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Molecular characterization of Alpine and Northern European populations of Arctic charr *Salvelinus alpinus* (Linnaeus, 1758) by means of nuclear and mitochondrial markers

Licia COLLI[1*], Riccardo NEGRINI[1], Andrea GANDOLFI[2], Fatima CHEGDANI[1], Elisabetta MILANESI[1], Marco PELLECCHIA[1], Francesco NONNIS MARZANO[3], Stella GRANDO[2] & Paolo AJMONE-MARSAN[1]

[1] Laboratory of Animal Genetics, Istituto di Zootecnica, Facoltà di Agraria, Università Cattolica del S. Cuore di Piacenza, via Emilia Parmense 84, 29122 Piacenza, Italy
[2] Department of Valorizzation of Natural Resources, Istituto Agrario S. Michele all'Adige – Fondazione Edmund Mach, Via E. Mach 1, 38010 S. Michele all'Adige (TN), Italy
[3] Department of Evolutive and Functional Biology, Università degli Studi di Parma, via G.P. Usberti 11/a, 43124Parma, Italy
[*] Corresponding author e-mail: *licia.colli@unicatt.it*

SUMMARY - *Molecular characterization of Alpine and Northern European populations of Arctic charr* Salvelinus alpinus *(Linnaeus, 1758) by means of nuclear and mitochondrial markers* - The Arctic charr, *Salvelinus alpinus*, is a Holarctic Salmonid species adapted to cold-water habitats and whose either anadromous or sedentary populations are characterized by an extremely variable morphology. The presence of *S. alpinus* in Southern Europe is deemed as a relict of the Pleistocenic glaciations which strongly influenced both the distribution and the genetic variability of the species, due to repeated isolation and bottleneck events. Previous genetic studies revealed a very low mitochondrial diversity in contrast with the high genetic variability highlighted by microsatellites both within and between populations. In this study, mtDNA control region sequences and AFLP polymorphisms were characterized in 537 specimens from 35 Alpine and five Scandinavian populations. The control region data were aligned with GenBank sequences to build a Median-joining network. The results of AFLP analyses were evaluated by Factorial Correspondence Analysis (individual level) and by Multi-Dimensional-Scaling (population level), while hidden genetic structures were investigated by a Bayesian clustering approach. Multivariate statistical analyses showed that individuals and populations mostly clustered according to the geographic origin. Furthermore, some populations displayed low levels of genetic polymorphism which is probably due either to past demographic fluctuations or to a strong impact of anthropic activities in the recent decades.

RIASSUNTO - *Caratterizzazione molecolare di popolazioni di Salmerino alpino* Salvelinus alpinus *(Linnaeus, 1758) delle Alpi e dell'Europa settentrionale tramite analisi con marcatori nucleari e mitocondriali* - Il salmerino alpino, *Salvelinus alpinus*, è un Salmonide oloartico adattato ad ambienti di acque fredde e presenta popolazioni, sia anadrome sia stanziali, caratterizzate da estrema variabilità morfologica. La presenza di *S. alpinus* nell'Europa meridionale viene fatta risalire agli effetti delle glaciazioni pleistoceniche che hanno influenzato fortemente la distribuzione geografica e la variabilità genetica della specie attraverso ripetuti eventi di isolamento e "colli di bottiglia". Precedenti indagini genetiche hanno rivelato una variabilità mitocondriale ridotta, in contrasto con l'elevata diversità genetica evidenziata con marcatori microsatellite. In questo studio, 537 esemplari provenienti da 35 popolazioni alpine e cinque scandinave sono stati caratterizzati tramite sequenziamento della regione di controllo mitocondriale e marcatori AFLP. L'allineamento delle sequenze mitocondriali con dati da GenBank ha permesso la costruzione di un Median-joining Network. I dati AFLP sono stati analizzati tramite Analisi Fattoriale delle Corrispondenze (livello di individuo) e Multi-Dimensional-Scaling (livello di popolazione), mentre la struttura genetica dei campioni raccolti è stata studiata tramite un approccio bayesiano di clustering. Le analisi di statistica multivariata hanno mostrato come individui e popolazioni tendano a formare gruppi in accordo con la provenienza geografica; in alcune popolazioni, inoltre, è stato riscontrato un ridotto polimorfismo genetico probabilmente causato da passate fluttuazioni demografiche o dall'effetto delle attività antropiche.

Key words: arctic charr, *Salvelinus alpinus*, AFLP, mitochondrial DNA, Alps, Scandinavia
Parole chiave: salmerino alpino, *Salvelinus alpinus*, AFLP, DNA mitocondriale, Alpi, Scandinavia

1. INTRODUCTION

The Arctic charr, *Salvelinus alpinus* (Linnaeus, 1758), is a species of the family Salmonidae characterized by an extreme morphological variability. Being adapted to cold-water habitats, the Arctic charr has a Holoarctic distribution and is widespread at very high latitudes and altitudes. The populations of the circumpolar area are anadromous, but at lower latitudes the migratory behavior is gradually lost, the species becoming sedentary and restricted to freshwater habitats. Both in Northern and in Southern Europe, some landlocked populations are

Tab. 1 - Lakes and countries of origin of Arctic charr samples, total numbers of analyzed specimens (n) and population ID labels. *Tab. 1 - Laghi e nazioni d'origine dei campioni di Salmerino alpino, numeri totali di esemplari analizzati (n) e sigle identificative delle popolazioni (Pop. ID).*

Lake of origin	Country of origin	n	Pop. ID
S. Giuliano	Italy	6	GIU
Costa Brunella	Italy	22	COB
Erdemolo	Italy	28	ERD
Corvo Maggiore	Italy	24	COM
Stellune	Italy	7	STE
Casarina	Italy	10	CAS
Molveno	Italy	2	MOL
Morgex	Italy	39	MOR
Brutto	Italy	13	BRU
Cece	Italy	13	CEC
Colbricon	Italy	2	COS
Tovel	Italy	30	TOV
Lagorai Maggiore	Italy	30	LAM
Iuribrutto	Italy	20	IUR
Barco	Italy	8	BAR
Bombasel	Italy	15	BOM
Lago d'Iseo	Italy	8	ISE
Lago Santo	Italy	11	SAN
Lago di Cavazzo	Italy	3	CAV
Grande di Rava	Italy	18	GRA
Lünersee	Austria	2	LUS
Oberer Plendeler See	Austria	6	OPI
Hintersteiner	Austria	11	HIN
Achensee	Austria	20	ASE
Rotfelssee	Austria	12	RFS
Wolfgang see	Austria	37	AUS
Plansee	Austria	15	PLS
Lunzer See	Austria	20	LUZ
Spuler See	Austria	2	SPS
Zurser See	Austria	12	ZUS
Kleiner Mühldorfer See	Austria	7	KMS
Drachensee	Austria	5	DRS
Stappitzer See	Austria	5	STS
Stapnik See	Austria	20	SKS
Bodensee	Germany	9	BOD
Saimaa	Finland	20	ENO
Toskaljärvi	Finland	20	TOS
Somasjärvi	Norway	20	SOM
Haukejavri	Norway	20	HAU
Buevattnet	Norway	20	BUE

known, which inhabit bodies of water not connected to the sea by rivers.

The presence of the species in Southern Europe is deemed as a relict of the last Pleistocenic glaciation. At the end of the Würmian phase, the ice cover extension decreased and the distribution area of the Arctic charr started to shrink northwards. The southernmost populations remained separated from the Northern conspecifics and adapted to live in the alpine lakes, thus leading to the present-day fragmented distribution pattern. On the southern side of the Alps, the only *S. alpinus* nucleus considered autochthonous is represented by the populations of Trentino-Alto Adige region. Although different authors are doubtful on the native status of these populations, Arctic charrs from this region have probably been used in historical times to introduce the species in several other Alpine lakes (Machino 1999; Piccinini *et al*. 2004).

Hence, the Pleistocenic climatic changes and the anthropic activities had a strong impact on *S. alpinus* geographic distribution and genetic variability through isolation and bottleneck events, and frequent changes in population densities due to exploitation/overfishing,

Previous genetic studies on European Arctic charrs revealed a contrasting scenario: on the one hand, the genetic variability detected at nuclear microsatellite loci was particularly high. The number of alleles per locus varied between six and 49, with values of overall expected heterozygosity between 0.72 ± 0.09 and 0.87 ± 0.04; the analysis of molecular variance assigned 19.20% of the total variation to the between-population and 63.04% to the within-population level, respectively (Brunner *et al*. 1998); on the other hand, both PCR-RFLP analyses (Volpe & Ferguson 1996; Brunner *et al*. 1998) and direct sequencing of the control region (Brunner *et al*. 2001) detected very low levels of mitochondrial DNA diversity (e.g. only three control region haplotypes were identified in *S. alpinus* specimens of Alpine provenance). Although low, the mitochondrial variability of the species seems to be geographically structured. In fact, Brunner *et al*. (2001) identified, within the Holoarctic range of the species, five major haplogroups, distributed at present in five distinct geographic areas and most probably derived from as many distinct glacial refugia. In particular, sequences belonging to only two different haplogroups, namely the Atlantic and the Siberian one, were detected in European Arctic charrs.

Here we present the results of a study on the molecular diversity of European *S. alpinus* by means of mitochondrial and nuclear AFLP markers, aiming at the detection of possible autochthonous, introduced and hybrid populations.

2. STUDY AREA

We analyzed 592 Arctic charr specimens from 40 different populations sampled in 35 Alpine (20 in Italy, 14 in Austria and one in Germany) and five Scandinavian (two in Finland and three in Norway) lakes (Tab. 1).

Information about the origin (native, restocked, hatchery stock etc.) and the migratory behavior (landlocked, sedentary or anadromous) of the populations was collected whenever possible.

3. METHODS

3.1. MtDNA analysis

A fragment of 552 base pairs, corresponding to the 5' end of the mitochondrial control region, was amplified in 112 individuals randomly selected from 12 Italian lakes (72 individuals), 12 Austrian lakes (35 individuals) and five Scandinavian lakes (15 individuals).

Amplification conditions and primer pair sequences are described in Brunner *et al*. (2001). The PCR products were purified with Wizard® SV Gel and PCR Clean-up System (Promega) and sequenced in the forward direction.

The software ClustalX (Thompson *et al*. 1997) was used to align the control region data with 142 publicly available GenBank sequences. Starting from a final alignment of 254 sequences 552 bp long, a Median-joining Network was built with the software Network ver. 4.5 (Bandelt *et al*. 1999; http://www.fluxus-engineering.com).

3.2. AFLP analysis

AFLP (Amplified Fragment Length Polymorphism) profiles were obtained by digestion with *Eco*RI and *Taq*I restriction enzymes and radioactive labeling following the procedure described by Ajmone-Marsan *et al*. (1997). The following four primer combinations, carrying three selective nucleotides each, were used: Eco32(AAC)/Taq32(AAC), Eco35(ACA)/Taq32(AAC), Eco45(ATG)/Taq32(AAC), Eco33(AAG)/Taq33(AAG). Polymorphic loci were detected and binary scored in a dominant manner as 1= presence of the band, 0= absence of the band or 2= missing data.

A Bayesian approach with a uniform distribution of allele frequencies was used to estimate allelic frequencies with the software AFLP-surv ver. 1.0 (Vekemans *et al*. 2002). Expected heterozygosity was calculated following Nei (1987). Estimates of the between-populations and within-population components of the genetic variance were calculated using both the Fst (Weir & Cockerham 1984) and Gst (Nei, 1973) indices.

Further analyses were performed at the level of single individuals by a Factorial Correspondence Analysis (FCA) using the software Genetix (Belkhir *et al*. 2004). Reynolds genetic distances (Reynolds *et al*. 1983) were calculated between populations with Arlequin ver. 3.11 (Excoffier *et al*. 2005) and represented by a tri-dimensional Multi-Dimensional Scaling plot (MDS; Kruskal 1964) obtained with the software Statistica ver. 7.0.

The clustering of individuals was investigated by a Bayesian approach with the software Structure ver. 2.1 (Pritchard *et al*. 2000) under the assumptions of Hardy-Weinberg equilibrium and complete linkage equilibrium, with a burn-in period of 20000 replicates and 50000 Monte Carlo Markov Chains iterations.

4. RESULTS

4.1. MtDNA

The fragment of the mitochondrial control region was successfully amplified for all the 112 selected specimens. All the genetic variation observed within the set of sequences was represented by four different single base-pair mutations (two transitions and two transversions) that identified five haplotypes: three of them (white circles in Fig. 1) had been previously identified in Alpine and Northern European populations by Brunner *et al*. (2001) and had been assigned to the Atlantic (one haplotype) and to the Siberian (two haplotypes) haplogroups, respectively. Within our sample, two new haplotypes were detected in Arctic charr individuals from the Italian lake of Erdemolo and the Austrian lake of Kleiner Mühldorfer See (black circles in Fig. 1). The Median-joining Network of figure 1 shows that one of these haplotypes belongs to the Atlantic group and the other is positioned at the branching point between the Atlantic and the Siberian haplogroups.

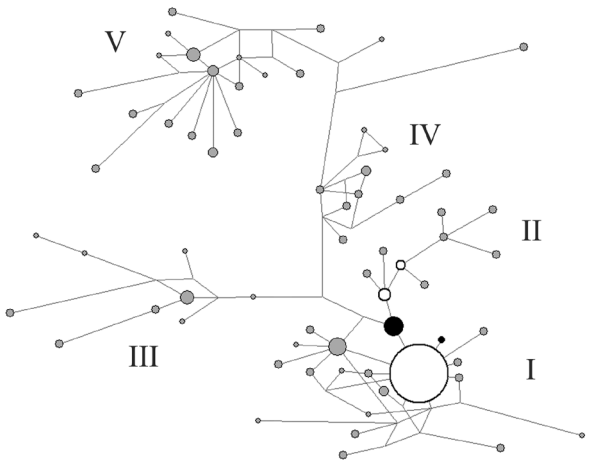

Fig. 1 - Median-joining Network of *S. alpinus* control region sequences. Roman numerals indicate the five major haplogroups detected by Brunner *et al*. (2001) as follows: I - Atlantic haplogroup; II- Siberian haplogroup; III - Beringian haplogroup; IV - Acadian haplogroup; V - Arctic haplogroup. The white circles represent previously described Atlantic and Siberian haplotypes that were also found within our samples, while the black circles highlight the two newly described haplotypes. Gray circles identify GenBank sequence data.

Fig. 1 - Median-joining Network delle sequenze della regione di controllo mitocondriale di S. alpinus. *I numeri romani indicano i cinque aplogruppi principali identificati da Brunner* et al. *(2001) come segue: I - aplogruppo "Atlantic"; II - aplogruppo "Siberian"; III - aplogruppo "Beringian"; IV - aplogruppo "Acadian"; aplogruppo "Arctic". I cerchi bianchi rappresentano aplotipi del gruppo atlantico e siberiano noti da ricerche precedenti e che sono stati identificati anche nei nostri campioni; i cerchi neri indicano i due nuovi aplotipi identificati, mentre i cerchi grigi rappresentano sequenze ottenute dalla banca dati GenBank.*

4.2. AFLP data

Clearly readable AFLP profiles were obtained from 537 individuals out of 592. Samples and markers with a level of missing data above 5% were removed from the definitive dataset. The four primer combinations detected a total of 77 polymorphic loci out of about 400 amplified AFLP bands, with an average of 19.3 markers per primer pair. Within population, the number of polymorphic loci varied from 30 to 77, with an average of 55 loci per population. The final matrix of binary data included 41,100 data-points with an overall percentage of missing data equal to 0.007%.

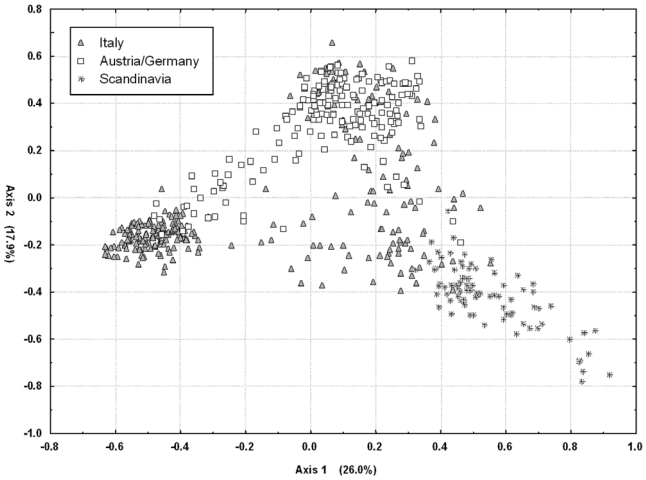

Fig. 2 - Two dimensional plot of the Factorial Correspondence Analysis of AFLP data relative to the first two axes. The values into brackets indicate the percentages of the total variance explained by each axis. Symbols indicate the origin of the individuals: grey triangles – Italian samples; white squares – Austrian/German samples; black stars – Scandinavian samples.

Fig. 2 - Rappresentazione bidimensionale dei risultati dell'Analisi Fattoriale delle Corrispondenze dei dati AFLP. I valori tra parentesi indicano le percentuali di varianza spiegata da ciascun asse. Nel grafico di dispersione gli individui sono rappresentati sui primi due assi principali con simboli diversi in base all'origine geografica: triangoli grigi – campioni italiani; quadrati bianchi – campioni austriaci/tedeschi; asterischi neri – campioni scandinavi.

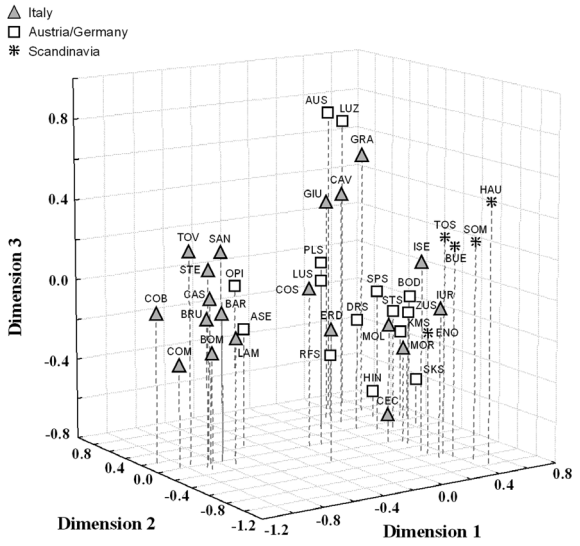

Fig. 3 - Multi-Dimensional Scaling plot of Reynolds genetic distances between populations. The populations are represented by different symbols according to the geographic origin: grey triangles – Italian samples; white squares – Austrian/German samples; black stars – Scandinavian samples. The correspondence between labels and single populations is detailed in Table 1.

Fig. 3 - Rappresentazione tridimensionale dei risultati dell'analisi Multi-Dimensional Scaling condotta a partire dalla matrice di distanze genetiche di Reynolds tra popolazioni. Simboli diversi identificano le popolazioni in base alla provenienza geografica: triangoli grigi – campioni italiani; quadrati bianchi – campioni austriaci/tedeschi; asterischi neri – campioni scandinavi. Per la corrispondenza tra le sigle e i laghi di origine delle popolazioni, si faccia riferimento alla Tabella 1.

Expected heterozygosity values ranged from 0.13 (TOV lake; Standard Error, S.E. 0.014) to 0.31 (BOD lake; S.E. 0.017). Concordant estimates of the between-populations component of genetic variance were obtained with the indexes Fst=0.23 and Gst=0.24, which assigned the 23/24% of the total variance to the inter-populations level.

The first and second axes of the FCA accounted for the 26.0% and 17.9% of the total variance, respectively, and identified three main clusters, where individuals and populations tended to group according to the geographic provenance (Fig. 2). In fact the Arctic charr specimens of Italian and Austrian/German origin clustered on the left and upper side of the graph, respectively, while the Northern European individuals were concentrated in the lower right corner. Nevertheless, the individuals belonging to some populations were either placed within a cluster not concordant with their region of origin (e.g. specimens from lakes GIU, GRA, OPI, IUR) or scattered between two different clusters (e.g. specimens from lakes ERD, MOR, BOD, ASE).

These results were further confirmed at the population level by the MDS plot (stress value 0.054) of figure 3 where the three main groups - Italian, Austrian/German and Scandinavian - are still identifiable, as well as the aforementioned "misplaced" populations. Moreover, the addition of the third dimension allowed to detect a further sub-clustering within the Austrian/German group, where the populations AUS, LUZ, GRA, CAV and GIU appear separated from the others.

When the Bayesian assignment test was performed setting the parameter K= 3, that is to say by hypothesizing the existence of three reference populations under the assumption of Hardy-Weinberg equilibrium, the analysis sorted the individuals into the same three main clusters already identified by the multivariate approaches; the same assignment procedure carried out with K= 4 confirmed the presence of a fourth group of populations exactly matching the sub-structuring revealed by the third MDS dimension (data not shown).

Since the software Structure also evaluates the composition of the genome of each single individual, the analysis revealed that the specimens, whose FCA plot points were scattered between the principal clusters, possessed hybrid genomes.

5. DISCUSSION AND CONCLUSIONS

Our analyses on sequence data confirmed the existence of a low level of genetic mitochondrial variability in the Alpine populations of *S. alpinus* already detected during previous studies (Brunner *et al*. 1998; 2001). Very few haplotypes were identified and most individuals, 80 out of 112, possessed identical control region sequences. Nevertheless our analyses showed that some novel mtDNA variation, although limited, can still be detected by widening the sampling area and that the Arctic charr populations from the north-eastern and southern side of the Alps possess some genetic uniqueness in terms of haplotypes. In fact, the three haplotypes described by Brunner *et al*. (2001) for the Alpine populations were all detected in France and Switzerland, while the very few analyzed specimens (n= 6) from Austria and Germany all possessed the most common sequence variant.

The Median-joining Network obtained from our fi-

nal alignment is consistent with the phylogeographic reconstructions and the subdivision into five major haplogroups proposed by Brunner *et al*. (2001). In fact, our mtDNA sequences of Scandinavian Arctic charrs fall within the Siberian haplogroup, while all the Alpine sequences belong to the Atlantic group. Moreover, the position of one of the newly described haplotypes seems particularly interesting, since it represents a connection between the aforementioned two haplogroups. The presence of this peculiar haplotype in Alpine Europe may have an important phylogeographic meaning and can help to unravel the post-glacial history of the species, but further and more detailed analyses are needed to settle this issue.

The evidence collected from AFLP analyses showed that also the genomic variability is geographically structured: the right-to-left disposition of the scatter points along the first axis of the FCA corresponds to a north-to-south pattern of diversity (Scandinavia-Austria/Germany-Italy) whose origin is probably due to the combined effects of paleogeographic events and genetic drift.

If we consider the three main clusters detected by the FCA, the differences in the relative width of their respective scatters of points suggest the existence of a higher variation within the Scandinavian populations which possess a wider scatter area compared to the less variable Alpine groups, whose points concentrate within narrower regions of the graph. The origin of both the misplaced populations, which group together with individuals of different geographic provenance, and of the hybrid individuals scattered between the main clusters can be traced back to the effects of human intervention. In fact, this was probably due to restocking practices in recent times or *de novo* introduction of Arctic charrs from foreign countries and thus characterized by a different but clearly identifiable genetic makeup.

Although the *S. alpinus* populations from Italy seem to possess low levels of genetic variability, an interesting evidence concerns their genetic distinctiveness. So far the ichthyologists' opinions about the existence of autochthonous Italian Arctic charrs were conflicting. Our molecular analyses seem to confirm the hypothesis of some populations (e.g. those from Lagorai Maggiore, Corvo Maggiore or Bombasel lakes) being Italian native. These are clearly distinct from other European conspecific groups and therefore need to be carefully managed and conserved.

Taken together, the evidence drawn from our molecular analyses indicates that the complex patterns of *S. alpinus* mitochondrial and nuclear genetic variability were probably shaped by the early influence of the Pleistocenic climate changes, that isolated groups of individuals and populations in different geographic areas and induced repeated demographic fluctuations and genetic drift. The effects of human activities, tied to introduction/restocking practices during the last decades, superimposed on this former scenario and deeply contributed to shape the genetics of the present day Arctic charr populations.

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