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Genetic structure in northeastern populations of the Alpine newt (*Triturus alpestris*): evidence for post-Pleistocene differentiation

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Abstract

Genetic variation in 13 populations of the Alpine newt, *Triturus alpestris*, was assessed at the northeastern margin of its range (southern Poland). Variation at six microsatellite loci was scored in 354 newts, and two mitochondrial DNA fragments (*c.* 2000 bp) were sequenced in a subset of 27 individuals. Significant differences in allele frequencies and the presence of private alleles determined genetic units corresponding to three separate mountain ranges, i.e. the Carpathian, Sudetes and Holy Cross Mountains. F_{ST}'s were three times greater in among than in within mountain range pairwise comparisons. An assignment test and pairwise F_{ST}'s suggested relatively high levels of gene flow at the local level, although the Sudetes populations revealed some subtle structuring. Genetic variation was lower in the Carpathians and Holy Cross Mountains. The geographic pattern of mitochondrial DNA variation indicated that these newt populations originated from a single glacial refugium/founder population, and that the colonization of southern Poland took place in an easterly direction. The data show that substantial neutral variation and between group divergence has accumulated relatively quickly in these low-vagility organisms. The Alpine newt case exemplifies species history as a factor determining patterns of genetic diversity in marginal populations.

Keywords: conservation, gene flow, population structure, *Triturus*

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Introduction

An understanding of the genetic structure of populations and gene flow among them is critical for the maintenance of ecologically and evolutionary viable species or populations (Frankham 2003; Avise 2004). In low-vagility organisms, medium-scale regional surveys investigating gene flow and population structure are most informative for understanding the processes governing their evolutionary trajectories. Considerable attention has been given to populations inhabiting the margins of species ranges. The risk of extinction in peripheral areas is highest and population densities are often lower than elsewhere (Hoffmann & Blows 1994; Lesica & Allendorf 1995), which predestines these populations to bottleneck events. This leads to the prediction that neutral genetic variation will be depleted in peripheral populations. Thus, a notion that these populations are unimportant for the evolutionary and conservation status of a species has persisted. This has been challenged by the idea that suboptimal environmental conditions in peripheral populations will promote adaptation to local conditions, providing a source of adaptively significant variation (García-Ramos & Kirkpatrick 1997). Therefore marginal populations may contribute to a species' evolutionary potential (Gaston 1998). The disagreement concerning the significance of these populations is as yet unresolved and requires further studies on the genetic variation in the periphery of species ranges. Another factor that may shape the pattern of genetic variation in peripheral areas is species history. Colonization processes, especially those that have taken place in formerly glaciated areas, are associated with loss of genetic variation and latitudinal trends in the levels of genetic variation (Hewitt 1999).

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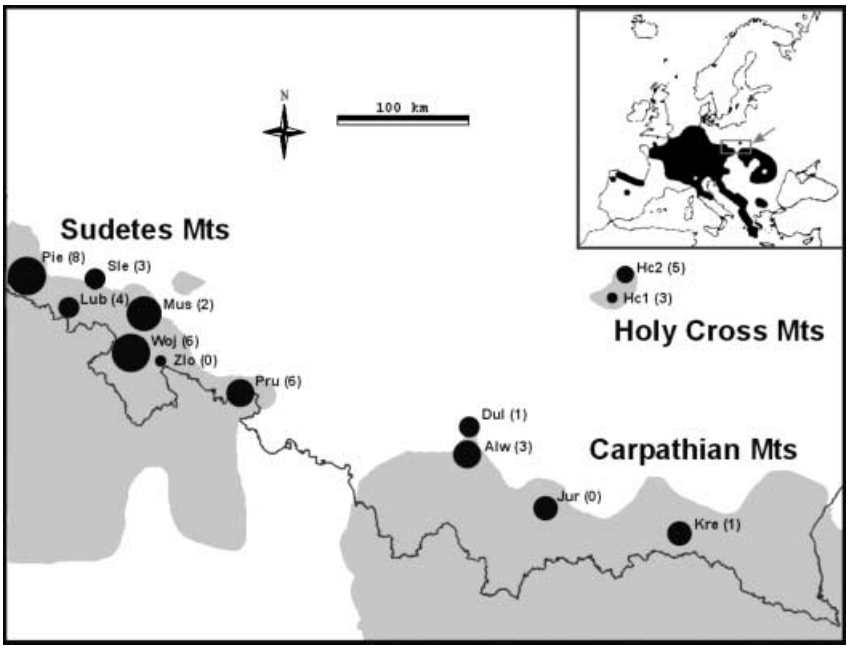

Fig. 1 Distribution of the Alpine newt *Triturus alpestris* in Poland and in Europe (shaded area). The locations of the studied populations are shown; the circles representing the populations are proportional to the total number of alleles in each population. The number of private alleles is given in parentheses. Population abbreviations as in Appendix. The solid line represents Poland's southern political border.

Low-vagility poikilotherms probably found it particularly hard to adjust to climatic change in short periods of time, thus pronounced range shifts must have occurred during the Pleistocene glaciations. The genetic signature of these range shifts is still in need of study. In this paper, we explore genetic variation in an amphibian, the Alpine newt, *Triturus alpestris* (Laurenti, 1768), at the margin of its range. Among amphibians, urodeles have limited dispersal capabilities and show substantial, and often ancient, genetic structure at a relatively small spatial scale (Arano *et al*. 1991; Ward *et al*. 1992; Weisrock *et al*. 2001; Babik *et al*. 2005).

The Alpine newt inhabits central and eastern Europe and some parts of the Iberian, Italian and Balkan peninsulas (Fig. 1, inset) (Gasc *et al*. 1997). The widespread nominative subspecies has a continuous distribution in central Europe, interrupted only by lowlands in the eastern part of its range. In southern Poland the Alpine newt is confined to the Carpathian, Sudetes and Holy Cross Mountains with lowland hiatuses of at least 100 km separating these groups of populations (Fig. 1). It is abundant in the western part of the Polish Carpathians, but becomes rare towards the east (Głowaciński & Rafiński 2003). Ecologically, it is a highly flexible species living in a wide altitudinal range and breeding in a variety of small water bodies (Juszczyk 1987; Roček *et al*. 2003). Previously we analysed the population genetic structure of the Alpine newt using allozyme markers (Pabijan *et al*. 2005). In Poland, the newt's distribution reaches its northeastern border and three distinct groups of populations inhabit separate mountain chains. The samples were collected at the beginning of the 1980s. On the basis of allozyme allele frequencies we postulated that populations from each separate mountain chain deserve management unit status. Now we extend the survey using recently acquired samples, and employ highly variable microsatellite markers and also mitochondrial DNA (mtDNA), aiming at a better understanding of genetic structure, history, and patterns of gene flow among the population groups, further validating the evolutionary and conservation status of these marginal populations.

Materials and methods

Laboratory methods

Tissue samples of 354 adult or larvae *Triturus alpestris* (toe or tail clips) from the Carpathians (four localities), Sudetes Mountains and their foreland (seven localities) and the Holy Cross Mountains (two localities), collected during the spring seasons in 2002–2004, were used for genetic analyses (Fig. 1, Appendix). DNA from frozen or alcohol-preserved tissues was extracted using a standard proteinase K-phenol–chloroform method. Multilocus genotypes were obtained from six microsatellite loci: *Ta1Ca1*, *Ta3Ca8*, *Ta4Ca4U*, *Ta1Caga4*, *Ta3Caga1*, *Ta3Caga2* (Garner *et al*. 2003). A seventh locus (*Ta2Caga3*) was discarded because of inconsistent amplification. These loci represent imperfect di- and/or tetranucleotide repeats and often possess mononucleotide runs, making them both compound and complex. Polymerase chain reaction (PCR) protocols followed Garner *et al*. (2003) with slight modification of the number of cycles (between 25 and 32 cycles depending on the quality of the DNA). Electrophoresis of PCR products was performed using the SEA 2000™ advanced submerged gel electrophoresis apparatus on EL 400, EL 600 or EL 800 Spreadex® gels

(Elchrom Scientific AG), depending on allele sizes. SYBR® Green I (Sigma) nucleic acid stain was used to visualize products under UV light. The M3 Marker (Elchrom Scientific AG was used as a size standard. Allele sizes were obtained using TOTAL-LAB 1.20 (Nonlinear Dynamics) and ascertained by eye.

The mtDNA analyses included at least one individual from every site (Appendix). For comparative purposes we also sequenced samples of *T. alpestris* from France (Ambleteuse, 3 individuals), and the Romanian (Retezat and Baiu Mountains, 4 individuals), and Ukrainian (Charnohora, 2 individuals) Carpathians. These specimens were collected between 1998 and 2005 (Appendix). The population from the Romanian Carpathians was originally intended as a reference sample representing a distant part of the mountain range. However, microsatellite loci amplified poorly in newts from this population, suggesting substantial genetic divergence from the Polish populations. Therefore, the Romanian newts were scored for mtDNA variation only. A 1146-bp fragment of the ND2 gene and 809-bp fragment of the ND4 gene were amplified and sequenced with primer pairs L3780/H5018 (Babik *et al*. 2005) and ND4/Leu (Arevalo *et al*. 1994), respectively. PCR, cycling schemes, PCR purification, and sequencing protocols were done according to Babik *et al*. (2005). Sequences were checked by eye and aligned manually in BIOEDIT 7 (Hall 1999).

Data analysis

Data were checked for misprint, scoring errors and deviations from Hardy–Weinberg proportions due to the presence of null alleles using MICRO-CHECKER (van Oosterhout *et al*. 2004). Deviations from Hardy–Weinberg equilibrium were also assessed by applying exact tests of Guo & Thompson (1992) in GENEPOP 3.3 (Raymond & Rousset 1995). Linkage disequilibria within populations were tested for each pair of loci using the Fisher exact test or its Markov chain Monte Carlo approximations in GENEPOP. *F*-statistics according to the formulas of Weir & Cockerham (1984) for each locus and across loci, global single locus gene diversities (H_T), allelic richness per locus and sample (R_S) and over all samples (R_T) were calculated in FSTAT (Goudet 2001). At this point one of the loci (*Ta4Ca4U*) was discarded due to significant linkage disequilibrium (see below), all the following analyses were conducted on a total of five loci. We tested for differences in R_S, F_{IS}, F_{ST}, H_O, H_E between groups (i.e. mountain ranges) using a procedure permuting samples among groups as implemented in FSTAT. Additionally, we formally assessed whether the intermountain range F_{ST}'s were higher than the values for intramountain comparisons by applying a permutational *t*-test. An analysis of molecular variance (AMOVA) approach as implemented in ARLEQUIN (Schneider *et al*. 2000) was used to partition the total variance in allele frequency data into among group, among populations within groups and within population covariance components. The sequential Bonferroni procedure (Rice 1989) was applied to adjust the significance level to $\alpha = 0.05$ whenever multiple tests were performed. Population structure within the Sudetes and Carpathian ranges was further assessed through an exact Bayesian analysis by enumerative calculation in BAPS (Corander *et al*. 2003).

The microsatellite markers differed dramatically in the amount of genetic variation attributable to each locus (see Results), thus the standardized genetic differentiation measure of Hedrick (2005) was employed in order to better evaluate whether the among-population differentiation is biologically significant. First, Nei's (1973) G_{ST} indices were calculated for each locus. Then, the maximum values for these indices were calculated, given the observed heterozygosity within subpopulations: $G_{ST(max)} = (1 - H_S)/(1 + H_S)$, where H_S is average subpopulation heterozygosity. The G_{ST} values were then standardized according to:

$$G'_{ST} = G_{ST}(1 + H_S)/(1 - H_S)$$

The extent of isolation-by-distance effects (IBD) was assessed by Mantel tests in GENEPOP with 10 000 permutations for all populations and for the Carpathian and Sudetes groups separately. Straight-line geographic distances were used in the analysis. Some authors (e.g. Funk *et al*. 2005), have used distances along landscape elements in assessing IBD, arguing that the latter are more realistic than straight line distances for species in mountainous habitat. However, detailed analyses of the distribution of Alpine newts in the Carpathian Mountains have shown that this species inhabits the entire landscape, often being quite abundant on mountain ridges as well as along valleys (Świerad 1988; Babik & Rafiński 2001). This, together with this species' tendency to breed in ephemeral wheel-ruts and puddles at all elevations, co-opted for the use of straight-line distances as a reasonable simplification.

Cavalli-Sforza chord distances were used in a nonmetric, multidimensional scaling procedure (NMDS) in order to explore the relationship between geography and genetic differentiation. NMDS is an ordination procedure in which interpopulational genetic differentiation is used to produce a clustering pattern similar to a geographic map (Lessa 1990; Jackman & Wake 1994). The technique does not necessarily reveal historical relationships between populations, but nonetheless provides a better fit to the data when populations have recently diverged and hierarchical relationships between them are difficult to confidently establish (Krauss 1996). The analysis was performed in STATISTICA (StatSoft 2001).

Gene flow among populations was assessed by pairwise F_{ST} in FSTAT. We used GENECLASS 2 (Piry *et al*. 2004) to assess contemporary migration rates using the Rannala & Mountain (1997) criterion, assigning individuals to populations where the probability of their multilocus genotype is the highest

Table 1 Single locus expected heterozygosity (H_E, upper rows) and allelic richness per locus and population (R_S, lower rows) and over all populations (R_T). Bottom row — average allelic richness over six loci in each population. Population abbreviations as in Appendix; number of newts sampled in each locality is given in parentheses

	Carpathians				Sudetes							Holy Cross		
	Alw (33)	Jur (24)	Dul (26)	Kre (24)	Woj (34)	Mus (30)	Zlo (11)	Lub (25)	Sle (32)	Pru (27)	Pie (38)	Hc1 (15)	Hc2 (35)	R_T
Ta1Ca1	0.477	0.438	0.511	0.509	0.652	0.538	0.591	0.655	0.446	0.688	0.732	0.429	0.457	
	2.000	2.000	2.000	2.000	2.999	2.603	3.000	3.000	3.166	3.815	3.944	2.000	2.000	3.212
Ta3Ca8	0.218	0.382	0.177	0.480	0.684	0.714	0.659	0.633	0.699	0.561	0.640	0.457	0.422	
	1.969	2.000	1.945	2.000	3.545	3.970	3.000	4.525	4.445	4.024	3.772	2.000	2.683	4.253
Ta3Caga2	0.462	0.440	0.489	0.444	0.471	0.506	0.509	0.428	0.507	0.425	0.496	0.203	0.386	
	2.000	2.000	2.000	2.000	2.546	2.000	2.000	2.691	2.000	2.653	3.653	2.746	2.000	2.509
Ta4Ca4U	0.218	0.402	0.183	0.469	0.691	0.716	0.659	0.520	0.690	0.539	0.666	0.457	0.416	
	1.969	2.000	1.954	2.000	3.868	3.967	3.000	3.769	3.883	3.344	4.388	2.000	2.532	4.265
Ta3Caga1	0.946	0.908	0.950	0.937	0.911	0.927	0.941	0.942	0.882	0.937	0.964	0.895	0.922	
	13.502	12.611	13.441	12.684	11.540	12.290	13.000	12.798	9.661	11.973	14.606	9.572	11.357	15.782
Ta1Caga4	0.899	0.947	0.914	0.891	0.960	0.964	0.800	0.822	0.744	0.894	0.943	0.868	0.888	
	12.191	14.309	11.567	12.186	15.590	16.208	8.000	7.552	9.570	12.471	14.116	8.272	10.072	14.228
mean R_S	4.560	4.735	4.462	4.457	5.436	5.565	4.339	4.656	4.438	5.191	6.032	3.606	4.156	

based on the population's allele frequency distribution. A total number of 1000 individuals were simulated and a threshold of 0.05 was used. Tests for recent effective population size reductions from the allele frequency data were conducted in BOTTLENECK (Cornuet & Luikart 1996) under the two-phased model of microsatellite mutation (Di Rienzo *et al*. 1994) with 70% SMM employed.

Results

The number of alleles per locus ranged from 6 (*Ta1Ca1*), and 8 (*Ta3Ca8*, *Ta3Caga2*, *Ta4Ca4U*) to 58 (*Ta3Caga1*) and 71 (*Ta1Caga4*). The total number of alleles across loci was 159. Locus *Ta3Caga1* showed a small though consistent heterozygote deficit, most likely due to null allele(s) across 8 populations (Pie, Lub, Mus, Woj, Pru, Alw, Kre, Hc1, estimated null allele frequencies available upon request). Several populations showed a significant excess of homozygotes at some loci after Bonferroni adjustment: Lub (*Ta4Ca4U*, *Ta1Caga4*), Pie (*Ta1Ca1*, *Ta3Caga2*), Woj (*Ta1Ca1*) and HC1 (*Ta1Caga4*). Significant linkage disequilibrium was detected between *Ta3Ca8* and *Ta4Ca4U* in all samples ($P < 0.001$), except for Dul, and between *Ta3Caga1* and *Ta1Caga4* in Pru ($P < 0.001$), after Bonferroni correction.

Table 1 presents measures of genetic diversity for loci and populations. Among loci, allelic richness varied between 3.212 (*Ta1Ca1*) to 15.782 (*Ta3Caga1*). Gene diversity and allelic richness were significantly higher in populations from the Sudetes Mountains (Table 2) than in those from the Carpathian or Holy Cross ranges. The lowest values were found in the Holy Cross Mountains (Hc1 and Hc2) and in two sub-Carpathian populations (Alw, Dul). The Sudetes populations had 132 alleles, while the Carpathians and Holy Cross populations contained 96 and 47 alleles, respectively. A total of 76 alleles were shared between the Sudetes and Carpathian populations, 33 between the Carpathian and Holy Cross populations, and 30 between the Sudetes and Holy Cross populations. Private alleles, i.e. those detected only in one of the three regions, were numerous in the Sudetes (53 alleles), but relatively few in the Carpathians (10) and Holy Cross Mountains (11). The numbers of private alleles per population

	R_S	H_O	H_E	F_{IS}	F_{ST}
Carpathians	5.597	0.531	0.567	0.063	0.023
Sudetes	6.261	0.642	0.703	0.087	0.043
Holy Cross	4.769	0.550	0.573	0.040	0.002
P	**0.044**	0.191	**0.013**	0.831	0.791

Variation among groups	0.16 (***P* < 0.001**)
Variation among populations within groups	0.14 (***P* < 0.001**)
Variation within populations	99.70 (***P* < 0.001**)

Table 2 Comparison of diversity indices among groups; two-sided *P* values after 10 000 permutations. R_S, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; F_{ST}, variance among subpopulations. Population differentiation as assessed by an analysis of molecular variance of microsatellite allele frequencies. Values are the percentages of variation explained by each covariance component. Significance of components based on 1000 permutations

Table 3 Comparison of Nei's (1973) G_{ST} values among loci and the maximum levels ($G_{ST(max)}$) this index can attain for the observed amount of genetic variation at each locus (Hedrick 2005). Values in parentheses are the percentages of the maximum differentiation attained after standardization of G_{ST} (see Methods)

	G_{ST}	$G_{ST(max)}$
Ta1Ca1	0.069	0.291 (23.6%)
Ta3Ca8	0.110	0.318 (34.5%)
Ta4Ca4U	0.132	0.385 (34.3%)
Ta3Caga2	0.116	0.324 (35.7%)
Ta3Caga1	0.036	0.037 (96.4%)
Ta1Caga4	0.049	0.059 (81.8%)

ranged between 0 and 8 (Fig. 1). One population in the Holy Cross Mountains (Hc2) showed a significant excess of heterozygosity ($P < 0.05$, one-sided Wilcoxon test), while another population in the Carpathians approached significance (Dul; $P = 0.07$) suggesting recent bottlenecks. Unfortunately, the power of the tests for population bottlenecks was diminished by the use of only five loci.

Overall, among population differentiation was significant, $F_{ST} = 0.078 \pm$ (SE) 0.017; with the loci containing the lowest number of alleles contributing the most to among population differences in allele frequencies (Table 3). However, after standardization, two highly variable loci (*Ta3Caga1*, *Ta1Caga4*) were shown to have come close to attaining the maximum differentiation possible, given the observed levels of heterozygosity (Table 3). Thus, despite low F_{ST} levels, populations were highly structured. AMOVA results showed that most of the variation was found within populations, however, among group (i.e. mountain ranges), and among population variance components both were significant (Table 2).

Table 5 Results of an assignment test on microsatellite genotypes of *Triturus alpestris*, values indicate the percentages of individuals assigned to their population of origin or geographically proximate populations. Population abbreviations as in the Appendix

	Pop. of origin	Nearest pop.		Next nearest		Unassigned
Alw	39.4	18.2	(Dul)	6.1	(Jur)	36.3
Jur	29.7	—	(Alw)	8.3	(Kre)	62.0
Dul	38.5	23.1	(Alw)	3.8	(Jur)	34.6
Kre	29.2	33.3	(Jur)	4.2	(Alw)	33.3
Woj	32.4	2.9	(Zlo)	20.6	(Mus)	44.1
Mus	60.0	10.0	(Woj)	—	(Sle)	30.0
Zlo	27.3	27.3	(Woj)	9.1	(Mus)	36.3
Lub	64.0	—	(Pie)	28.0	(Sle)	8.0
Sle	28.2	18.7	(Lub)	9.4	(Mus)	43.7
Pru	22.2	—	(Zlo)	7.4	(Woj)	70.4
Pie	57.9	5.3	(Lub)	—	(Sle)	36.8
Hc1	26.7	52.3	(Hc2)	6.7	(Dul)	13.3
Hc2	62.9	14.3	(Hc1)	—	(Dul)	22.8

Pairwise F_{ST} values were relatively low, indicating substantial historical gene flow (Table 4). A substantial proportion of intraregional comparisons were not significantly different from zero (Table 4). Intermountain range F_{ST}'s were shown to be approximately three times higher than intramountain range comparisons (mean intermountain range pairwise F_{ST}: $0.096 \pm$ (SE) 0.006; mean intramountain range F_{ST}: 0.037 ± 0.005; $P < 0.0001$, permutational t-test). In the assignment test, 40.7% of individuals were assigned to their population of origin. Some newts were assigned to localities that are geographically proximate to their true sampling sites (Table 5). However, many were assigned to distant populations (unassigned column in Table 5) which,

Table 4 Matrix of pairwise F_{ST} values and corresponding straight-line geographic distances (in km, below diagonal) for 13 populations of the Alpine newt in southern Poland. Bold type indicates comparisons that were significantly different from 0 after 1000 permutations and Bonferroni correction. Population abbreviations as in the Appendix

	Carpathians				Sudetes							Holy Cross	
	Alw	Jur	Dul	Kre	Woj	Mus	Zlo	Lub	Sle	Pru	Pie	Hc1	Hc2
Alw	—	**0.017**	0.003	**0.024**	**0.086**	**0.103**	**0.078**	**0.045**	**0.115**	**0.058**	**0.059**	**0.151**	**0.096**
Jur	69	—	**0.028**	0.009	**0.068**	**0.090**	**0.039**	**0.044**	**0.124**	**0.040**	**0.042**	**0.176**	**0.121**
Dul	13	73	—	**0.023**	**0.078**	**0.093**	**0.075**	**0.052**	**0.094**	**0.059**	**0.057**	**0.122**	**0.064**
Kre	160	92	160	—	**0.048**	**0.071**	0.021	**0.029**	**0.068**	**0.039**	**0.029**	**0.154**	**0.103**
Woj	208	275	208	368	—	**0.025**	0.023	**0.039**	**0.065**	**0.027**	**0.015**	**0.167**	**0.128**
Mus	191	256	190	349	28	—	0.030	**0.073**	**0.069**	**0.054**	**0.048**	**0.136**	**0.106**
Zlo	190	254	190	349	18	23	—	0.036	**0.074**	0.023	0.026	**0.163**	**0.118**
Lub	258	324	259	417	50	70	69	—	**0.081**	0.017	**0.016**	**0.190**	**0.140**
Sle	217	284	213	372	42	32	51	56	—	**0.100**	**0.074**	**0.140**	**0.117**
Pru	136	202	138	296	70	57	52	121	88	—	0.007	**0.186**	**0.135**
Pie	290	357	290	450	84	101	103	32	80	154	—	**0.168**	**0.127**
Hc1	151	158	137	178	301	276	286	344	289	242	369	—	0.003
Hc2	140	142	129	161	302	279	290	344	290	240	372	16	—

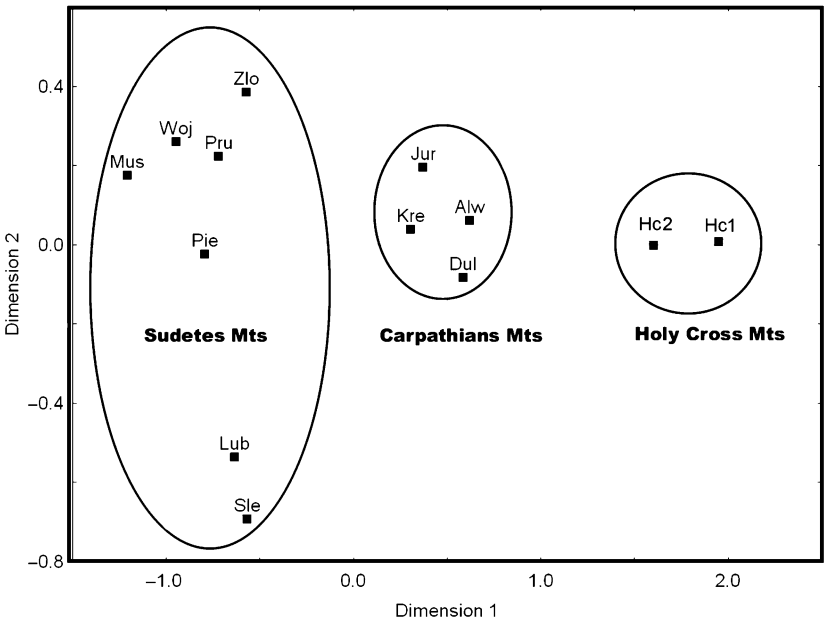

Fig. 2 Two-dimensional scaling of 13 populations of the Alpine newt based on a matrix of Cavalli-Sforza chord distances computed from 5 microsatellite loci; stress = 0.048; for abbreviations see Appendix.

given the limited dispersal capabilities of newts, is rather associated with the occurrence of alleles at high frequency across populations.

The Mantel test revealed a weak positive relationship between geographic and genetic distances based on all populations ($r = 0.031$; $P = 0.003$). At a regional scale, there was no correlation between geographic and genetic distances in the Sudetes samples ($r = 0.001$; $P = 0.474$, 7 populations), or the Carpathian populations ($r = 0.007$; $P = 0.086$; 4 populations). The Carpathian and Holy Cross populations were clearly separated from each other in the NMDS analysis; the Sudetes cluster, distinct from the rest, was composed of two subgroups (Fig. 2). The Bayesian analysis of genetic structure revealed a single cluster in the Carpathians encompassing all populations ($P < 0.001$). Three clusters were found within the Sudetes range ($P < 0.001$): two clusters were represented by single populations (Lub and Sle) while the third contained the remaining populations (Pie, Mus, Pru, Woj, Zlo).

Sequences for mitochondrial ND2 and ND4 fragments (1957 bp combined) were obtained for 27 individuals and were represented by seven haplotypes (GenBank accession no.: DQ282132–DQ282143, Appendix). Two frequent haplotypes, differing by only two substitutions in ND4, were found. One of these was limited to all Sudetes and French samples (hap A), the other was found in the northern Carpathians including the Holy Cross and Ukraine samples (hap B), except for a single individual from Alw (hap C) which was a singleton variant of hap B. The Romanian samples constituted four haplotypes differing by 30–36 amino acid substitutions (15.9–16.8%) from the Polish samples. Pairwise nucleotide *p*-distances between Romanian and Polish haplotypes varied between 8.3 and 8.7%.

Discussion

The overall level of genetic variation revealed by microsatellites in the Alpine newt was much higher than that reported in skin-grafting and allozyme studies (Rafiński 1974; Pabijan *et al*. 2005). Exceptionally high levels of variation are reported here for two of the loci (*Ta3Caga1* and *Ta1Caga4*), consistent with the extraordinary microsatellite variation seen in other taxa (e.g. van Oppen *et al*. 1997; Streiff *et al*. 1998) but not in amphibians (Newman & Squire 2001). However, the microsatellite loci applied in our study differed by as much as an order of magnitude in the level of variation as expressed by the number of alleles per locus. This likely reflects the underlying differences in the mutation rate between loci, which in turn may be influenced by the repeat structure or position in the genome (Li *et al*. 2002). The variation seen in the remaining loci is rather moderate and comparable to that of microsatellite loci used in other amphibians (see review in Newman & Squire 2001).

Genetic units in the Alpine newt in Poland

Significant regional (i.e. between mountain ranges) components were detected in the overall variation, reflected in pronounced differences in allelic and gene diversity between populations and higher level groupings. Pairwise F_{ST} values between mountain ranges were three times higher than intramountain range values. Thus, the highly variable markers used here have corroborated previous conclusions (Rafiński 1974; Pabijan *et al*. 2005) of the existence of three discrete genetic units in a relatively small portion of this species' range. Two independent lines of evidence support the genetic units identified in this study. Rafiński (1974) discovered

interregional (but not intraregional) genetic divergence of these newts through skin grafting experiments. Pabijan *et al*. (2005) defined conservation units, corresponding to the mountain ranges in southern Poland, on the basis of significant differences in allozyme allele frequencies. In all, these peripheral population groupings seem to have diverged from each other as a result of the interwoven effects of isolation, genetic drift, and possibly natural selection. Consequently, the conservation of these units may be beneficial to the protection of the evolutionary processes that are likely to generate future evolutionary diversity in this species. We suggest that these three population groupings be managed separately, on the basis of divergence in the nuclear genome, where loci controlling traits of adaptive significance occur.

Generally an inverse relationship exists between organismal vagility and population structure as measured by F_{ST} (Avise 2004). Thus, gene flow between populations of relatively sedentary organisms should decrease with increasing geographic distance between them. In agreement, our data show low levels of gene flow among three allopatric groups of populations. In fact, interregional recruitment of individuals is highly unlikely given the geographic distances between regions and the limited dispersal capabilities of newts (Joly & Miaud 1989; Arntzen & Wallis 1991). The overlap in sets of alleles seen in the now completely isolated groups may be the result of constraints on repeat numbers in the microsatellite loci or back mutation (Nauta & Weissing 1996). However, we think that it is more plausible that they share a recent common ancestry (see below) and therefore high frequency alleles are found across populations. Additionally, we have sequenced a subset of microsatellite homozygotes from all three mountain ranges and have found no evidence for size homoplasy (M. Pabijan, unpublished), although this is by no means conclusive.

The substantial variation and divergence among groups of populations in three types of nuclear markers (microsatellites, allozymes and skin grafting), compared to the lack of resolution in the mtDNA, emphasizes the need to use more than one type of genetic marker before making judgements on the evolutionary or conservation status of marginal populations (cf. Monsen & Blouin 2003). If only the mtDNA had been studied, we would have postulated a single genetic unit for the Alpine newt in this area and recommended very different management practices.

Regional genetic structure

Given the strong differentiation between Alpine newt populations inhabiting different mountain ranges, we now focus our discussion on within mountain range variation. At this level, isolation by distance effects were weak and may have been confounded by the complex structure of mountainous habitat, which on the one hand may physically impede gene flow on account of inaccessible terrain, but on the other may facilitate migration via some landscape elements, e.g. through valleys (Funk *et al*. 2005; Spear *et al*. 2005). However, the Carpathians in southern Poland are for the most part rather gentle sloping, low mountains, and detailed studies have shown that the Alpine newt and other amphibian species, such as the endemic Carpathian newt *Triturus montandoni*, inhabit both mountain ridges and valleys (Świerad 1988; Babik & Rafiński 2001). Locally, their distributions seem to be determined by the presence of breeding habitat, which, for the Alpine newt, can be anything from wheel ruts to permanent ponds and even slow-moving streams (Juszczyk 1987; Świerad 1988). In agreement, levels of gene flow between local populations of Alpine newts in the Carpathians and Holy Cross Mountains are considerable, as we have failed to detect any substantial differentiation within these areas, this being despite distances of up to 160 km between sampling sites. A bottleneck event was inferred for one population in the Holy Cross Mountains (Hc2), while another population in the Carpathians approached significance (Dul). These bottlenecks could be due to inbreeding depression, recent habitat fragmentation, the presence of null alleles, founder effects, or a combination of these factors. Inbreeding depression and recent habitat fragmentation seem unlikely due to the considerable gene flow occurring between populations of newts in these areas. Null alleles, although detected in many of our populations, were not inferred in Hc2 and Dul. Recent founder events may be involved as both of these populations are located at the margin of the species distribution and thus may have just recently been colonized.

Generally, among population differentiation is higher in the Sudetes than in the remaining mountain ranges. Two populations, Lub and Sle, clearly separate from the remaining Sudetes samples as shown in the NMDS and Bayesian analyses, indicating subtle genetic substructuring in this mountain range. Sle seems to be quite distinct and genetically isolated, in accordance with its semi-isolated position at the margin of the Sudetes range. There are also pronounced differences in the number of private alleles between the Sudetes samples, e.g. three populations contain most of the diversity in the Sudetes (Pie, Woj and Pru). The allelic diversity of a population is thought to be primarily determined by the number of populations contributing immigrants (Saccheri *et al*. 1998), suggesting that these populations are well connected with neighbouring populations and exhibit considerable gene flow.

Although few microsatellite studies investigating gene flow between populations of mountain dwelling amphibians are available for comparison, all exhibited higher F_{ST}'s than those shown for the Alpine newt in southern Poland (Call *et al*. 1998; Monsen & Blouin 2003; Funk *et al*. 2005; Martínez-Solano *et al*. 2005; Spear *et al*. 2005). In part, this

may be due to the extremely high variation revealed in two of our loci. Measures of among population variation for multiple alleles have been shown to be particularly dependant on the level of variability, their magnitudes decreasing with increasing variation (Hedrick 1999). After standardization, the genetic differentiation seen in our most variable loci was quite high and in one case approached nonoverlapping sets of alleles for subpopulations, indicating a biologically significant structure despite low F_{ST} values. Nonetheless, our data point to relatively high dispersal in mountainous habitat, contrary to what is expected for generally philopatric amphibian species (Shaffer *et al*. 2000; Tallmon *et al*. 2000; Beebee 2005; but see Marsh & Trenham 2000).

Population history

All three allopatric population groups must have diverged relatively recently, after the Last Glacial Maximum. Present-day Poland was uninhabitable for newts during the last glacial period, so all populations must have been established postglacially. They were derived from a single refugial population as evidenced by a single mtDNA lineage detected in these populations. One of three detected haplotypes was found exclusively in the Sudetes, while the other (and its singleton variant) was specific for the Carpathians and Holy Cross Mountains. The Sudetes haplotype was also found in specimens from northwestern France, so the extent of the Sudetes genetic unit may in fact encompass a much larger area. On the contrary, our Carpathian unit apparently does not inhabit the entire Carpathian range, as demonstrated by a very divergent mtDNA lineage found in the southern Carpathians (and microsatellite amplification problems). Because range expansions are thought to involve repeated founder events reflected in decreased genetic variation and weak population structure, which is especially apparent in formerly glaciated areas (Hewitt 1999, 2000), we hypothesize that a single widespread mtDNA lineage, closely related to our haplotypes A–C, colonized a large expanse of central Europe extending from northwestern France to western Ukraine. A clear west–east gradient in the number of private alleles and allelic richness across southern Poland points to an easterly direction of post-Pleistocene expansion. Similar patterns of genetic variation, suggesting species history as the overriding factor shaping the genetic diversity in marginal populations, have been shown for at least two other amphibian species (Garner *et al*. 2004; Martínez-Solano *et al*. 2005).

An important implication of this scenario is that these genetic units have diverged from one another in the relatively short amount of time of *c*. 10 000 years. The generation time in Alpine newts is highly dependent on elevation but can be averaged as 5 years using data from Miaud *et al*. (2000). Therefore, *c*. 2000 generations have passed since colonization took place. However, divergence between contemporary population groups can only have started after gene flow was curbed, i.e. when the distribution of northeastern populations began to resemble that of today. The timing of this event is speculative at best, but evidence exists for dry climatic conditions between 8000 and 4500 cal year BP (Seppa & Poska 2004), which may have disconnected a previously contiguous (and maybe more extensive) distribution. Support for this hypothesis comes from ecological observations of urodeles which have shown that local climatic fluctuations involving dryness may substantially restrict gene exchange even on a very local scale (e.g. Maiorana 1977). Accordingly, the southernmost populations of Alpine newts, inhabiting the driest and most unpredictable environments, also have the largest between-population differentiation in allozyme frequencies (Kalezic & Hedgecock 1980; Kyriakopoulou-Sklavounou 2000). If divergence between contemporary population groups was driven by climatic change during the Holocene, then *c*. 900–1600 generations have passed during which a substantial amount of neutral differences have accumulated. Thus, the Alpine newt has acquired substantial variation in some parts of its genome despite its 'northern purity' in mtDNA (Hewitt 2000), and also evidences the evolutionary potential of marginal populations.

Conclusions

This work focused on the population genetic structure of a low-vagility organism from a regional perspective. Despite geographic proximity, three unique genetic units with incipient evolutionary trajectories were distinguished at the northeastern border of the Alpine newt's range. This conclusion was based on divergence in nuclear loci, while very little variation was seen in mtDNA. Gene flow within these mountainous regions was unexpectedly high. These genetic units must have differentiated after a post-Pleistocene colonization/expansion, most probably from a western refugium, as suggested by the longitudinal gradient in genetic variation.

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Appendix

Collection information and mitochondrial DNA haplotypes found in each sampling site

Population name	Locality/region	Number of individuals	mtDNA haplotypes*	Geographic coordinates	Collector and year of collection
Alw	Alwernia; N Carpathians	33	C (1)	50°40′N, 19°31′E	MP, 2002
Jur	Jurków; N Carpathians	24	B (1)	49°40′N, 20°15′E	A. Osikowski, 2002
Dul	Dulowa; N Carpathians	26	B (2)	50°80′N, 19°31′E	MP, 2002
Kre	Krempna; N Carpathians	24	B (1)	49°31′N, 21°31′E	WB, 2002
Woj	Wojborz; Sudetes Mts	34	A (1)	50°31′N, 16°38′E	M. Ogielska, 2002
Mus	Muszkowice; Sudetes Mts	30	A (1)	50°38′N, 16°58′E	MP & Z. Boratyński, 2002
Zlo	Złoty Stok; Sudetes Mts	11	A (1)	50°26′N, 16°52′E	M. Ogielska, 2003
Lub	Lubawka; Sudetes Mts	25	A (1)	50°44′N, 15°51′E	WB & M. Liana, 2004
Sle	Ślęża; Sudetes Mts	32	A (1)	50°53′N, 16°43′E	WB & M. Liana, 2004
Pru	Prudnik; Sudetes Mts	27	A (1)	50°19′N, 17°34′E	M. Liana, 2002
Pie	Piechowice; Sudetes Mts	38	A (1)	50°51′N, 15°35′E	MP & Z. Boratyński, 2002
Hc1	Holy Cross Mts	15	B (1)	51°01′N, 20°53′E	MP, 2003
Hc2	Holy Cross Mts	35	B (5)	50°51′N, 20°53′E	MP, 2003
Romania	Retezat Mts, S Carpathians	2	3, 4 (2)	45°20′N, 22°42′E	MP, 2003
Romania	Baiu Mts, S Carpathians	2	1, 2 (2)	45°25′N, 25°25′E	MP, 1998
Ukraine	Charnohora, E Carpathians	2	A (2)	48°12′N, 24°28′E	M. Bonk, 2005
France	Ambleteuse, NW France	3	A (3)	50°26′N, 01°36′E	JW Arntzen, 2003

*number of individuals sequenced in parentheses.