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Marked genetic structuring and extreme dispersal limitation in the Pyrenean brook newt *Calotriton asper* (Amphibia: Salamandridae) revealed by genome-wide AFLP but not mtDNA

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Abstract

Direct estimation of dispersal rates at large geographic scales can be technically and logistically challenging, especially in small animals of low vagility like amphibians. The use of molecular markers to reveal patterns of genetic structure provides an indirect way to infer dispersal rates and patterns of recent and historical gene flow among populations. Here, we use mitochondrial DNA (mtDNA) sequence data and genome-wide amplified fragment length polymorphism markers to examine population structure in the Pyrenean brook newt (*Calotriton asper*) across four main drainages in the French Pyrenees. mtDNA sequence data (2040 bp) revealed three phylogroups shallowly differentiated and with low genetic diversity. In sharp contrast, variation in 382 amplified fragment length polymorphism loci was high and revealed a clear pattern of isolation by distance consistent with long-term restriction of gene flow at three spatial scales: (i) among all four main drainages, (ii) between sites within drainages, and (iii) even between adjacent populations separated by less than 4 km. The high pairwise F_{ST} values between localities across numerous loci, together with the high frequency of fixed alleles in several populations, suggests a combination of marked geographic isolation, small population sizes and very limited dispersal in *C. asper*. The contrasting lack of variation detected in mtDNA sequence data is intriguing and underscores the importance of multilocus approaches to detect true patterns of gene flow in natural populations of amphibians.

Keywords: AFLP, amphibian, dispersal, genetic structure, genome scan, phylogeograph

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Introduction

Dispersal of individuals among populations is a central parameter in ecological and evolutionary dynamics of natural populations (Clobert *et al.* 2001; Nathan *et al.* 2003), and is critical for population viability in species with fragmented distributions (Hanski & Ovaskainen 2000). However, direct estimation of dispersal and connectivity in natural populations is often difficult to obtain and requires labour-intensive experimental designs at local geographic scales (McGarigal & Cushman 2002; Bowne & Bowers 2004; Smith & Green 2005). Molecular markers can provide data on population genetic structure from which to infer patterns of gene flow and dispersal, and investigate mechanisms of evolutionary divergence (Slatkin 1985; Bohonak 1999). This can be particularly valuable in animals of small body size, small local population sizes and low vagility like many amphibians. Because amphibian populations are often vulnerable to anthropogenic activities and have been shown to experience widespread population declines in fragmented populations (Alford & Richards 1999; Stuart *et al.* 2004; Cushman 2005), landscape level

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studies of genetic variation are crucial to understanding their ecological and evolutionary dynamics and assessing their long-term viability.

Most studies of genetic population structure in amphibians have focused on mitochondrial DNA (mtDNA) sequence data and microsatellites (reviews in Vences & Wake 2007; Zeisset & Beebee 2008), while genome-level assessments of genetic variation using amplified fragment length polymorphism (AFLP) are scarce despite their potential for elucidating patterns of genetic structure at various geographic scales (Bensch & Akesson 2005). Here, we combine mtDNA sequence with genome-wide AFLP loci to examine patterns of gene flow in the Pyrenean brook newt *Calotriton asper*. The species is found in fast-flowing streams, alpine lakes and caves across most of the French, Andorran and Spanish Pyrenees, typically ranging in altitude from 500 m to 2500 m (Montori *et al*. 2002). A genetically and morphologically distinct population in the Montseny Mountains of Spain was recently described as a separate species (*Calotriton arnoldi*), but otherwise no significant genetic structure has been found within *C. asper* using either mtDNA sequence data (Carranza & Amat 2005) or allozymes (Gasser & Clergue-Gazeau 1981; Montori *et al*. 2008). A limited survey by Schlegel *et al*. (2009) found two nucleotide changes in the control region sequence of six individuals from three French localities, but the limited sampling precludes any further inference. This lack of genetic structure is in contrast with the high levels of phenotypic variation found across the species range, which includes differences in size, skin texture and colouration (Clergue-Gazeau & Martínez-Rica 1978; Clergue-Gazeau & Bonnet 1980; Serra-Cobo *et al*. 2000).

This unexpected genetic uniformity in the species has been ascribed to the potentially high rates of juvenile dispersal during the 2-year-long terrestrial stage following metamorphosis and before individuals return to water for the remainder of their life cycle (Montori 1988). The fact that *C. asper* inhabits high-altitude lakes and streams in areas that were recently glaciated, has also been proposed as evidence for its purportedly high dispersal capacity (Carranza & Amat 2005). However, in the absence of direct measures of individual dispersal and multilocus genetic data to complement existing mtDNA and allozymic surveys, the temporal and spatial scales of *C. asper* dispersal and gene flow have remained a matter of speculation.

Methods

Field sampling

Between 2006 and 2007, specimens of *Calotriton asper* were captured at 17 different localities throughout the French Pyrenees (Table 1, Fig. 1A), encompassing most of the streams and caves where the species is known to occur in the region (O. Guillaume, personal observation). Following capture in the field, specimens were transported to the facilities of the CNRS Station of Experimental Ecology in Moulis, France, and are kept alive in aquaria to date.

DNA extraction and mtDNA sequencing

Genomic DNA was extracted from fresh tail clippings using a Qiagen™ (Valencia, California) Mini Kit and following the manufacturer's tissue-extraction protocol. The following regions of the mtDNA were amplified for a total of 2040 base pairs (bp): a 374 bp fragment of the cytochrome *b* gene using primers *Cytb1EuprF* and *Cytb2EuprR* from Carranza & Amat (2005) and condi-

Table 1 Sampling localities for *Calotriton asper* individuals in four main regions of the French Pyrenees: Aquitanie (IR, OL), Lourdes (BM, GL), Garonne (PL, BD, LA, AR, CO, SI, VI, RI) and Aude (AU, CA, CR, FD, VA)

Locality (code)	*n*	Elevation (*m*)	Latitude	Longitude	Type
Irati (IR)	15	1097	43.047542º	−1.057151°	Stream
Olhadoko (OL)	13	673	42.990381º	−0.949525°	Stream
Betharram (BM)	30	773	43.100823º	−0.191621°	Cave
Genie Longue (GL)	16	658	43.051385º	−0.147640°	Stream
Pas du Loup (PL)	18	506	43.009400º	0.998330°	Cave
Bernard (BD)	26	554	42.999328º	1.534566°	Cave
Labouiche (LA)	17	504	43.000278º	1.574080°	Cave
Arcouzan (AR)	9	1160	42.801249º	1.116890°	Stream
Courbiere (CO)	6	1596	42.850657º	1.451806°	Stream
Siech (SI)	7	797	42.882640º	1.545493°	Cave
Vicdessos (VI)	7	748	42.767173º	1.487772°	Cave
Ribaui (RI)	14	797	42.790770º	1.343743°	Stream
Auriac (AU)	7	516	42.930817º	2.492486°	Stream
Cailla (CA)	23	788	42.805993º	2.190989°	Stream
Cass-Rats (CR)	19	521	42.876078º	2.315065°	Stream
Font de Dotz (FD)	4	524	42.877961º	2.356708°	Shallow cave
Valmanya (VA)	10	951	42.534668º	2.544748°	Stream

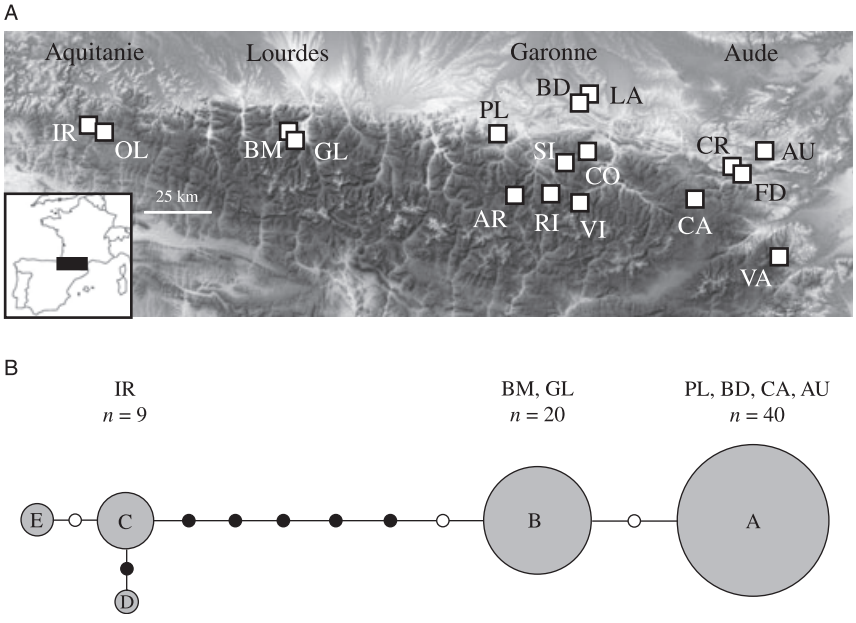

Fig. 1 (A) Geographic distribution of *Calotriton asper* localities sampled for this study. Locality codes correspond to names on Table 1. (B) Median-joining network of mitochondrial DNA haplotypes based on 2,040 bp of sequence. Grey circles represent haplotypes, with size proportional to frequency. Black dots along branches represent nucleotide changes or unsampled haplotypes, and white dots represent insertion-deletions (see also Table S2).

tions in Carranza *et al.* (2000); 895 bp encompassing 710 bp of the NADH dehydrogenase subunit 4 (ND4) gene, tRNA histidine (tRNA-his, 69 bp), tRNA serine (tRNA-ser, 67 bp) and tRNA leucine (tRNA-leu, 49 bp), using primers in Arevalo *et al.* (1994) and conditions in Martínez-Solano *et al.* (2006); and 771 bp of the control region using primers and conditions from Steinfartz *et al.* (2000). Sequences were automatically aligned using SEQUENCHER 4.1. (GeneCodes) and variable sites were checked visually for accuracy. Coding gene sequences were unambiguously translated into their amino acid sequence and no double peaks were observed in the chromatographs, suggesting that sequences were of mitochondrial origin and not pseudogenes. Haplotype and nucleotide diversity indices were calculated in DNASP (Rozas *et al.* 2003), and we constructed a network of haplotypes using the reduced median-joining algorithm in the program NETWORK v. 4.5.1.0 (Forster *et al.* 2007). We obtained sequence for a total of 69 individuals from various populations representing the different watersheds, which were distributed as follows: Irati (IR; $n = 9$), Betharram (BM; $n = 10$), Genie Longue (GL; $n = 10$), Pas du Loup (PL; $n = 10$), Bernard (BD; $n = 10$), Cailla ($n = 13$) and Auriac ($n = 7$).

AFLP profiling and analysis

Amplified fragment length polymorphism profiles were generated using a protocol modified slightly from Vos *et al.* (1995). A detailed laboratory protocol and sample electropherogram plots are provided in the Appendix S1 and Fig. S1 (Supporting information), respectively. In brief, whole genomic DNA was digested with restriction enzymes *Eco*RI and *Mse*I (*Tru9*) and fragments were ligated to oligonucleotide adapters with T4 DNA ligase. A random subsample of fragments was obtained through a pre-selective amplification reaction using primers E_T and M_C, followed by four selective amplifications using primer pairs E_{TCT}/M_{CCA}, E_{TGA}/M_{CCA}, E_{TAG}/M_{CGA}, and E_{TGC}/M_{CGA}, with each E primer fluorescently labelled. Eight pairs of selective amplification primers were tested, but only those producing repeatable and unambiguously scorable profiles were used in the analysis. Selectively amplified fragments were run in an ABI 3700 genetic analyzer (Applied Biosystems) with a LIZ500 size standard. Peaks were visualized using GENEMAPPER 3.7 (Applied Biosystems) and scored manually by a single observer (B. Milá). Only unambiguously scorable loci and individuals were included in the analysis and peaks found in less than 2% of individuals were excluded. Methodological error rate was assessed by running a subset of 10 individuals twice from the preselective amplification step. The average per-locus genotyping error rate for the AFLP loci selected, measured as recommended by Bonin *et al.* (2004), was low at 0.8%.

We estimated allelic frequencies using Zhivotovsky's (1999) Bayesian method with uniform prior distributions and assuming Hardy–Weinberg genotypic proportions. Genetic diversity (H_e), and per-locus F_{ST} values based on allele frequencies where calculated using the method by Lynch & Milligan (1994) as implemented in the program AFLP-SURV v. 1.0 (Vekemans *et al.* 2002). A matrix of pairwise population F_{ST} values using the F_{ST} analogue Φ_{PT} was calculated with GENALEX 6.0 (Peakall & Smouse 2006). Φ_{PT} is calculated as $V_{AP}/(V_{AP} + V_{WP})$

where V_{AP} is the variance among populations and V_{WP} is the variance within populations. Probability values of pairwise Φ_{PT} were based on 9999 permutations.

To assess genetic structure among samples, we conducted a principal coordinate analysis (PCoA; Orloci (1978)) on a genetic distance matrix generated from the binary presence–absence matrix as implemented in GENALEX 6.0. We also examined patterns of population structure using the Bayesian assignment probability test in the program STRUCTURE 2.2 (Pritchard *et al.* 2000). This program uses a Bayesian approach to generate posterior probabilities of assignment of individuals to each of a given number of groups (K) independently of the sampling site of origin. As recommended for dominant markers, we applied a model of no admixture with correlated allele frequencies (Pritchard & Wen 2004) and the optimal value of K was calculated following the method by Evanno *et al.* (2005).

To examine the pattern of isolation by distance, we tested the correlation between a matrix of pairwise $F_{ST}/(1 - F_{ST})$ values among localities and a matrix of the natural log of Euclidean geographic distances between localities using a Mantel test with 10 000 permutations of Spearman rank correlation coefficient with a one-tailed exact test, as implemented in the program ISOLDE in the package GENEPOP (Raymond & Rousset 1995). Due to the often abrupt topography of the Pyrenees, Euclidean geographic distances among localities may not be a realistic estimate of the distance an individual might travel, therefore we also estimated a real-distance measure that takes into account topographical relief between sites using the method by Beyer (2004) (Table S1, Supporting information).

We assessed genetic structure with an AMOVA and used allele frequencies to test the significance of the variance components associated with various hierarchical levels of genetic structure: within populations (Φ_{PT}, equivalent to Φ_{ST}), among populations within groups (Φ_{PR}, equivalent to Φ_{SC}) and among groups (Φ_{RT}, equivalent to Φ_{CT}) by means of nonparametric permutation methods (Excoffier *et al.* 1992) as implemented for dominant markers in GENALEX 6.0. Here, we examined variance components according to two designs, one grouping localities into the four main geographic watersheds (Aquitanie, Lourdes, Garonne and Aude), and the second one grouping localities into the main genetic units identified by the principal coordinate analysis described before.

Results

Variation in mtDNA sequence

Variation in the 2040 bp of mtDNA sequence examined in 69 individuals was low, with six nucleotide changes in ND4 (all synonymous transitions), two indels in the control region and one indel in tRNA-his. No variation was found in cyt *b*, tRNA-leu or tRNA-ser. The single haplotype found for cyt *b* corresponds to one of 12 detected by Carranza & Amat (2005) on the Spanish side of the Pyrenees (H3, GenBank accession DQ092243), which were themselves very similar and shown to diverge among them by only 0.56–0.84%.

Despite the low levels of variation, sequence data revealed three phylogroups corresponding to Aquitanie, Lourdes and the Garonne/Aude drainages, respectively (Fig. 1B). Including indels as changes for the purpose of calculating sequence divergence, IR individuals diverged from the Lourdes individuals (BM and GL) by six to eight changes, and BM and GL were divergent from all other populations to the east (Garonne and Aude) by a single change (Fig. 1B). Haplotype frequencies per population are provided in the Table S2 (Supporting information). Genetic diversity in all populations was zero except for IR, where haplotype diversity (h) and nucleotide diversity (π) in the ND4 gene were 0.222 and 0.00031, respectively. All sequences have been deposited in GenBank under accession nos: GU067485–GU067489.

Variation in AFLP loci

A total of 382 genome-wide amplified fragments were unambiguously scored for 241 individuals using four pairs of selective primers. Out of the total 382 loci, 103

Table 2 Primers used to generate AFLP profiles in *Calotriton asper*, and variability of loci

Combo	E primer	M primer	Total scorable loci	Mono-morphic loci	Variable loci	% Variable	Size range of peaks scored (bp)
C1	E-TCT	M-CCA	69	62	7	10	68-368
C2	E-TGA	M-CCA	91	59	32	35	56-358
C3	E-TAG	M-CGA	125	92	33	26	54-382
C4	E-TGC	M-CGA	97	66	31	32	95-443
Total			382	279	103	27	

Table 3 Genetic diversity in *Calotriton asper* populations estimated from 103 AFLP variable loci

Locality (code)	*n*	No. alleles*	No. alleles (freq.>5%)†	% polymorphic loci‡	No. private alleles§	Local alleles (<25%)¶	Local alleles (<50%)**	H_e††	SE (H_e)
Irati (IR)	15	51	51	49.5	0	9	19	0.053	0.014
Olhadoko (OL)	13	52	52	50.5	0	10	20	0.051	0.013
Betharram (BM)	30	64	62	60.2	1	16	29	0.026	0.010
Genie Longue (GL)	16	63	63	61.2	0	17	29	0.056	0.014
Pas du Loup (PL)	18	50	50	48.5	2	4	13	0.019	0.008
Bernard (BD)	26	55	53	51.5	1	0	5	0.053	0.013
Labouiche (LA)	17	52	52	50.5	0	0	4	0.038	0.011
Arcouzan (AR)	9	62	62	60.2	0	1	11	0.104	0.018
Courbiere (CO)	6	52	52	50.5	0	0	4	0.057	0.014
Siech (SI)	7	57	57	55.3	0	2	7	0.051	0.013
Vicdessos (VI)	7	55	55	53.4	0	1	5	0.048	0.013
Ribaui (RI)	14	61	61	59.2	0	2	9	0.105	0.018
Auriac (AU)	7	46	46	44.7	0	0	1	0.006	0.005
Cailla (CA)	23	52	50	48.5	0	0	4	0.032	0.010
Cass-Rats (CR)	19	49	49	47.6	0	0	4	0.039	0.011
Font de Dotz (FD)	4	53	53	51.5	0	0	5	0.040	0.012
Valmanya (VA)	10	53	53	51.5	2	2	10	0.018	0.008

*Total number of alleles per locality.
†Number of alleles with frequency greater than 5%.
‡Percentage of loci (of frequency >5%) that are polymorphic out of the total 103 loci.
§Number of alleles unique to a single population.
¶Number of locally common alleles (of frequency >5%) occurring in 25% or less of the populations.
**Number of locally common alleles (of frequency >5%) occurring in 50% or less of the populations.
††Mean expected heterozygosity.
Allele frequencies are calculated according to the method by Lynch & Milligan (1994)

(27%) were polymorphic, with each primer combination used in the selective amplification showing levels of polymorphism ranging between 10% and 35% (Table 2). The number of polymorphic loci per locality ranged from 44% to 61%, which is relatively high, but heterozygosity values per locality were low, reflecting the high rate of allele fixation in many populations (Table 3). In addition, a large proportion of loci were found only at local geographic scales. For example, about 26% of loci in the Lourdes drainage (BM and GL) were not found in 75% of the remaining populations, and 47% were not found in 50% of the remaining localities (Table 3).

A PCoA of AFLP variation revealed marked structure both between and within the main drainages. A plot of the two principal coordinates (accounting for 49% and 25% of the variance, respectively), showed a clear separation between Aquitanie (IR and OL) and Lourdes (BM and GL) regions, whereas the Garonne and Aude drainages formed a single cluster, except for two well-differentiated populations, PL and VA (Fig. 2A). A second analysis including all the Garonne and Aude localities except PL and VA, revealed further structure in this region (Fig. 2B), with an almost complete separation of the Aude localities (Auriac, Cailla, Cass-Rats and Font de Dotz) from the Garonne localities (BD, LA, AR, RI, CO, SI and VI), and a striking degree of separation between the two cave populations BD and LA not just from the rest but from each other, despite being separated by only 3.2 km.

A Bayesian assignment analysis using the program STRUCTURE 2.2 produced similar results to those from PCoA. Analysis of all individuals from all localities grouped their genotypes with 100% posterior probability of assignment to an optimal K of 5 (upper panel in Fig. 3A), which corresponds to the same five clusters identified by the PCoA (Aquitanie, Lourdes, Garonne-Aude, PL and VA). Separate analysis of each of the five clusters (lower panel in Fig. 3A) revealed optimal K values of 1 for Aquitanie, PL and VA, whereas the two Lourdes populations (BM, a cave; and GL, a stream) showed significant separation (optimal $K = 2$). The Aude-Garonne region was in turn divided into five clusters, one corresponding to the four Aude localities (AU, CA, CR and FD), and the other four corresponding to different areas of the Garonne region (Fig. 3). These correspond to an east–west separation of the high-elevation Garonne sites, with AR on the west, the localities of CO and SI to the east, and RI and VI showing a mixture of individuals from the east and the west

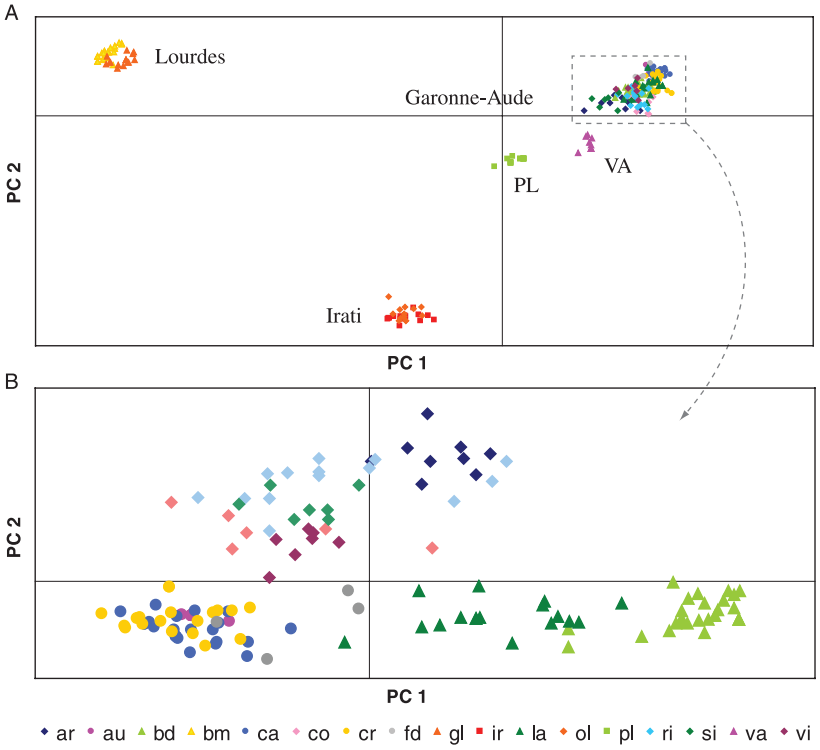

Fig. 2 Principal coordinates analysis (PCoA) of genetic variation in 103 AFLP loci in *Calotriton asper*. (A) Genetic structuring among all 241 individuals in all 17 localities. The variance explained by PC1 and PC2 is 49% and 25%, respectively. (B) Genetic structuring among individuals from the Garonne-Aude region (139 individuals in 11 localities, excluding PL and VA). The variance explained by PC1 and PC2 is 42% and 22%, respectively.

(Fig. 3B). The other two Garonne clusters correspond to the two low-elevation and closely located caves of BD and LA.

This strong pattern of genetic structuring is reflected in high F_{ST} values between populations (Table 4), with an overall F_{ST} across all loci of 0.861 (SD = 0.016). F_{ST} values reached as much as 0.96 across watersheds (e.g. between BM and PL), and showed high values even between closely located populations (Table 4), such as the caves of BD and LA (F_{ST} = 0.47; 3.2 km), the BM cave and GL stream (F_{ST} = 0.39; 6.6 km), and the streams at IR and OL (F_{ST} = 0.14; 10.8 km). Importantly, high F_{ST} values between localities were due to the contribution of several loci, many of them fixed, and were not due to just one or a few outliers (Table 5). This was particularly apparent in populations across drainages. For example, of the 64 variable loci in the pairwise comparison between IR and GL, two stream sites with similar sample sizes and separated by 74 km, 51 loci (80%) had F_{ST} values greater than 0.50, and 41 (64%) were fixed (F_{ST} = 0.80) in one of the two populations (Fig. 4, Table 5).

As shown by an AMOVA, most of the genetic variance (65%) was partitioned among regions, with 24% corresponding to variance among populations within regions, and only 10% to within-population variation (Table 6). Grouping populations into the six genetic regions identified by the PCoA analysis increased the among-region component to 78%, with the remaining of the variance being shared equally by the other two components (Table 6).

Population differentiation followed a general pattern of isolation by distance, and a Mantel test of the correlation between a matrix of $F_{ST}/(1 - F_{ST})$ values and a matrix of the natural logarithm of Euclidean geographic distances was highly significant (10 000 permutations of Spearman rank correlation coefficient, $P < 0.0001$) (Fig. 5). A similar Mantel test using real (topography-adjusted) distances had the same P value.

Discussion

Genetic structure and dispersal in Calotriton asper

Variation in AFLP loci revealed high levels of genetic structure across the distribution of *Calotriton asper* in the French Pyrenees at various geographic scales. Marked genetic differentiation exists at the scale of different drainages, but also between localities separated by just a few kilometres. Even though levels of polymorphism in AFLP were relatively high and within the range found in previous studies conducted in species across vertebrate groups (see summary table in Milot *et al.* (2007)), the high F_{ST} values across many loci and the high degree of allelic fixation in several populations (yielding very low heterozygosity values) suggests the role of long-term isolation and small-effective population size. High F_{ST} values among distant populations

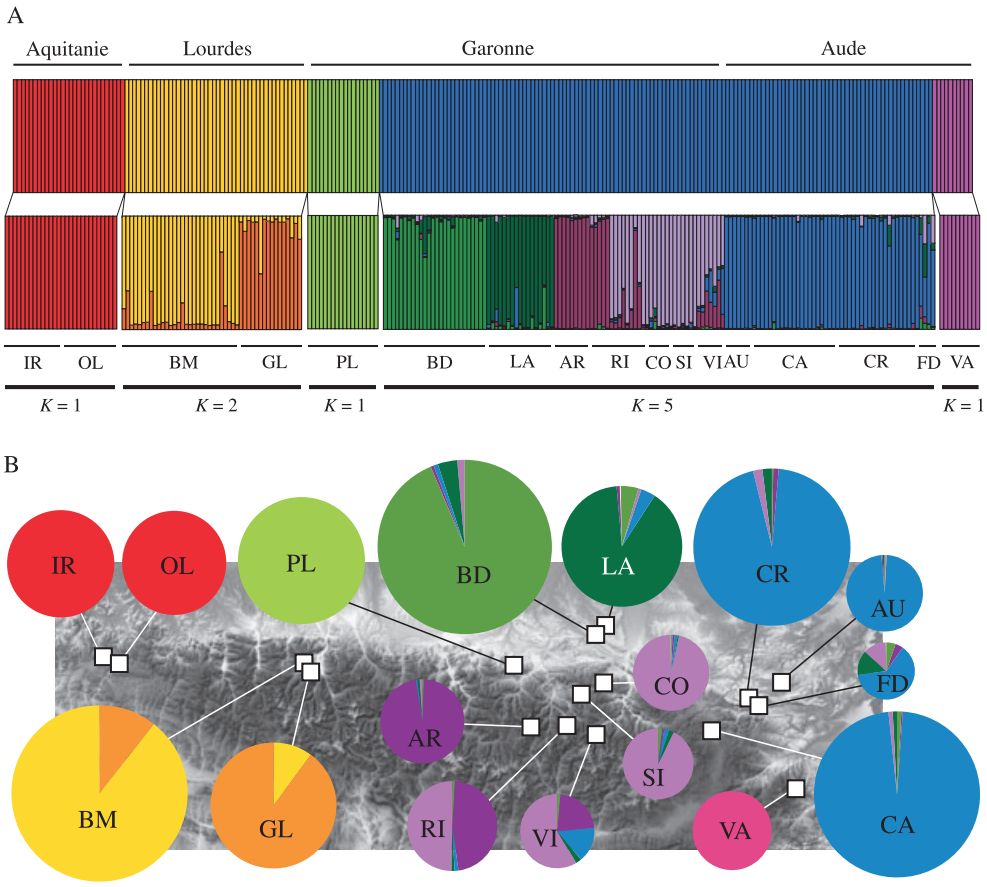

Fig. 3 Spatial structure of AFLP data in *Calotriton asper* according to a Bayesian assignment probability analysis using the program STRUCTURE 2.2. A. Posterior assignment probabilities per individual. Each vertical bar represents an individual, with each colour corresponding to the posterior probability of assignment to each of a number of clusters (*K*). Upper panel: Analysis of all individuals from all localities grouped their genotypes with 100% posterior probability of assignment to an optimal $K = 5$. Lower panel: Separate analysis of each of the five clusters revealed the following optimal *K* values: Aquitanie $K = 1$, Lourdes $K = 2$, Garonne-Aude $K = 5$, Pas du Loup $K = 1$, Valmanya $K = 1$. B. Average posterior assignment probabilities to STRUCTURE clusters averaged across *C. asper* individuals in each locality, overlaid on a map of the Pyrenees. Each colour in each pie diagram represents the percent posterior probability of assignment to a given cluster, averaged across all individuals in that population.

and drainages, and the clear pattern of isolation by distance, suggests that divergence among drainages and localities is driven mainly by geographic barriers to dispersal, and only low-elevation populations within the Aude region appear to be connected by considerable levels of gene flow. However, high levels of genetic differentiation among some very closely located sites, such as those in BM and GL, and BD and LA, demonstrate high overall dispersal limitation in *C. asper*, especially into and out of cave populations.

The fact that populations in high-elevation areas of the Pyrenees (thought to be partly covered by glaciers during the last glacial maximum) are genetically differentiated from adjacent lowland populations (see upper and lower Garonne sites in Fig. 3), and that this level of genetic divergence is unlikely to be of postglacial origin, suggests that at least some individuals could have survived glacial periods in high-elevation refuges, although no firm evidence of this is currently available.

Given that *C. asper* is a cold-adapted stenothermal organism suggests that life history traits may be an important factor in driving divergence in this species. A strong association between low basal metabolic rate (BMR) and dispersal limitation has been documented in amphibians (Bernardo *et al.* 2007). A combination of low dispersal rates and the existence of isolated populations of small effective population size susceptible to accelerated differentiation by genetic drift (Frankham *et al.* 2002), probably accounts for the high levels of genetic structure found here.

Our results are in contrast to those found in the few surveys of AFLP variation in urodeles conducted to date. Studies at local scales have found either no differentiation or F_{ST} values at least an order of magnitude

Table 4 Pairwise values of Φ_{PT} (a statistic analogous to F_{ST}) among *Calotriton asper* localities (below diagonal), and probability values based on 9999 permutations (above diagonal)

	IR	OL	BM	GL	PL	AR	BD	LA	SI	CO	VI	RI	AU	CA	CR	FD	VA
IR		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
OL	0.141		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.006	0.001
BM	0.945	0.943		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
GL	0.926	0.921	0.390		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
PL	0.935	0.935	0.960	0.951		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
AR	0.849	0.838	0.913	0.872	0.799		0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
BD	0.912	0.906	0.938	0.924	0.897	0.614		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
LA	0.905	0.899	0.938	0.921	0.898	0.556	0.466		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SI	0.883	0.873	0.934	0.903	0.903	0.465	0.714	0.616		0.001	0.002	0.001	0.001	0.001	0.001	0.004	0.001
CO	0.880	0.872	0.940	0.913	0.920	0.455	0.703	0.577	0.386		0.001	0.001	0.001	0.001	0.001	0.009	0.001
VI	0.897	0.887	0.941	0.912	0.919	0.420	0.688	0.590	0.494	0.526		0.001	0.002	0.001	0.001	0.004	0.001
RI	0.843	0.832	0.906	0.868	0.822	0.248	0.589	0.474	0.360	0.287	0.344		0.001	0.001	0.001	0.001	0.001
AU	0.934	0.928	0.957	0.941	0.966	0.591	0.760	0.656	0.694	0.667	0.654	0.436		0.001	0.001	0.003	0.001
CA	0.927	0.923	0.948	0.934	0.931	0.643	0.732	0.629	0.675	0.625	0.572	0.514	0.428		0.001	0.002	0.001
CR	0.911	0.906	0.943	0.925	0.920	0.596	0.727	0.560	0.616	0.521	0.554	0.469	0.429	0.290		0.001	0.001
FD	0.902	0.892	0.942	0.916	0.936	0.446	0.627	0.454	0.527	0.479	0.463	0.309	0.599	0.384	0.363		0.001
VA	0.923	0.917	0.960	0.946	0.965	0.756	0.882	0.880	0.857	0.825	0.872	0.723	0.944	0.900	0.877	0.888	

Table 5 Number of loci contributing to the paiwise F_{ST} values between selected pairs of populations of *Calotriton asper*

	Variable loci	No. loci $F_{ST} = 0$	No. loci $F_{ST} > 0.10$	No. loci $F_{ST} > 0.50$	Fixed loci ($F_{ST} > 0.80$)
IR vs. GL	64	2	61	51	41
IR + OL vs. BM + GL	68	1	62	53	38
IR + OL vs. PL	54	3	47	43	34
PL vs. AR	39	0	33	17	11
BM vs. GL	12	4	5	1	1
BD vs. CA	22	2	16	8	3
BD vs. LA	18	3	10	3	0

The first column shows the number of loci used in each pairwise comparison, out of 103 total variable loci. The last column shows the numer of loci fixed in either population and absent in the other one.

lower (Curtis & Taylor 2003; Lowe *et al.* 2006, 2008; Kinkead *et al.* 2007). A study of *Salamandra atra* across its range in central Europe (about twice the size of *C. asper*'s), found moderate levels of structure among subspecies and the significant separation of some isolated populations (Riberon *et al.* 2004). However, levels of structure were overall lower than those found with mtDNA sequence (Riberon *et al.* 2001) and small sample sizes, limited analysis, and the use of different restriction enzymes in the AFLP procedure, allows for only superficial comparison.

With respect to other amphibians, AFLP surveys in anurans have found considerably lower levels of structure. Studies in frogs at local scales (within a watershed or less than 40 km) have revealed little differentiation among populations (Curtis & Taylor 2003; Measey *et al.* 2007; Lowe *et al.* 2008). At larger geographic scales, a study on *Gastrophryne carolinensis* across SE North America found no significant structure (Makowsky *et al.* 2009), and two studies using variation in random amplified polymorphic DNA markers (a similar technique to AFLP) found considerable levels of structure, although much lower than in *C. asper*. These include a study of *Ascaphus truei* in British Columbia (Ritland *et al.* 2000), which detected a maximum F_{ST} among regions of 0.18, and a study of *Rana lessonae* across Northern Europe, which found differentiation only at large scales but also considerable overlap among regions as revealed by a principal components analysis (PCA) (Snell *et al.* 2005). Our results are perhaps most comparable in terms of the magnitude of AFLP differentiation to those by Rudh *et al.* (2007) in their study of the poison frog *Dendrobates pumilio* in the Bocas del Toro archipelago of Panama, where F_{ST} values reached 0.47 between island populations separated by about 25 km of sea water. Another comparable study in

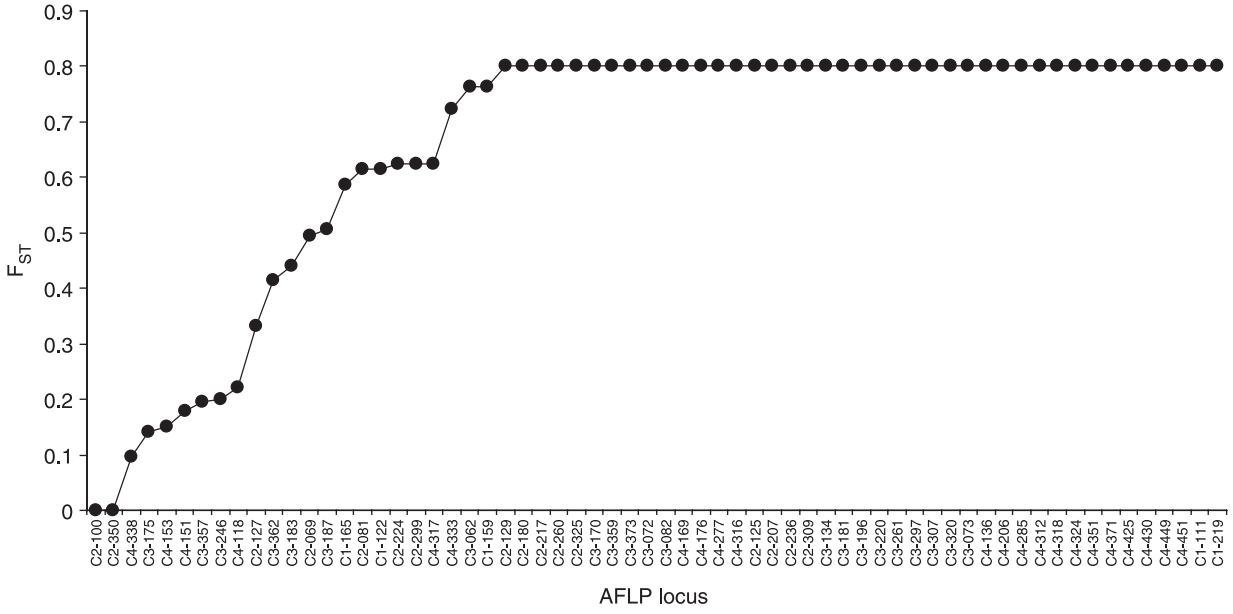

Fig. 4 F_{ST} values per locus in the pairwise comparison between localities Irati (IR) and Genie Longue (GL). Loci with $F_{ST} = 0.80$ are fixed at one of the two populations (frequency = 1.0) and absent in the other one.

Table 6 Analysis of molecular variance (AMOVA) of AFLP variation in *Calotriton asper* when individuals from 17 localities are grouped into the four main watersheds (Aquitanie, Lourdes, Garonne and Aude), and into the six groups identified by a principal coordinates analysis (Aquitanie, Lourdes, Garonne, Aude, Pas du Loup and Valmanya, see Fig. 2)

	d.f.	SS	Est. variance	% Variance	Value	*P* value
Four regions (watersheds)						
Among regions (Φ_{RT})	3	2557.08	13.58	65%	$\Phi_{RT} = 0.653$	0.001
Among pops./regions (Φ_{PR})	13	878.51	5.04	24%	$\Phi_{PR} = 0.699$	0.001
Within pops. (Φ_{PT})	224	486.82	2.17	10%	$\Phi_{PT} = 0.895$	0.001
Total	240	3922.42	20.79			
Six regions (PCoA clusters)						
Among regions (Φ_{RT})	5	3094.28	15.66	78%	$\Phi_{RT} = 0.779$	0.001
Among pops./regions (Φ_{PR})	11	341.31	2.27	11%	$\Phi_{PR} = 0.511$	0.001
Within pops. (Φ_{PT})	224	486.82	2.17	11%	$\Phi_{PT} = 0.892$	0.001
Total	240	3922.42	20.10			

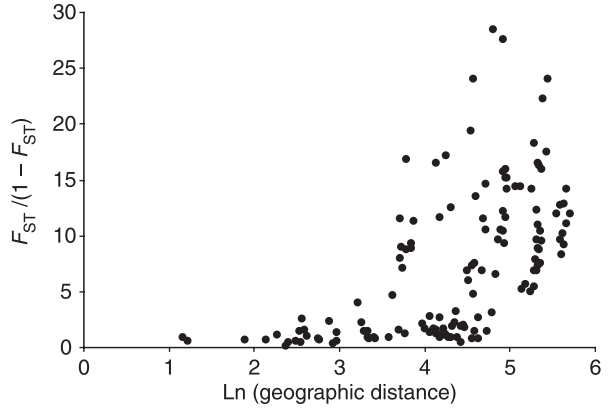

Fig. 5 Pairwise values of genetic differentiation (calculated as $F_{ST}/[1 - F_{ST}]$) against geographic distance to assess the pattern of isolation by distance in *Calotriton asper*.

reptiles found differences across Caribbean species of *Anolis* that are similar to those found among populations of *C. asper* according to a PCA of AFLP variation similar to the one used here (Ogden & Thorpe 2002). Clearly, additional studies of AFLP variation in amphibians and reptiles are needed to assess its potential relative to other techniques in many taxa (Bensch & Akesson 2005).

Incongruence of ALFP and mtDNA/allozymic variation

Given the high levels of variation and population divergence found with genome-wide AFLP loci, the low levels of variation in mtDNA and allozymes is surprising. The relative substitution rate of mtDNA and nuclear DNA in amphibians is among the highest reported for vertebrates, with anurans reaching a 16:1 ratio

(Crawford 2003), and there is little reason to believe that *C. asper* would depart substantially from this pattern. Therefore, the incongruence between the high variation in genomic DNA and low variation in mtDNA and allozymes was unexpected. A selective sweep on an mtDNA gene and allozymic loci could potentially account for the low variation, and selection on mtDNA has been reported in many taxa (Jansa *et al.* 2003; Ruiz-Pesini *et al.* 2004; Zink 2005; Bazin *et al.* 2006; Grant *et al.* 2006). However, the fact that present-day populations thrive across a broad altitudinal range (from under 500 m to above 2000 m), encountering very different environments and thus selective regimes, weakens support for the role of selection as a main driver of this pattern.

An alternative reason for the incongruence is the possibility that AFLP variation is abnormally high due to unique characteristics of the urodele genome. The salamander genome is the largest reported for any organism (7291 cM in species of *Ambystoma*, Voss *et al.* (2001)), and it is estimated that about 95–99% of it is noncoding, or satellite DNA (Cavalier-Smith 1985), also known as 'junk DNA'. Satellite DNA in eukaryotic genomes contains tandemly arrayed, highly repetitive DNA sequences that can account for most of the DNA in the genome (Li & Graur 1991). Because the amount of satellite DNA is negatively correlated with developmental rate, which in turn is known to be slow in cold-adapted species (Pagel & Johnstone 1992), the proportion of satellite DNA in *C. asper*'s genome could be particularly high. The presence of a large number of repetitive DNA sequences containing by chance the restriction sites targeted by the enzymes employed in the AFLP procedure could potentially increase the probability of sampling genomic polymorphism.

The striking degree of differentiation of some *C. asper* populations (Aquitanie, Lourdes, PL), as indicated by a random sample of genome-wide DNA fragments, suggests that genetic divergence affects the large majority of their genome. However, further analysis with microsatellite loci and nuclear sequence will be necessary to fully assess variation in the nuclear genome. In any case, the fact that individuals from at least some of these populations can interbreed in captivity indicates that important coding regions of their genomes remain relatively conserved, so that whether or not these populations constitute different evolutionary units (and even species) remains unclear until proper controlled tests are conducted. Unfortunately, the low variation in mtDNA and the nature of AFLP data preclude the estimation of divergence times between populations, so that the amount of time necessary to accumulate such high levels of polymorphism remains unknown.

Nevertheless, our results are unambiguous with respect to the fact that gene flow is highly restricted among populations of *C. asper*, and demonstrate that an assessment of genetic structure using only mtDNA data would have severely underestimated levels of population divergence and led to erroneous inferences about gene flow and patterns of dispersal behaviour in this species.

Implications for population viability

The lack of appreciable gene flow among many populations of *C. asper* in the French Pyrenees and the low genetic diversity detected in many of them, suggests that long-term viability of at least some isolated populations could be at risk. Loss of genetic diversity in fragmented amphibian populations has been documented (Beebee 2005), and the correlation between inbreeding, reduced fitness and population declines (Rowe & Beebee 2003; Andersen *et al.* 2004) are a cause for concern. Predicted increases in global temperatures could negatively affect viability if individuals are incapable of dispersing to areas of suitable climate (higher elevations, Massot *et al.* (2008)). Moreover, because stenothermal organisms can be well adapted to conditions at very specific altitudes, dispersal to areas of different conditions could result in metabolic depression and lower fitness (Bernardo & Spotila 2006). Although predictions on the potential impacts of future climate change on amphibian populations should be made with caution (Kearney *et al.* 2009), the combination of small population size, restricted gene flow and low genetic diversity suggest a high degree of vulnerability in geographically restricted amphibian species like *C. asper*.

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B.M.'s research focuses on inferring mechanisms of population divergence and speciation in vertebrates using phylogeography, population genetics and ecomorphology. S.C. uses molecular and phenotypic approaches to investigate the ecology, systematics and taxonomy of amphibians and reptiles. O.G. studies the biology of cave-dwelling urodeles and is an expert in the natural history of *Calotriton asper*. J.C. studies the evolution of animal dispersal using demographic, physiological, behavioural and genetic approaches.

Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1 Detailed AFLP protocol.

Fig. S1 Image of sample AFLP electropherogram plots for *C. asper*.

Table S1 Geographic distances between *C. asper* localities (in km)

Table S2 MtDNA haplotype frequencies per population

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Supplementary Materials

Marked genetic structuring and extreme dispersal limitation in the Pyrenean brook newt *Calotriton asper* (Amphibia: Salamandridae) revealed by genome-wide AFLP but not mtDNA

Borja Milá, Salvador Carranza, Olivier Guillaume and Jean Clobert

Appendix S1.

Detailed AFLP protocol (modified from Vos *et al.* (1995) by Darren Irwin, University of British Columbia, and Borja Milá):

1. DNA extraction: Genomic DNA was extracted from fresh tissue samples using a Qiagen Mini-Kit (tissue protocol). DNA concentration (estimated using a Nanodrop™) of all samples was within the range of 13 to 400 ng/µl (some samples were diluted to fall within this range).

2. Cutting with restriction enzymes: The enzyme cocktail (per reaction) was composed of 6.9 µl of ddH_2O, 2 µl of EcoRI-buffer (10x), 1 µl of BSA (1µg/µl), 2.5 units of EcoRI, 2.5 units of MsE1 (Tru9), and 10 µl of DNA, for a total volume of 20 µl. Incubation at 37ºC for 1 hour.

3. Ligation of adaptors: Ligation cocktail (per reaction) is composed of 4.2 µl of ddH_2O, 0.5 µl of Promega Ligation buffer (10x), 0.025 µl of E-adaptor, 0.25 µl of M-adaptor, and 0.05 units of Promega T4 ligase. 5 µl of this cocktail was added to the 20 µl from the cutting reaction above and incubated at 37ºC for 3 hours. The E-adaptor is a 1:1 mix of E-adaptor 1 (5'-CTCGTAGACTGCGTACC-3') and E-adaptor 2 (5'-AATTGGTACGCAGTCTAC-3'). The M-adaptor is a 1:1 mix of M-adaptor 1 (5'-GACGATGAGTCCTGAG-3') and M-adaptor 2 (5'-TACTCAGGACTCAT-3'). Before use, adaptors were denatured at 80˚C for 5 min at 80˚C and put immediately on ice. The product of the ligation was diluted 10x.

4. Pre-amplification reaction: The PCR cocktail contained (per reaction) 1.8 µl of ddH_2O, 2 µl of $MgCl_2$ (25mM), 2 µl of PCR buffer (10x), 4 µl of dNTP (1mM), 0.06 µl of E_T primer (5'-GACTGCGTACCAATTCT-3'), 0.06 µl of M_C primer (5'-GATGAGTCCTGAGTAAC-3'), 0.4 units of high quality Taq polymerase, and 10 µl of the diluted cutting-ligation product, for a total volume of 20 µl. The PCR cycle was as follows: [94˚C - 2 min] + [94˚C -30 s, 56˚C - 30 s, 72˚C - 60 s] x 20 cycles + [72˚C - 10 min]. The product from the pre-amplification reaction was diluted 10x and kept at -20˚C.

5. Selective amplification reaction: The PCR cocktail contained (per reaction) 3.3 µl of ddH_2O, 1 µl of $MgCl_2$ (25 mM), 1 µl of PCR buffer (10x), 2 µl of dNTP (1mM), 0.06 µl of E-primer (100 µM) (5'-GACTGCGTACCAATTCXXX-3'), 0.06 µl of M-primer (100 µM) (5'-GATGAGTCCTGAGTAAXXX-3'), 0.4 units of high quality Taq polymerase, and 2.5 µl of DNA template (diluted pre-amp reaction), for a total volume of 10 µl. The PCR cycle was as

follows ("touch-down" PCR): [94˚C - 2 min] + [94˚C -30 s, 65˚C-0.7˚C/cycle - 30 s, 72˚C - 60 s] x 12 cycles + [94˚C -30 s, 56˚C - 30 s, 72˚C - 60 s] x 23 cycles [72˚C - 10 min]. The E primer 5'-end was labeled with fluorescent dye 6-FAM. The three bases at the 3'-end of the E and M primers "XXX" are arbitrarily selected (for example, primer E_{TGA} is the EcoRI primer with TGA at the 3' position). In this study we used the following four primer pairs: E_{TCT}/M_{CCA}, E_{TGA}/M_{CCA}, E_{TAG}/M_{CGA}, and E_{TGC}/M_{CGA}. The product of the PCR reaction was diluted 20x (50x in some cases).

6. Visualization of AFLP profiles: About 2 µl of the diluted product of the selective amplification was run in an ABI 3700 genetic analyzer with a LIZ500 size standard. Profiles were analyzed using Genemapper 3.7. Figure S1 illustrates a section of an AFLP profile from this study. Not all loci are easily scorable, and many were excluded from analysis when excessive variation in peak size or shape made scoring ambiguous. Extensive positive and negative controls were carried out throughout the procedure. For more information contact the corresponding author (and see also Bonin *et al.* (2005)) .

Figure S1. Screen-capture image of the electropherogram plots from AFLP profiles in the program Genemapper 3.7 for three individuals of *Calotriton asper* (BM08, OL02 and BD28, where the first two letters represent sampling site). Plots show AFLP results for primer pair C2, and each peak corresponds to an amplified fragment or "locus". The top panel corresponds to individual BM08, middle panel to OL02, and bottom panel to BD28. The axis along the top of each panel indicates locus size. In addition to several monomorphic loci (e.g. locus 200), three polymorphic loci are shown here: locus 207 (present only in BM08), locus 217 (present only in OL02), and locus 224 (absent in OL02).

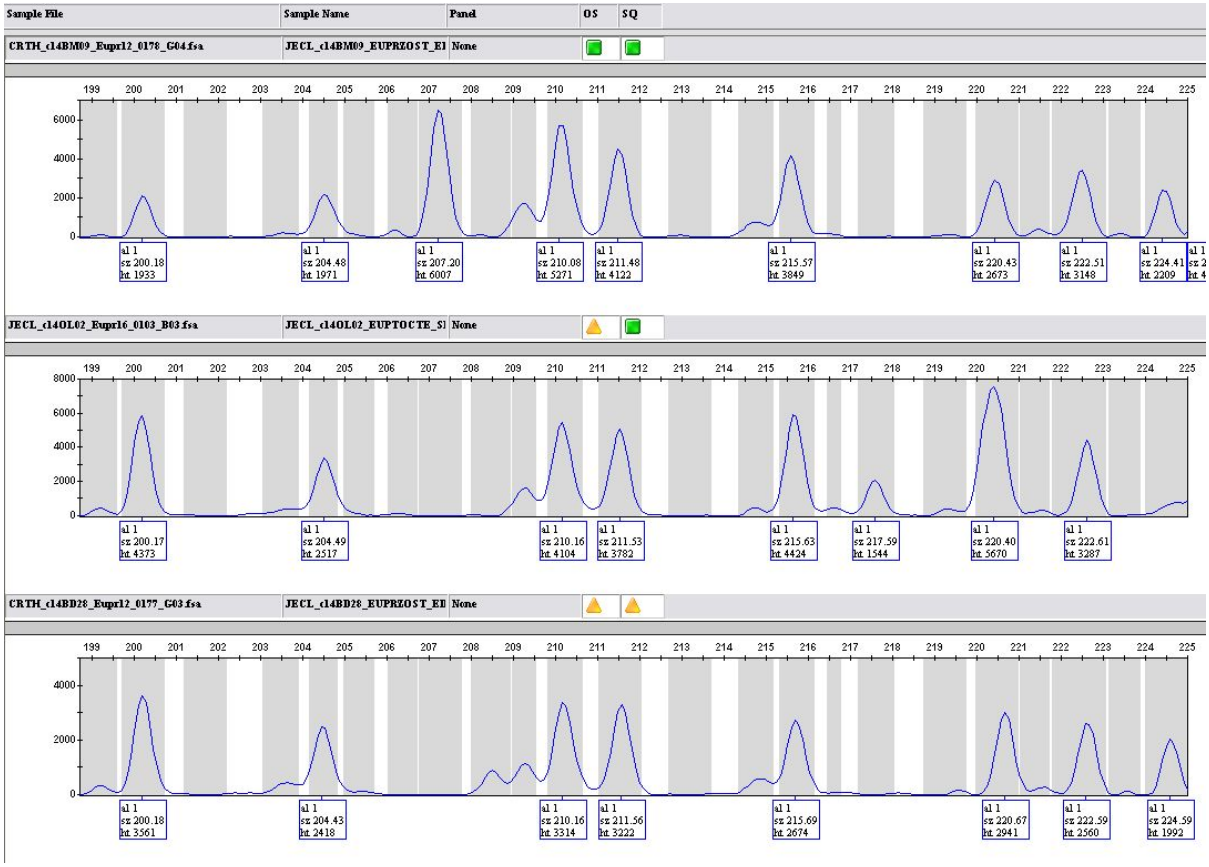

Table S1. Geographic distances between *C. asper* localities (in km). Below diagonal: Euclidean distances. Above diagonal: real distances taking into account topography.

	IR	OL	BM	GL	PL	AR	BD	LA	SI	CO	VI	RI	AU	CA	CR	FD	VA
IR		14.9	106.2	111.4	251.8	268.0	317.5	322.3	319.4	308.2	313.6	295.7	435.0	398.9	413.5	418.6	445.6
OL	10.8		93.8	98.5	238.6	254.1	304.2	309.1	305.9	294.6	299.8	281.9	421.6	385.3	400.1	405.2	431.6
BM	70.7	63.0		8.1	146.2	164.4	211.8	216.6	214.4	203.6	209.7	191.8	329.4	294.0	308.2	313.3	342.2
GL	74.1	65.7	6.6		140.4	157.9	206.1	211.0	208.4	197.4	203.3	185.4	323.7	288.0	302.4	307.5	335.8
PL	167.6	158.9	97.5	93.6		29.3	65.7	70.5	68.8	58.8	66.9	50.1	183.2	148.2	162.1	167.1	198.1
AR	179.7	170.1	111.9	107.0	25.1		56.6	61.1	53.4	41.5	45.6	27.8	169.2	131.6	147.0	152.1	177.9
BD	211.4	202.7	141.2	137.3	43.8	40.6		4.8	14.4	20.8	29.0	34.6	117.6	83.8	96.8	101.8	136.2
LA	214.6	205.9	144.4	140.5	47.0	43.4	3.2		14.8	23.7	30.4	38.1	112.8	79.2	92.0	97.0	131.9
SI	213.2	204.1	143.8	139.5	46.9	36.2	13.0	13.3		12.1	15.8	27.2	116.1	79.6	94.3	99.4	129.6
CO	206.0	196.8	137.0	132.5	41.0	28.0	17.9	19.4	8.5		11.1	15.1	127.8	90.7	105.8	110.9	139.3
VI	210.2	200.8	142.1	137.3	48.2	30.6	26.1	26.9	13.7	9.7		17.9	124.7	86.3	102.2	107.3	132.5
RI	198.2	188.7	130.0	125.2	37.3	18.6	28.0	30.0	19.4	11.1	12.1		141.7	103.8	119.4	124.5	150.4
AU	290.0	281.1	219.8	215.9	122.4	113.5	78.7	75.5	77.6	85.6	84.3	95.3		40.0	22.7	17.8	48.9
CA	266.7	257.5	197.3	193.0	100.1	88.0	57.8	54.9	53.5	60.7	57.8	69.4	28.3		17.5	22.1	54.6
CR	276.0	267.0	206.2	202.1	108.6	98.4	65.3	62.1	63.0	70.7	68.8	80.1	15.7	12.8		5.1	50.4
FD	279.4	270.4	209.5	205.4	112.0	101.9	68.6	65.4	66.4	74.1	72.2	83.5	12.6	15.8	3.4		47.9
VA	300.4	290.7	232.7	227.9	137.3	120.9	97.6	95.0	90.7	96.4	90.6	102.7	44.3	41.9	42.4	41.2	

Table S2. MtDNA haplotype frequencies per population. Dataset includes 2,040 bp of mtDNA, including control region, cyt *b*, ND4, control region and three tRNAs (see text for details). Haplotype B is due to a single indel in the control region; haplotype C is due to 5 synonymous transitions in ND4; haplotype D is due to one synonymous transition in ND4; and haplotype E is due to a single indel in the control region. The sequences of the five haplotypes have been deposited in Genbank under accessions GU067485- GU067489.

Haplotype	**IR**	**BM**	**GL**	**PL**	**BD**	**CA**	**AU**	Totals
A	0	0	0	10	10	13	7	40
B	0	10	10	0	0	0	0	20
C	6	0	0	0	0	0	0	6
D	1	0	0	0	0	0	0	1
E	2	0	0	0	0	0	0	2
Totals	9	10	10	10	10	13	7	