Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Biotechnology 113 (2004) 43–53

JOURNAL OF Biotechnology

www.elsevier.com/locate/jbiotec

# Experience from Lipizzan horse and salmonid species endemic to the Adriatic river system Examples for the application of molecular markers for preservation of biodiversity and management of animal genetic resources

Peter Dovc*, Simona Sušnik, Aleš Snoj

*University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Groblje 3, 1230 Domzale, Slovenia*

Received 9 September 2003; received in revised form 23 March 2004; accepted 1 April 2004

## Abstract

Management of breeding- and free-living populations, traditionally based on phenotypic traits, relays more and more on availability of reliable information about the basic population genetic parameters as heterozygosity, mean number of alleles per locus, percentage of polymorphic loci, population structuring, genetic distances and others. Therefore, the application of molecular markers, revealing a great deal of phenotypically hidden information, becomes inevitable for population analysis. Conservation geneticists use this information for implementation of appropriate management policies. Application of molecular markers in Lipizzan horse breed, which is an example for a pedigreed breeding population, and in two endangered salmonid fish populations in Slovenia, are presented. In the Lipizzan horse breed, an insight in the population structure, overall heterozygosity, relationship between population parameters and phenotypic traits and reliability of pedigree data was gained by using molecular markers. Marble trout and Adriatic grayling were selected as examples for free-living populations, seriously endangered by human activity in the past. Development of informative molecular markers, their application and suggestions for appropriate conservation actions are described.

*Keywords:* Biodiversity; Animal genetic resources; Pedigreed population; Free-living populations; Molecular markers; Preservation biology

## 1. Introduction

Permanent reduction of biological diversity on earth, which is mainly due to loss of specific habitats, pollution, introduced species and overexploitation, is

---

* Corresponding author. Tel.: + 386-1-7217-836; fax: +386-1-7241-005.

*E-mail address:* peter.dovc@bfro.uni-lj.si (P. Dovc).

0168-1656/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2004.06.005

rapidly progressing. A large number of species have gone to extinction and many others have been reduced to the point where they urgently require human intervention to protect them from extinction (Lawton and May, 1995). Free-living populations as well as their domesticated counterparts are affected and therefore, appropriate conservation measures have been developed for both situations. Conservation of domesticated and free-living populations contrasts importantly in several regards: in domesticates, time perspectives are short, whereas wildlife conservation is open-ended; in domesticates conservation ex situ is applicable, in wild species conservation in situ is the only possible solution and finally, generation of genetic variation and selection in domesticates are controlled by man, whereas wild species living in natural communities are dependent upon variability generated by their genetic system and are kept in balance with environmental changes by natural selection (Frankel, 1974). However, recent development of genome resource banks for wildlife is beneficial and equal or even more important than its use in livestock species. Genetic management of rare populations held in captivity as well as frozen repositories could help to insure wild populations against natural and human-induced catastrophes (Wildt, 2000). Molecular markers have been successfully used to resolve taxonomic uncertainties, but there are only a limited number of cases where genetic management is being used to address inbreeding (e.g. Florida panther) and loss of genetic diversity in wild populations (e.g. red-cockaded woodpecker) (Frankham, 1999). Even though, the study of gene diversity in free-living, natural populations remains in the centre of interest, special concern also has to be assigned to conservation of breeds and genetic diversity within domesticated animals. Due to the fact that livestock breeding strategies depend on the availability of a large gene pool, from which useful traits can be selected, conservation of genetic resources becomes pivotal role in planning further development of animal breeding, considering all existing breeds, regardless of their usefulness under present conditions. Application of numerous molecular markers for population analysis offers fast and reliable way to obtain information about the basic population parameters, further more, it can also reveal a great deal of phenotypically hidden information. Highly informative molecular markers (e.g. microsatellites) are of particular importance, especially in the case when analysis of phenotypic traits is unable to reveal population structure.

The availability of molecular data has also tremendous importance for phylogenetic studies. Because phylogeny is “the stream of heredity,” only genetically transmitted traits are informative in phylogeny estimation (Avise, 1994). In order to propose appropriate conservation plans for domesticated populations, it is of crucial importance to know the history of the breed, possibly underpinned by molecular data. In this regard, mitochondrial DNA (mtDNA), characterized by maternal inheritance, absence of recombinations and relatively fast mutation rate has represented the most informative genomic element for testing out the what, where and with less confidence the when of livestock domestication (MacHugh and Bradley, 2001). However, dealing with a single segregating locus and not being necessarily concordant with the origin of the nuclear genome are the widely perceived drawbacks of the mtDNA phylogeny, which make the application of nuclear markers inevitable (Allendorf et al., 2001). Among them are microsatellites, and more recently SNPs (single nucleotide polymorphisms), the most frequently used molecular markers, which allow estimation of basic population parameters, due to their co-dominant mode of inheritance. However, the possibility of crossing over among nuclear loci can reduce the informativity of these markers, representing a complication which can be counteracted by the usage of a larger number of marker loci and asssuming that crossing over is a rare event.

In this short review we present examples and our experience in application of genetic markers for preservation of well documented pedigreed Lipizzan horse population and usefulness of genetic markers in description of free-living, endangered (most frequently due to anthropogenic activity) populations of salmonids that need to be protected from further destruction or extinction. In both cases, molecular data can be used first for breed- or population description and further for conservation plan proposals including monitoring of the efficacy of conservation actions.

### 1.1. *Example of the Lipizzan horse breed*

The history of the Lipizzan breed, at present the oldest European cultural horse breed, goes back to 1580 when the original stud of Lipica was established. The stud at Slovenian Karst produced riding and light carriage horses for the Habsburg court (Dolenc, 1980). Due to well preserved studbook records, Lipizzan horse breed represents a unique example of pedigreed domestic animal population. The basic population was formed from nine stallions and 24 mares, imported from Spain, and from a number of mares belonging to the local breed of white Karst horses. Later, imports of Andalusians, Barbs, Italian- and also Arab horses were important source for formation of the Lipizzan gene pool (Nürnberg, 1993). The Lipizzans deserve a special attention also because they represent an important gene pool, where genes from several historical breeds (some of them are already extinct) are preserved. However, they represent small population, which could potentially be threatened by stochastic effects. The available studbook documentation enables reliable reconstruction of the breed history using different methods for pedigree analysis (Zechner et al., 2002). By the end of the 19th century, six classical Lipizzan stallion lines (Conversano, Favory, Maestoso, Neapolitano, Pluto and Syglavy) and 18 classical mare family lines were established (Nürnberg, 1998). Later on two more stallion lines (Incitato and Tulipan) and 20 mare family lines were added. Inspite of the extensive pedigree data, the question about the real genetic diversity within the breed, as well as genetic structure of the population, remained unanswered. Therefore, it was an appealing idea to apply molecular genetic markers in order to get more objective estimation of genetic variability within the breed, degree of heterozygosity, number and distribution of mtDNA haplotypes and finally also about the reliability of pedigree records.

Recent analyses of large data sets of horse mtDNA revealed great heterogeneity of mtDNA haplotypes within breeds and suggested that several distinct populations were involved in the domestication of the horse (Vila et al., 2001; Jansen et al., 2002). Therefore, the use of mtDNA haplotypes as breed specific markers in horse is not applicable. However, huge variety of mtDNA haplotypes, present within almost all breeds, allows elucidation of the population structure using mtDNA data. Lipizzan horse breed became the first pedigreed horse population where contribution of different maternal lines was examined by DNA sequence analysis (Kavar et al., 1999), allowing an insight into genetic diversity of founder animals. The pedigree data suggested very broad pool of mtDNA haplotypes as a consequence of numerous imports during the history of the breed. Indeed, 37 mitochondrial haplotypes, belonging to all four main equine mtDNA clusters, were identified in 56 Lipizzan maternal family lines (Kavar et al., 2002). In order to pay tribute to the cultural value of the Lipizzan breed, names of the founder mares were assigned to the mtDNA haplotypes, found in classical mare family lines. All major equine haplotype groups were also represented in the Lipizzan horse breed (Kavar et al., 1999, 2002) with the exception of some exotic haplotypes, found in glacial specimens, historical bone samples and in specimens belonging to the population of Przewalski horses (Vila et al., 2001; Jansen et al., 2002). A high degree of similarity between mtDNA haplotypes found in the Lipizzan horse breed and in Arabian horses (Bowling et al., 2000), confirmed historical gene flow from the Arab- to the Lipizzan population. Lipizzan data also support the domestication hypothesis, based on the assumption that domestic horse breeds originate from a large number of different ancestral populations. Comparison of molecular and pedigree data revealed, that some mares, founders of maternal lines, shared the same mtDNA haplotype (haplotype Capriola is present in 13 and haplotype Batosta in four mare family lines). The most common haplotype was Capriola (26.4%), followed by Batosta (10.1%) and Allegra (5.5%). The distribution of mtDNA haplotypes among different Lipizzan studs reflects the intensity of exchange of breeding animals during the history of the breed. A good coverage of classical mare family lines was typical for traditional studs (Lipica, Piber, Monterotondo), whereas more remote studs in Romania, Slovakia and Croatia, which were not included so frequently in these exchanges, showed very specific selection of only locally present mare family lines. However, more surprising was the fact that in some maternal lines more than one haplotype was found (Fig. 1). This is an unambiguous proof that some pedigree errors occurred during the history of the breed. The minimal number of maternal pedigree errors in the whole Lipizzan pedigree was estimated to be 25, however it is possible that some pedigree errors remained hidden allowing even higher real number of errors. Based on this data, the proportion of animals having maternal parentage in their pedigree in disagreement with mtDNA haplotype in the present Lipizzan population is about 11%. Similar finding was observed also in the population of Thoroughbred horses (Hill

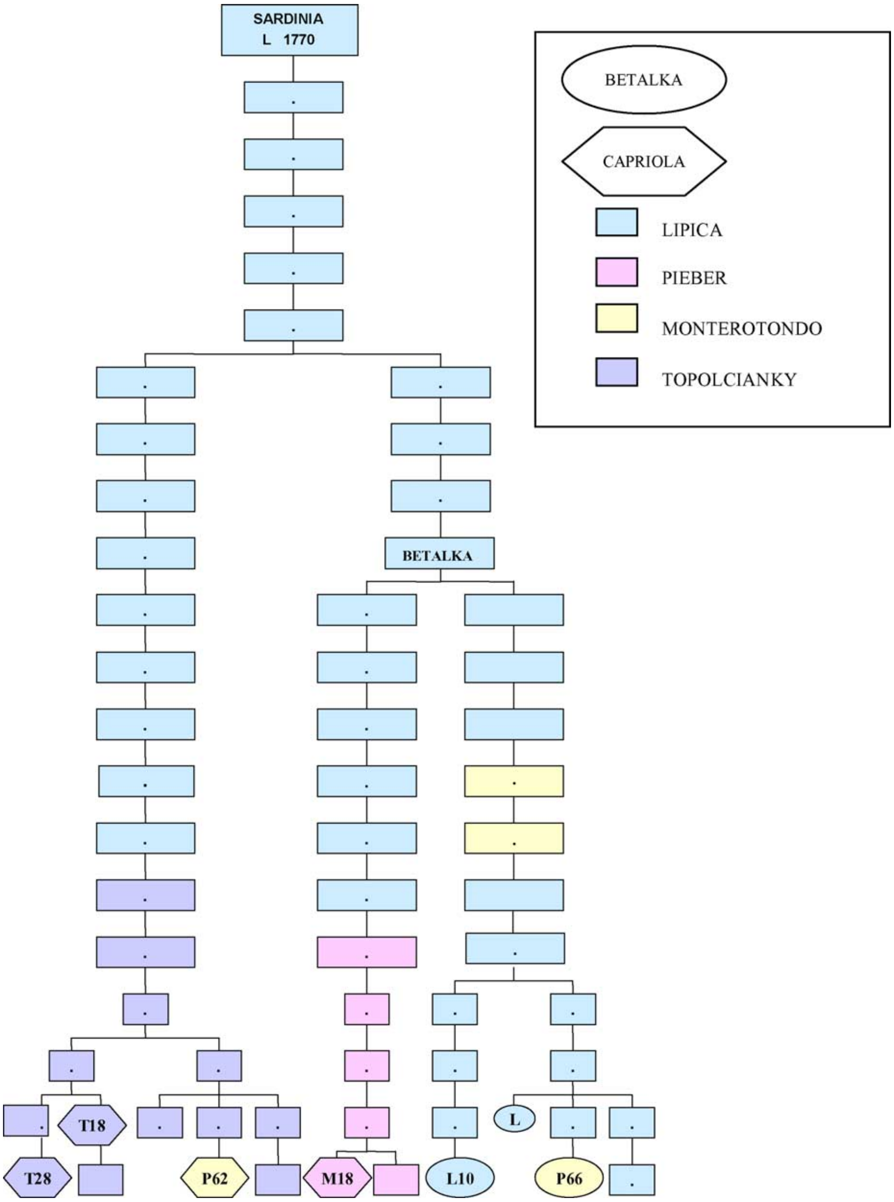

Fig. 1. An example of “historical” matrilineal pedigree error in the Lipizzan horse breed. Pedigree of the maternal line Sardinia L 1778, harbouring two different mtDNA haplotypes is shown in the diagram. Two haplotypes, Betalka (shown as ellipses) and Capriola (hexagons), were found in animals from the last two generations of the maternal line Sardinia L 1778. Ellipses and hexagons demonstrate presence of both mtDNA haplotypes, Capriola and Betalka in the same maternal line. The haplotype Capriola seems to be the correct haplotype, characteristic for the maternal line Sardinia L 1778, whereas the presence of the second haplotype, Betalka, represents an error in the matrilineal pedigree. Not analysed samples are shown as rectangles. Location of the animals is colour coded (see legend).

et al., 2002), where the proportion of pedigree errors in maternal parentage was detected in eight out of 19 mare family lines. The drawback of the method is that the exact identification of wrong entry into the pedigree is not possible without multi generational material, which is normally not available. Therefore, only very recent errors (in the current or in one to two generations before) can be traced to their origin. In some recent cases such errors were also successfully confirmed using microsatellite markers.

Microsatellite data were used for estimation of microsatellite heterozygosity and genetic distances among Lipizzan studs and between Lipizzans and some other horse breeds. Analysis revealed clear clustering among studs and relative high degree of heterozygosity, comparable with other breeds, lagging only slightly behind much larger populations as for instance Holsteiner, Hannoveraner and Quarter Horses. From the point of the general usefulness of microsatellite markers in different horse breeds it is important to stress that two MS loci, routinely used for parentage testing in horse (HMS3 and ASB2) have mutated priming sites in Lipizzans, with the consequence that null alleles and high discrepancy between expected and observed heterozygosity appeared at these loci in Lipizzans (Achman et al., 2001). The effect of inbreeding and microsatellite heterozygosity on morphological traits was also studied (Curik et al., 2003) and no overall large effects were found in Lipizzan horse. However, the usefulness of microsatellite markers for controlling heterozygosity, inbreeding and paternity was confirmed.

The availability of molecular data will enable consideration of rare mtDNA haplotypes and microsatellite alleles present in low frequencies as an important source of genetic diversity within the breed and will, hopefully, contribute to their preservation. This is especially important in a small, structured population for which the Lipizzan horse population is an outstanding example.

### *1.2. Examples from salmonids endemic to the Adriatic river system*

The Adriatic district harbours a variety of freshwater endemic fishes (Behnke, 1968). Due to specific paleogeography and especially to the history of the hydrographical systems, they split from their Danube relatives approximately five million years ago (after the Mediterranean Messinian salinity crisis) and during their long-lasting isolation developed distinct forms exhibiting pronounced phenotypic characteristics (Bianco, 1990). Some of these taxa are considered to be on the way to their speciation and therefore represent an extremely sensitive and vulnerable biological system which is due to hybridisation with Danube relatives, induced by anthropogenic activities, highly endangered.

#### *1.2.1. Marble trout*

A project for rehabilitating marble trout (*Salmo marmoratus*) is used as an example to illustrate a successful application of molecular markers to preservation of free-living animals.

The marble trout has a restricted geographical range in the Po river basin in northern Italy and in the Adriatic river basin in former Yugoslavia and Albania (Povž, 1995), where it parapatrically coexist with indigenous brown trout (*Salmo trutta*; Giuffra et al., 1996). Natural hybridization between the two species was minimal (limited only to their contact zones) or none, maintaining undisturbed parallel evolution of both species. The situation has drastically changed when foreign strains of brown trout were introduced into the marble trout living space in the beginning of the last century. They built a reproductive bridge between the incipient species, which resulted in uncontrolled and unstoppable interspecies hybridization. In the late 1980s, hybrid swarms dominated the majority of Slovenian Adriatic river system and only few upper reaches were still inhabited with marble trout. An action plan for marble trout rehabilitation started in 1993 (Povž, 1995; Crivelli et al., 2000). Its strategy was to establish a captive breeding programme with wild genitor stocks from pure marble trout populations, in order to release genetically pure individuals in the hybridization zone until almost all foreign alleles had been eliminated (Crivelli et al., 2000). In this sense, a diagnostic test for discrimination of marble trout from the hybrid swarms was required. Species determination based on morphology is due to susceptibility of morphological characters to environmental effects and invasive sampling inappropriate in such cases. Alternatively, molecular genetic approach can account for a variety of polymorphic characters that are not under selection pressure, and material collection is harmless for animals. Hence, genetic markers were

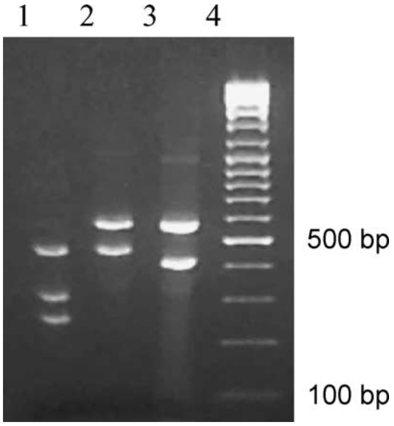

Fig. 2. According to Bernatchez (2001), the marble trout is considered an independent phylogeographic lineage of brown trout *S. trutta*, which is further divided into four other lineages inhabiting the main river systems in Europe: the Atlantic, Danubian, Adriatic and Mediterranean. Restriction analysis of mtDNA control region, using *Alu* I endonuclease, provides a simple diagnostic method for discrimination between the haplotypes determining Atlantic/Danubian/Mediterranean (lane 1), Adriatic (lane 2) and the marble trout lineage from the Soča river basin (lane 3); 100 bp marker is shown in the lane 4.

applied for establishing a diagnostic tool for marble trout. In the early 1990s, the first information on marble trout genetic uniqueness in comparison to brown trout, inferred from mitochondrial DNA control region, was given (Bernatchez et al., 1992; Giuffra et al., 1994), describing a group of haplotypes, which were marble trout characteristic, and accordingly designated as Ma (*marmoratus*). This group of haplotypes, which can be easily detected by *Alu* I restriction analysis (Fig. 2), have been later described also in marble trout from the Soča river basin in Slovenia (Snoj et al., 2000), indicating this marker might be considered as one of criteria for evaluating genetic purity of marble trout throughout the North Adriatic region. The genetic structure of marble trout in Slovenia was surveyed and compared to brown trout also at the biochemical level (Berrebi et al., 2000). In this regard, 31 presumptive enzyme loci were investigated and four of them (AAT-1*, LDH-5*, SOD* and TF*) turned out as diagnostic for marble trout. The existing set of marble trout diagnostic markers was later supplemented by three microsatellite loci from BFRO series (BFRO 1, 2 and 3; Snoj et al., 1997, 2000; Sušnik et al., 1997), all obtained from the marble trout microsatellite DNA library (Snoj et al., 1996), enabling discrimination between marble and brown trout.

Using the collection of marble trout informative markers, eight genetically pure populations of marble trout, separated from downstream hybrid populations by impenetrable barriers, were revealed in the Soča river basin (Berrebi et al., 2000; Pleško and Budihna, 2000; Snoj et al., 2000) providing material applicable for conservation strategies. Recent study (Fumagalli et al., 2002), based on 13 microsatellite loci, revealed that microsatellite allele number and heterozygosities of these eight populations were remarkably low compared to genetic variation at microsatellite loci commonly observed in other fish species (DeWoody and Avise, 2000). Given the extremely low levels of genetic variability detected in those populations, identification of new genetic source of marble trout would be beneficial in order to augment the gene pool of genitor stocks and to maximize genetic diversity of reintroduced populations. Population disequilibria (Berrebi et al., 2000) and strong correlation between genetic and morphological markers characteristic for marble trout (Delling et al., 2000), detected in the zone of hybridization indicate that panmixia has not yet been reached there. This means that genetically pure individuals of marble trout probably still exist in this zone, representing, therefore, a potential source of a new genetic variation. Assortative mating and genome incompatibilities between the hybridizing taxa might be a possible reason for such fragmentation (Leary et al., 1995). For evaluating genetic purity of marble trout extracted directly from hybridization zone, available microsatellite markers may not be a sufficient approach: they were established in the populations exhibiting only a limited amount of genetic variation, and in addition, microsatellites represent neutral markers with few or no impact on phenotype. Therefore, an additional supporting diagnostic tool may need to be developed. From this perspective, RAPD markers might represent an acceptable alternative. Although this fingerprinting method has been criticized a lot, mainly due to questionable reproducibility and prevalently dominant inheritance, its main advantage in this very case seems to be in their random distribution over the genome, comprising also coding regions, which may lead to the discovery of causative genes for phenotypic traits. A first attempt in this direction has already been made: 200

decamer oligonucleotides have been tested on marble trout from the upper Soča river system, brown trout of different origins and F1 hybrids, obtained by controlled hybridisation of marble and brown trout (Jug et al., 2004). Twenty nine of these primers yielded products showing clear and reproducible inter-specific differences. The RAPD markers obtained by this analysis have already been applied as a supporting tool for management arrangements in hybridisation zones.

At the end, it has to be stressed that in spite of undisputed usefulness of the molecular techniques, the use of unlinked molecular markers in order to discriminate purebreds from hybrids has to be cautiously interpreted; namely, rather than the overall genotypic origin of a particular organism, they can only reliably reflect their own origin. The origin of the rest of the genome, as inferred from molecular data, should be considered an estimate, whereby the estimate's accuracy depends on the number and distribution of the marker loci used. Therefore, molecular markers should be used as an additional diagnostic criterion, which follows previous phenotypic selection. One should always be aware that the final goal of conservation projects as the described "marble trout project" is the restoration of the indigenous population in its original habitat, which means a reintroduction of individuals with characteristic phenotype and capability to transmit it to their offspring. The combination of phenotypic and genetic selection is, consequently, inevitable, while any selection considering only one of the two criteria is insufficient.

### *1.2.2. The case of grayling*

Grayling (*Thymallus thymallus*), a European salmonid species is native in the northern part of the Adriatic river system in Italy and Slovenia. In this habitat, grayling evolved in a distinct race, hereafter referred to as Adriatic grayling, that morphologically differs from its trans-Alpine counterparts inhabiting the adjacent Sava river system.

During the last few decades, grayling throughout Europe have been seriously affected by environmental degradation, over-fishing and in some regions also by depredation by piscivorous birds (Uiblein et al., 2000). Due to a constant population decline, stocking has been the ultimate conservation action. Non-native strains were usually used as stocking material, which seriously threatened the genetic integrity and existence of some native stocks. Particularly in Slovenia, strains originating from the Sava river have been translocated into the Soča river (Slovenian part of the Adriatic drainage), which caused a widespread introgression and appearance of hybrid swarms. Due to the grayling natural range being restricted to the middle part of streams, the entire native population has been jeopardized by hybridization, and no isolated parental populations, potentially representing a breeding material for repopulation, existed. Before undertaking any action plan for grayling restoration in the Soča river, the main idea was to explore the possibilities for using molecular markers to identify non-introgressed remains of indigenous grayling for future management and supportive breeding. For this purpose, the first set of microsatellite markers for grayling was established (Snoj et al., 1999; Sušnik et al., 1999a, 1999b, 2000). Mitochondrial DNA and additional microsatellites, primarily designed for other salmonid species, were also applied in population and phylogenetic studies of grayling originating from different locations across Europe (Gross et al., 2001; Weiss et al., 2002; Koskinen et al., 2002a, 2002b, 2002c). Using mtDNA and 15 microsatellite markers, genetic distinctness of the grayling population from the upper Soča river system was demonstrated (Sušnik et al., 2001, in press). Nucleotide divergency between Adriatic grayling and the one of Danubian origin was, based on mtDNA sequences, more than 3%, providing evidence for a long-lasting and independent evolutionary history of grayling inhabiting the north Adriatic region. Microsatellite analysis provided relatively high number (12) of alleles, diagnostic for the Adriatic grayling. Data analysis revealed high correlation of these alleles with morphological characters specific for the Adriatic grayling, (Fig. 3), indicating long-lasting parallel development of novel forms of unlinked characters. Extended microsatellite analysis based on multilocus genotypes revealed extensive introgressive hybridisation between the indigenous and introduced parental lineages and among the hybrid swarms as well. On the basis of private allele distribution and admixture coefficient, it was estimated that only 50–60% of the original gene pools remained in the Soča river and only few non-introgressed indigenous individuals could be identified.

Prior to antropogenically induced introgression, the Adriatic grayling used to be reproductively isolated from other conspecific populations and therefore,

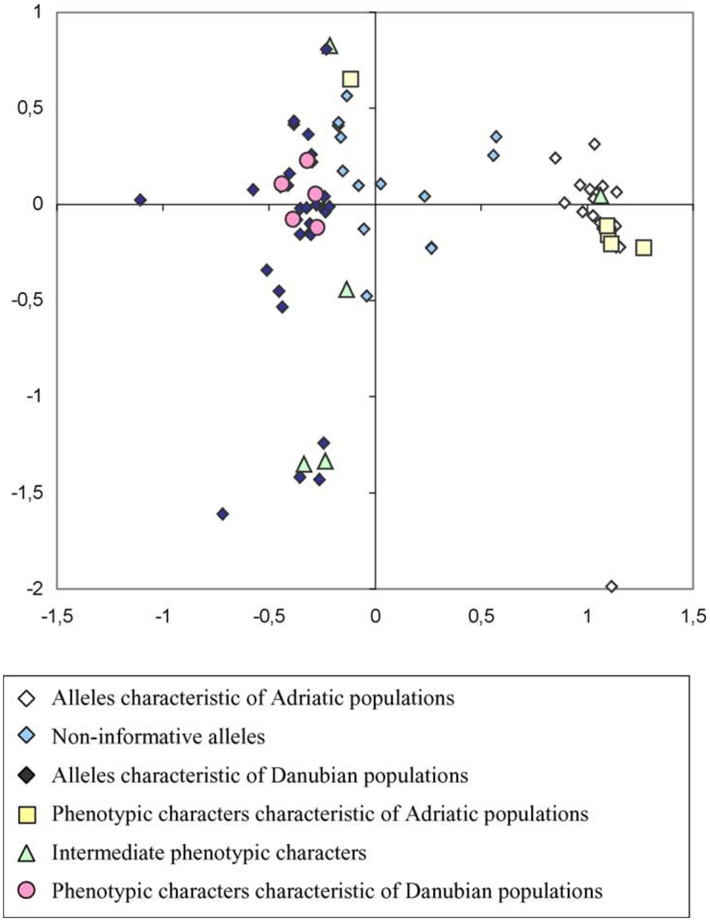

Fig. 3. Diagram of microsatellite allele distribution including distribution of phenotypic variables according to factorial correspondence analysis of Danubian and Adriatic populations of grayling in Slovenia (Sušnik et al., in press). FCA analysis was performed in Genetix program (Belkhir, 1998). Five phenotypic characters were assessed: the shape of the hump, body colour, the colour of the tail, magenta stain, and number of black spots.

represents an important component in the evolution of the species, which allowed its consideration as an evolutionary significant unit (ESU; Waples, 1995). Nowadays, when the population is highly admixed, the question raises whether it deserves to be protected or not? According to Allendorf et al. (2001), conservation of hybrids in cases like this should be considered, because it is the only available option to avoid the complete loss of the hybridized population. According to this view, preservation strategies for the indigenous grayling in the Soča river have been established proposing selection of non-hybridized individuals from the hybrid population to be used as founders for new populations or for captive breeding. Sorting of appropriate material should not only rely on results obtained by genotyping but should consider specific phenotypic characters as well. The apparent correlation between genetic markers and phenotypic characters implies that, in spite of a high level of introgression, the integrated and preserved genome architecture of the remnant indigenous population can still be found in the Soča river. Proper management, supported by selection of breeding stocks for desired genotype and phenotype, could, therefore, provide the only satisfactory solution for conservation of the Adriatic grayling.

## 2. Conclusion

Preservation of biodiversity in free-living as well as in domesticated species requires a profound knowledge about the basic population parameters and population structure. Efforts in reaching this goal can be substantially supported by the application of molecular markers. Modern biotechnology offers a whole pallet of methods for production and high throughput analysis of different types of DNA markers. Presented examples demonstrate the usefulness of molecular markers for preservation of biodiversity in pedigreed and free-living populations. In all three cases, the application of molecular markers considerably contributed to better understanding of population structure and basic population parameters. In the case of Lipizzan horses molecular information will facilitate management of genetic pool in terms of preservation of genetic diversity within the breed and help to prevent undesired loss of rare haplotypes and alleles. Both examples with salmonid fish demonstrated the power of molecular data for rescuing highly endangered populations, even if the hybridization process was already in progress. Marble trout in the Soča river shows also, that even in the case when pure populations are still available, preservation of specific genes from the hybridization zone may be important because of their impact on adaptive capacity. As inferred from mtDNA genetic variants, the Adriatic grayling was recognized as evolutionary distinctive population demonstrating highly restricted gene-flow from other lineages within grayling species and as such justified to be considered at least as an ESU. However, by the introduction of alochthonous populations, the status of the Adriatic grayling has changed. It does not represent reproductively isolated unit anymore, but rather a community of individuals with various levels of introgression and probably equal ability to mate with any other individuum. Nevertheless, the Adriatic population still possess characteristic distinctive distribution of allele and haplotype frequencies, which allows the Adriatic grayling to be according to definition introduced by Moritz (1994) recognized as management unit (MU). As such, this population deserves adequate management and conservation of the extant status.

In the future, the new generation of molecular markers, tightly linked to the loci encoding important physiological, morphological and, especially in livestock species, with quantitative trait loci will allow design of more powerful strategies for preservation of biodiversity and hence more efficient and sustainable breeding strategies.

## References

Achman, R., Huber, T., Wallner, B., Dovč, P., Müller, M., Brem, G., 2001. Base substitutions in the sequences flanking microsatellite markers HMS3 and ASB2 interfere with parentage testing in the Lipizzan horse. Anim. Genet. 32 (1), 52.

Allendorf, F.W., Leary, R.F., Spruel, P., Wenburg, J.K., 2001. The problems with hybrids: setting conservation guidelines. Trends Ecol. Evolut. 16 (11), 613–622.

Avise, J.C., 1994. Molecular Markers. In: Natural History and Evolution. Chapman & Hall, New York.

Behnke, R.J., 1968. A new subgenus and species of trout (*Salmo* (*Platysalmo*) *platycephalus*), from south-central Turkey with comments on the classification of the subfamily Salmoninae. Mitteilungen aus dem Hamburgischen Museum und Institut 66, 1–15.

Belkhir, K., 1998. GENETIX, logiciel sous WindowsTM pour la génétique des populations. http://www.univ-montp2.fr/ssgenetix/genetix.htm. Laboratoire Génome et Populations, CNRS UPR 9060, Université Montpellier II, Montpellier, France.

Bernatchez, L., 2001. The evolutionary history of brown trout (*Salmo trutta* L.) inferred from combined phylogeographic, nested clade and mismatch analyses of mitochondrial DNA variation. Evolution 55, 351–379.

Bernatchez, L., Guyomard, R., Bonhomme, F., 1992. DNA variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. Mol. Ecol. 1, 161–173.

Berrebi, P., Povz, M., Jesensek, D., Cattaneo-Berrebi, G., Crivelli, A.J., 2000. The genetic diversity of native, stocked and hybrid populations of marble trout in the Soca river, Slovenia. Heredity 85, 277–287.

Bianco, P.G., 1990. Potential role of the palaeohistory of the Mediterranean and Paratethys basin on the early dispersal of Euro-Mediterranean freshwater fishes. Ichthyol. Explorat. Freshwaters 1, 167–184.

Bowling, A.T., Del Valle, A., Bowling, M., 2000. A pedigree-based study of mitochondrial D-loop DNA sequences variation among Arabian horses. Anim. Genet. 31 (1), 1–7.

Crivelli, A., Poizat, G., Berrebi, P., Jesenšek, P., Rubin, J.F., 2000. Conservation biology applied to fish: the example of a project for rehabilitating the Marble trout (*Salmo marmoratus*) in Slovenia. Cybium 24 (3), 211–230.

Curik, I., Zechner, P., Sölkner, J., Achman, R., Bodo, I., Dovč, P., Kavar, T., Marti, E., Brem, G., 2003. Inbreeding, microsatellite heterozygosity, and morpohological traits in Lipizzan horses. J. Hered. 94 (2), 125–132.

Delling, B., Crivelli, A.J., Rubin, J.-F., Berrebi, P., 2000. Morphological variation in hybrids between *Salmo marmoratus* and alien

*Salmo* species in the Volarja stream, Soca river basin, Slovenia. J. Fish Biol. 57, 1199–1212.

DeWoody, J.A., Avise, J.C., 2000. Microsatellite variation in marine, freshwater and anadromous fishes compared to other animals. J. Fish Biol. 56, 461–473.

Dolenc, M., 1980. Lipica. Ljubljana, Mladinska knjiga.

Frankel, G.H., 1974. Genetic conservation: our evolutionary responsibility. Genetics 78, 53–65.

Frankham, R., 1999. Quantitative genetics in conservation biology. Genet. Res. 74, 235–244.

Fumagalli, L., Snoj, A., Jesenšek, D., Balloux, F., Jug, T., Duron, O., Brossier, F., Crivelli, A.J., Berrebi, P., 2002. Extreme genetic differentiation among the remnant populations of marble trout (*Salmo marmoratus*) in Slovenia. Mol. Ecol. 11, 2711–2716.

Giuffra, E., Bernatchez, L., Guyomard, R., 1994. Mitochondrial control region and protein coding genes sequence variation among phenotypic forms of brown trout *Salmo trutta* from northern Italy. Mol. Ecol. 3, 161–171.

Giuffra, E., Guyomard, R., Forneris, G., 1996. Phylogenetic relationships and introgression patterns between incipient parapatric species of Italian brown trout (*Salmo trutta* L. complex). Mol. Ecol. 5, 207–220.

Gross, R., Kühn, R., Baars, M., Schröder, W., Stein, H., Rottmann, O., 2001. Genetic differentiation of European grayling populations across the Main, Danube and Elbe drainages in Bavaria. J. Fish Biol. 58, 264–280.

Hill, E.W., Bradley, D.G., Al-Barody, M., Ertugrul, O., Splan, R.K., Zakharov, I., Cunningham, E.P., 2002. History and integrity of thoroughbred dam lines revealed in equine mtDNA variation. Anim. Genet. 33 (4), 287–294.

Jansen, T., Forster, P., Levine, M.A., Oelke, H., Hurles, M., Renfrew, C., Weber, J., Olek, K., 2002. Mitochondrial DNA and the origins of the domestic horse. Proc. Natl. Acad. Sci. 99 (16), 10905–10910.

Jug, T., Dovc, P., Pohar, J., Snoj, A., 2004. RAPD analysis as a tool for discrimination marble trout from hybrids (marble trout × brown trout) in the zones of hybridisation. J. Anim. Breed. Genet. 121, 156–162.

Kavar, T., Brem, G., Habe, F., Sölkner, J., Dovč, P., 2002. History of Lipizzan horse maternal lines as revealed by mtDNA analysis. Genet. Sel. E 34 (5), 635–648.

Kavar, T., Habe, F., Brem, G., Dovč, P., 1999. Mitochondrial D-loop sequence variation among the 16 maternal lines of the Lipizzan horse breed. Anim. Genet. 30, 423–430.

Koskinen, M.T., Knizhin, I., Primmer, C.R., Schlotterer, C., Weiss, S., 2002c. Mitochondrial and nuclear DNA phylogeography of Thymallus spp (grayling) provides evidence of ice-age mediated environmental perturbations in the world's oldest body of fresh water, Lake Baikal. Mol. Ecol. 11, 2599–2611.

Koskinen, M.T., Nilsson, J., Veselov, A.J., Potutkin, A.G., Ranta, E., Primmer, C.R., 2002b. Microsatellite data resolve phylogeographic patterns in European grayling, *Thymallus thymallus*, Salmonidae. Heredity 88, 391–402.

Koskinen, M.T., Piironen, J., Primmer, C.R., 2002a. Genetic assessment of spatiotemporal evolutionary relationships and stocking effects in grayling (*Thymallus thymallus*, Salmonidae). Ecol. Lett. 5, 193–205.

Lawton, J.H., May, R.M., 1995. Extinction Rates. Oxford University Press, Oxford.

Leary, R.F., Allendorf, F.W., Sage, G.K., 1995. Hybridization and introgression between introduced and native fish. Am. Fish Soc. Symp. 15, 61–101.

MacHugh, D.E., Bradley, D.G., 2001. Livestock geneic origins: goats buck the trend. Proc. Natl. Acad. Sci. 98 (10), 5382–5384.

Moritz, C., 1994. Applications of mitochondrial-DNA analysis in conservation – a critical review. Mol. Ecol. 3, 401–411.

Nürnberg, H., 1993. Der Lipizzaner: mit einem Anhang über den Kladruber. Magdeburg, Weastarp Wissenschaften.

Nürnberg, H., 1998. Auf den Spuren der Lipizzaner. Über die Gestüte und Nachzuchtgebiete des Lipizzaners nach 400 Jahre Traditionszucht. Hildesheim, Zürich. Olms Presse, New York.

Pleško, S., Budihna, N., 2000. Repopulation of marble trout (*Salmo marmoratus* Cuvier, 1817) in the upper Soča river basin (Slovenia) – Action plan. J. Freshwater Biol. (Quaderni ETP) 29, 45–50.

Povž, M., 1995. Status of freshwater fishes in the Adriatic catchment of Slovenia. Biol. Conserv. 72, 171–177.

Snoj, A., Jug, T., Melkič, E., Sušnik, S., Jesenšek, D., Budihna, N., Pohar, J., Dovč, P., 2000. Mitochondrial and microsatellite DNA analysis of marble trout in Slovenia. J. Freshwater Biol. (Quaderni ETP) 29, 5–11.

Snoj, A., Pohar, J., Dovč, P., 1996. A set of new microsatellite DNA markers for *Salmo marmoratus* Cuvier 1817. Proceedings of the 3rd International Conference, Gozd Martuljek, Slovenija, September 21–26. Life Sci., p. 94.

Snoj, A., Pohar, J., Dovc, P., 1983. The first microsatellite DNA marker for marble trout. J. Anim. Sci. 75.

Snoj, A., Sušnik, S., Pohar, J., Dovč, P., 1999. The first microsatellite marker (BFRO 004) for grayling, informative for its Adriatic population. Anim. Genet. 30, 74–75.

Sušnik, S., Berrebi, P., Dovc, P., Hansen, M.M., Snoj, A. Genetic introgression between wild and stocked salmonids and the prospects for using molecular markers in population rehabilitation: the case of the Adriatic grayling (*Thymallus thymallus* L. 1785) Heredity (in press).

Sušnik, S., Snoj, A., Dovč, P., 1999a. Microsatellites in grayling (*Thymallus thymallus*): comparison of two geographically remote populations from the Danubian and Adriatic river basin in Slovenia. Mol. Ecol. 8, 1756–1758.

Sušnik, S., Snoj, A., Dovč, P., 1999b. A new set of microsatellite markers for grayling: BFRO014, BFRO015, BFRO016, BFRO017 and BFRO018. Anim. Genet. 30, 478.

Sušnik, S., Snoj, A., Dovč, P., 2001. Evolutionary distinctness of grayling (*Thymallus thymallus*) inhabiting the Adriatic river system, as based on mtDNA variation. Biol. J. Linn. Soc. 74, 375–385.

Sušnik, S., Snoj, A., Jesenšek, D., Dovč, P., 2000. Microsatellite DNA markers (BFRO010 and BFRO011) for grayling. J. Anim. Sci. 78, 488–489.

Sušnik, S., Snoj, A., Pohar, J., Dovc, P., 1997. The microsatellite marker (BFRO 002) characteristic for different geographically remote brown trout, *Salmo trutta* L., populations. Anim. Genet. 28, 372.

Uiblein, F., Jagsch, A., Kössner, G., Weiss, S., Gollmann, P., Kainz, E., 2000. Untersuchungen zu lokaler Anpassung, Gefährdung und Schutz der Äsche (*Thymallus thymallus*) in drei Gewässern in Oberösterreich. Österreichishe Fischerei 53, 89–165.

Vila, C., Leonard, J.A., Götherström, A., Marklund, S., Sandberg, K., Linden, K., Wayne, R.K., Ellgren, H., 2001. Widespread origins of domestic horse lineages. Science 291, 474–477.

Waples, R.S., 1995. Evolutionary significant units and the conservation of biological diversity under the Endangered Species Act. In: Nielsen, J.L., Powers, G.A. (Eds.), Proceeding of Symposium 17 on Evolution and the Aquatic Ecosystem: defining Unique Units in population Conservation. American Fisheries Society, Bethesda, Maryland, pp. 8–27.

Weiss, S., Persat, H., Eppe, R., Schlötterer, C., Uiblein, F., 2002. Complex pattern of colonization and refugia revealed for European grayling *Thymallus thymallus*, based on complete sequencing of the mitochondrial DNA control region. Mol. Ecol. 11, 1393–1407.

Wildt, D.E., 2000. Genome resource banking for wildlife research, management, and conservation. ILAR J. 41 (4), 228–234.

Zechner, P., Solkner, J., Bodo, I., Druml, T., Baumung, R., Achmann, R., Marti, E., Habe, F., Brem, G., 2002. Analysis of diversity and population structure in the Lipizzan horse breed based on pedigree information. Liver Prod. Sci. 77 (2–3), 137–146.