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A molecular study of diversity in European
water fleas *Daphnia pulicaria*

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Ph.D. Dissertation Thesis



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Abstract

Water fleas of the *Daphnia pulex* species complex inhabit diverse environments throughout the northern hemisphere, they have enormous dispersal capacity and show striking patterns of phenotypic diversity, which makes them interesting study subjects. My main intention was to better understand diversity in the water flea *D. pulicaria* Forbes. Therefore I used molecular markers. *D. pulicaria* is widely distributed across the temperate and arctic regions of North America and Eurasia, and it shows a remarkable phenotypic polymorphism in Europe. An unpigmented morphotype inhabits lowland regions throughout Europe and mountain lakes in the High Tatra, a carotenoid pigmented morphotype coexists with the unpigmented one in the High Tatra, and a melanic morphotype occurs in the Pyrenees. As yet no study assessed genetic differentiation between the morphotypes. My study therefore had three primary objectives. I first used DNA sequence variation at the mitochondrial DNA (mtDNA) ND5 gene to reconstruct phylogenetic relationships among *D. pulicaria* from different parts of its distribution and to investigate their relationships to other members in the *D. pulex* complex. The main finding was that the carotenoid and melanin pigmented populations from the European mountain lakes were not related to the unpigmented populations inhabiting the same lakes and the lowland regions throughout Europe, but were more closely related to *D. pulicaria* sampled from Canada and Greenland. This Eastern Nearctic lineage was not found in Europe before. My second objective was to test for a concordance of the morphotype divergence across different genetic markers. I quantified variation at eight microsatellite loci and found that each population was fixed for a single multilocus genotype and that genotypes were not shared among the morphotypes despite the coexistence in the High Tatra, confirming that their genetic divergence is not restricted to mtDNA. I concluded that at least two divergent species are confused under the name *D. pulicaria* in Europe, which differ in pigmentation and life history, and coexist in the same lakes without loss of the genetic integrity. My final research objective was to assess the utility of diapausing egg banks of *D. pulicaria* in the High Tatra lakes as repositories of genetic information. Very low numbers of resting eggs and their poor physical condition yielded no amplifiable DNA, making tracking long-term genetic shift in these ultra-oligotrophic lakes unfeasible. Autochthonous recovery of extinct genotypes is therefore unlikely, which underscores the importance of immigration and local selection in the spatial patterning of genotypes. In general, my study shows that a considerable part of *Daphnia* diversity, previously attributed to ecophenotypy by morphological taxonomy, has been missed.

Chapter 1

General introduction

Introduction

Water fleas of the genus *Daphnia* (Crustacea: Cladocera) are ubiquitous residents of inland waters within all continents of the globe and play an important ecological role in lake and pond ecosystems. As the primary grazers of algae and the primary food source of fish, they are key members of aquatic food webs (Colbourne et al. 2005). Many attributes (e.g. short generation time, asexual reproduction) make water fleas ideal model species in freshwater and general ecology (Peters and de Bernardi 1987; Kalff 2002), and they have been successfully used in a number of evolutionary studies of speciation, adaptation, and population genetics (Colbourne et al. 1988; Weider et al. 1999; Schwenk et al. 2004; Paland et al. 2005). Recently *Daphnia* became the subject of genomic research (Colbourne et al. 2005).

The use of genetic markers in the last fifteen years has radically changed species diversity estimates in cladoceran crustaceans. Flaws in taxonomic assignments caused by morphological stasis, phenotypic plasticity, hybridisation, and morphological convergence linked to the occupation of similar habitats have resulted in a serious underestimation of the species diversity. Many species once thought to be cosmopolitan are now viewed as cryptic species assemblages of regionally restricted lineages (Hebert and Finston 1996; Taylor et al. 1998). For example, the *Daphnia* fauna of North America is now known to include a minimum of 34 species instead of the 15 previously assigned based on morphology (Hebert 1995) and the global total could reach as many as 200 species (Hebert and Taylor 1997).

Molecular markers provide an essential tool for assessing the genetic variation in natural population (Awise 1994; Sunnucks 2000). Nuclear microsatellite DNA loci (the non-coding tandem repeats of short sequence motifs; Goldstein and Schlötterer 1999) are increasingly employed to assess genetic variation within and evolutionary relationship among populations (Cavalli-Sforza 1998; Driscoll et al. 2002). Because of their genomic abundance and hypervariability due to the length polymorphism, microsatellites allow discrimination even between closely related populations and permit simultaneous scoring of a large number of linkage independent loci. Over five hundred microsatellite loci have recently been isolated and characterised for *D. pulex*, which largely facilitated the use of these markers for *Daphnia* research (Colbourne et al. 2004). As a consequence, within the past decade microsatellites have developed into one of the most popular genetic markers

in ecological and evolutionary genetic study of this group (Pálsson 2002; Limburg and Weider 2002; Fox 2004; Brede et al. 2006).

Modern molecular biological technologies have allowed the identification and routine application of markers that provide information about genetic variation, typically in the form of restriction sites or nucleotide sequences. For this class of data, the mutational changes among the alleles (or haplotypes) provide information about their historical relationship, making it feasible to reconstruct the allelic phylogeny in the form of a gene tree. Because of the maternal, no recombination transmission, and rapid evolution the mitochondrial DNA (mtDNA) became the most broadly used molecular markers, for which gene tree can be recovered (Avice 2000).

The general objective of my study was to apply both these classes of molecular markers to assess the diversity and biogeography of the water flea *D. pulicaria* Forbes, a member of the *D. pulex* complex (sensu Colbourne and Hebert, 1996). This species complex has a circumpolar distribution, extending through all of arctic and temperate America, Europe and Asia (Colbourne et al. 1998; Weider et al. 1996, 1999a,b). Although in recent years the *D. pulex* complex has received considerable attention from molecular phylogenetic studies (Colbourne et al., 1998; Weider et al., 1999a,b; Paland et al., 2005), its species delimitation remains controversial. Genetic lineages within the *D. pulex* complex are very distinct, suggesting that speciation has been proceeding since the Pliocene (Colbourne and Hebert, 1996). However, the slow rate of morphological evolution relative to the underlying molecular divergence has caused considerable taxonomic confusion, leading to the presence of geographically widespread taxa with negligible morphological variation that show high levels of sequence diversity (Colbourne et al., 1998).

Daphnia pulicaria, as currently recognized, has a circumarctic geographic range (Hrbáček, 1959). It is widely distributed across the temperate and arctic regions of North America. In Eurasia it is confined to the west of the Ural Mountains, with a center in middle Europe and extending northwards to Norway and eastwards to western Russia (Hobaek and Weider, 1999). Recent phylogenetic studies of mitochondrial DNA (mtDNA) sequences (Colbourne et al., 1998) and restriction fragment length polymorphism data (RFLP; Weider et al., 1999a) of members of the *D. pulex* complex have shown that *D. pulicaria* is not a monophyletic species. The American lineages of *D. pulicaria* belong to a different main clade of the *D. pulex* complex, the *pulicaria* group following the nomenclature devised by Colbourne et al. (1998), than the European populations, which are included in the highly divergent *tenebrosa* group (Colbourne et al. 1998). Three morphotypes defined based

on variation in photoprotective pigmentation but lacking any other prominent morphological differences exist in neighboring sympatry in European temperate regions. A transparent (unpigmented) morphotype inhabits lowland regions throughout Europe and mountain lakes in the High Tatra Mountains, an orange (carotenoid-pigmented) morphotype coexists syntopically with the transparent morphotypes in the High Tatra, and a black (melanic) morphotype occurs in glacial lakes in the Pyrenees. Pigmentation may be adaptive in crustaceans and play a role in protection against harmful UV-radiation and predation pressure. However, no study has assessed genetic differentiation between the morphotypes of *D. pulicaria*. On the other hand, ecological studies showed that the morphotypes are differentiated in terms of their life-history characteristics. The black and orange morphotypes over-winter as parthenogenetic females (Gliwicz et al., 2001; Ventura and Catalan, 2005), whereas the transparent morphotype produce diapausing eggs (ephippia), which hatch in the summer (Haney and Buchanan, 1987; Gliwicz et al., 2001). This well coordinated reproduction is especially pronounced in syntopic populations of the transparent and orange morphotypes in the High Tatra (Gliwicz et al. 2001), and its correlation with pigmentation variation suggests that these phenotypes may be associated with evolutionary subdivisions (see Tarjuelo et al. 2004). These characteristics have made water fleas *D. pulicaria* an interesting model for a molecular study of the historical relationships and diversification.

In my thesis I for the first time include populations belonging to all three pigmentation morphotypes distinguished in Europe and occupying different habitats, including lowland regions throughout Europe and alpine lakes in the High Tatra and Pyrenees. My objectives were to reconstruct phylogeny of *D. pulicaria* from different parts of the distribution range and their relationships to other lineages in the *D. pulex* complex; and to determine whether different colour morphotypes found among *D. pulicaria* are genetically differentiated; and to assess the utility of diapausing egg banks of *D. pulicaria* in the High Tatra lakes as repositories of genetic information.

Outline of the thesis

In this thesis I have generated molecular data to describe the diversity and phylogenetic relationships among *D. pulicaria* that live in different habitats (lowland ponds and reservoirs versus alpine lakes), differ in pigmentation (melanin and carotenoid based versus transparent) and are ecologically divergent (over wintering as adults versus resting eggs). I used nucleotide sequence data on 672bp of the mtDNA *ND5* gene and allele distribution data for eight

polymorphic microsatellite loci. The ND5 gene has been successfully used in earlier studies to address the questions on the phylogeny and biogeography of the *D. pulex* complex, and it has proven powerful in resolving the evolutionary diversity of the members of this complex (Colbourne et al. 1998; Weider et al. 1999a,b). A number of microsatellite markers were described for the *D. pulex* complex (Colbourne et al. 2004) and were shown to differentiate between populations at different geographic scales (Pálsson 2000), which facilitated their application in my study.

Chapter 2 addresses the phylogenetic diversity and relationships in *D. pulicaria* throughout its circumpolar distribution with the emphasis on the European populations. Data on the nucleotide variation at the ND5 gene are gathered for 65 isolates of *D. pulicaria* and other members of the *D. pulex* complex from 45 different localities in Europe, North America and the arctic islands. Populations of *D. pulicaria* belonging to all three pigmentation morphotypes distinguished in Europe were for the first time included in a phylogenetic study. A phylogenetic hypothesis is presented and used to clarify whether the phenotypic subdivision of European *D. pulicaria* is related to genetic divergence. A historical biogeographic scenario is proposed that accounts for the distribution of evolutionary lineages present in Europe, and the implications for the taxonomic status of *D. pulicaria* are discussed. The manuscript giving the results of this work has been submitted for publication..

Chapter 3 gives the results of a microsatellite analysis of a total of 111 *D. pulicaria* individuals representing all different morphotypes, which were collected from 13 lakes in the High Tatra in Slovakia and Poland, and from three lakes in the Pyrenees in Spain. Differences in allelic composition among morphotypes are assessed statistically with the aim to address the question whether their differentiation is supported by genetic divergence at highly variable nuclear markers, and to test for a concordance of the morphotype differentiation across different genetic markers. The results of this study provide the foundation of the manuscript that has been submitted for publication.

The research objective of the study presented in Chapter 4 is to assess the utility of *D. pulicaria* diapausing egg banks in the High Tatra lakes as repositories of genetic and ecological information. Two morphotypes coexist in these lakes and the ability to track long-term genetic shifts would provide insights in the dynamics of their coexistence. The amount and quality of DNA that can be recovered from the ancient eggs and used for PCR amplification is evaluated and discussed in relation to the potential role of the resting

eggs in autochthonous recovery of extinct genotypes and populations. The results of this study have been accepted for publication in the journal *Biológia, Bratislava*.

The final conclusions of the thesis are summarized in chapter 5.

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Chapter 2

Cryptic intercontinental colonization in water fleas *Daphnia pulicaria* inferred from phylogenetic analysis of mitochondrial DNA variation

Submitted for publication

Cryptic intercontinental colonization in water fleas *Daphnia pulicaria* inferred from phylogenetic analysis of mitochondrial DNA variation

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Abstract

The water fleas of the *Daphnia pulex* complex play a key role in freshwater ecosystems throughout the northern hemisphere. Despite the fact that they have been the subject of study for numerous biological disciplines, their phylogeny and species delimitation remain controversial. We used DNA sequence variation of the mitochondrial ND5 gene to reconstruct the phylogenetic relationships of *D. pulicaria* Forbes, a widespread member of this complex from North America and Europe. Populations from the two continents respectively split into two evolutionary lineages, Eastern Nearctic and European, which each belong to another main clade within the *D. pulex* complex (the *pulicaria* and *tenebrosa* groups, respectively). Unexpectedly, melanin and carotenoid pigmented *D. pulicaria* populations from European high-mountain lakes were not allied with the unpigmented populations inhabiting the same lakes and the lowland ponds and reservoirs throughout Europe, but were included with the samples from Canada and Greenland in the Eastern Nearctic lineage. Until now populations belonging to this lineage were known only from Canada and North Atlantic islands, but not from mainland Europe. Two divergent species are therefore confused under the name *D. pulicaria* in Europe. The close phylogenetic relationships of European populations with those from Canada and Greenland suggest that the Nearctic species is of recent origin in Europe via intercontinental dispersal from the North America. It has evolved melanin and carotenoid pigmentation as adaptations against the UV light stress, which enable it to share habitat occupied by the unpigmented European species. The Nearctic *D. pulicaria* thus provides a new model for studying successful intercontinental invasion. In general, our study demonstrates that a considerable part of the diversity among widespread taxa of cladoceran crustaceans has been overlooked in morphological taxonomies.

Keywords: Arctic; colonization; *Daphnia pulex* complex, mtDNA, ND5

1. Introduction

Water fleas (Crustacea: Cladocera) of the *Daphnia pulex* species complex (sensu Colbourne and Hebert, 1996) are ubiquitous residents of inland waters throughout the temperate and arctic regions of the northern hemisphere (Hobaek and Weider, 1999). Many characteristics make these organisms perfect subjects for ecological and evolutionary research (Colbourne et al. 2005). As the primary consumers of algae and a food of fish, they are key members of freshwater ecosystems and serve as model species for freshwater and general ecology (Peters and de Bernardi, 1987; Kalff, 2002). Largely because of the wide geographic distribution, phenotypic variation and capability of sexual and asexual reproduction they have been effectively used in evolutionary studies of speciation and adaptation (Schwenk et al. 2004; Paland et al., 2005), and recently become the subject of genomic research (Colbourne et al., 2005). However, exploration of the full potential of this emerging model organism is hampered by poorly resolved evolutionary relationships and species boundaries within the *D. pulex* complex (Colbourne et al., 1998).

Although in recent years this species complex has received considerable attention from molecular phylogenetic studies (Colbourne et al., 1998; Weider et al., 1999a,b; Paland et al., 2005), its species delimitation remains the subject of controversy. Genetic lineages within *D. pulex* complex are very distinct, suggesting that speciation has been proceeding since the Pliocene (Colbourne and Hebert, 1996). However, the slow rate of morphological evolution relative to the underlying molecular divergence has caused considerable taxonomic confusion, leading to the presence of geographically widespread taxa with negligible morphological variation that show high levels of sequence diversity (Colbourne et al., 1998; Weider and Hobaek 2003).

Daphnia pulicaria Forbes is one such taxon. The species, as currently recognized, has a circumarctic geographic range (Hrbáček, 1959). It is widely distributed across the temperate and arctic regions of North America. In Eurasia it is confined to the west of the Ural Mountains, with a center in middle Europe and extending northwards to Norway and eastwards to western Russia (Hobaek and Weider, 1999). Recent phylogenetic studies of mitochondrial DNA (mtDNA) sequence (Colbourne et al., 1998) and restriction fragment length polymorphism data (RFLP; Weider et al., 1999a) for the members of the *D. pulex* complex have shown that *D. pulicaria* is not a monophyletic species. The American lineages of *D. pulicaria* belong to a different main clade of the *D. pulex* complex, the *pulicaria* group following the nomenclature devised by Colbourne et al. (1998), then the European

populations, which are a part of the highly divergent *tenebrosa* group (Colbourne et al., 1998). However, these studies were primarily concerned with phylogenetic relationships and diversity within the entire species complex, and their sampling coverage of *D. pulicaria* in temperate regions of Europe was too limited to provide much insight into its evolutionary history.

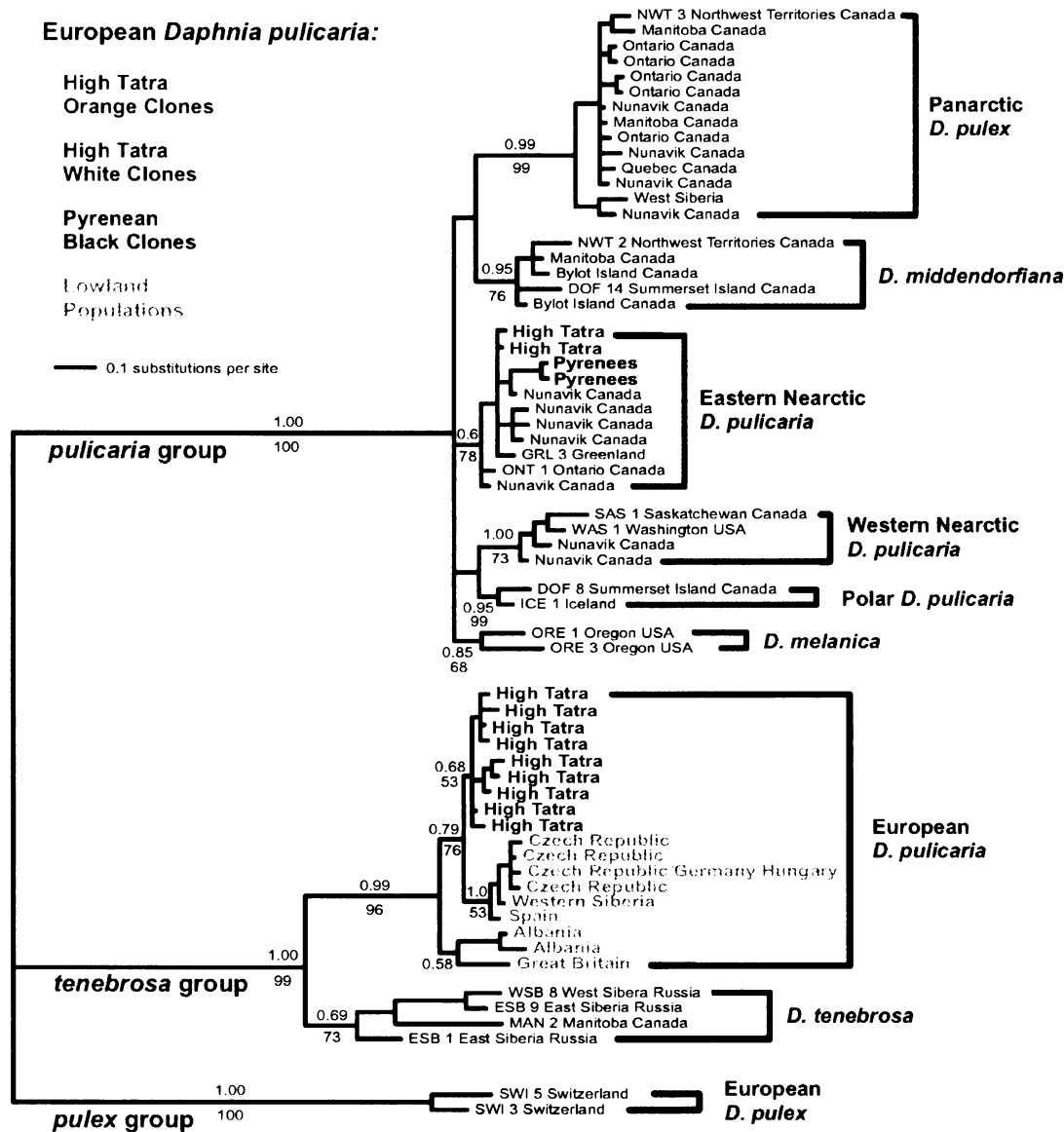


Fig. 1. Bayesian reconstruction of the phylogenetic relationships of the European high-mountain and lowland *Daphnia pulicaria* with the major lineages within the primarily Nearctic *pulicaria* and the circumarctic *tenebrosa* groups of the *D. pulex* complex. The tree is rooted with the *pulex* group. Statistical support for major clades is expressed as the Bayesian posterior probabilities and as percentage bootstrap values estimated using the maximum likelihood.

Daphnia pulicaria has been found in a wide range of habitats throughout Europe, ranging from lowland fishponds and reservoirs to glacial lakes in high mountains and shallow ponds in the arctic regions. Three morphotypes based on variation in photoprotective pigmentation but lacking other prominent morphological differences exist in neighboring sympatry in Europe. A transparent (unpigmented) morphotype inhabits lowland regions throughout Europe and mountain lakes in the High Tatra (western part of the Carpathian Mts), an orange (carotenoid-pigmented) morphotype coexists with the transparent morphotypes in the High Tatra (Litzyński, 1917), and a black (melanic) morphotype occurs in glacial lakes in the Pyrenees (Ventura and Catalan, in press).

Although there is variation in pigmentation in other *Daphnia*, genetic differentiation between the color morphs has not been demonstrated (Schwenk et al., 2004). Pigmentation plays a role in protection against harmful UV-radiation and predation pressure, and it has been shown to have a genetic basis in isopod crustaceans (Hargeby et al., 2004). As yet no study assessed genetic differentiation between the morphotypes of *D. pulicaria*. However, ecological studies showed that pigmentation morphotypes are differentiated in terms of their life-history characteristics. The black morphotype and the orange morphotype start new-year generation in the spring from subitaneous eggs released by over-wintering females (Gliwicz et al., 2001; Ventura and Catalan, in press), while in the transparent morphotype the new-year generation was recruited from diapausing eggs, which hatched in summer (Haney and Buchanan, 1987; Gliwicz et al., 2001). This synchronized reproduction is especially pronounced in syntopic populations of the transparent and orange morphotypes in the High Tatra (Gliwicz et al. 2001), and its correlation with pigmentation variation suggests that these phenotypes may be associated with evolutionary subdivision (Tarjuelo et al. 2004).

In the present study we analyze mtDNA sequence variation of *D. pulicaria* in North America and Europe. We for the first time include populations belonging to all three pigmentation morphotypes distinguished in Europe and occupying different habitats, including lowland regions throughout Europe and alpine lakes in the High Tatra and Pyrenees. Our objectives were to reconstruct phylogeny of *D. pulicaria* from different parts of the distribution range and their relationships to other lineages in the *D. pulex* complex, and to clarify whether the phenotypic (pigmentation and ecological) subdivision of *D. pulicaria* is related to genetic divergence.

2. Materials and methods

2.1. Sampling

Daphnia pulicaria individuals were collected from 45 different localities in Europe and North America (Table 1). Water fleas were collected from lowland fishponds and reservoirs, high-mountain lakes, and arctic ponds and rockpools by horizontal sweeps on the lakeshore or by plankton net tows from an inflatable boat in the lake center, and were stored in 95% ethanol at 4 °C until analysis. To complete the dataset and provide a wider phylogenetic framework for our study we included 18 homologous sequences (Table 1) described by Colbourne et al. (1998) to represent all members of the *D. pulex* complex analyzed by these authors, including six constituent clades of the *pulicaria* group (termed eastern, western and polar lineage of North American *D. pulicaria*, Panarctic *D. pulex*, *D. middendorffiana* and *D. melanica*) and *D. tenebrosa* of the *tenebrosa* group. Sequences of European *D. pulex* were used to root the trees because they form a third clade (the *pulex* group) within the *D. pulex* complex, sister to the *tenebrosa* and *pulicaria* groups, and represent an outgroup to the other lineages (Colbourne et al., 1998; Hobaek and Weider, 1999).

Table 1. Samples of the *Daphnia pulex* complex used in this study.

Taxa and collection localities	Sequence ^a	Data source
<i>pulicaria</i> group		
Panartic <i>D. pulex</i>		
Churchill (Site 1), Manitoba, Canada	DQ779137	This study
Churchill (Site 2), Manitoba, Canada	DQ779139	This study
Kuujuarapik (Site 1), Nunavik, Canada	DQ779122	This study
Kuujuarapik (Site 2), Nunavik, Canada	DQ779131	This study
Kuujuarapik (Site 3), Nunavik, Canada	DQ779123	This study
Kuujuarapik (Site 4), Nunavik, Canada	DQ792505	This study
Rimouski, Quebec, Canada	DQ779125	This study
Windsor (Site 1), Ontario, Canada	DQ779135	This study
Windsor (Site 2), Ontario, Canada	DQ779136	This study
Windsor (Site 3), Ontario, Canada	DQ779132	This study
Windsor (Site 4), Ontario, Canada	DQ779133	This study
Windsor (Site 5), Ontario, Canada	DQ779134	This study
Kachkovsky Bay, Western Siberia	WSB 1	Colbourne et al. (1998)
Tuktoyaktuk, Northwest Territoris, Canada	NWT 3	Colbourne et al. (1998)

Table 1. (continued)

Taxa and collection localities	Sequence ^a	Data source
<i>D. middendorffiana</i>		
Bylot Island (Site 1), Nunavut, Canada	DQ779126	This study
Bylot Island (Site 2), Nunavut, Canada	DQ779127	This study
Churchill (Site 3), Manitoba, Canada	DQ779138	This study
Summerset Island, District of Franklin, Canada	DOF 14	Colbourne et al. (1998)
Tuktoyaktuk, Northwest Territories, Canada	NWT 2	Colbourne et al. (1998)
Eastern Neartic <i>D. pulicaria</i>		
Kuujjuarapik (Site 5), Nunavik, Canada	DQ779119	This study
Kuujjuarapik (Site 6), Nunavik, Canada	DQ779128	This study
Kuujjuarapik (Site 7), Nunavik, Canada	DQ779129	This study
Kuujjuarapik (Site 8), Nunavik, Canada	DQ779130	This study
Kuujjuarapik (Site 9), Nunavik, Canada	DQ779124	This study
Estat Lake, Pyrenees, Catalonia, Spain	DQ779112	This study
Sottlo Lake, Pyrenees, Catalonia, Spain	DQ779113	This study
Redon Lake, Pyrenees, Catalonia, Spain	DQ779113	This study
Vyšné Temnosmrečinské Lake, High Tatra Mts., Slovakia	DQ779098	This study
Czarny Staw pod Rysmi, High Tatra Mts., Poland	DQ779097	This study
Zelené Krivánske Lake, High Tatra Mountain, Slovakia	DQ779097	This study
Godhavn, Greenland	GRL 3	Colbourne et al. (1998)
Redchalk Lake, Ontario, Canada	ONT 1	Colbourne et al. (1998)
Western Neartic <i>D. pulicaria</i>		
Kuujjuarapik (Site 10), Nunavik, Canada	DQ779120	This study
Kuujjuarapik (Site 11), Nunavik, Canada	DQ779121	This study
Redberry Lake, Saskatchewan, Canada	SAS 1	Colbourne et al. (1998)
Washington Lake, Washington, USA	WAS 1	Colbourne et al. (1998)
Polar Neartic <i>D. pulicaria</i>		
Summerset Island, District of Franklin, Canada	DOF 8	Colbourne et al. (1998)
Western Iceland	ICE 1	Colbourne et al. (1998)
<i>D. melanica</i>		
Florence, Oregon, USA	ORE 1	Colbourne et al. (1998)
Florence, Oregon, USA	ORE 3	Colbourne et al. (1998)
<i>tenebrosa</i> group		
<i>European D. pulicaria</i>		
Czarny Staw pod Rysmi, High Tatra Mts., Poland	DQ779101	This study
Ladové Lake, High Tatra Mts., Slovakia	DQ779104	This study
Litvorové Lake, High Tatra Mts., Slovakia	DQ779105	This study
Malé Hincovo Lake, High Tatra Mts, Slovakia	DQ779099	This study
Nížné Žabie Bielovodské Lake, High Tatra Mts., Slovakia	DQ779103	This study

Table 1. (continued)

Taxa and collection localities	Sequence ^a	Data source
Veľké Žabie Javorové Lake, High Tatra Mts., Slovakia	DQ779106	This study
Vyšné Žabie Bielovodské Lake, High Tatra Mts., Slovakia	DQ779102	This study
Welki Staw Polski, High Tatra Mts., Poland	DQ779100	This study
Zelené Javorové Lake, High Tatra Mts., Slovakia	DQ779107	This study
Bohdaneč reservoir, Czech Republic	DQ779108	This study
Chmelnice pound, Czech Republic	DQ779109	This study
Malá Kuš pound, Czech Republic	DQ779110	This study
Římov reservoir, Czech Republic	DQ779111	This study
Slapy reservoir, Czech Republic	DQ779111	This study
Embalse Lake, San Rafael de Matallana, Spain	DQ779114	This study
Hortobágy pound, Hungary	DQ779111	This study
Konstanz Lake, Germany	DQ779111	This study
Ohrid Lake, Albania	DQ779115,	
	DQ779116	This study
Petchora Delta, Western Siberia	DQ779118	This study
Queen Elisabeth reservoir, London, England	DQ779117	This study
D. tenebrosa		
Belyi Island, Western Siberia	WSB8	Colbourne et al. (1998)
Churchill (Site 3), Manitoba, Canada	MAN 2	Colbourne et al. (1998)
Kolyma Delta (Site 1), Eastern Siberia	ESB9	Colbourne et al. (1998)
Kolyma Delta (Site 2), Eastern Siberia	ESB 1	Colbourne et al. (1998)
pulex group		
European D. pulex		
Basel, Switzerland	SWI 5	Colbourne et al. (1998)
Basel, Switzerland	SWI 3	Colbourne et al. (1998)

Classification and terminology follows those established by Colbourne et al. (1998) and later applied by e.g. Weider et al. (1999a,b).

^a Sequences described by Colbourne et al. (1998), unavailable from GenBank, have been obtained from the authors and are labeled in accordance with their study.

2.2. PCR amplification and sequencing analysis

DNA was extracted by incubating single animals in 30µl of a 6% (w/v) suspension of Chelex 100 resin (Bio-Rad, Hercules, CA) at 55 °C for 3 hours and then boiling for 9 minutes followed by cooling at room temperature, centrifuging at 14 000 rpm for 2 minutes, and cooling at 4°C overnight. A fragment (~850 bp) including part of the gene coding for the NADH dehydrogenase subunit 5 (ND5) was amplified by PCR following methods described in Colbourne et al. (1998) and Weider et al. (1999a) and using primers DpuND5a

and DpuND5b (Colbourne et al., 1998; Weider and Hobaek, 2003). The resulting PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and both strands were directly sequenced with the same primers as those used for the amplification and the Dye Terminator Quick Start Kit for cycle-sequencing (Beckman Coulter, USA). The extension products were run on a Beckman Coulter CEQ 8000 automated capillary sequencer. Sequences were aligned manually to each other and to the sequences of the *D. pulex* complex described by Colbourne et al. (1998). All polymorphisms were additionally checked by visual inspection of automated sequencer chromatograms.

2.3. Phylogenetic analyses

Estimates of nucleotide divergence between different sequences were calculated with the PAUP* software package, version 4.0b10 (Swofford, 2003), using a corrected distance based on the gamma-HKY model of sequence evolution, which accounts for unequal nucleotide frequencies and excess transitions, and models among-site rate heterogeneity in substitution rate by the gamma distribution (Hasegawa et al., 1985). The gamma-HKY model was determined to be the appropriate model for our dataset by the hierarchical likelihood ratio test of goodness-of-fit of 56 different nested models to the data, as implemented in MODELTEST program, version 3.7 (Posada and Crandall, 1998).

The phylogenetic relationships among the sequences were reconstructed using PAUP* by analysis of the distance matrix by the neighbor-joining (NJ) algorithm and using the maximum-parsimony (MP; heuristic search) optimality criterion. Phylogenetic analyses using the maximum-likelihood (ML) criterion were performed with the algorithm implemented in the PHYML software, version 2.4.4, that simultaneously adjusts tree topology and branch lengths to maximize tree likelihood (Guindon and Gascuel, 2003), and using the gamma-HKY evolutionary model with the following base frequencies: A = 0.20, C = 0.20, G = 0.22 and T = 0.39, a transition/transversion ratio of 5.69, and a gamma distribution shape parameter α equaling to 0.33. To quantify the confidence in the partitioning within the ML tree we performed the nonparametric bootstrap test as applied to phylogeny by Felsenstein (1985) using 1000 replications.

Bayesian phylogenetic analysis was performed with MrBayes program, version 3.1.1 (Hüelsenbeck and Ronquist, 2001; Ronquist and Hüelsenbeck, 2003), assuming the gamma-HKY model of sequence evolution (two substitution types with gamma-distributed rates across sites) and flat Dirichlet distributions of prior probability densities on the substitution rates and nucleotide frequencies, the uniform prior distributions on the shape parameter

of the gamma distribution of rate variation and on the tree topology, and the unconstrained exponential prior distribution on the branch lengths. The analysis was done using several independent runs starting from different random trees, each with 4 Markov chains under the Metropolis coupling to improve mixing. Each chain was initiated with a burn-in period of 2,500 updates and the total length of each analysis was 10 million updates with trees sampled every 100 generations. The analysis was considered to have converged upon a stationary distribution if the independent runs generated similar posterior distributions.

To compare competing phylogenetic hypotheses we performed the test of the statistical significance of differences in log-likelihoods between topologies as proposed by Shimodaira and Hasegawa (1999) (SH test), which allows for simultaneous comparison of multiple topologies and inclusion of the ML tree (Goldman et al., 2000; Shimodaira and Hasegawa, 1999). We used the PAUP* implementation of the SH test, which generates the test distribution by a nonparametric bootstrap resampling of the sitewise log-likelihoods (the RELL approximation). Using the SH test, we examined the monophyly of European *D. pulicaria* by taking the ML tree estimated under the gamma-HKY model and evaluating it against an alternative ML topology estimated under the constraint of the European sequences of *D. pulicaria* forming a clade.

3. Results

3.1. Sequence variation

A nucleotide sequence of 672 bp of the ND5 gene was obtained for each individual, which translated to 223 amino acids. In the complete dataset, there were 217 variable characters, of which 177 were phylogenetically informative and revealed 62 distinct haplotypes. Third codon positions accounted for most of the variation with 66.4% of variable sites located at third position, 21% at first position, and 12.6% at second position. Because of the absence of stop codons and of indels, we considered it likely that the sequences represented a part of the functional mtDNA *ND5* gene, rather than a nuclear pseudogene (Zhang and Hewitt, 1996).

3.2. Phylogenetic reconstruction

A fifty-percentage majority-rule consensus of trees sampled from the posterior distribution in the Bayesian analysis showing posterior probabilities and ML bootstrap values for internal branches is presented in Fig. 1. Phylogenetic reconstructions obtained with the ML, NJ

and MP approaches showed very similar topologies to the Bayesian tree and they consistently recovered all nine evolutionary lineages within the *D. pulex* complex, which fell into three major clades (*pulex*, *tenebrosa* and *pulex*). These groups corroborate those previously identified by Colbourne et al. (1998) and Weider et al. (1999a) with a limited sampling in Europe. The haplotypes from North America were scattered throughout the *pulex* group. Twelve haplotypes from Manitoba, Nunavik, Churchill, Ontario, and Quebec in Canada were included in the Panarctic *D. pulex* lineage, three haplotypes from the Manitoba and Bylot Island in the *D. middendorffiana* lineage, two haplotypes from Nunavik in the Western Nearctic *D. pulex* lineage, and five haplotypes from Nunavik in the Eastern Nearctic *D. pulex* lineage (Fig. 1).

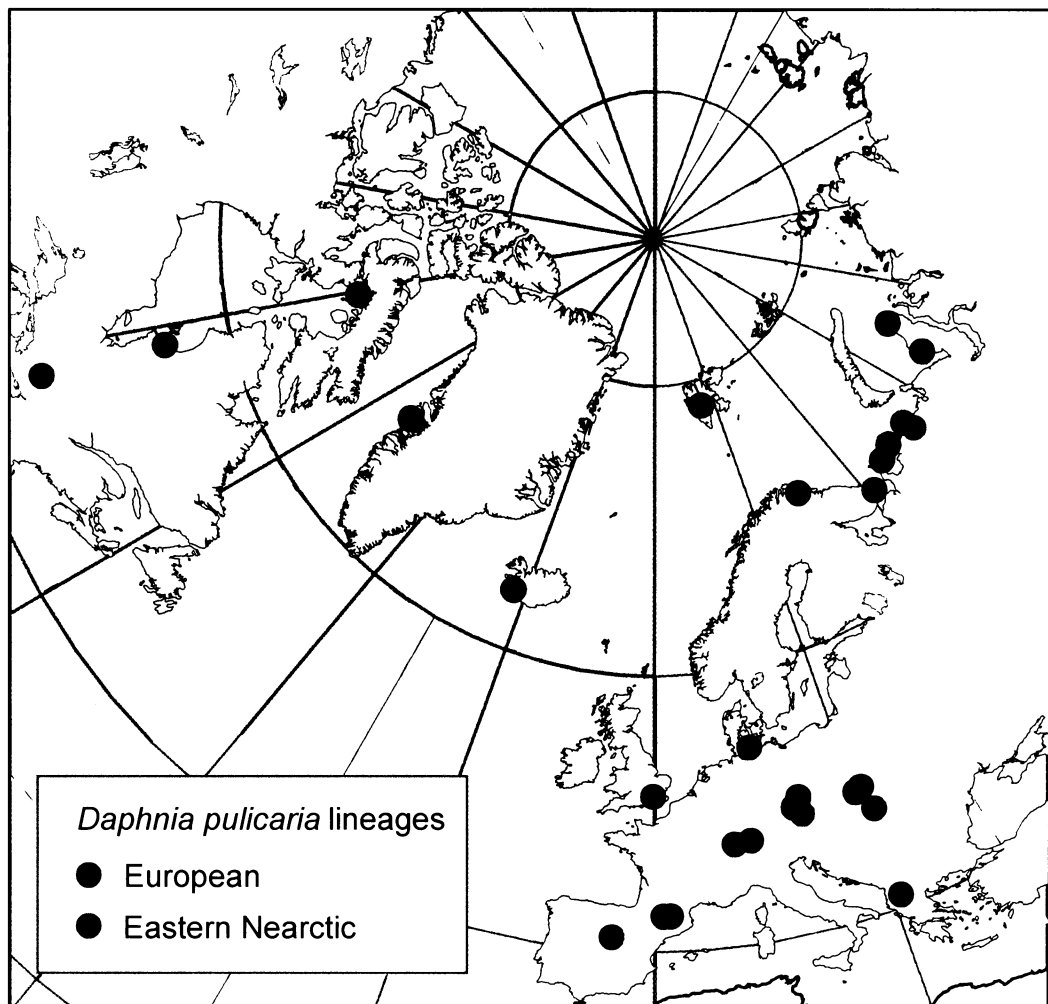


Fig. 2. Geographic distribution of the Eastern Nearctic *Daphnia pulex* lineage (*pulex* group) and the European *D. pulex* lineage (*tenebrosa* group) based on the results of the present study and of Colbourne et al. (1998) and Weider et al. (1999a).

Unexpectedly, in none of the phylogenetic reconstructions did European *D. pulicaria* form a monophyletic group with respect to the other evolutionary lineages of the *D. pulex* complex because the haplotypes of *D. pulicaria* sampled in Europe were clustered within the *tenebrosa* group as well as in the *pulicaria* group (Figs. 1 and 2). The transparent morphotype individuals from the High Tatra were included in a well-supported clade with the individuals sampled from the European lowland populations (mean sequence divergence of 2.1%) and together they constituted the European *D. pulicaria* lineage within the *tenebrosa* group (Fig. 1). The orange morphotype individuals from the High Tatra and black morphotype individuals from the Pyrenees were phylogenetically closely related to each other (mean sequence divergence of 1.4%). However, they were highly divergent from the High Tatra transparent morphotype and from the lowland populations (mean sequence divergence of 30.7-32.2%), and were unambiguously identified as members of the predominantly North American *pulicaria* group. Together with the sequences from Quebec, Ontario and Greenland (mean sequence divergence of 0.7-1.2%) they were included in the Eastern Nearctic *D. pulicaria* lineage of this group (Fig. 1).

The tree partitions that rendered the European *D. pulicaria* polyphyletic received high posterior probability in the Bayesian analyses and were well supported by the bootstrap test (Fig. 1). The SH likelihood test indicated that the topology where the European *D. pulicaria* were constrained to form a monophyletic group (-Ln likelihood = 2928.3883) was significantly less likely given our data (one-tailed test, $P < 0.05$) than the unconstrained ML topology (-Ln likelihood = 2836.3344).

4. Discussion

Our analyses of mtDNA variation within *D. pulicaria* provide clear evidence that the European populations belong to two major phylogenetic groups of the *D. pulex* complex. The posterior probability distributions, nonparametric bootstrap test and the likelihood-based SH test consistently showed that the populations belonging to the *tenebrosa* group do not form a clade with those included in the *pulicaria* group.

A widespread survey of mtDNA variation in the *D. pulex* complex demonstrated that *pulicaria* group lineages were restricted primarily to the Nearctic, with some limited colonization at the northernmost fringe of Europe, while lineages of the *tenebrosa* group were widespread across the circumarctic (Colbourne et al., 1998; Weider et al., 1999a). The two groups were known to overlap only in arctic Europe and western arctic Canada (Weider et al.,

1999a). Europe was thus considered to be occupied primarily by the *tenebrosa* group with the European *D. pulicaria* lineage widely distributed throughout the temperate zone from central Europe to western Russia, and the *D. tenebrosa* lineage in the European arctic (Hobaek and Weider, 1999).

Consistent with this view, all our European lowland samples from Great Britain and Spain across central Europe into the Balkans belonged to the European *D. pulicaria* lineage. High mountain populations of the transparent morphotype from the High Tatra were also included in this lineage. However, the High Tatra populations of the orange morphotype and the Pyrenean black morphotype populations were not allied with the *tenebrosa* group but were included in the *pulicaria* group. Until now this lineage was not found on mainland Europe. It was known only from Canada and Greenland, with a few representatives further east on Iceland and the arctic archipelago Svalbard (Weider et al., 1999a; Hobaek and Weider, 1999). Our results instead demonstrate that the distribution of the Eastern Nearctic lineage extends much further south, being present not only on the Arctic islands but also at much lower latitudes in the Western and Central European mountains (Fig. 2).

The *pulicaria* and *tenebrosa* groups represent clearly separated ancient evolutionary lineages with the time of divergence in the Pliocene (5-2.2 millions years ago; Colbourne et al., 1998). The results of our study show that the geographic distributions of these two groups overlap not only in the European Arctic but also in European mountain regions. The close relationships of haplotypes from the European populations with haplotypes from Canada and Greenland (Fig. 1) suggest that the Eastern Nearctic *D. pulicaria* lineage is of recent origin in Europe. Water fleas most likely colonized Iceland and Svalbard across Greenland from the high Nearctic shortly after the last glacial retreat (Weider and Hobaek, 1997; Weider et al., 1999a). It is therefore possible that the Eastern Nearctic lineage dispersed across the North Atlantic and further southwards towards mainland Europe. Its distribution in Europe after deglaciation thus could have been wider than at present and only following the Holocene warming it retreated northwards and into high altitudes. A similar scenario has been suggested to explain arctic-alpine distribution of other species in Europe (Abbott and Brochmann 2003; Schoenswetter & al. 2006; Skrede et al., 2006). As another possibility the Eastern Nearctic *D. pulicaria* may have crossed the Atlantic several times and via different routes. The dispersal capabilities of water fleas are greatly enhanced by the passive transport of resistant diapausing eggs that are encased in hard capsules (ephippia), and new populations can be established from ephippia that disperse over long distances via vectors such as migratory birds, wind and ocean currents (Weider et al. 1999a,b; Havel and Medley

2006). The colonization of mainland Europe by the Eastern Nearctic lineage thus may be the result of a direct trans-Atlantic transport from Eastern North America rather than a southward dispersal from the Arctic. The RFLP data of Weider et al. (1999a) showed that Svalbard is occupied by a distinct mtDNA haplotype lineage from those found in Greenland and Iceland, which suggests a direct transfer from the Canadian arctic and supports the view that a long-distance dispersal from the Nearctic was not a unique event. Although our present data are inconclusive in this respect, they show that the colonization of Europe by the Nearctic *D. pulicaria* is recent and the founder populations have not had sufficient time to phylogenetically diverge from their source populations in North America. Recent molecular evidence for postglacial trans-Atlantic migrations has also been provided for several Arctic plant species occurring on both sides of the Atlantic (Abbott and Brochmann 2003). Consequently, the Atlantic Ocean does not appear to have been an impenetrable dispersal barrier for the freshwater and terrestrial organisms that are passively transported as resistant propagules (eggs, seeds). Recently, several instances of intercontinental introductions to North America of exotic cladoceran species have been documented (Havel and Medley 2006). *Daphnia*'s enormous dispersal potential therefore raises the intriguing possibility that some gene flow may still be occurring in *D. pulicaria* between the two continents and contribute against the population divergence.

The Eastern Nearctic lineage and European lineage coexist in the High Tatra where they even inhabit the same lakes (Table 1). Sympatric occurrence of differentiated forms provides the opportunity of assessing their genetic isolation. Our recent study (S. Marková et al., unpublished data) has revealed high degree of differentiation at eight polymorphic microsatellite loci between the sympatric populations of the two lineages, which corroborates their genetic integrities. The work conducted by Gliwicz et al. (2001) showed that this genetic isolation is coupled with life history divergence. Although both lineages produce single generation each year, their reproduction is temporarily separated. In the Nearctic lineage the new generation starts in spring primarily from subitaneous eggs released by overwintering females, while in the European lineage it is recruited from diapausing eggs that hatch in summer. This synchronized reproduction is highly adaptive because it reduces resource competition between the lineages and enables their coexistence (Gliwicz et al. 2001), and it helps to maintain their integrities despite geographic contact.

The deep phylogenetic history of the Eastern Nearctic *D. pulicaria* lineage and European *D. pulicaria* lineage, the genetic isolation between their syntopic populations and their ecological differentiation demonstrate that the two lineages indeed represent

different species. The melanin and carotenoid pigmentation therefore are different adaptations that the colonizing Nearctic species evolved against the ultraviolet light stress. It enables it to explore upper and lighter water strata, and share habitats occupied by the unpigmented European species. The ability to develop photoprotective pigmentation therefore likely represents important physiological trait that facilitated successful colonization of the European continent by the Nearctic species. The Nearctic *D. pulicaria* thus provides a new model for studying successful intercontinental invasion by passively transported organisms. Further study with more extensive sampling will estimate likely source populations of the colonists and timing of the migration.

The fact that two species are confused under the name *Daphnia pulicaria* Forbes in Europe has been overlooked in previous studies, likely owing to the lack of prominent morphological differences other than pigmentation. Lityński (1917) has noted occurrence of orange and transparent color morphs in the High Tatra, and Gliwicz et al. (2001) described from the same lake different life history strategies, which he later matched with the two color morphs (Z. M. Gliwicz, personal communication). However, these phenotypes have been interpreted as a variation of a single species and attributed to environmental factors rather than genetic divergence (Lityński, 1917; Gliwicz et al., 2001).

Apparently, a considerable part of *Daphnia* diversity has been missed or attributed to ecophenotypy by morphological taxonomies. Together with recent findings in North America (Taylor et al., 1998; Hebert et al., 2003) and arctic Europe (Schwenk et al., 2004), our study show that widespread and morphologically uniform *Daphnia* species are likely to harbor cryptic evolutionary subdivisions. Species delimitation and relationships within the entire *D. pulex* complex thus need revision. These results will have important implications for the use of *Daphnia* as model organisms.

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Chapter 3

Microsatellite variation reveals genetic divergence among colour morphotypes of *Daphnia pulicaria* from European high-mountain lakes

Submitted for publication

Microsatellite variation reveals genetic divergence among colour morphotypes of *Daphnia pulicaria* from European high–mountain lakes

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Abstract

We used microsatellite variation to study the genetic differentiation within the European water flea *Daphnia pulicaria* (Crustacea: Cladocera). Three pigmentation morphotypes of this species inhabit glacial lakes in European high-mountain regions: Populations of the black morphotype occur in the Pyrenees and have allopatric distribution relative to populations of the orange and white morphotypes, which coexist in the High Tatra. We showed that each population was fixed for one of the nine multilocus genotypes detected with eight microsatellite loci. The genotypes were not shared among the three morphotypes, which showed fixed heterozygosity at polymorphic loci. Neighbour-joining trees as well as a multivariate Factorial Correspondence Analysis consistently showed evidence of three clusters significantly separating the morphotypes from each other. Geographical isolation could account for the genetic divergence of the black Pyrenean populations, consistent with other alpine disjunct species. Genetic differentiation between the white and orange morphotypes coexisting in sympatry and syntopy in the High Tatra and differing in life-history strategies (e.g. over wintering as diapausing eggs or active adults, respectively) suggests a reproductive isolation, possibly maintained due the low incidence of sex. Our study demonstrates that the alpine *D. pulicaria* is not a homogeneous species, and has differentiated into forms with distinctive genetic, pigmentation, and ecological characteristics. Together with recent findings in North America and European arctic, these results argue that widespread and morphologically uniform *Daphnia* species are likely to harbour cryptic evolutionary diversity, shaped not only by genetic drift but also as a result of adaptation.

Keywords: carapace pigmentation, *D. pulicaria*, microsatellites, parthenogenesis, High Tatra, Pyrenees

1. Introduction

Flaws in taxonomic assignments caused by morphological stasis, phenotypic plasticity, hybridization, and morphological convergence linked to the occupation of similar habitats have resulted in a serious underestimation in species numbers in zooplankton. Many species once thought to be cosmopolitan are now viewed as cryptic species assemblages of regionally restricted lineages (Hebert and Finston 1996; Taylor et al. 1998). For example, the *Daphnia* fauna of North America is now known to include a minimum of 34 species instead of the 15 previously assigned based on morphology (Hebert 1995) and the global total could reach as many as 200 species (Hebert and Taylor 1997). As a result of availability of novel genetic markers, the study of species diversity in cladoceran crustaceans has undergone a phase of rapid progress, which has increased the knowledge of genetic structuring and speciation in this group.

The *Daphnia pulex* complex is one group of arctic and alpine freshwater animals that has been the target of numerous genetic studies (Dufresne and Hebert 1995, 1997; Colbourne et al. 1998; Weider et al. 1999a,b). This complex has been proposed as an example of rapid ecological speciation and recent radiation (Colbourne et al. 1998; Pfender et al. 2000). Nine lineages have been identified, many of which dominate ponds and lakes in arctic and alpine environments (Colbourne et al. 1998). These environments differ from temperate habitats in a number of physical (e.g. ultraviolet radiation, temperature, nutrient input) and biological regimes (e.g. community structure; Baron et al. 1991), and offer many opportunities for adaptation and diversification (Schwenk et al. 2004). Arctic and high-mountain *Daphnia* have developed various strategies to minimise damage from harmful ultraviolet (UV) radiation. They often synthesise or concentrate compounds such as melanin and carotenoids that absorb the UV radiation energy, thereby increasing their survivorship under high light intensity (Hebert and Emery 1990).

Black pigmentation caused by melanin deposition is common to *D. pulex* group living in alpine and arctic region (Borgeraas and Hessen 2002). This pigmentation has evolved repeatedly in the group and is found in a number of species inhabiting shallow water bodies with high UV radiation exposure, including *D. melanica* in sand dunes in North America (Hebert 1995), *D. middendorffiana* and *D. tenebrosa* in the arctic regions (Colbourne et al. 1998), and *D. pulicaria* in the alpine lakes in Europe (Ventura and Catalan 2005). In small and shallow ponds, there is a correlation between the extent of carapace melanisation and UV radiation, whereas in deeper lakes both pigmented and unpigmented clones can co-occur,

which has been explained by the ability of unpigmented individuals to seek a refuge in deeper parts of the lake (Hebert and McWalter 1983; Haney and Buchanan 1987; Rautio and Korhola 2002; Ventura and Catalan 2005).

An alternative mechanism of protection against UV radiation in crustaceans is deposition of carotenoids. These pigments are acquired from food and accumulated in the lipid reserves, thereby playing a dual role as absorbents of UV radiation and as major anti-oxidants (Hessen et al. 1999). The carotenoid-based orange coloration is widespread in some groups of crustaceans (e.g. copepods; Hairston 1976), and it less often occurs also in cladocerans (Hessen and Sørensen 1990; Hessen 1996).

Intraspecific variation in pigmentation has been observed in alpine populations of *D. pulicaria*. Differently pigmented populations of this species have been found in the High Tatra Mountains (Western Carpathians in Slovakia and Poland) and the Pyrenees (north-eastern part of the Iberian Peninsula). A black pigmented (melanic) morphotype has been reported from the Pyrenees (Ventura and Catalan 2005) and an unpigmented transparent (hereafter referred to as white) morphotype and an orange (carotenoid-coloured) morphotype exist in sympatry and syntopy in the High Tatra (Gliwicz et al. 2001; Gliwicz, M., pers. comm.). Ecological studies by Gliwicz et al. (2001) and Ventura and Catalan (2005) showed that the morphotypes are also differentiated in terms of their ecological characteristics. The black morphotype and the orange morphotype start new-year generation in the spring from subitaneous eggs released by over-wintering females, while in the white morphotype the spring generation was recruited from diapausing eggs. Analyses of allozyme and mitochondrial DNA variation suggested that the High Tatra *D. pulicaria* are composed of multiple clones, which are closely related to the arctic lineages of *D. tenebrosa*. However, the individual clones could not be assigned to either different morphotypes or locally adapted populations (Černý 1995; 1999), and no information exists on the genetic variation of the Pyrenean *Daphnia* or their relationships to the High Tatra populations.

In the present study we describe variation in 18 populations of *D. pulicaria* from the High Tatra and Pyrenees. We used microsatellite markers that differentiate between *Daphnia* populations at different geographic scales (Pálsson 2000), and we applied the genetic information to assess the evolutionary differentiation and relationships among the different colour morphotypes of *D. pulicaria*.

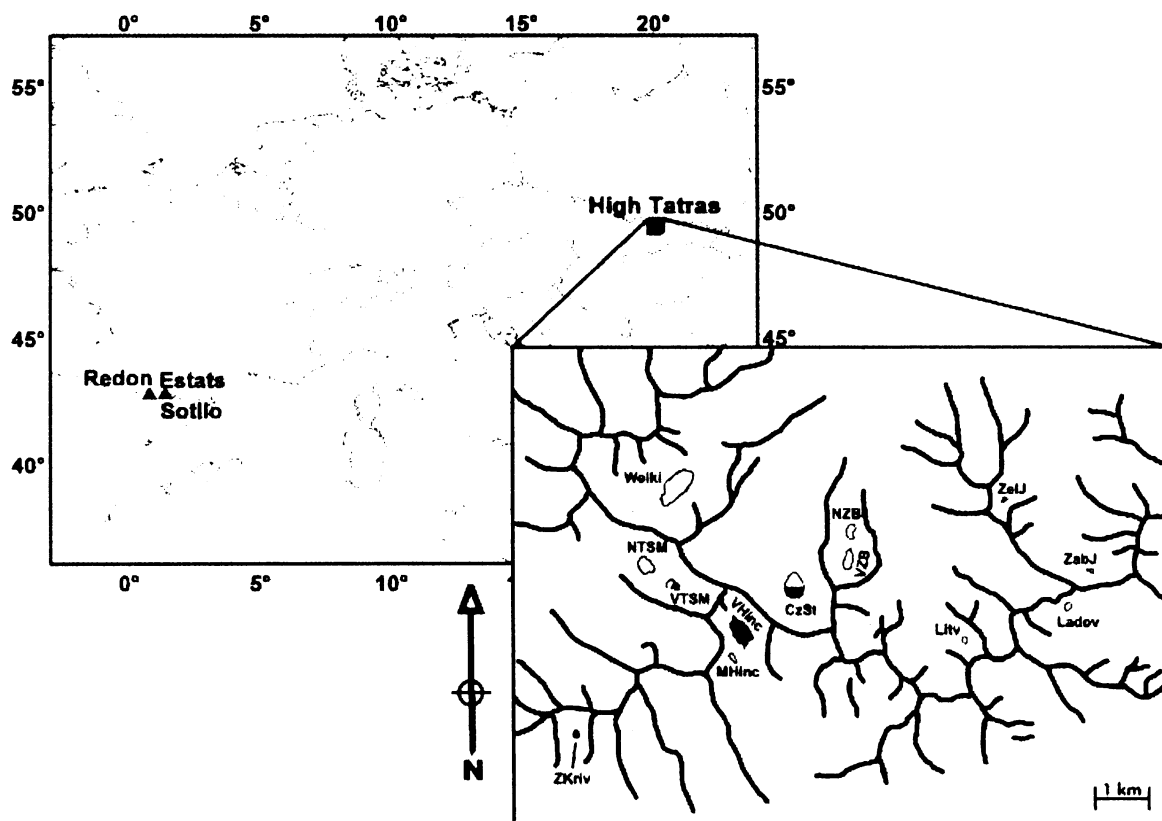


Fig. 1. Geographical location of sampling sites. Population codes are given in Table 1. A close-up map of the High Tatra localities (inset) shows lakes sampled for the white (empty lake symbols) and orange morphotypes (black-filled symbols) and lakes not inhabited by *Daphnia pulicaria* (grey-filled symbols).

2. Materials and methods

2.1. Sample collection

A total of 111 *D. pulicaria* individuals representing the white, orange and black morphotypes were collected during the summer and autumn of 2003 from 13 lakes in the High Tatra Mountains in the Western Carpathians in Slovakia and Poland, and from three lakes in the Pyrenees in north-eastern Spain (Table 1; Fig.1). All known High Tatra populations were included in this study (Marková et al. in press.) whereas only a proportion (approximately 20%) of the lakes inhabited by the species were successfully sampled in the Pyrenees. Water fleas were collected by plankton net tows from an inflatable boat in the lake centre or by horizontal sweeps on the lakeshore, and were stored in 95% ethanol at 4 °C until analysis. Because orange and white morphotypes coexist in sympatry in VTSM and CZSt in the High Tatra, and *Daphnia* lose their coloration while stored in ethanol, individuals from these two lakes were sorted according to morphotypes immediately upon sampling, and treated as separate populations in subsequent analyses (Table 1).

Table 1. Geographical details for *Daphnia pulicaria* populations sampled in this study and the distribution of microsatellite genotypes (Table 2). Absolute genotypes frequencies in each population are given in parentheses.

Population (Population code)	Region	Country	Lat.	Long.	Genotype
White Morphotpe					
Czarny Staw pod Rysmi (CzStwh)	High Tatra	Poland	49.19	20.08	B (2)
Vyšné Temnosmrečianske (VTSMwh)	High Tatra	Slovakia	49.19	20.04	A (2)
Nížné Temnosmrečianske (NTSM)	High Tatra	Slovakia	49.19	20.03	C (2)
Nížné Žabie Bielovodské (NZB)	High Tatra	Slovakia	49.19	20.09	B (2)
Vyšné Žabie Bielovodské (VZB)	High Tatra	Slovakia	49.19	20.09	B (2)
Welki Staw, dolina					
Pienci Polskich Stawov (Welki)	High Tatra	Poland	49.21	20.04	B (2)
Malé Hincovo (MHinc)	High Tatra	Slovakia	49.17	20.06	B (23)
Zelené Javorove (ZelJ)	High Tatra	Slovakia	49.21	20.14	D (2)
Litvorové (Litv)	High Tatra	Slovakia	49.18	20.13	B (23)
Ladové, Veľká studená dolina (Ladov)	High Tatra	Slovakia	49.18	20.00	B (2)
Žabie Javorove (ZabJ)	High Tatra	Slovakia	49.19	20.17	B (2)
Orange Morphotype					
Zelené Krivánske (Zkriv)	High Tatra	Slovakia	49.16	20.01	F (23)
Czarny Staw pod Rysmi (CzStor)	High Tatra	Poland	49.19	20.08	F (2)
Veľké Hincovo (VHinc)	High Tatra	Slovakia	49.18	20.06	F (2)
Vyšné Temnosmrečianske (VTSMor)	High Tatra	Slovakia	49.19	20.04	E (2)
Black Morphotype					
Estats (Estats)	Pyrenees	Spain	42.66	1.39	I (8)
Sotllo (Sotllo)	Pyrenees	Spain	42.65	1.38	H (8)
Redon (Redon)	Pyrenees	Spain	42.64	0.78	G (8)

2.2. Genetic analyses

DNA was extracted using the IsoQuick Nucleic Acid Extraction Kit (ORCA research, USA) following the manufacturer’s instruction. Genetic variation and relationships among morphotypes were assessed using eight microsatellite loci Dp512, Dp513, Dp514, Dp514alt, Dp519, Dp522, Dp523 and Dp525 described by Colbourne et al. (2004). All loci consisted of dinucleotide motifs except for Dp514 and Dp514alt that contained trinucleotide repeats. Forward primers were end-labelled with fluorescent dyes (Life Technologies, Univ. of Oklahoma, USA). Polymerase chain reaction (PCR) amplifications were carried out as described by Pálsson (2000) and Colbourne et al. (2004) and at the *Daphnia* Genomics Consortium website (<http://daphnia.cgb.indiana.edu/tools/microsats>). Sizes of amplified microsatellite alleles were scored using a denaturing polyacrylamide gel and visualized using a FM-BIO III scanner (Hitachi).

Previous analyses using 11 allozyme loci revealed that *D. pulicaria* populations in the High Tatra lakes were composed of a single clone and all analysed individuals were identified as diploid and heterozygous at polymorphic loci (Černý 1995; Černý, M., unpubl.). To assess the level of clonal variation detectable with microsatellite markers we screened 23 individuals from each MHinc and Litv white morphotype populations, 23 from the ZKriv orange morphotype population, and eight from each Redon and Sotllo black morphotype populations. All individuals from the same lakes shared identical combination of alleles at all microsatellite loci, suggesting they likely belonged to the same clone, and supporting the results obtained with allozymes. Therefore for further large-scale analyses with 18 populations from 16 lakes, we used two individuals from each of the other populations.

Data analyses—To investigate the relationships among *D. pulicaria* individuals from different or same morphotype, we estimated pairwise genetic distances among their multilocus genotypes, using the Allele Shared Distance (D_{AS} ; Jin and Chakraborty 1994) which counts the proportion of shared alleles between the genotypes, and the Cavalli-Sforza's Chord Distance (D_{SE} ; Cavalli-Sforza and Edwards 1967) which is an Euclidean distance assuming divergence in allele frequencies by genetic drift. Genetic distances were calculated with POPULATIONS, version 1.2.28 (Langella 2003), and were used to construct unrooted neighbour-joining trees (Saitou and Nei 1987) using the PAUP* software package, version 4.0 Beta (Swofford 2003). To quantify the confidence in the partitioning within the trees we performed the nonparametric bootstrap test, calculated between individuals, with 1000 replications using MSANALYZER, version 2.39 (Dieringer and Schlötterer 2003).

To further assess the extent of genetic differentiation among morphotypes we carried out a Factorial Correspondence Analysis (FCA) available in GENETIX Version 4.04 (Belkhir et al. 2003). The FCA is a multivariate analysis that allows projection of individuals in a multidimensional space according to their similarity in the allelic state for each allele, i.e. whether it is heterozygote, homozygote or if the given allele is absent (She et al. 1987). The advantage of the FCA is that every individual can be represented using each allele as an independent variable, and it is therefore suitable to visualize the relationships between multilocus genotypes.

Table 2. Alleles detected at eight microsatellite loci screened for variation in *Daphnia pulicaria* from the High Tatra and Pyrenees and their distribution among the nine multilocus genotypes (A–I). Numbers indicate sizes of alleles (in base pairs). x = allele was present in the genotype.

Genotype	Dp513				Dp512				Dp514alt																					
	113	115	119	121	128	130	138		127	130	133	148	151	163	169	175	187													
White Morphotype																														
A	x				x	x				x	x																			
B	x		x		x	x				x				x																
C	x		x		x	x				x					x															
D	x				x	x				x							x													
Orange Morphotype																														
E	x				x		x			x			x																	
F	x				x		x			x			x																	
Black Morphotype																														
G	x		x		x					x																				
H		x	x		x				x					x																
I		x		x	x					x		x																		
Genotype	Dp522				Dp519				Dp525				Dp523				Dp514													
	116	120	122	124	146	148		114	123		128	134	136	140	99	100	101	102												
White Morphotype																														
A	x		x		x			x	x			x			x				x	x										
B	x		x		x			x	x			x			x				x	x										
C	x		x		x			x	x			x			x				x	x										
D	x		x		x			x	x			x			x				x	x										
Orange Morphotype																														
E	x				x			x	x			x							x											
F	x				x			x	x				x						x											
Black Morphotype																														
G		x		x										x																
H		x		x										x					x											
I		x		x										x					x	x										

3. Results

3.1. Gene and genotypic diversity

In total, 32 alleles were detected at the eight microsatellite loci assessed in 111 individuals of *D. pulicaria* from 11 white and four orange morphotype populations in the High Tatra and three black morphotype populations in the Pyrenees. All loci were polymorphic across morphotypes. The overall number of alleles per locus ranged from two at Dp519 and Dp525 to nine at Dp514alt with an average of four (Table 2). Five loci were polymorphic in the white morphotype (two to five alleles), six in the orange morphotype (two to three alleles) and four in black morphotype (two to five alleles; Table 2). All alleles at polymorphic loci except the most common allele at each locus were present only in heterozygous genotypes. All individuals in each morphotype therefore were heterozygous at all loci that showed more than one allele in that morphotype (Table 2).

Altogether nine eight-loci genotypes were resolved (Table 2), four (A-D) in the white morphotype, two (E and F) in the orange morphotype and three in the black morphotype (G-I). Each population was fixed for a single genotype, but genotypes varied between populations (Table 1). Of six genotypes detected among the 15 High Tatra populations (A-F), only two were present in multiple populations. Genotype B was shared by CzStwh, NZB, VZB, Welki, MHinc, Litv, Ladov and ZabJ white morphotype populations, and genotype F by ZKriv, CzStor and VHinc orange morphotype populations. Remaining four High Tatra genotypes (A, C-E) were unique to the population (Table 1). The three genotypes (G-I) detected among the three Pyrenean populations of the black morphotype were all unique to a population (Table 1).

3.2. Morphotype differentiation

Microsatellite variation was not randomly distributed among differently pigmented morphotypes (Table 2, 3). No shared genotypes were observed among the populations from different morphotypes, and 20 morphotype-specific private alleles were found, with nine private alleles of the total 18 (50.0%) in the black morphotype, six of 15 (40.0%) in the orange morphotype, and five of 17 (29.4%) in the white morphotype (Table 3).

Neighbour-joining trees constructed using D_{AS} and D_{SE} genetic distances showed identical topologies, which revealed significant grouping among the multilocus genotypes (Fig. 2).

Table 3. Genetic diversities within morphotypes and mountain regions.

	No. of populations	No. of genotypes	No. of alleles	No. of private alleles	No. of polymorphic loci
High Tatra					
White morphotype	11	4	17	5	1
Orange morphotype	4	2	15	6	1
Total	15	6	23	14	2
Pyrenees					
Black morphotype	3	3	18	9	4
Total	18	9	32	N/A	8

Three groups were consistently supported by >90% bootstrap values where genotypes from populations of the same pigmentation type were clustered; the three black morphotype populations from the Pyrenees, the four orange morphotype populations from the High Tatra, and the 11 white morphotype populations from the High Tatra.

4. Discussion

Three pigmentation morphotypes of *D. pulicaria* have been distinguished among populations inhabiting glacial lakes in European high-mountain regions: Populations of the black morphotype occur in the Pyrenees and have allopatric distribution relative to populations of the orange and white morphotypes, which coexist in sympatry and syntopy in the High Tatra. In the present study, we quantified genetic variation at eight microsatellite loci in 18 populations of *D. pulicaria* to address the question whether the phenotypic differentiation of these morphotypes is supported by genetic divergence.

4.1. Genetic isolation and reproduction

Microsatellite results unambiguously revealed genetic differentiation of the three *D. pulicaria* morphotypes. Neighbor-joining trees showed strong evidence of three clusters separating the Pyrenean black morphotype, the High Tatra orange morphotype and the High Tatra white morphotype, using either D_{AS} or D_{SE} . This result was further supported by the FCA projection of individual genotypes in a multidimensional space, which clearly differentiated between the morphotypes based on their allelic composition. Taken altogether, these results demonstrate a genetic isolation between the three morphotypes.

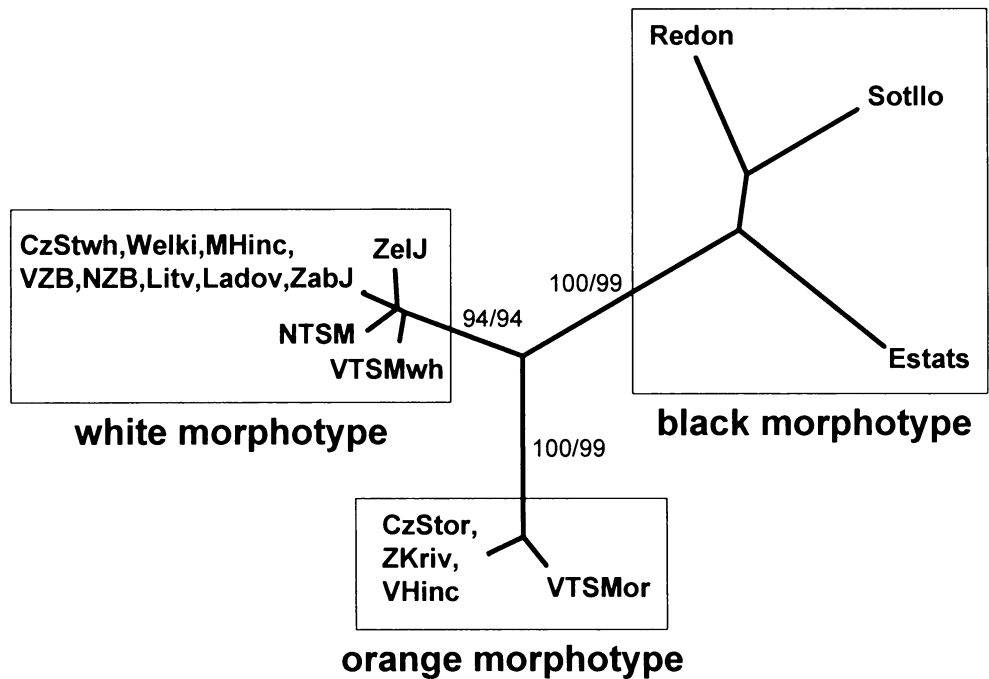


Fig. 2. Unrooted neighbour-joining tree inferred from microsatellite data using Cavalli-Sforza's Chord Distance (D_{SE}), illustrating relationships between multilocus genotypes detected among 18 populations of *Daphnia pulicaria* from High-Mountain lakes in Europe. Population codes are given in Table 1. Tree topology is identical with that based on the Allele Shared Distance (D_{AS} ; not shown). Variation in pigmentation scored as three different morphotypes is reported on the tree. The numbers along branches indicate percentage bootstrap support >90% (1000 replications) obtained using D_{SE}/D_{AS} . The tree yielded three well supported groups: the black morphotype group including three populations from the Pyrenees, the orange morphotype group constituted by four populations from the High Tatra, and the white morphotype group including 11 High Tatra populations.

The disjunct distribution may itself account for the observed genetic isolation of the black-pigmented Pyrenean populations from the orange and white morphotype populations in the High Tatra. A similar west/east subdivision is displayed by other European species with disjunct alpine distributions, e.g. perennial *Ranunculus glacialis* (Schönswetter et al. 2003) and lepidopteran *Erebia epiphron* (Schmitt et al. 2005), supporting the geographic component to the differentiation. However, within the High Tatra the genetic distinctiveness of differently pigmented populations is maintained despite the fact that the orange and white morphotypes coexist in sympatry within same lake (Fig. 1). Arctic species of *D. pulex* complex, to which *D. pulicaria* belongs, reproduce as a rule by obligate parthenogenesis, which is thought to facilitate better dispersal and colonization of new habitats (Weider et al. 1987). Adaptation of obligate asexuality has also been attributed to the short season (Brooks 1957) in arctic and alpine lakes, where organisms gain an advantage by avoiding the cost of production of males. The microsatellite data for the populations surveyed in this study

strongly suggest that parthenogenesis is likely the primary mode of reproduction of *D. pulicaria* in the High Tatra, as was implied by an earlier study of allozyme variation (Černý 1995). Each population was fixed for a single genotype at eight microsatellite loci, despite the fact that at least four loci were polymorphic in each population (Table 2). Moreover, all individuals in each morphotype were heterozygous at all loci that showed more than one allele in that morphotype. Although sample sizes were low for many populations, this genetic uniformity and fixed heterozygosity at polymorphic loci was observed for all populations including the three populations with relatively large sample sizes (23 individuals; Table 1), which is consistent with obligate parthenogenesis or very low incidence of sex in these populations (Gregorius 2005).

Although few males have ever been observed in the High Tatra populations (Lityński 1917, Černý 1995; Gliwicz et al. 2001), some Pyrenean populations of *D. pulicaria* produce males regularly (Ventura and Catalan 2005). The presence of males does not necessarily imply sexual reproduction, however, because even obligate parthenogens are known to produce viable males (Innes and Hebert 1988). Several mechanisms could potentially act to prevent or limit gene exchange between sympatric orange and white morphotypes in spite of occasional production of males. The males produced by obligate asexual lineages may no longer produce functional sperm as has been observed in arctic obligate parthenogens (Dufresne, F., unpubl.). Alternatively, there might be prezygotic or postzygotic barriers to reproduction that might act on population specialization. Although the present data are inconclusive in this respect, they do strongly suggest that the white and orange morphotypes represent genetically differentiated and reproductively isolated evolutionary lineages despite their syntopic occurrence.

4.2. Adaptive role of pigmentation polymorphism

Pigmentation in aquatic invertebrates is often under strong environmental selection and its variation may therefore indicate adaptive responses (Hebert and Emery 1990; Hargeby et al. 2004). The presence of melanin in the carapace, typical of the black morphotype, provides protection against harmful UV radiation (Hebert and Emery 1990; Rautio and Korhola 2002). Many cladoceran species are capable of synthesizing melanin (Hebert and Emery 1990). However this biosynthesis is energetically demanding and might result in selective disadvantage of melanic morphs co-occurring in deep ponds with unpigmented clones that may escape into lower and darker water strata under UV radiation (Hebert and Emery 1990). Therefore, the absence of melanin synthesis in the High Tatra lakes may be an adaptive

response to different UV regime then in the Pyrenees, associated with lower UV exposure in the High Tatra lakes, which are situated at 400-900 meters lower altitude; and different depth of the High Tatra lakes, most of which are 10 to 60 meters deeper than Pyrenean lakes. Furthermore, the photoprotective function of melanin may in part be compensated by the carotenoid-based coloration in the orange morphotype, restoring its UV tolerance (Stephan et al. 2001). Hence, these results suggest that differences in UV radiation and competition regimes most likely act synergistically in shaping pigmentation polymorphism in the alpine *D. pulicaria*.

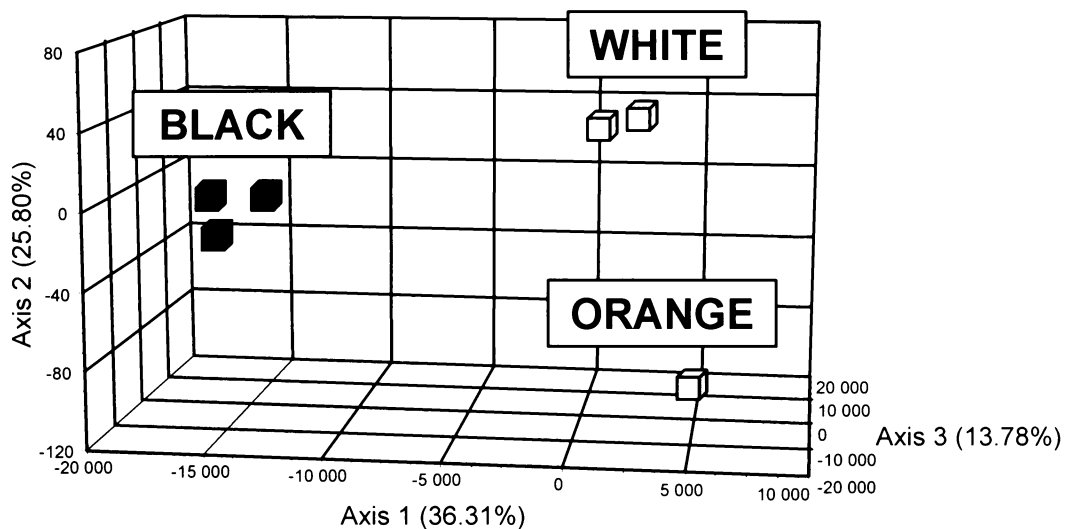


Fig. 3. Genetic differentiation among multilocus genotypes of differently pigmented *Daphnia pulicaria* from European High-Mountain lakes based on Factorial Correspondence Analysis (FCA) of allelic composition at eight microsatellite loci. Symbols may overlap for genotypes with similar FCA values.

4.3. Ecological divergence

The available evidence suggests that the three morphotypes of *D. pulicaria* constitute discrete evolutionary units based not only on pigmentation and genetic differentiation but also in terms of their ecological and life history characteristics. Previous studies described two alternative life history strategies in *D. pulicaria* from the High Tatra (Gliwicz et al. 2001), which were recently matched with the two differently pigmented morphotypes (Gliwicz, Z. M., pers. comm.). In the orange morphotype the new-year generation started in spring from subitaneous eggs released by over-wintering females, while in the white morphotype it is recruited from diapausing eggs over-wintering in ephippia, which hatched in the summer following the abundance peak of the spring generation of the orange morphotype. This synchronised reproduction in the High Tatra lakes is highly adaptive because it

is associated with optimised resource utilization and lower predation risk from *Cyclops* (Gliwicz et al. 2001). Populations of the black morphotype from the Pyrenees have a similar life cycle to that of the orange morphotype in the High Tatra, with over-wintering adults and few ephippia produced (Ventura and Catalan 2005).

Within each morphotype, the spatial partitioning of genotypes identified with microsatellites supports further ecological separation. Although different genotypes occurred in lakes separated by short distances (hundreds of meters) and connected by streams (e.g. Estats and Sotillo, or VTSM and NTSM), we never observed more than a single genotype of the same morphotype within one lake. This apparent lack of clonal diversity in the alpine *D. pulicaria* populations contrasts with much higher estimates obtained for arctic parthenogenetic *Daphnia*, which varied from 1.5 ± 0.2 for the *D. pulicaria* group to 2.4 ± 0.0 for *D. tenebrosa* group (Weider et al. 1999b). The dispersal potential of *D. pulicaria* via diapausing eggs should allow for dispersal events between lakes connected by streams (Marková, S., unpubl.), enough to permit genotype sympatry at the microgeographical level. Therefore, the spatial partitioning of genotypes within each morphotype is most probably the result of strong local selection among genotypes and competitive exclusion of immigrant clones (De Meester et al. 2002), and it underscores the importance of ecological specialization for the distribution of genetic variation in these alpine *Daphnia*.

In conclusion, the present study shows that the alpine *D. pulicaria* is not a homogeneous species, and has differentiated into forms with distinctive genetic, pigmentation and ecological characteristics. Although lacking any other morphological differences, the genetic isolation between these morphotypes coupled with ecological differentiation suggests that they may represent different species. Together with recent findings in North America (Taylor et al. 1998; Hebert et al. 2003) and arctic Europe (Schwenk et al. 2004), our results show that widespread and morphologically uniform *Daphnia* species are likely to harbour cryptic evolutionary diversity. Divergent and disruptive selection caused divergence and reproductive isolation of ecologically differentiated arctic *Daphnia* lineages (Schwenk et al. 2004), and these processes likely have been important in the differentiation of the alpine *D. pulicaria*. Additionally, demographic and biogeographic history may have played an important role, e.g. the genetic change might be associated with an episode of population growth in a newly colonized habitat. Extending the sampling with more localities throughout the arctic-alpine distribution of *D. pulicaria* and application of genetic markers amenable to phylogenetic analysis (e.g. DNA sequences) will be

necessary before establishing the role of dispersal and selection in shaping its evolutionary diversity.

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Chapter 4

Are they still viable? Physical condition and abundance of *Daphnia pulicaria* resting eggs in sediment cores from lakes in the High Tatra Mountains

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Are they still viable? Physical condition and abundance of *Daphnia pulicaria* resting eggs in sediment cores from lakes in High Tatra Mountains

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Abstract: All species of *Daphnia* (Cladocera) produce, at some stage in their life cycle, diapausing eggs, which can remain viable for decades or centuries forming a “seed bank” in lake sediments. Because of their often good preservation in lake sediment, they are useful in paleolimnology and microevolutionary studies. The focus of this study was the analysis of cladoceran resting eggs stored in the sediment in order to examine the ephippial eggs bank of *Daphnia pulicaria* Forbes in six mountain lakes in the High Tatra Mountains, the Western Carpathians (northern Slovakia and southern Poland). Firstly, we analyzed distribution, abundance and physical condition of resting eggs in the sediment for their later use in historical reconstruction of *Daphnia* populations by genetic methods. To assess changes in the genetic composition of the population through time, we used two microsatellite markers. Although DNA from resting eggs preserved in the High Tatra Mountain lake sediments was extracted by various protocols modified for small amounts of ancient DNA, DNA from eggs was not of sufficient quality for microsatellite analyses. Distribution curves of resting eggs from sediment cores correspond to the environmental changes that have occurred in the High Tatra Mountains area during last two centuries (atmospheric acid deposition, fish introduction) and demonstrate their influence on natural populations. Evaluation of ephippia physical condition (the most common category was empty ephippial covers) suggests that the majority of resting eggs hatched to produce a new generation of *Daphnia* or may be due to failed deposition of resting eggs by *Daphnia* to the chitinous case. In conclusion, age, low quantity and poor physical condition of resting eggs from these Tatra lake sediments proved to be unsuitable not just for use in genetic analyses, but also the possibilities of autogenous restoration of *Daphnia* populations from the resting egg banks in the Tatra sediments are negligible.

Key words: *Daphnia*, resting eggs, microsatellite markers, alpine lakes, High Tatra Mountains.

Introduction

Mountain lakes in the High Tatra Mountains (Mts) represent unique ecosystems in remote areas, and have been the object of investigations for more than two centuries (WIERZEJSKI, 1882, 1883; MINKIEWICZ, 1917; LITYŃSKI, 1913, 1917). One of few dwellers adapted for harsh condition of these mountain lakes is *Daphnia* spp. (Crustacea, Anopoda). LITYŃSKI (1913) found *Daphnia pulicaria* Forbes, 1893 in High Tatra lakes, and it was initially described as *Daphnia wierzejskii* Lityński, 1913. During later detailed sampling, MINKIEWICZ (1917) recorded the presence of *Daphnia* in 23 of 82 sampled Tatra lakes. In recent years, *Daphnia* have been found to inhabit just 13 Tatra lakes (STUHLÍK & MARKOVÁ, unpublished data). The main reasons for these extinctions are lake acidification and fish introduction (KOPÁČEK & STUHLÍK, 1994; GLIWICZ & ROWAN, 1984). The Tatra Mts are one of

the most severely acidified European alpine ecosystems (KOPÁČEK et al., 2004).

The purpose of this paper is to attempt the historical reconstruction of *Daphnia pulicaria* populations by using ephippia occurrence in the sediments of Tatra lakes. A feature of *Cladocera* is the production of diapausing eggs at some stage in their life cycle. The resting eggs are contained and protected within a resistant chitinous structure named an ephippium (VOLLMER, 1912). The study of resting eggs and egg banks is important for a variety of disciplines, such as taxonomy, ecological biogeography, paleolimnology, natural conservation, evolutionary ecology and community and population ecology (BRENDONCK & DE MEESTER, 2003). *Cladocera* provide useful insights into the past conditions of lakes (FREY, 1976). *Daphnia* ephippia are often well preserved in the lake sediments (JEPPESEN et al., 2002) and are used in paleolimnological and microevolutionary studies for analyses of past environ-

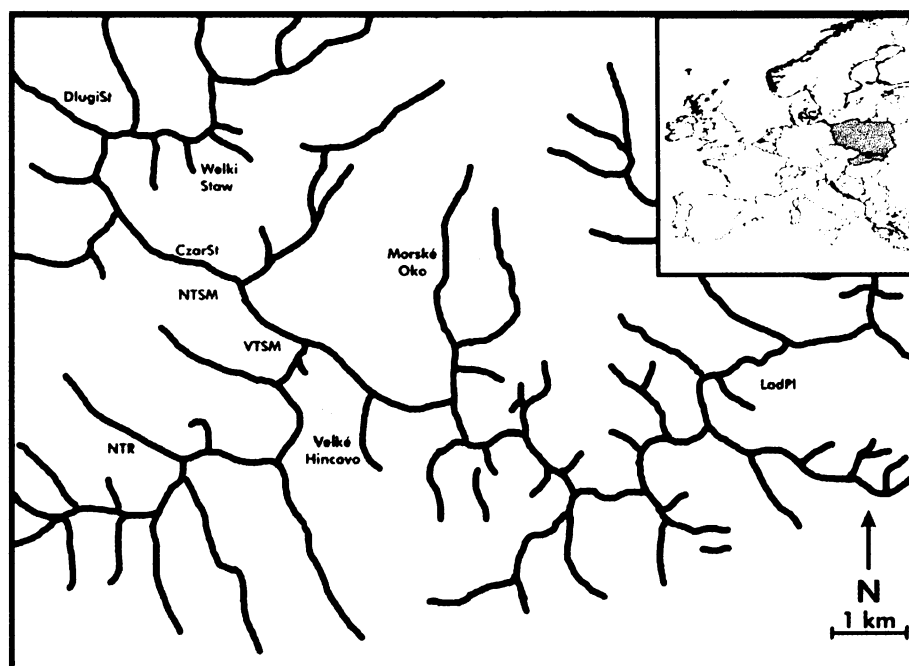


Fig. 1. Distribution of alpine lakes in the High Tatra Mountains. Lakes Velké Hincovo, Welki Staw and Morské Oko serve as orientation; other marked lakes were used in ephippia analyses. Bold lines designate mountain-ridges. Short Lakes name correspond to those in Table 1.

mental changes in lake conditions and their influence on microevolutionary processes in natural populations.

The focus of our study was to analyse distribution, abundance and physical condition of resting eggs in the sediment, and then using microsatellite markers to assess change in genetic composition of the *Daphnia* population through time. DNA from resting eggs deposit in the High Tatra sediments was extracted by following various protocols for small amounts of DNA and the viability of DNA was tested by genetic analyses. We discuss our results in light of the past environmental changes that have occurred in the High Tatra area during the last two centuries, and we demonstrate how egg bank propagules may allow one to examine the influence of these changes on *Daphnia* populations.

Finally, based on the physical condition of resting eggs we considered the possibility for natural reestablishing of *Daphnia* populations in lakes where they have gone extinct, through the use of resting egg banks.

Study sites

The Tatra Mts belong to the Carpathian chain and are situated on the Slovak-Polish border (20° E, 49°15' N; KOPÁČEK et al., 2004; Fig. 1). For the purpose of analysing resting eggs deposited in the sediment we selected six lakes. These six lakes were chosen as representatives of lakes found by MINKIEWICZ (1917) to contain *Daphnia*. In some of these lakes *Daphnia* has become extinct while others presently support *Daphnia* populations. These lakes also present a variety of physical characteristics that may have influenced

persistence or loss of *Daphnia* (see Tab. 1). Four of the lakes selected are on the Slovak side: Nižné Terianske pleso (NTR); Nižné Temnosmrečinské pleso (NTSM); Vyšné Temnosmrečinské pleso (VTSM); Ladové pleso in Velká Studená dolina valley (LadPl) and two are on the Polish side: Długi Staw Gąsienicowy (DługiSt); Czarny Staw Polski (CzarSt) of the High Tatra Mts (Fig. 1). All of these lakes are of glacial origin.

Material and methods

Lake sediment sampling and ephippia classification

A Kajak core sampler (diameter 60 mm) was used to collect one sediment core from the deepest part of each lake during the summer of 2003. The upper 2 cm of the surface sediment was divided immediately in the field into 0.5 cm sections and the rest of the cores into 1 cm thick sections. Samples were transported to the laboratory and stored in the dark at 4°C in plastic bags until analysis. Ephippia were collected from the sediment sections by sieving through a 100 µm mesh sieve, and subsequently decapsulated under a binocular microscope and resting eggs were removed. The number of resting eggs found in each slice was recorded, and all ephippial manipulations followed a set of protocols appropriate for ancient DNA studies (THOMAS, 1994; COOPER & POINAR, 2000). Based on observations of ephippia physical conditions within these sediment cores, five categories were created for classification of resting eggs. For each section, physical conditions of ephippia and resting eggs were assessed and they were classified into five categories (Fig. 2): 1 – Empty ephippial cover, 2 – Degraded eggs “brown clumps”, 3 – Degraded eggs “red clumps”, 4 – Empty egg membrane and 5 – Eggs. The first category represents ephippia which, when opened, were found to be without resting eggs (Fig. 2 a). Clumps of

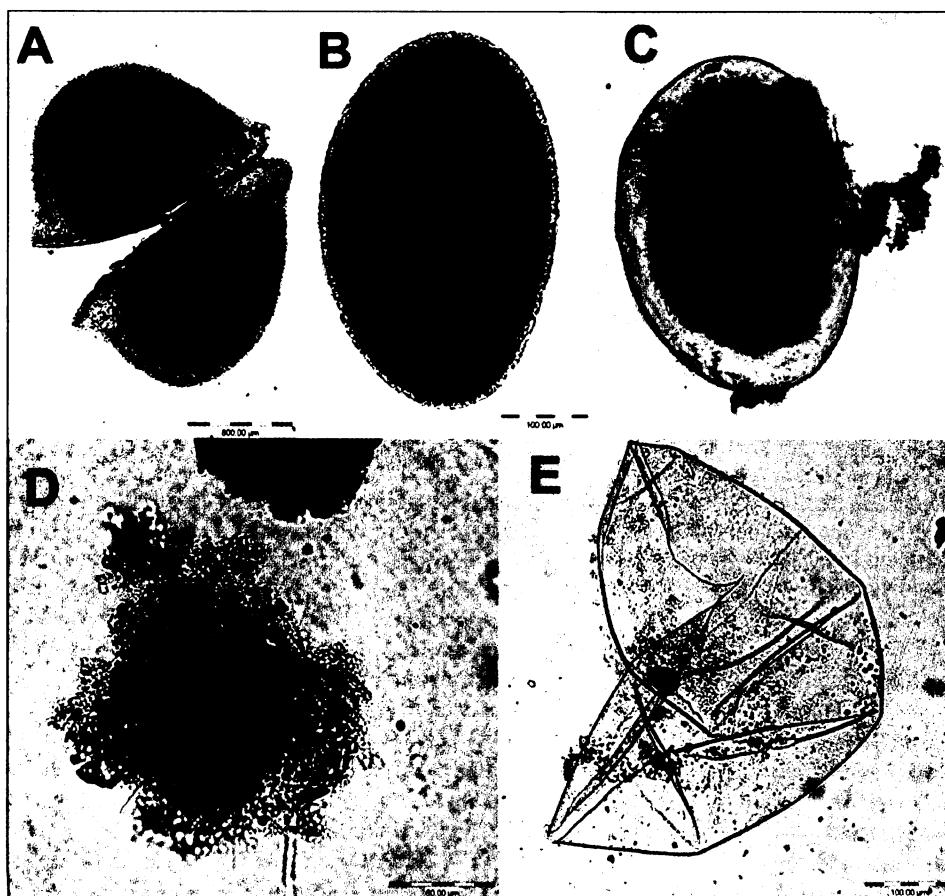


Fig. 2. Evaluation categories of resting eggs in the sediment of the High Tatras lakes: A – Empty ephippial cover (category 1); B – Eggs (category 5); C – Degraded eggs (“red clumps”) (category 3); D – Degraded eggs (“brown clumps”) (category 2); E – Empty egg membrane (category 4).

degraded eggs, which exhibited differences in colour, were characterized by categories 2 (brown) and 3 (red) (Fig. 2 d, c). Category 4 represents empty egg covers of degraded eggs (Fig. 2 e), while category 5 signifies resting eggs in good condition, which were subsequently used in genetic analyses (Fig. 2 b).

Lakes categories of sensitivity to acidification

The acid-base status of the lakes in the 1980s (FOTT et al., 1994) was used to divide the lakes into three main categories of sensitivity to acidification: non-sensitive lakes ($\text{pH} > 6$; Acid Neutralizing Capacity (ANC) $> 25 \mu\text{eq L}^{-1}$), where the species composition of planktonic Crustacea has not changed; acid-sensitive lakes ($5 < \text{pH}$; ANC $0\text{--}25 \mu\text{eq L}^{-1}$), where planktonic Crustacea were present in the past, but are now absent; and extremely sensitive lakes ($\text{pH} < 5$; ANC $< 0 \mu\text{eq L}^{-1}$), where only one species (*Chydorus sphaericus* Müller, 1785) remains. Only one of the six lakes in this study is classed as sensitive to acidification, according to this definition; Długi Staw Gąsienicowy (DługiSt).

Genetic analyses

Three methods were used for extraction of DNA from resting eggs: 1 – 20 μL of 5% CHELEX 100 solution (Bio-Rad, Hercules, CA), following the protocol of WALSH et

al. (1991), 2 – use of the Forensic extraction KIT 1 (Invitex, Germany) following the procedures of LIMBURG & WEIDER (2002), and 3 – use of the IsoQuick Nucleic Acid Extraction Kit (ORCA research, USA) following the manufacturer's instructions. During all work with ancient DNA from sediments, appropriate guidelines were followed (COOPER & POINAR, 2000). All PCR reactions were prepared in an isolated DNA work area (all manipulations were performed in a fume hood, cleaned with decontamination spray (Cambio Ltd) for removal of DNA each time before PCR preparation); all PCR reactions were run with negative controls, none of which gave any indication of contamination problems. Amplification of PCR products was also carried out independently in two laboratories. Two pairs of microsatellite primers, Dpu122 (available at GenBank/ EMBL under accession: AF233363; COLBOURNE et al., 2004), Dpu6 (GenBank AY057864; COLBOURNE et al., 2004) were used to amplify 133 and 138 bp fragments. Forward primers were end-labelled with fluorescent dyes (Life Technologies, Univ. of Oklahoma, USA). PCR reactions were carried out in 25 μL volumes with a final concentration of 1.2 μM of each primer, 0.2 mM dNTPs, 0.5 mM MgCl_2 , 0.25 units of *Taq* DNA polymerase (MBI Fermentase). PCR conditions consisted of an initial denaturation step (3 min at 94°C) followed by 40 cycles of: 45 s denaturation at 94°C , 1 min

Table 1. Lakes physical characteristic.

Lake abbrev.	Lake name	Latitude	Longitude	Altitude (m a.s.l.)	Lake volume ($1 \times 10^3 \text{ m}^3$)	Max depth (m)	Lake area (ha)	Fish	Sensitivity to acidification	Daphnia currently present
NTSM	Nížné Temno-smrečianske	49.192900	20.030600	1674	2161	41	13.2	absent	non-sensitive	Yes
VTSM	Vyšné Temno-smrečianske	49.189100	20.039500	1716	460	20	4.51	absent	non-sensitive	Yes
LadPl	Ladové pleso in Veľkej studenej Valley	49.184100	20.162900	2057	114	18	1.7	absent	non-sensitive	Yes
NTR	Nížné Terianske	49.169800	20.014300	1941	879	43	4.9	absent	non-sensitive	No
DługiSt	Długi Staw in Gąsienicowa Valley	49.227300	20.01700	1784	81	11	1.6	absent	sensitive	No
CzarSt	Czarny Staw Polski	49.204600	20.027700	1722	2826	50	12.7	present	non-sensitive	No

Main physical characteristics and *Daphnia* status for the six lakes in the present study (adapted from KOPACEK et al., 2004). The GPS data come from the EMERGE database (web side: http://www.mountain-lakes.org/emerge/districts/ta_map/index.html). For definition of Sensitivity to acidification, see page 4.

annealing at 53°C for Dpu6 and 54°C for Dpu122, 1 min extension at 72°C, followed by a final 10 min 72°C extension. Microsatellite alleles were amplified resolved on a denaturing polyacrylamide gel and visualized using FMBIO scanner (Hitachi).

Results and discussion

Lake physical characteristic, distribution and physical conditions of ephippia

Nížné Temnosmrečinské pleso and Vyšné Temnosmrečinské pleso

Nížné Temnosmrečinské pleso and Vyšné Temnosmrečinské pleso are situated in the Temné Smrečiny valley and are connected by a stream which flows from Vyšné Temnosmrečinské pleso to Nížné Temnosmrečinské pleso. These lakes are representatives of the few Tatra lakes still supporting *D. pulicaria*. The abundance of ephippia was higher in Nížné Temnosmrečinské pleso (maximum of 266 ephippia per layer; 10.88% ephippia total) compared to Vyšné Temnosmrečinské pleso (maximum of 9 ephippia per layer; 28.13% total). The level of this difference is surprising given that both lakes presently support *Daphnia* populations, but can potentially be explained by two main factors. Firstly, there are the morphological characteristics of lakes; Nížné Temnosmrečinské pleso (13.2 ha) is approximately three times the area of Vyšné Temnosmrečinské pleso (4.5 ha) and the lake volume is almost five times larger in Nížné Temnosmrečinské pleso ($2161 \times 10^3 \text{ m}^3$) than in Vyšné Temnosmrečinské pleso ($460 \times 10^3 \text{ m}^3$; Tab. 1). The second important factor is that freshly produced ephippia are finely vacuolated, which makes them float at the water surface; these could then be transported by the stream that connects the two lakes, from Vyšné Temnosmrečinské pleso to Nížné Temnosmrečinské pleso. Assessment of ephippia transport by this stream was made during an experiment in September 2003, when plankton nets mounted within the stream collected 787 ephippia in three hours (MARKOVÁ, unpublished data). An addi-

tional factor that may help explain the large difference in ephippia in sediments from these two lakes are the physical characteristics of the catchment areas surrounding them (VTSM: bare rocks 40%, moraine 34%, meadow 26%; NTSM: bare rocks 35%, moraine 15%, meadow 50%); these differences lead to a higher input of nutrients into Nížné Temnosmrečinské pleso compared to Vyšné Temnosmrečinské pleso. Vyšné Temnosmrečinské pleso is also dominated by a high, north-facing cliff, which throws almost half of the lake into shadow.

Ladové pleso and Długi Staw Gąsienicowy

Of the six lakes in this study, Ladové pleso and Długi Staw Gąsienicowy are the smallest in terms of lake area and volume and are highest in altitude (Tab. 1). Both are situated above the tree line and have no sources of water inputs except rainfall and melting snow. Three different sections of ephippia abundance were recorded across the whole core from Ladové pleso. Firstly, the section with the highest abundance of ephippia was from 16 cm and below (range from 103 to 254 ephippia per layer). The second section, with medium abundance was from 4 cm to 15 cm (range from 11 to 68 ephippia per layer) and the third section, with lowest abundance, was from 3 cm to top of core (range from 0 to 8 ephippia per layer; Fig. 5). The core with the lowest abundance of ephippia was from Długi Staw Gąsienicowy, with a total of 20 ephippia. The peak of abundance was recorded at 11 cm (7 ephippia); rest of core had ephippia abundance from 0 to 3 ephippia per layer. *Daphnia* are recently presented in Ladové pleso, but they are not present in Długi Staw Gąsienicowy. The only reference to the occurrence of *D. pulicaria* in Długi Staw Gąsienicowy was made by LITYŃSKI (1917), from samples collected from 1910 to 1915. Since then, the presence of *Daphnia* has never been confirmed in this lake. As we use a dating scale (albeit only as a rough guide) we can expect that the 5 cm layer approximately corresponds with year 1910. The suspected timing of the loss of *Daphnia* from the water column therefore appears to be confirmed by this study.

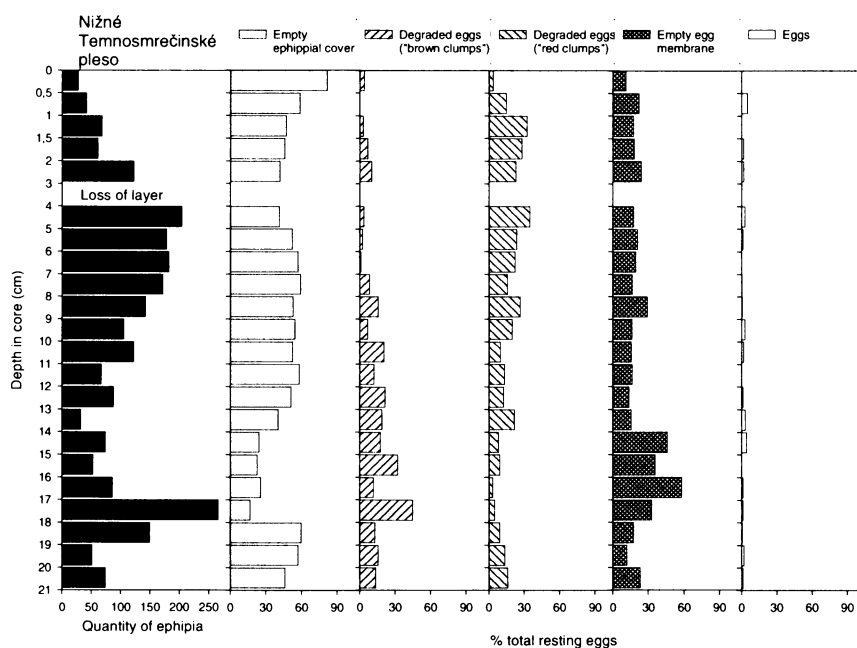


Fig. 3. Ephippia abundance in the sediment core from Nižné Temnosmrečinské pleso. The first column indicates the total quantity of ephippia in each layer, while the other columns show total % of resting eggs in the five different categories (Empty ephippial cover, Degraded eggs ("brown clumps"), Degraded eggs ("red clumps"), Empty egg membrane, Eggs).

Czarny Staw Polski

Czarny Staw Polski is situated on Polish side of the High Tatra Mts. The core from this lake has third highest abundance of ephippia of all six analysed cores (443 ephippia in total). The distribution of ephippia across the whole core has two different sections. The first has very low ephippia abundance from the surface down to 7 cm (0 to 6 ephippia per layer), and the second has an abundance of 15 to 54 ephippia per layer (from layer 7 cm down; Fig. 7).

Nižné Terianske pleso

The ephippia abundance in most of the core layers from Nižné Terianske pleso was very low (from 0 to 10 ephippia per layer) with two marked peaks, one in the layer at 6 cm (20 ephippia), the second in the layer at 19 cm (21 ephippia). Ephippia were completely absent from the upper 6 cm of the core. The reference to *Daphnia* occurrence in Nižné Terianske pleso again comes from MINKIEWICZ (1917), who relied on data from WIERZEJSKI (1882, 1883); DADAY (1896) and LITYŃSKI (1913). As we used a sedimentation rate for the core used in this study as a rough guide, the last record of ephippia present comes from the end of the 19th century, which roughly corresponds with the last record of *Daphnia* being present. Lack of ephippia in the upper layers of the core could also be due to low abundance of *Daphnia* in the lake combined with the sampling effect (i.e. a patchy distribution of resting eggs in the sediment or limited sample size).

For classification of physical condition of ephippia categories 1 to 5 were established, ranked in order of

their usability in later genetic analyses of ancient *Daphnia* populations (Fig. 2). The most common category was category 1 (Empty ephippial covers), which was found in almost every layer. Categories 2 (Degraded "brown clumps"), 3 (Degraded "red clumps") and 4 (Empty egg membrane) were present in most layers from all lakes except Vyšné Temnosmrečinské pleso, where these three categories were not recorded. Eggs (category 5) were the least common category, being absent or found in very low abundance in most layers. All five categories of resting eggs were found in Nižné Temnosmrečinské pleso, but the most abundant category was category 1, which made up around 50% of the total number of ephippia in most core sections. Category 5 was of low abundance, and made up only 0–6% of the total (Fig. 3). Only two categories of ephippia were present in the sediment core from Vyšné Temnosmrečinské pleso. Category 5 was present in only a few core sections (maximum at 1.5 cm; 50% of total), while category 1 was abundant and present in almost all layers (Fig. 4). In Ladové pleso, all five categories were observed, with category 1 being most abundant throughout the core (at least 60% of total counts in most layers) and category 5 being least common (0–6%; Fig. 5). In the Długi Staw Gąsienicowy core, most layers were dominated by only a single category of ephippia (categories 1 to 4). This was also the only core from which category 5 was completely absent (Fig. 6). In lakes Czarny Staw Polski and Nižné Terianske pleso the most abundant categories of resting eggs were categories 1 (30–100% of total ephippia in each layer) and 2 (15–100%). Again, category 5 had low abundance with

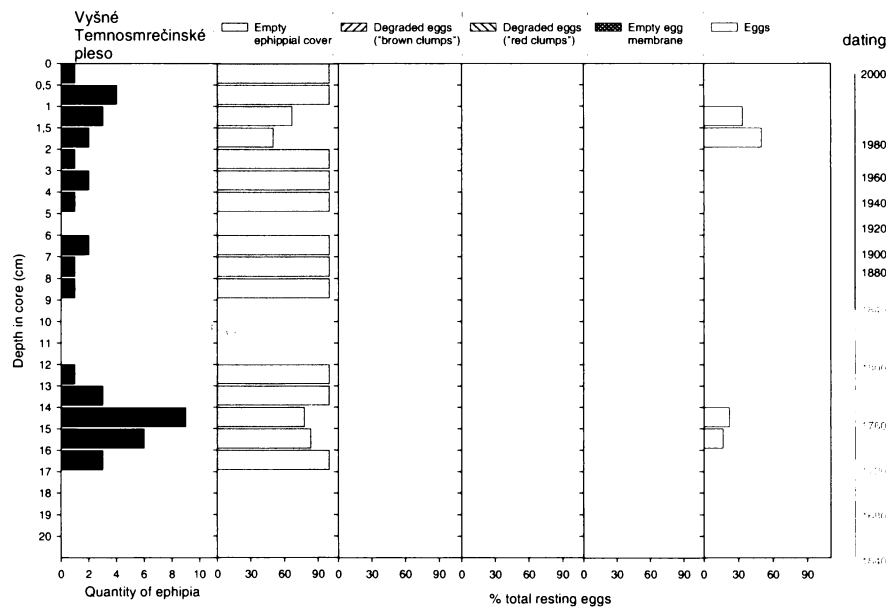


Fig. 4. Ephippia abundance in the sediment core from Vyšné Temnosmrečinské pleso. The first column indicates the total quantity of ephippia in each layer, while the other columns show total % of resting eggs in the five different categories (Empty ephippial cover, Degraded eggs (“brown clumps”), Degraded eggs (“red clumps”), Empty egg membrane, eggs). The dating scale is intended only as a rough estimate.

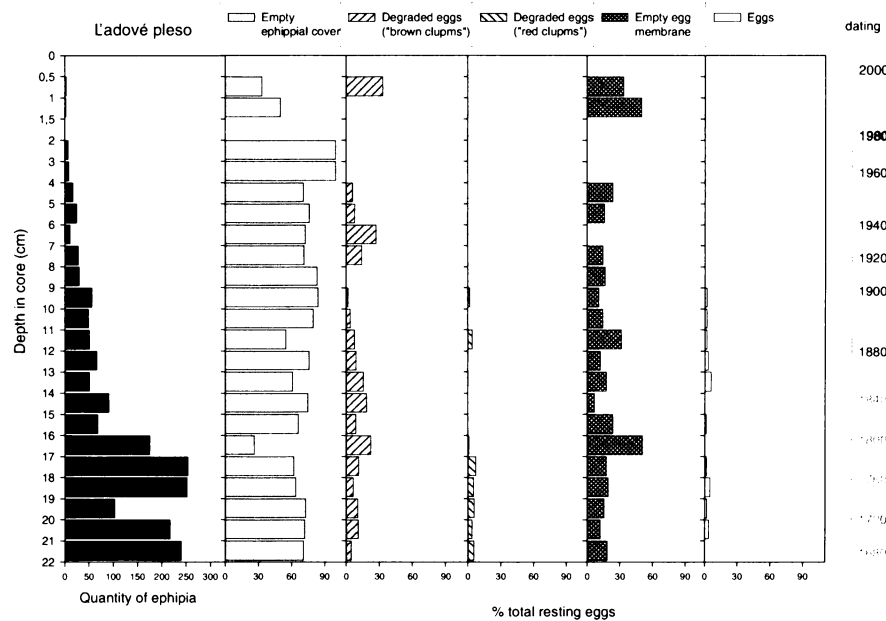


Fig. 5. Ephippia abundance in the sediment core from Ladové pleso. The first column indicates the total quantity of ephippia in each layer, while the other columns show total % of resting eggs in the five different categories (Empty ephippial cover, Degraded eggs (“brown clumps”), Degraded eggs (“red clumps”), Empty egg membrane, Eggs). The dating scale is intended only as a rough estimate.

maximum totals found at 1.5 cm in the Czarny Staw Polski core (50% of total) and at 13 and 16 cm for Nižné Terianske pleso 50% of total; Figs 7, 8).

Sedimentation rates are known for four of the six lakes in this study (VTSM, LadPl, DlugiSt and NTR; Figs 4–6, 8); (NTR data from ŠPORKA et al., 2002; NTR, VTSM, LadPl and DlugiSt data

from APPLEBY & PILIPOSIAN, 2006). In order to get more accurate sedimentation rates applicable to our data we used mean values of sedimentation rate from different cores. For Nižné Terianske pleso and Ladové pleso the mean sedimentation rate calculated from two (TERI 93/2 and TERI 96/7) and six cores (LADO00/1, LADO00/2, LADO00/2, LADO003,

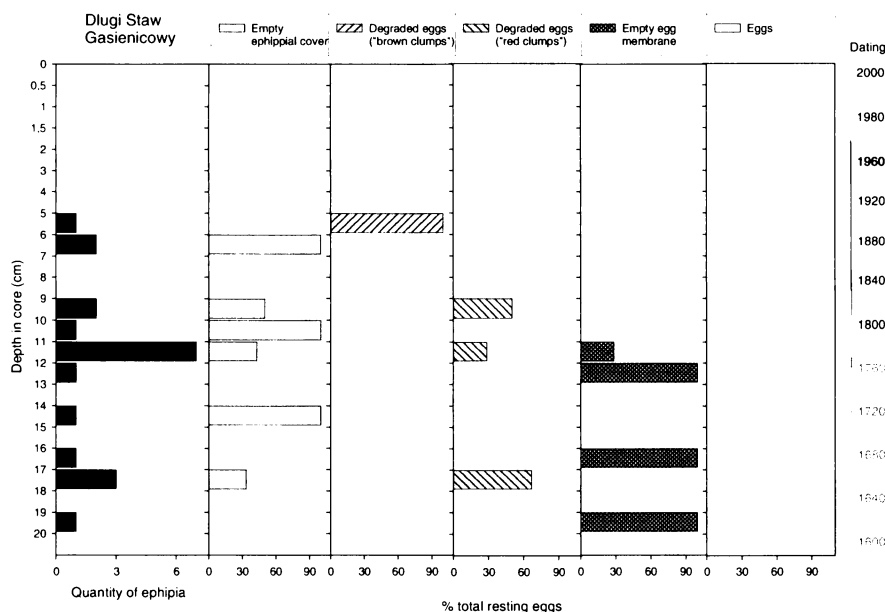


Fig. 6. Ephippia abundance in the sediment core from Długi Staw Gąsienicowy. The first column indicates the total quantity of ephippia in each layer, while the other columns show total % of resting eggs in the five different categories (Empty ephippial cover, Degraded eggs ("brown clumps"), Degraded eggs ("red clumps"), Empty egg membrane, Eggs). The dating scale is intended only as a rough estimate.

LADO00/4, LADO00/5, LADO00/6), respectively (APPLEBY & PILIPOSIAN, 2006). As these rates were based on different cores than those used in this study, these figures should only be used as an approximate guide where the dating scales are intended only as a rough estimate (Figs 4-6, 8).

Changes in genetic composition of *Daphnia* populations

Only resting eggs of category 5 recovered from sediment cores were used for genetic analyses. In total only 94 resting eggs from six High Tatra cores correspond to category 5; in contrast to cores from Lake Belauer See, where an enormous number of *Daphnia* resting eggs are preserved chronologically in the sediment (LIMBURG & WEIDER, 2002). The 94 resting eggs analysed by genetic methods corresponds to 1.9% of the total ephippia count (4,948 ephippia) obtained from six High Tatra lakes sediments. The successful hatching of most *Daphnia* resting eggs is restricted to ca. 60-70 years, or less (WEIDER et al., 1997; CÁCERES, 1998; HAIRSTON et al., 1999), which approximately corresponds to layers 0 to 5 cm in the High Tatra cores. A total of just 17 ephippia from six cores correspond to be 60-70 years old; in cores from lakes Długi Staw Gąsienicowy, Ładové pleso and Nižné Terianske pleso resting eggs of this age were not found. The successfully analysed ancient resting eggs from the Belauer See correspond to an age range of ca. 159-195 years (LIMBURG & WEIDER, 2002), where just 33 resting eggs from six High Tatra cores correspond to this age (0 resting eggs from DługiSt and just 1 resting egg from NTR). Although sediment area picked up

with the Kajak corer from High Tatra lakes sediments is same as with the Ekman corer used on the Belauer See (ca. 28.3 cm³), the sedimentation rate of these lakes is markedly different. Belauer See has been classified as hypereutrophic during the past several decades (LIMBURG & WEIDER, 2002), whereas all surveyed High Tatra lakes are oligotrophic. While an age range of ca. 159-195 years corresponds to a depth range of 1-1.30 m from Belauer See, in High Tatra lakes sediments same age range represent just 0.064 m to 0.08 m for Nižné Terianske pleso; 0.13 m to 0.16 m for Ładové pleso; 0.087 m to 0.11 m for Długi Staw Gąsienicowy and 0.095 m to 0.12 m for Vyšné Temnosmrečinské pleso of depth range (APPLEBY & PILIPOSIAN, 2006).

DNA was extracted by appropriate methods for ancient DNA: the Chelex method, the Invisorb extraction KIT for ancient DNA and the IsoQuick Nucleic Acid Extraction Kit. During all extraction work with DNA from sediments, rules for work with ancient DNA were followed closely. Conditions for PCR reactions were optimized using fresh ephippia. The age of eggs used in genetic analyses ranged from 10 to almost 500 years (18 cm in the NTR core). Two microsatellite markers employed in this study readily amplified DNA from fresh ephippia. However, despite extensive efforts, neither of these markers was successful in amplifying ancient DNA from resting eggs collected from sediment cores. The PCR success of ancient microsatellite sequences was considerably reduced also in the work of LIMBURG & WEIDER (2002), when compared with results obtained from fresh resting eggs, but sediments from the Belauer See contain an enormous number of

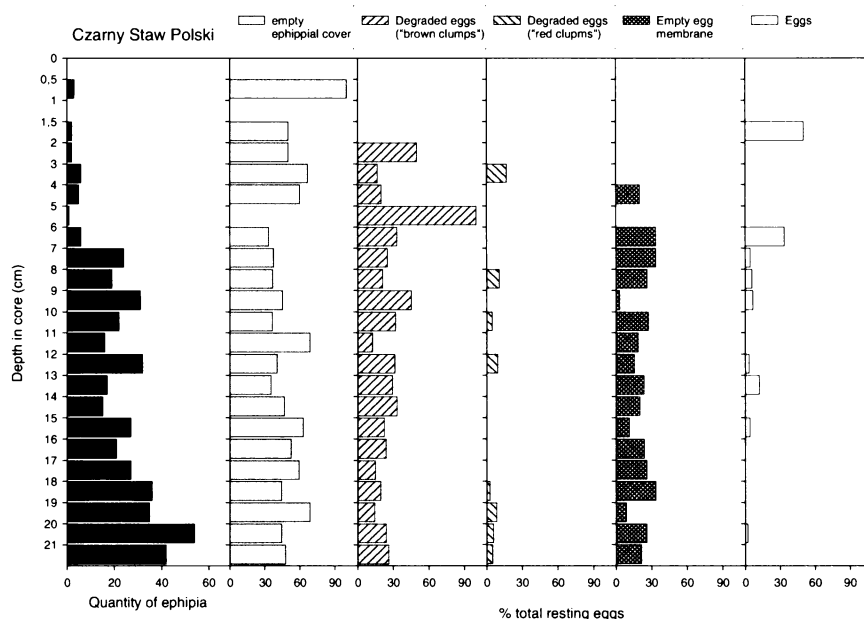


Fig. 7. Ehippia abundance in the sediment core from Czarny Staw Polski. The first column indicates the total quantity of ehippia in each layer, while the other columns show total % of resting eggs in the five different categories (Empty ehippial cover, Degraded eggs ("brown clumps"), Degraded eggs ("red clumps"), Empty egg membrane, Eggs).

Daphnia resting eggs and so they were successful in amplifying microsatellite alleles from more than 100 ancient resting eggs. Unfortunately, the quality of DNA extracted from High Tatra ancient resting eggs does not appear to be of sufficient quality to allow amplification and subsequent genetic analyses.

The changes in genetic composition of *Daphnia* populations through time could not be assessed because of an insufficient number of suitable ehippia and failure in amplifying microsatellite alleles from ancient resting eggs. Although 94 resting eggs were analysed, none of them were useful for microsatellites analyses. The six analysed High Tatra sediments proved to be unsuitable for any genetic analyses.

Influence of environmental changes on resting egg distribution

Atmospheric acid deposition

As noted by ŠPORKA et al. (2002), BATTARBEE et al. (2002) and KOPÁČEK et al. (2004), the High Tatra Mts are one of Europe's regions of highest acid deposition. Gradual acidification of the High Tatra lakes began around 1860. Values of pH and water alkalinity declined rapidly in the 1950s after a century of moderate declines, and reached their minima in the 1980s (KOPÁČEK et al., 2003). Reversal of lake acidification started in the late 1980s and progressed rapidly throughout the 1990s (KOPÁČEK et al., 2004).

In the Nižné Temnosmrečinské pleso core, the striking decline in ehippia abundance from 2–3 cm to the surface could correspond with the high levels of

acidification in 1977–1987 (KOPÁČEK et al., 2004). Although chemical reversal from acidification started in the late 1980s, biological recovery may be substantially delayed with respect to actual deposition rate, as well as due to slow dispersal of species formerly inhabiting the lake (KOPÁČEK et al., 2002).

Lakes Długi Staw Gąsienicowy and Ładové pleso exhibited gradual acidification between 1860 and 1950, and underwent significant changes during the peak of acidification (1950s – 1980s) and its later reduction (1980s onward; KOPÁČEK et al., 2003). Only Długi Staw Gąsienicowy is classed as sensitive to acidification, according to the definition of sensitivity to acidification (FOTT et al., 1994). The other five lakes are classed as non-sensitive (Tab. 1). Lakes with a smaller area, such as Długi Staw Gąsienicowy and Ładové pleso, will generally be more sensitive to acidification and the effects will be exhibited earlier than in large lakes (KOPÁČEK et al., 2004). Recovery of the original status of small lakes can also take longer, particularly in lakes in which inputs are limited to rainfall and meltwater. Lakes Długi Staw Gąsienicowy and Ładové pleso have a very low nutrient input, because the main part of their catchment consists of bare rock and moraine (ŁadPl: catchment area 13 ha, bare rocks 32%, moraine 53%, meadow 15%; DługiSt: catchment area 65 ha, bare rocks 44%, moraine 27%, meadow 29%; KOPÁČEK et al., 2000). Rocky lakes should be the most acidic among the alpine lakes (KOPÁČEK et al., 2000).

The curve of ehippia abundance in the Czarny Staw Polski indicates very low fluctuation between depths of 20 and 7 cm (from 15 to 36 ehippia pro

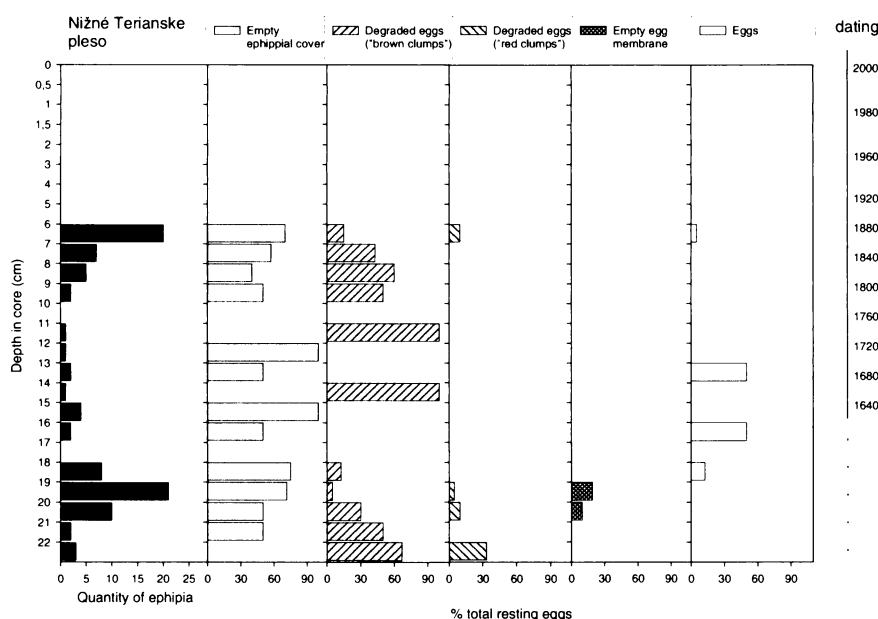


Fig. 8. Ephippia abundance in the sediment core from Nizné Terianske pleso. The first column indicates the total quantity of ephippia in each layer, while the other columns show total % of resting eggs in the five different categories (Empty ephippial cover, Degraded eggs ("brown clumps"), Degraded eggs ("red clumps"), Empty egg membrane, Eggs). The dating scale is intended only as a rough estimate.

layer). It suggests fairly steady population size during that time. The marked decline in ephippia abundance (from 6 cm to the top), may correspond with two noted events (acidification and fish introduction), which took place in the High Tatra area at the end of the 19th century. However, as there are no data on sedimentation rates in the Czarny Staw Polski, we can only roughly estimate, base on known sedimentation rates of surrounding lakes, that the 6 cm layer corresponds with the end of 19th century and gradual acidification which began in the 1860s in the High Tatra area.

The complete absence of ephippia from the upper 6 cm of the core from Nizné Terianske pleso can be also connected with the beginning of gradual acidification of the High Tatra Mts at the end of the 19th century.

The distribution curve of ephippia from Vyšné Temnosmrečinské pleso does not show as strong effect of acidification event as are evident in other High Tatra cores, probably because of very low ephippia abundance across the whole Vyšné Temnosmrečinské pleso core (0 to 9 ephippia per layer).

Fish introduction

The majority of mountain lakes in the Tatra Mts remained fish-free until the end of the 19th century, when stocking with brook charr (*Salvelinus fontinalis* Mitchill, 1815) and trout species (mainly three species, with *Salmo trutta* morph *fario* L., 1758 being most abundant) became very common (DAWIDOWICZ & GLIWICZ, 1983).

Of the six lakes involved in this study, Czarny Staw Polski is the only one in which fish are known

to have been introduced, although the exact date is unknown. The marked decline of ephippia abundance at 6 cm may be due to the introduction of fish to the lake. The introduction of salmonids to the mountain lakes caused a drastic change in their zooplankton communities (GLIWICZ & ROWAN, 1984; CAMMARANO & MANCA, 1997). A similar situation with an identical trend in the ephippia abundance curve was described by GLIWICZ (1980, 1985); following fish introduction to the Zielony Staw Gąsienicowy, where the quantity of ephippia in sediment cores fell from 120 to 10 per cm⁻³. Due to its large size, *D. pulicaria* is extremely vulnerable to selective predation by charr (GLIWICZ 1963, 1980, 1985; DAWIDOWICZ & GLIWICZ, 1983). Extinction of cladocerans becomes evident a few years after a major stocking (GLIWICZ & ROWAN, 1984).

Disappearance of *Daphnia* from Długi Staw Gąsienicowy could also be linked to fish introduction to the lake. Intensive fish introductions were made to several lakes on Polish side of the Tatra Mts in the 1890s, for example Zielony Staw Gąsienicowy, the neighbouring lake of Długi Staw Gąsienicowy in the Dolina Gąsienicowa valley (GLIWICZ & ROWAN, 1984). However, no records exist to describe any fish introductions to Długi Staw Gąsienicowy.

Changes in climate

The effects of climate on mountain lakes occur mainly through changes in temperature, precipitation and wind regimes that affect duration of snow and ice cover, catchment hydrology and mixing and stratification of the water column. These factors control many biologi-

cal and chemical processes, such as primary production, nutrient cycling, and water column pH, and as such may have great effects on habitat characteristics, biological life cycles and species distribution.

AGUSTÍ-PANAREDA & THOMPSON (2002) and BATTARBEE et al. (2002) presented data for air temperatures from European alpine and arctic lakes for the period 1781 to 1997 AD, which included Nižné Terianske pleso. These studies indicate that temperatures of area in which our study lakes are situated began to rise towards the end of the 18th century reaching some of the highest recorded over the measurement period. From 1800 to 1997 a general cooling trend was recorded (AGUSTÍ-PANAREDA & THOMPSON, 2002). Using the sedimentation rates and ephippia deposition patterns from the Vyšné Temnosmrečinské pleso as a rough guide, the highest abundance (at 14 cm in VTSM and at 17 cm in NTSM) appears to correspond to a warm period around 1790–1800 (AGUSTÍ-PANAREDA & THOMPSON, 2002). In the core from Ladvé pleso the highest abundance of ephippia was found deep within the core (17 cm), which could also correspond to the warm period in the 1790s (Fig. 5). Similarly, in Nižné Terianske pleso, Czarny Staw Polski and Długi Staw Gąsienicowy the highest abundance of ephippia could correspond to a warm period around the years 1790–1800.

Pasturing

Sheep pasturing is often associated with increases of nutrient inputs to the lakes. Sheep pasturing was most intensive in the beginning of the 19th century in the High Tatra area, declined continuously from middle of 19th century, and was finally forbidden after 1949 and 1955 when the Tatra Mountains were designated as a national park in Slovakia and Poland, respectively (VOLOŠČUK, 1994). Records on sheep pasturing in the Temné Smrečiny valley do exist (VOLOŠČUK, 1994). The considerable increase in ephippia abundance from 10 cm to 4 cm in Nižné Temnosmrečinské pleso could be connected with influence of pasturing on resting eggs abundance.

Possibility of natural reestablishing of *Daphnia* populations

In total just 17 resting eggs from six High Tatra cores were recovered from the layers corresponding to ca. 60–70 years, the age restricted to the successful hatching of most *Daphnia* resting eggs. The DNA from all 17 resting eggs used in genetic analyses was found to be degraded, and the viability of these resting eggs is therefore similarly negligible.

In the three lakes (NTR, DługiSt, CzarSt), where *Daphnia* populations have recently disappeared, there appears to be very little possibility of autogenous restoration of *Daphnia* populations from the resting egg banks in the High Tatra sediments. However, natural repopulation of *Daphnia* in these lakes may be possi-

ble by colonisation from neighbouring lakes in which *Daphnia* still exist, although it is unknown how likely this is.

Conclusions

A total 4,948 ephippia from six High Tatra sediments were analysed in this study. The highest abundance of ephippia (all categories) across the whole core was recorded in Nižné Temnosmrečinské pleso (2,444 ephippia in total), the second highest abundance was in the core from Ladvé pleso (1912 ephippia in total) and the third was in the core from Czarny Staw Polski 443 ephippia in total). Very low total numbers of ephippia were found in cores from Nižné Terianske pleso, Vyšné Temnosmrečinské pleso and Długi Staw Gąsienicowy; 89, 40 and 20 ephippia, respectively.

In order to classify physical condition of ephippia, for their use in later genetic analyses of ancient *Daphnia* populations, five categories were established. The most common category was category 1 (Empty ephippial covers), which was found in almost every layer of all six analysed cores. Less common were resting eggs from categories 2 (Degraded eggs “red clumps”), 3 (Degraded eggs “brown clumps”) and 4 (Empty egg membrane) and the least common was category 5 (Eggs).

The 94 resting eggs from the category 5 were used in genetic analyses, with ages ranging from 10 to almost 500 years. DNA from resting eggs was extracted by three different methods appropriate for ancient DNA. In this study two microsatellite markers were employed, but neither of them was successful in amplifying microsatellite alleles of ancient DNA from resting eggs. The quality of DNA extracted from ancient resting eggs from High Tatra sediment cores does not appear to be of sufficient quality for genetic analyses. The poor condition and low number of resting eggs from the six High Tatra cores in this study unfortunately did not allow the reconstruction of *Daphnia* population genetic structure across long term time periods.

Environmental changes such as atmospheric acid deposition, fish introduction and partly also changes in climate and pasturing are likely to have influenced the distribution curves of resting eggs. In this way, resting eggs can be used as an indicator of past changes to lake environments. Ephippia abundance in cores from the six lakes provides a record of the gradual water acidification in the period 1860–1950, with significant changes during the period of greatest acidification, 1950–1980s. Acidification has eliminated the cladoceran *D. pulicaria* from several High Tatra lakes lying above the tree line. The dramatic change in ephippia abundance in the sediment from Czarny Staw Polski is probable due to the introduction of fish. The peaks recorded in the ephippia abundance curve of High Tatra sediment cores may also corresponded with the warm period that occurred in the 1790s. The increase of ephippia abundance on

the beginning of the 19th century in the High Tatra area can be also associated with sheep pasturing and subsequent increases of nutrient inputs to the lake.

Low numbers of resting eggs, together with their generally poor physical condition suggested that there is very little possibility of population recovery from resting eggs present in the lake sediments. In lakes that have lost their original populations, natural repopulation of *Daphnia* may be possible by colonization from neighbouring lakes in which *Daphnia* still exist.

Resting eggs from six High Tatra cores does not allow one to study *Daphnia* population genetic structure, across long term time periods.

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Chapter 5

General conclusions

Conclusions

The results of chapters 2 and 3 show that the phenotypic divergence between *D. pulicaria* morphotypes is related to genetic differentiation. Populations of different morphotypes did not share mitochondrial nor nuclear genotypes and this association between pigmentation and molecular markers was complete in my datasets. Furthermore, the melanin and carotenoid pigmented morphotypes were included in the Eastern Nearctic lineage and not with the unpigmented morphotype in the European lineage. This deep phylogenetic history, the genetic differentiation between syntopic populations and their life history divergence demonstrate that the two lineages represent different species. I therefore conclude that the variation at the phenotypic traits (pigmentation and life history) in European *D. pulicaria* is primarily determined by ancestry and most likely represents adaptive divergence of evolutionarily divergent species, rather than environmentally induced phenotypic plasticity of a single species. The phenotypic difference between species is particularly pronounced in the High Tatra where it contributes to their resource partitioning and therefore coexistence. The melanin and carotenoid pigmentation then represent different adaptations that the Eastern Nearctic lineage evolved to cope with the ultraviolet light stress.

Phylogenetic analyses in chapter 2 further demonstrated that this lineage is of recent origin in Europe, most likely as the result of a long-distance dispersal from the North America. My results thus give substantial support to the hypothesis that the Atlantic Ocean has not been a barrier to postglacial dispersal for passively transported organisms.

From the results of chapter 4 it can be concluded that egg banks in the ultra-oligotrophic lakes in the High Tatra contain very low numbers of resting eggs (a total of 94 recovered eggs compared to thousands of eggs available from a hypereutrophic Konstaz Lake), which are in poor physical condition and yield no amplifiable DNA, making tracking long-term genetic shifts unfeasible. Autochthonous recovery of extinct genotypes is therefore unlikely, which underscores the importance of immigration and local selection in the spatial patterning of genotypes observed among lakes (chapter 3).

Apparently, a considerable part of the evolutionary diversity among cladoceran crustaceans has been overlooked in morphological taxonomies or interpreted as a variation of a single species and attributed to environmental factors. This finding should prompt a taxonomic revision and it has important implications for the use of *Daphnia* as a model organism.