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Integrating genetic, demographic and ecological issues for the conservation of the Alpine newt in central Italy

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In this paper, we used a multidisciplinary approach to investigate the present conservation status of the Alpine newt in central Italy, particularly of the relict population from the Laga Mts. With respect to other European populations studied, those from peninsular Italy show a degree of genetic differentiation (using mitochondrial cytochrome-*b* gene sequences and 23 allozyme loci) resembling that found among several species of salamanders (p distance = 5.6%; D_{Nei} = 0.30), clearly individuating these populations as a distinct Evolutionary Significant Unit, thus in need of separate conservation efforts. From both the genetic and the demographic point of view, the population from the Laga Mts. appears to be under serious threat of disappearance, showing the lowest levels of genetic diversity observed for the species and an adult population size well below the estimated threshold value for a Minimum Viable Population. Finally, the analysis of habitat correlates of newt abundance suggest that human activities in the area (tree-cutting and grazing) may be important causal factors. Therefore, possible ways in which these activities could have an impact on the Alpine newt populations are also discussed.

Introduction

Despite considerable debates during the last few decades about the relative role of environmental, demographic and genetic stochastic processes in driving populations to extinction (e.g. Lande 1988, Caro & Laurenson 1994, Caughley 1994, Frankham *et al*. 2002), there is now a general agreement that all of them can affect the probability of long-term population persistence (e.g. Beeby 1993, Caughley & Sinclair 1994). This is particularly true for small and isolated populations impacted by deterministic factors, such as habitat degradation and loss (e.g. Young & Clarke 2000). In spite of this, research integrating important disciplines such as population ecology and genetics has generally been scarce (Young & Clarke 2000; but *see* e.g. Scribner *et al*. 2001). These are of particular relevance for conservation studies focusing on organisms, like amphibians, which have generally shown complex population structures, a

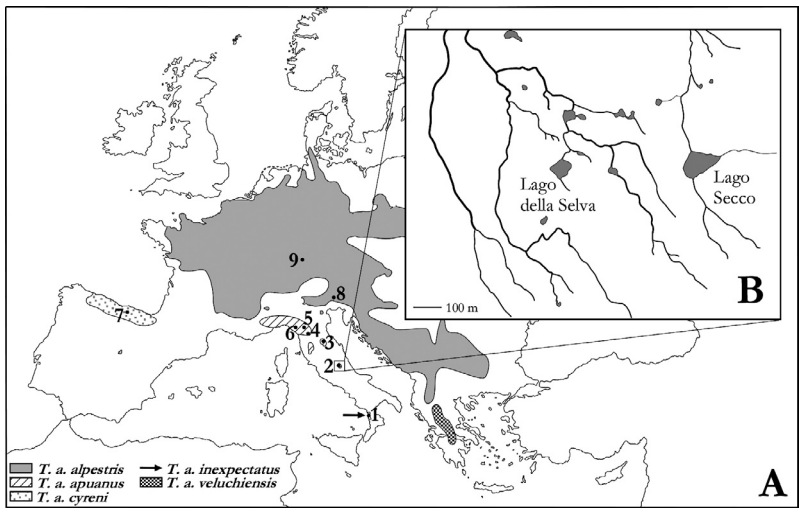

Fig. 1. (**A**) Geographic range of *Triturus alpestris* (modified from Denöel *et al.* 2001), showing the approximate distribution of the main subspecies and the geographic location of the nine samples analysed for the study of genetic variation (samples numbered as in Table 1). (**B**) Map of the surface hydrography of the study site showing location of the two breeding ponds of Alpine newts in the area.

high degree of population fragmentation and small effective population size (e.g. Jehle & Arntzen 2002), and which are thus particularly susceptible to the above mentioned stochastic processes. Moreover, an increasing number of studies concerning the population genetic structure of these organisms are giving evidence of a previously unsuspected cryptic biodiversity, at both inter- and intra-specific levels (Duellman 1993, Veith 1996, Borkin 1999, Hanken 1999, Frost 2002), thus unequivocally highlighting the importance of ascertaining what we are going to conserve before any management plan can be implemented (e.g. Canestrelli *et al*. 2006).

Triturus alpestris Laurenti is a medium-sized newt mainly distributed in central and south-central Europe (Fig. 1). In Italy the species shows a highly fragmented distribution, and is represented by three subspecies: *T. a. alpestris*, found in Alpine and pre-Alpine areas of north-eastern Italy; *T. a. apuanus*, ranging from the Maritime Alps to the north-central Apennines; *T. a. inexpectatus* which is limited to a few breeding sites in Calabria. Some uncertainty exists about the taxonomical assignment of the isolated population from the central Apennines (Laga Mountains; Latium), upon which this study is mainly focused. In fact, some authors refer to this population as belonging to the subspecies *T. a. apuanus* (e.g. Ambrogio & Gilli 1998, Bologna *et al*. 2000), whereas other authors have recognized it under the subspecies *T. a. inexpectatus* (e.g. Griffiths 1995, Gasc *et al*. 1997).

The Alpine newt is one of the rarest amphibian species of south-central Italy (Bologna *et al*. 2000). Because of its likely relictual origin (Capula & Bagnoli 1983), the relatively recent discovery of an Alpine newt population in two isolated high-altitude ponds in the Laga Mountains (central Italy; Bagnoli & Canini 1982) was considered of particular interest (e.g. Bruno 1983, Lanza 1983), and gave rise to the creation of a WWF oasis aimed at its protection (the WWF oasis of "Lago Secco"). However, field studies carried out since 1998 have documented the dramatic decline and the almost complete disappearance of the species in at least one of the two ponds (Bagnoli *et al*. 2000), while no studies have to date investigated the demographic and ecological parameters of the population or levels of genetic variability.

The main aim of this paper is to assess the present conservation status of the *T. alpestris* population from the Laga Mountains, as well as to consider management priorities. To this end, we present studies on this population, concerning: (i) genetic differentiation with respect to other cospecific populations; (ii) the levels of population genetic variability; (iii) the distribution, abundance and demographic structure of the population; and (iv) the role of some environmental parameters in determining patterns of species distribution within a breeding pond. Since the methodological approach used in this paper also led us to achieve relevant results for the Alpine newt populations from peninsular

Italy as a whole, the conservation implications of these results will also be discussed.

Materials and methods

Study area and population trend

The study area (Fig. 1) is located in central Italy, within the 'Gran Sasso e Monti della Laga' National Park (42°42´N, 13°19´E) at the altitude between 1070 m and 2020 m a.s.l. It is characterized by a dense hydrographical network of brooks, streams, springs, temporary pools, floodplains and permanent ponds. Vegetation is extremely diversified and rich in endemisms (Tondi & Plini 1995), although the landscape is dominated by beeches, *Fagus sylvatica* L., alternated with natural meadow-pastures. This is an area of particular biogeographical interest because, also due to the climatic conditions (Tondi & Plini 1995), relict populations of several species having typical Alpine distribution can be found there (Silvetti 1998). Largely due to these considerations, the area has also been protected by the creation of a WWF Oasis, and is now a 'Site of Community Importance' (European Commission Habitat Directive 92/43/EEC).

Two main human activities have long been carried out in the area: grazing and tree-cutting. These activities have led to an increasing amount of debris and organic charge on the local wetlands, and may well have contributed to their general deterioration and, in some cases, disappearance (Bagnoli *et al*. 2000).

In the early 1980s, a population of *T. alpestris* — syntopic with two other newt species: *T. carnifex* and *T. vulgaris* — was found in the two major ponds in the area (Lago Secco and Lago della Selva) (Bagnoli & Canini 1982). Whereas the presence of the latter species was an expected finding, *T. alpestris* represented a southward extension of the species range in Italy and was also considered of great interest because of the unusual abundance of paedomorphic adults (about 85%), especially when considering the moderate pond depth (Capula & Bagnoli 1983). At this time, according to Capula and Bagnoli (1983), in both ponds *T. alpestris* was the most abundant newt species. However, further field surveys carried out during 1998 and 2000 (C. Bagnoli unpubl. data; Bagnoli *et al*. 2000), showed that within one of these ponds (Lago Secco; from here on referred to as Secco) the species was experiencing a dramatic decline, leading to the presence of *T. alpestris* being 23% and 8.6% of the total number of newts caught in 1998 and 2000, respectively. By contrast, in the other pond (Lago della Selva; from here on referred to as Selva) no significant decrease was found, and the species was still the most abundant newt species. Interestingly, the two ponds were also differently affected by human activities in the area. Whereas the shoreline of Secco and its surroundings were homogeneously modified by tree-felling and grazing, only a small portion of the Selva surroundings (on the north-east side) had been affected by these activities, probably because this site is located on a steeper slope and is thus less easily accessible.

Genetic variation

mtDNA

The analysis of mtDNA sequence variation was conducted on specimens sampled from the population from the Laga Mts., as well as 8 populations belonging to the subspecies *T. a. apuanus*, *T. a. inexpectatus*, *T. a. cyreni* and *T. a. alpestris*. The geographic origin of samples studied and sample size are shown in Table 1 and Fig. 1. DNA was extracted from frozen toes, clipped during previous field activities (for populations 1, 3 and 8), or from alcohol-preserved tissues (for all other populations) following standard Sambrook *et al*. (1989) extraction protocol. A fragment of 499 bp of the mitochondrial cytochrome-*b* gene was amplified by means of the polymerase chain reaction (PCR). PCR amplification and sequencing were initially carried out using the primers pair MVZ15 and MVZ16 (Moritz *et al*. 1992). Sequences obtained with these primers were used to design the following primers, that were used for the amplification and sequencing of all further individuals: CytbTritF (5´-ACGCAAYATRCACATCAACGG-3´) and CytbTritR (5´-GGAGTGACTATAGARTTTGCTGGG-3´).

PCR cycling procedure was: 95 °C for 5 min followed by 33 cycles of 93 °C for 1 min, 52 °C for 45 s, 72 °C for 1 min 30 s and a single final step at 72 °C for 10 min. Sequences were obtained using an ABI PRISM 377 DNA sequencer (PE Applied Biosystems) following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. All specimens analysed were double sequenced.

The sequencing chromatograms were analysed with the program CHROMAS (Technelysium Pty Ltd., Australia). Alignments were carried out using the software CLUSTALX 1.81 (Thompson *et al*. 1997). All haplotype sequences were deposited in GenBank (Accession Numbers: DQ093821–DQ093824)

Pairwise sequence divergence (uncorrected *p* distances) and phylogenetic analyses were computed using the software PAUP* 4.0b10 (Swofford 2003). Phylogenetic trees were inferred using Neighbour Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML). The unweighted MP tree was obtained using the exhaustive search. The optimal model of sequence evolution for ML and NJ analyses was assessed using Akaike Information Criterion (AIC) as implemented in the software Modeltest 3.6 (Posada & Crandall 1998) associated with PAUP*. This analysis supported the General Time Reversible model (Rodriguez *et al*. 1990) as the best fit substitution model for the data, with estimates of the substitution rates to be: A to C, 3.888; A to G, 7.173; A to T, 2.315; C to G, 0.4041; C to T, 7.173; G to T, 1.00. Nodal support for the inferred trees was evaluated by the bootstrapping method with 1000 pseudoreplicates. *Salamandrina perspicillata* was used as an outgroup (GenBank Accession Number: AY695905).

Allozymes

Allozyme analysis was performed using tissue samples obtained from toe-clipping procedures (*see* next section). Altogether 28 specimens of *T. alpestris* from the Laga Mts., 12 from Verghereto and 10 from Campone were analysed (*see* Table 1).

Horizontal electrophoresis was carried out on 10% starch gel. Enzyme systems analysed and their putative encoding loci are listed in Table 2 together with electrophoretic conditions. Isozymes were numbered in order of decreasing mobility from the most anodal. Alleles at each locus were designated by their mobility (in mm, standardized conditions) in relation to the most common one (100) in the sample from Campone.

BIOSYS-2 software (Swofford & Selander 1999) was used to calculate allele frequencies and estimate population genetic variability as mean observed heterozygosity (H_o), unbiased estimate of expected heterozygosity (H_e; Nei 1978), percentage of polymorphic loci and mean number of alleles per locus (*A*). Possible departures from the expected Hardy-Weinberg equilibrium (HW) were assessed for each polymorphic locus in each population using Fisher's exact significance tests (Finney 1948), applying Levene's correction for small sample size (Levene 1949). The level of genetic divergence between the three populations studied was evaluated by

Table 1. Sampling location and sample size of the nine populations surveyed of *Triturus alpestris* for the analysis of cytochrome-*b* mtDNA sequence variation. * Samples studied also at allozyme level.

Locality	Subspecies	*n*	Lat. (N)	Long.	Elevation (m a.s.l.)
1. Fagnano	*T. a. inexpectatus*	2	39°34´	16°3´E	850
2. Laga Mts.	*T. a. apuanus*	28*	42°42´	13°19´E	1500
3. Verghereto	— „ —	12*	43°47´	12°0´E	812
4. Greve	— „ —	3	43°35´	11°19´E	430
5. Lago del Greppo	— „ —	9	44°6´	10°40´E	1442
6. Levigliani	— „ —	5	44°2´	10°20´E	550
7. Lago de la Ercina	*T. a. cyreni*	4	43°16´	4°58´W	1100
8. Campone	*T. a. alpestris*	10*	46°15´	12°49´E	430
9. St. Gallen	— „ —	4	47°15´	9°8´E	830

calculating the standard Nei's (1972) genetic distance value.

Demographic and ecological investigations

Species distribution and relative abundance

From 20 April to 15 July 2003, we conducted a Visual Encounter Survey (VES; Crump & Scott 1994), in order to ascertain the present distribution of the species in the study area and its relative abundance as compared with those of the other newt species present in the area (*T. carnifex* and *T. vulgaris*). This method was also chosen in order to identify population trends, since it has been already used in previous studies carried out on the *T. alpestris* population from the area (Bagnoli *et al*. 2000). In total, VES accounted for 450 hours of field work. Land searches were conducted through a 'random walk design' (Crump & Scott 1994), searching under logs, barks, stones and within the leaf litter, while pond searches were conducted through dipnetting (3 m maximum dipnet length), without regard to previous accounts about presence/absence of the species in the site.

Population abundance and demographic parameters

Bearing in mind the results of the VES phase, we focused on Selva to estimate the abundance and demographic parameters of the Alpine newt population. Newt abundance was estimated through a mark–recapture investigation, using Petersen estimator as modified by Chapman (Chapman 1951) and the standard error of the estimate assessed with Seber equation (Seber 1982).

Newts were caught on days 22 and 26 September 2003 by a standardized dipnetting from the shore, with each dipnet sweep 3-m wide.

Table 2. Enzymes studied in *Triturus alpestris*, their commission number (EC), encoding loci, buffer systems, and kind of electrophoretic migration (+ = anodic; – = cathodic).

Enzyme	EC number	Encoding loci	Buffer system	Migration
Lactate dehydrogenase	1.1.1.27	*Ldh-1*	4	+
		Ldh-2	4	+
Malate dehydrogenase	1.1.1.37	*Mdh-1*	1	+
		Mdh-2	1	+
Malate dehydrogenase ($NADP^+$)	1.1.1.40	*Mdhp-1*	1	+
Isocitrate dehydrogenase	1.1.1.42	*Icdh-1*	1	+
		Icdh-2	1	+
6-Phosphogluconate dehydrogenase	1.1.1.44	*6Pgdh*	1	+
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	Gapdh	4	+
Superoxide dismutase	1.15.1.1	Sod-1	3	+
		Sod-2	3	+
Purine nucleoside phosphorylase	2.4.2.1	*Np*	3	+
Aspartate transaminase	2.6.1.1	*Aat-1*	2	+
		Aat-2	2	+
Alanine transaminase	2.6.1.2	*Alat-1*	2	+
		Alat-2	2	+
Adenylate kinase	2.7.4.3	*Adk*	2	+
Adenosine deaminase	3.5.4.4	*Ada*	1	+
Carbonic anhydrase	4.2.1.1	*Ca*	3	+
Phosphoglucomutase	5.4.2.2	*Pgm-1*	4	+
		Pgm-2	4	+
Glucose phosphate isomerase	5.3.1.9	*Gpi*	3	–
Mannose phosphate isomerase	5.3.1.8	*Mpi*	2	+

Buffer systems: 1 = Phosphate-Cytrate pH 6.3 (Harris 1966); 2 = Continuous Tris/Citrate pH 8.0 (Selander *et al.* 1971); 3 = Tris/Versene/Borate pH 8.0 (Brewer & Sing 1970); 4 = Tris/Maleate pH 7.4 (Brewer & Sing 1970).

Newts captured were anaesthetized by dipping them in a 0.02% solution of MS222 prepared with water taken from the pond (Donnely *et al.* 1994), marked by toe-clipping with an individual marking code and then released at the collection point. Larvae were not taken into consideration for population size estimate, and were therefore not marked. For each collected specimen the following information was noted: collection place to the nearest 0.5 m, age class (adult, juvenile [without swollen cloaca], larva), and, if adult, sex and phenotype (i.e. if metamorphic or paedomorphic). Demographic parameters estimated were sex ratio (adults only), juvenile/adult ratio and phenotypic ratio (paedomorphic/metamorphic; adults only).

Habitat correlates of newt abundance

A stepwise multiple regression was used to examine relationships between newt abundance and environmental parameters at Selva. We focused analyses on a strip comprising a 3-m-wide riparian zone of the water body. Five potential explanatory variables were chosen *a priori*: deep-water temperature (3 m from the shoreline; from here on, DT), water column (3 m away from the shoreline; from here on, WD), percentage of water surface covered by vegetation (from here on, VC), distance from shoreline to the surrounding tree-line (from here on, SV), slope (from here on, SL). These variables were chosen because they are affected by human activities in the area or because of their likely biological relevance.

The strip was partitioned into sectors of 5-m-long shoreline. For each sector: VC was estimated over the entire sector, DT was measured once at the centre of the sector, five measures of SV, WD and SL were taken and then averaged over the sector, whereas newt abundance was expressed as the average number of captures per meter during mark–recapture investigation. Measures of SV ≥ 30 m were cumulatively set to 30 m.

Before carrying out the stepwise multiple regression, explanatory variables chosen for the analysis were examined for collinearity by calculating the Spearman r correlation coefficients among all variable pairs. If collinearity was evident between two variables (Spearmann $|r| \geq 0.85$), the variable considered to have least biological relevance was removed from further analysis. Statistically significant factors ($P < 0.05$) were added using the forward selection procedure by order of F values.

All statistical analyses were carried out using the software package SPSS-12.

Results

Genetic variation

mtDNA

Amplified cytochrome-*b* partial gene yielded unambiguous sequences of 499 bp without indels, for all the 77 specimens analysed. Four distinct haplotypes were found, defined from 34 variable positions (30 at the third position, 4 at the first). Each sample studied showed a single fixed haplotype, revealing the lack of intra-population variation at the level of this mitochondrial gene fragment.

The geographic distribution of haplotypes was markedly non-random. The Iberian sample belonging to the subspecies *T. a. cyreni* (from Lago de la Ercina) and the central European samples belonging to the subspecies *T. a. alpestris* (from St. Gallen and Campone), showed distinct haplotypes (*h1* and *h2* respectively) not found in peninsular Italy. Within the samples from this region, two distinct haplotypes were found, *h3* and *h4*. The former was found only in the population from Fagnano (subspecies *T. a. inexpectatus*), whereas all samples studied from north and central Apennine, comprising the sample from the Laga Mts., shared the single haplotype *h4*.

Neighbour-Joining, Maximum-Parsimony and Maximum-Likelihood analyses all showed the same topology (Fig. 2). Two distinct groups of haplotypes can be recognized, both with high bootstrap support, one comprising those found within Spanish and central European samples (*h1* and *h2* respectively), the other comprising those found in peninsular Italy (*h3* and *h4*). As shown in Table 3, sequence divergence between these two groups ranged between 5.4% and 5.8%,

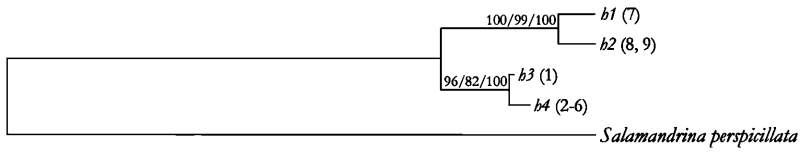

Fig. 2. Neighbour-joining tree of cytochrome-*b* haplotypes (499bp) found within the studied samples of *Triturus alpestris*. Localities where each haplotype was found are in brackets (numbered as in Table 1). Bootstrap supports over 1000 pseudoreplicates are shown at the nodes for Neighbour-Joining, Maximum Likelihood and Maximum Parsimony analyses (NJ/ML/MP).

whereas within them it was 0.6% and 1.8% for haplotype pairs *h3–h4* and *h1–h2* respectively.

Allozymes

Allozyme analysis revealed that nine out of twenty-three loci analyzed (*Ldh1*, *Mdhp1*, *6Pgdh*, *Sod-2*, *Np*, *Aat-1*, *Aat-2*, *Pgm1* and *Mpi*) were polymorphic. However in all the three populations surveyed, three of these loci (*6Pgdh*, *Sod-2*, *Np*) lacked the intra-population variation. All other loci were seen to be monomorphic for the same allele in all populations. Allele frequencies at polymorphic loci are given in Table 4. No significant deviations were found from the expected HW equilibrium.

Six loci (*Ldh1*, *6Pgdh*, *Sod-2*, *Np*, *Aat-1*, and *Mpi*) were fully diagnostic between Campone and the two samples from peninsular Italy (Laga Mts. and Verghereto), while the most common allele at locus *Mdhp-1* at Campone was found only at Verghereto at low frequency (0.04). Pairwise values of Nei's (1972) genetic distances were $D_{Nei} = 0.300$ and $D_{Nei} = 0.297$ for the population pairs Campone–Laga Mts. and Campone–Verghereto, respectively; whereas the population pair Laga Mts.–Verghereto showed a genetic distance of $D_{Nei} = 0.006$.

Table 3. Sequence divergence (*p* distance) among the four cytochrome-*b* haplotypes (499 bp) found within the studied samples of *Triturus alpestris*.

	h1	*h2*	*h3*	*h4*
h1	–			
h2	0.018	–		
h3	0.056	0.054	–	
h4	0.058	0.056	0.006	–

Table 4. Allele frequencies at the 9 polymorphic loci and estimates of genetic variability for the three populations sampled of *Triturus alpestris*.

Locus	Allele	Population		
		Laga Mts. (*N* = 28)	Verghereto (*N* = 12)	Campone (*N* = 10)
Ldh-1				
	95	0.09	0.17	–
	100	–	–	0.60
	104	0.91	0.83	–
	110	–	–	0.40
Mdhp-1				
	100	–	0.04	0.55
	107	1.00	0.96	0.45
6Pgdh				
	100	–	–	1.00
	110	1.00	1.00	–
Sod-2				
	100	–	–	1.00
	110	1.00	1.00	–
Np				
	95	1.00	1.00	–
	100	–	–	1.00
Aat-1				
	100	–	–	1.00
	103	1.00	0.80	–
	108	–	0.20	–
Aat-2				
	100	1.00	0.70	1.00
	115	–	0.30	–
Pgm-1				
	100	0.86	0.88	1.00
	105	0.14	0.12	–
Mpi				
	95	–	–	0.20
	100	–	–	0.60
	104	1.00	1.00	–
	106	–	–	0.15
	110	–	–	0.05
H_e		0.018 (0.013)	0.060 (0.027)	0.071 (0.039)
H_o		0.020 (0.014)	0.057 (0.027)	0.057 (0.034)
%		8.7	21.7	13.0
A		1.1 (0.1)	1.2 (0.1)	1.2 (0.1)

H_e = unbiased estimate of expected heterozygosity (Nei 1978). H_o = average observed heterozygosity. % = percentage of polymorphic loci. *A* = mean number of alleles per locus. Standard errors in parentheses.

Estimates of genetic variability are given in Table 4. Regarding all the parameters estimated, the population from the Laga Mts. was less variable. For instance the expected heterozygosity found in this population was about one third of that found in the population from Verghereto and about one quarter of that found in Campone

Demographic and ecological investigations

As a result of the VES, the only waterbodies in the area where the species was found were Secco and Selva, whereas on land only juveniles were found and only in the vicinity of Selva.

Compared with the other syntopic newt species (*T. carnifex* and *T. vulgaris*), the Alpine newt represented 53.4% and 2.8% of the total number of catches made during the VES activities at Selva (n = 204) and Secco (n = 108), respectively, the difference being highly significant ($\chi^2 = 78.8$, $P < 0.01$). At Secco the proportion of Alpine newts was also significantly lower than that found at the same site in 2000 (8.6%, n = 222; $\chi^2 = 3.9$, $P < 0.05$; Bagnoli *et al*. 2000), a difference that was not found at Selva (59.6%, n = 240; $\chi^2 = 1.8$, NS).

A total of 500 Alpine newts were caught during mark–recapture activities at Selva (recaptures not considered), of which 103 (20.6%) were larvae, 198 (39.6%) juveniles and 199 (39.8%) adults. The male:female ratio was 1:1.4, whereas 51.2% of the adult specimens collected were paedomorphic (phenotypic ratio 1:1). Neither sex ratio nor phenotypic ratio was significantly different from the estimates of these parameters made in September 2000 by Bagnoli *et al*. (2000) ($\chi^2 = 0.18$ and $\chi^2 = 0.26$ respectively, both not significant).

Using Chapman's modification of the Petersen estimator (Chapman 1951), with the standard error estimated from the Seber equation (Seber 1982), the population size was estimated as 4571 ± 1381 (larvae excluded). However, if only the actively reproductive population is considered, the estimate was 1254 ± 383.

Alpine newt abundance along the 3-m-wide riparian strip (A_{alp}) at Selva was markedly non-homogeneous. In fact, along 15 m (three 5-m-long sectors) of the NE side of the pond no Alpine newts were found. By contrast, 61.8% of all Alpine newts were caught along 50 m (ten 5-m-long sectors) of the SW side of the pond. In the other sectors, intermediate values of A_{alp} were observed. On the NE side and the SW side of the pond, respectively, average values (± standard deviation) of the environmental parameters measured were: DT = 24.7 ± 1.0 °C and 13.8 ± 0.6 °C, SV = 20.3 ± 8.3 m and 3.7 ± 1.9 m, WD = 6.7 ± 2.9 cm and 40.3 ± 22.8 cm, VC = 0% and 13.3% ± 20.7%, SL = 1.4% ± 0.6% and 7.2% ± 4.8%.

Stepwise multiple regression of A_{alp} and environmental variables (Table 5), revealed that A_{alp} was positively related to water column depth (WD) and slope (SL), and negatively related to water temperature (DT). These variables cumulatively explained 83% of the variance, whereas VC and SV did not significantly add explanatory power to the model.

Discussion

Although specifically designed for the study of conservation status and threats to the relict population of *Triturus alpestris* from central Italy, our investigation has important consequences for conservation of peninsular populations of the Alpine newt as a whole. In fact, the study of genetic variation at mitochondrial level has indicated these populations as having a high level of genetic differentiation (5.4%–5.8% of sequence divergence) as compared with that of the other European samples studied. Similar or lower levels of genetic divergence at cytochrome-*b* gene sequence have also been reported among congeneric species of European salamandrids, such as *Salamandra lanzai–S. atra* (6%; Nascetti *et al*. 2005) or *Triturus marmoratus–T. pygmaeus* (1.2%; Garcia-Paris *et al*. 2001). Also at the allozyme level, the recorded genetic differentiation of $D_{Nei} = 0.30$, with six diagnostic loci, is particularly high for two conspecific lineages of salamander (e.g. Nascetti *et al*. 1996, García-Paris *et al*. 2000), and clearly suggest the necessity of further investigation aimed at conclusively ascertaining the correct taxonomic assignment of the Italian populations of Alpine newt. Studying

the phylogenetic relationship within the genus *Triturus*, Macgregor *et al*. (1990) found D_{Nei} being 0.11 between *T. a. alpestris* and *T. a. apuanus*. However, since the list of the analysed loci and the table of allele frequencies were not published, we are unable to compare our data with theirs. Although taxonomic conclusions based only upon genetic distance data should be avoided, especially when only fully allopatric populations were studied (e.g. Ferguson 2002), our results clearly individuate populations from peninsular Italy as a distinct Evolutionary Significant Unit (ESU *sensu* Moritz 1994), thus deserving separate conservation efforts.

At least two distinct lines of evidence strongly suggest the need of conservation planning (still lacking) for the Alpine newt in Italy: the highly fragmented pattern of distribution, and the paucity of intra-population genetic variation.

The highly fragmented pattern of distribution of the Alpine newt in peninsular Italy seems to be of clear relictual derivation (Lanza & Poggesi 1971). Postglacial altitudinal migrations would have followed the altimetric plain raising and the establishment of favourable conditions at higher altitude areas (Lanza 1966, Capula & Bagnoli 1983). This scenario is also supported by our results concerning the paucity of the among-population genetic differentiation, which, even taking account of the vagaries of molecular clocks (Ayala 1997), strongly suggest that the present pattern of distribution of the species could be of recent (post-Würmian) origin. Apart from its historical origin, fragmentation has important implications for conservation. Isolated populations are exposed to increased fragility in the face of human disturbance and, especially when small, of environmental, demographic and genetic stochasticity (e.g. Young & Clarke 2000).

Each studied sample of *Triturus alpestris* showed a single fixed mtDNA haplotype, revealing the lack of intra-population variation at this marker. The reduction of genetic diversity associated with severe population bottlenecks is stressed for mitochondrial genes because of their reduced effective population size as compared with that of nuclear ones (Nei *et al*. 1975). Therefore, demographic processes, such as population bottlenecks, could have played an important role in shaping the observed pattern of mtDNA diversity. However, all populations from the northern and central Apennines have been shown to share the same haplotype, so that an historical founder effect, predating events which led to the present fragmented distribution in this area, could have concurred in originating this pattern of high homogeneity at the mtDNA level.

The genetic differentiation between the two populations from the Laga Mts. and Verghereto was found to be very low also at the allozyme level (D_{Nei} = 0.006), thus agreeing with mtDNA data in depicting a recent divergence. However, at the allozyme level the two populations seem to be different in terms of genetic diversity estimates, H_e being 0.018 and 0.060, respectively. Arano *et al*. (1991) found levels of H_e ranging from 0.06 and 0.07 among populations of *T. a. cyrenii* (Spain), and from 0.09 and 0.13 among populations of *T. a. alpestris* (central Europe), whereas among populations of *T. a. veluchiensis* (Greece), Kyriakopoulou *et al*. (1997) found H_e ranging from 0.10 and 0.14. In a recent meta-analysis, Spielmann *et al*. (2004) showed that heterozygos-

Table 5. Stepwise multiple regression analysis, with abundance of Alpine newts as a dependent variable and environmental parameters as independent variables. Model R^2 = 0.829.

Environmental variable	Code (units)	Beta in	Partial corr.	*F*	*P*
Water column depth (3 m away from the shoreline)	WD (cm)	0.511	0.729	38.981	< 0.001
Slope	SL (%)	0.465	0.714	32.027	< 0.001
Deep water temperature (3 m away from the shoreline)	DT (°C)	–0.207	–0.372	4.413	0.045
Percentage of water surface with emergent vegetation (within the 3-m-wide strip)	VC (%)	–0.208	–0.334	2.439	0.130
Distance of the shoreline from the surrounding tree-line	SV (m)	0.134	0.195	1.112	0.301

ity was on average 35% lower in taxa threatened with extinction than in related non-threatened ones, and suggested that these differences in heterozygosity indicate lowered evolutionary potential, compromised reproductive fitness, and elevated extinction risk. The heterozygosity we found in the population of *Triturus alpestris* at the Laga Mts. was never above 40% of that found elsewhere for this species (although some caution is needed when comparing results based on multilocus genotypes gathered from different labs). Furthermore, the population from Verghereto showed a genetic variability comparable to that of the other Alpine newt populations but about three times higher than that observed at the Laga Mts., in spite of the fact that both of them share the same evolutionary history (founder effect and post-Würmian habitat fragmentation). This finding strongly suggests that the lower variability at the Laga Mts. is of very recent, and hence anthropogenic, origin. Following these arguments, from a genetic point of view the Alpine newt population at the Laga Mts. appears under serious threat of disappearance.

Further evidence for such a worrying conservation status of the Alpine newt population under study, comes from demographic and ecological investigations. Within the study area, the species was historically found only at Lago Secco and Lago della Selva, a fact that has been confirmed by the present study. However, whereas in both ponds it was once the most abundant newt species (Capula & Bagnoli 1983), now it is still so only at Lago della Selva. At Lago Secco the species has undergone a rapid decline, which has led to an estimate of its relative abundance (with respect to the total number of newts caught) being 25% in 1998 (C. Bagnoli unpubl. data), 8.6% in 2000 (Bagnoli *et al*. 2000), and 2.8% in 2003 (this study). On the contrary, no sign of either a declining trend or changes in demographic structure were observed during the last six years for the Alpine newt at Lago della Selva (Bagnoli *et al*. 2000; C. Bagnoli unpubl. data and this study). Also, Lago della Selva is presently by far the most important breeding site for the species in the area, and at this site we estimated an adult deme size of 1254 ± 383 individuals. For vertebrate taxa, Reed *et al*. (2003) estimated the Minimum Viable adult Population (MVPa; that having 99% probability of persistence for 40 generations) to be of at least 7000 individuals. Therefore, the studied population appears to be under serious threat of disappearance also at demographic level. Furthermore, since it appeared stable over time at Lago della Selva, the poor conservation status of the Alpine newt in the area appears mainly linked to its decline at Lago Secco and its probable disappearance from the other small ponds in the area (on this subject *see* also Valente 1998 and Canestrelli *et al*. 2005).

Although the causes of such a poor conservation status are difficult to assess unequivocally, our analysis of the habitat correlates of newt abundance support that human activities could have played an important role. Previous studies have suggested that grazing and tree cutting, which lead to an increasing amount of debris on local wetlands, could be responsible for their general deterioration and in some case disappearance (Silvetti 1998, Bagnoli *et al*. 2000). Our results suggest that these activities can affect the Alpine newt population not only by reducing the ability of ponds to refill, thus causing the abolition of potential and/or effective breeding sites, but also by leading to less favourable environmental conditions, such as a reduced water column depth (*see* results concerning WD and SL in Table 5). Several studies investigated the habitat characteristics and preferences of the Alpine newt (e.g. Fasola 1993, Denoël & Andreone 2003, Denoël & Schabetsberger 2003), with results differing among study sites, even within the same study (e.g. Denoël & Schabetsberger 2003). At high-altitude breeding sites, such as that in the Laga Mts., factors affecting water level could be very effective in affecting Alpine newt populations. For instance, Nagl and Hofler (1997) showed that Alpine newt larvae are extremely sensitive to UV-B radiation, both from artificial radiation and direct sunlight, and that wherever possible, they actively escape exposure. They also noted that in ponds involved in events causing water-level reduction (in their case study, cattle watering), Alpine newt larvae had epithelial damage resembling that caused by UV and none of the larvae survived until metamorphosis. Interestingly, at the side of Selva where Alpine newts were absent (NE side), which is also the only

side whose surroundings were affected by tree-cutting and grazing, water column depth was on average very low (6.7 ± 2.9 cm, 3 m away from the shoreline), vegetation cover was absent, and the water surface was completely unshaded.

The above mentioned human activities can also affect daily, seasonal and annual temperature regimes in the study site both directly, by altering daily exposure of the pond to direct sunlight, and indirectly, by leading to decreased water column depth. The effects of temperature on amphibian physiological functions have long been documented (e.g. Feder & Burggren 1992), and more recently, Denoël *et al*. (2005) also reported important effects of ambient temperature on the sexual behaviour of the Alpine newt. Moreover, our results showed a significant (negative) correlation of temperature with Alpine newt abundance, thus suggesting a possible further way in which human activities can impact the resident Alpine newt population, by contributing towards sub-optimal temperature regimes.

In conclusion, although the Alpine newt population from the Laga Mts. is within an area which benefits from three distinct kinds of protection (National Park, WWF oasis and Site of Community Importance), the geographic isolation, low genetic variability, reduced population size and recent decline, are all features indicating the poor conservation status of this population. This has also appeared linked to the human activities in the area, thus suggesting that more effective planning of these activities should be urgently considered in order to assure even the short term persistence of the population. However, since from both the genetic and demographic standpoints the studied population appeared at serious threat of disappearance, we urge that other conservation practices, such as habitat restoration and population restocking, should be considered. The extreme paucity of genetic differentiation between populations from the Laga Mts. and Verghereto, as well as the higher level of genetic diversity found in the latter, suggest that, should a restocking operation be judged appropriate, populations from the northern Apennines could be suitable source populations, allowing to satisfy both the *accuracy* and *functionality* criteria required for an appropriate genetic restoration plan (e.g. Falk *et al*. 2001).

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