in *Saxifraga cernua*. *Molecular Ecology*, **13**, 81–86.

Lougheed SC, Gibbs HL, Prior KA, Weatherhead PJ (2000) A comparison of RAPD versus microsatellite DNA markers in population studies of the massasauga rattlesnake. *Journal of Heredity*, **91**, 458–463.

Mockford SW, Snyder M, Herman TB (1999) A preliminary examination of genetic variation in a peripheral population of Blanding's turtle, *Emydoidea blandingii*. *Molecular Ecology*, **8**, 323–327.

Nilsson S (1847) *Däggdjuren*, 2nd edn. C. W. K. Gleerup, Lund, Sweden.

Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, **4**, 347–354.

Pamilo P, Nei M (1988) Relationships between gene trees and species trees. *Molecular Biology and Evolution*, **5**, 568–583.

Paxton RJ, Thorén PA, Tengö J, Estoup A, Pamilo P (1996) Mating structure and nestmate relatedness in a communal bee, *Andrena jacobi* (Hymenoptera, Andrenidae), using microsatellites. *Molecular Ecology*, **5**, 511–519.

Peakall R, Smouse PE (2001) *GENALEX: Genetic Analysis in Excel. Population genetic software for teaching and research*. Australian National University, Canberra. http://www.anu.edu.au/BoZo/GenAlEx/.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences of the USA*, **94**, 9197–9201.

Remington CL (1968) Suture–zones of hybrid interaction between recently joined biotas. *Evolutionary Biology*, **2**, 321–328.

Rosenberg NA, Nordborg M (2002) Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. *Nature Reviews Genetics*, **3**, 380–390.

Schiemenz H (1995) *Die Kreuzotter Spektrum*. Akademischer Verlag, Heidelberg, Germany.

Schneider S, Kueffer J-M, Roessli D, Excoffier L (1997) *ARLEQUIN: A software for population genetic data analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**, 6531–6535.

Wilson AC, Cann RL, Carr SM *et al.* (1985) Mitochondrial DNA and two perspectives on evolutionary genetics. *Biology Journal of the Linnean Society*, **26**, 375–400.

Yue G, Li Y, Chen F *et al.* (2002) Comparison of three DNA marker systems for assessing genetic diversity in Asian arowana (*Scleropages formosus*). *Electrophoresis*, **23**, 1025–1032.

Martin Carlsson is interested in phylogeography in general, with a particular interest in genetic and social structure of Reptiles.
