Master thesis

Daphnia hybridization in canyon-shaped reservoirs

(Hybridizace perlooček rodu *Daphnia* v přehradních nádržích)

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ABSTRACTS

Part 1

Canyon-shaped reservoirs, characteristic by elongated morphology and by ecologically diverse conditions along both horizontal and vertical reservoir axes, are good model systems for ecological studies of zooplankton communities. Presence of ecological gradients improves habitat differentiation and may facilitate coexistence of related species. Here we provide detailed study of genetic structure of the *Daphnia longispina* complex inhabiting three canyon-shaped reservoirs. Using 12 microsatellite loci, we assessed taxonomic and genetic structure of assemblages composed by populations of *D. galeata, D. cucullata*, *D. longispina* and their interspecific hybrids. We focused on detailed taxon determination and on patterns of hybridization and introgression. High number of distinct hybrid genotypes in the samples suggested high frequency of hybridization; despite this, later generation hybrids were rare.

In one reservoir inhabited by a single species, *D. galeata*, we also tested if environmental gradients may cause intra-population genetic diversification similar to spatial differentiation in species composition. Subpopulations along horizontal gradient in the reservoir, as well as those in different layers of the stratified water column, were significantly genetically differentiated. We propose that this structuring of *D. galeata* population is caused by local adaptation and clonal selection facilitated by presence of ecological gradients. On the other hand, we did not observe such intraspecific differentiation in a reservoir inhabited by several taxa of the complex, although *D. galeata* was present along the whole reservoir.

Part 2

Daphnia galeata, *D. longispina* and *D. cucullata* (Crustacea: Cladocera) are closely related species often producing interspecific hybrids in natural populations. Common inconsistencies among species-specific markers using for their determination were traditionally attributed to the complexity of their relationships and to the occasional introgression. In order to test this hypothesis, we used several different approaches for the taxon identification. Using allozyme electrophoresis and ITS-RFLP, we identified more than 1200 individuals from ten localities situated in the Czech Republic as parental species or hybrids. 444 animals were additionally analyzed and identified by analysis of 12 microsatellite loci. The data set was further extended by samples from 19 sites across the whole Europe, in which the taxon was estimated using microsatellites and ITS-RFLP. Results of microsatellite analysis corresponded well with allozymes. However, two sites from the

Czechia and three sites from other European countries exhibited consistent discrepancies between ITS-RFLP and other markers. Although some marker disagreement could have been caused by occasional introgression, more serious deviations observed in ITS-RFLP more likely suggest a long-term maintenance of introgressed alleles in genomes of parental species, in which evolutional mechanisms such a gene conversion and meotic drive could support the maintenance of "alien" alleles.

Finally, we compared data from molecular markers with identification based on phenotypic characteristics of photographed animals for a randomly selected set of 240 *Daphnia* individuals, and quantitatively evaluated the body shape variation by geometric morphometrics. Morphological identification, at least when based on photographs, gave substantially worse results than any molecular method. The least successful was differentiation between *D. galeata*, pelagic *D. longispina*, and their hybrids; these taxa showed particularly high degree of overlap of their body shape.

ABSTRAKTY

Část 1

Korytovité nádrže jsou charakteristické svým protáhlým tvarem a výskytem rozličných ekologických podmínek na podélné i svislé ose. Rozdílnost habitatů v různých částech nádrže umožňuje soužití většího množství druhů a korytovité nádrže se proto hodí jako modelový systém pro studium ekologie zooplanktonu. V této práci se zabýváme studiem detailní struktury společenstev perlooček z druhového komplexu *Daphnia longispina* ve třech korytovitých nádržích. Za pomoci dvanácti mikrosatelitových lokusů jsme popsali detailní genetickou strukturu populací druhů *D. galeata, D. cucullata*, *D. longispina* a jejich mezidruhových hybridů, přičemž jsme se zaměřili především na determinaci a výskyt hybridů, zpětných kříženců a jedinců nesoucí známky introgrese. Přestože k hybridizaci mezi těmito druhy dochází relativně často, což také potvrdila přítomnost mnoha geneticky odlišných kříženců v jednotlivých populacích, hybridi dalších generací byli poměrně vzácní.

Na vzorcích z jedné z nádrží obývané pouze druhem *D. galeata* jsme rovněž testovali, zda přítomnost ekologických gradientů může způsobit vnitrodruhovou genetickou diferenciaci podobně jako tomu je na úrovni druhů. Subpopulace z jednotlivých odběrových míst se mezi sebou skutečně signifikantně geneticky lišily. Domníváme se, že příčinou by mohla být adaptace na lokální podmínky a klonální selekce.

Část 2

Daphnia galeata, *D. longispina* and *D. cucullata* (Crustacea: Cladocera) jsou blízce příbuzné druhy perlooček, které mezi sebou v přirozených podmínkách běžně hybridizují. Jelikož byli dokumentováni i hybridi dalších generací a jedinci nesoucí známky introgrese, má se za to, že hybridizace a horizontální přenos genů jsou hlavní příčinou nesouladu některých druhově specifických markerů používaných k jejich identifikaci. S použitím analýzy mikrosatelitů, alozymové elektroforézy a ITS-RFLP jsme tuto hypotézu testovali. Taxon jsme určili u více než 1200 jedinců z deseti různých lokalit z České republiky alespoň dvěma zmíněnými metodami. 444 jedinců bylo identifikováno s použitím všech tří metod. Metodou ITS-RFLP a analýzou mikrosatelitů byli navíc určeni jedinci z dalších devatenácti odběrových míst z celé Evropy. Výsledky analýzy mikrosatelitů se velmi dobře shodovaly s výsledky alozymové elektroforézy. U metody ITS-RFLP byla úspěšnost výrazně nižší. Přestože by se některá chybná určení dala vysvětlit současnou introgresí, odchylky pozorované u metody ITS-RFLP jsou pravděpodobně mnohem staršího původu. Cizí alely

mohly být v genomu rodičovských druhů šířeny mechanismy jako je genová konverze nebo meiotický tah (*meiotic drive*).

Na závěr jsme porovnali výsledky molekulárních metod s výsledky určení druhů na základě morfologických znaků. Determinace na základě morfologie se ukázala být k tomuto účelu nejméně spolehlivou metodou. Důvodem může být velká morfologická podobnost mezi jedinci různých druhů, zvláště pak druhů *D. galeata* a *D. longispina*. Tuto domněnku potvrdily i výsledky analýzy tvarů.

CONTENTS

GENERAL INTRODUCTION

When I was in my first year of the bachelor study, my parents asked me why I decided to be a hydrobiologist. I told them that the world would always need people with knowledge of aquatic environment. I also said that freshwater water bodies are full of exciting animals such as fish, crayfish and sponges and that I would NEVER have to spend my whole free time in a laboratory if I studied hydrobiology. After having said that, I spent four years sitting near the flow-box with a pipette in my hand, studying ecology of organisms which are not larger than a pinhead and of which most people never heard. However, I learnt that importance and beauty of the organism does not correlate with its size and with its popularity in human community.

* * *

In spring 2004, I joined a small team together with my supervisor Adam, with Jaromír Seďa and Jiří Macháček from the Institute of Hydrobiology, Czech Academy of Sciences, and with Ivana Vaníčková from the Department of Ecosystem Biology, Faculty of Sciences, University of South Bohemia, investigating distribution of three Palearctic *Daphnia* species - *D. galeata*, *D. cucullata* and *D. longispina* and their hybrids on ecological gradients in reservoirs. All three closely related species hybridize with each other in the natural ecosystems and their hybrids are commonly found living in the syntopy with one or both parental species. Hybrid populations are maintained by parthenogenesis and the hybrids may occur in densities similar to, or exceeding, those of their parental species. Thus, hybrids can become equivalent competitors to the pure species.

Canyon-shaped reservoirs, which have been studied already for decades by limnologists from the Institute of Hydrobiology in České Budějovice, were chosen as model systems in the project. These reservoirs are artificial water bodies with elongated morphology and depth increasing towards to the dam. In contrast to the typical lakes, they are characteristic by point source of nutrients (river inflow), which contributes to maintenance of relatively strong longitudinal ecological gradients. The presence of stable gradients may improve habitat differentiation and coexistence of zooplankton species, including different *Daphnia*. Unlike other standing waters, spatial differentiation of species composition is observable both on vertical gradients in deep parts of reservoirs and along their longitudinal axes.

During the study, we compared results of taxon identification provided simultaneously by two methods available for determination of species and interspecific hybrids in *Daphnia* -

by allozyme electrophoresis and by ITS-RFLP. Some of the results, already included in my bachelor thesis, were later published in cooperation with Norwegian and Swiss colleagues (Skage et al. 2007, *Hydrobiologia*). The publication also included a new ITS-RFLP protocol, which was developed to avoid a flaw in the originally proposed method.

Subsequently, I used this improved ITS-RFLP method in my work, and contributed data to a recently published paper summarizing main results of the whole project – spatial distribution of taxa within canyon-shaped reservoirs in time and space (Petrusek et al. 2008b, *Philosophical Transactions of the Royal Society B*). Data describing fit between allozymes and ITS-RFLP are also included in this thesis (Chapter 2).

In order to obtain data describing detailed structure of *Daphnia* assemblages from three selected reservoirs, part of the lab work for my thesis was elaborated in the laboratory of the Department of Ecology and Evolution of the Goethe University, Frankfurt am Main, in cooperation with Anne Thielsch, Robert Kraus, Nora Brede and Klaus Schwenk. We used set of twelve microsatellite markers for this purpose and the original dataset describing roughly the spatial distribution of species and interspecific hybrids was thus upgraded. Based on microsatellite data, we were also able to assess clonal diversity within separate taxa and frequency of hybridization. Microsatellite analysis also allowed us to investigate intraspecific spatial distribution and clonal selection on the gradients of a reservoir in which only a single species, *D. galeata*, was present.

My thesis is written in a form of two manuscripts. We will eventually attempt to publish them, although it is likely that in a different form. Hypotheses formulated in the thesis try to answer the questions we asked at the beginning of the project, as well as to resolve problems we had to face. Although I discussed my ideas with the co-authors, none of them except my supervisor could comment the thesis chapters after they have been written. Thus, some conclusions, and possibly even the focus of the manuscripts, may be changed and further analyses will be done before submitting any results for the publication. Please, note that parts discussing the results reflect my personal opinion, and do not necessarily agree with the views of other co-authors.

DETAILED GENETIC ARCHITECTURE OF SPATIALLY STRUCTURED HYBRIDIZING POPULATIONS OF THE *DAPHNIA LONGISPINA* COMPLEX

(Chapter 1)

with

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ABSTRACT

Canyon-shaped reservoirs, characteristic by elongated morphology and by ecologically diverse conditions along both horizontal and vertical reservoir axes, are good model systems for ecological studies of zooplankton communities. Presence of ecological gradients improves habitat differentiation and may facilitate coexistence of related species. Here we provide detailed study of genetic structure of the *Daphnia longispina* complex inhabiting three canyon-shaped reservoirs. Using 12 microsatellite loci, we assessed taxonomic and genetic structure of assemblages composed by populations of *D. galeata, D. cucullata*, *D. longispina* and their interspecific hybrids. We focused on detailed taxon determination and on patterns of hybridization and introgression. High number of distinct hybrid genotypes in the samples suggested high frequency of hybridization; despite this, later generation hybrids were rare.

In one reservoir inhabited by a single species, *D. galeata*, we also tested if environmental gradients may cause intra-population genetic diversification similar to spatial differentiation in species composition. Subpopulations along horizontal gradient in the reservoir, as well as those in different layers of the stratified water column, were significantly genetically differentiated. We propose that this structuring of *D. galeata* population is caused by local adaptation and clonal selection facilitated by presence of ecological gradients. On the other hand, we did not observe such intraspecific differentiation in a reservoir inhabited by several taxa of the complex, although *D. galeata* was present along the whole reservoir.

Key words: microsatellite analysis, genotypic richness, hybridization, spatial differentiation, clonal selection

INTRODUCTION

Members of the genus *Daphnia* (Crustacea: Cladocera) are small planktonic crustaceans inhabiting various water bodies across all continents. Their reproductive mode, cyclical parthenogenesis, allows experimental work with easy-to-maintain clonal lineages. *Daphnia* therefore has been used as model organism in many ecological and evolutionary studies. During the last two decades, interspecific hybrids between some *Daphnia* species have been discovered, and especially within the *D. longispina* species complex, hybridization became popular and intensively studied topic (e.g. Wolf 1987, Spaak & Hoekstra 1995, Spaak et al. 2004).

Closely related Palearctic species of this complex, *Daphnia galeata*, *D. cucullata,* and *D. longispina* (known also as *D. hyalina*; see Petrusek et al. 2008a) are known to hybridize with each other (Wolf & Mort 1986, Schwenk 1997, Jankowski & Straile 2004). Their hybrids can reproduce parthenogenetically and they may therefore maintain large populations during the season of favorable conditions. Classical models considering hybrids as low-fitness organisms unable to compete with parental species (e.g. tension zone model, Barton & Hewitt 1985) thus cannot be used to describe coexistence of hybrids and parental species in *Daphnia*. It has been also shown that at least some F_1 hybrids are able to reproduce sexually and later generation hybrids may occur (e.g. Schwenk & Spaak 1995, Taylor & Hebert 1993, Spaak et al. 2004). However, constrains regulating intra-specific breeding and mechanisms preventing horizontal gene-flow (introgression) seem to exist within the *D. longispina* species complex. For instance, hatching success and survival rate of interspecific hybrids is lower than in parental species (Schwenk et al. 2001). Due to this, backcrossing is relatively rare (Spaak 1996, Jankowski & Straile 2004) and the horizontal gene-flow is not frequent enough to fuse gene-pools of parental species. It has been therefore proposed that reproductive isolation effectively exists among hybridizing species within the complex (Keller et al. 2007). However, other studies suggested that the evolution of the complex could have a reticulate character (Schwenk 1997, Gießler 1997).

Ecological studies, usually investigate short-term coexistence of hybrids and parental species, impact of environmental factors on success and stability of hybrid zones and local population dynamics. Traditional hybrid zones models assume repetitious hybridizing events in the zone of overlap, which compensates handicap of lower fitness (e. g. Barton & Hewitt

1985, Moore 1997, Harrison 1986). However, hybrid populations in the *D. longispina* species complex can be maintained by asexual reproduction so the grow rate of hybrids is comparable to the parental species. In such cases, structure of the assemblage is mostly influenced by exogenous factors. The Temporal hybrid superiority hypothesis (Spaak & Hoekstra 1995) thus has been proposed to explain coexistence of hybrids and their parental species by the fluctuations of environmental conditions, which may temporally favor hybrid genotypes. Size-selective fish predation pressure (Spaak & Hoekstra 1997, Declerck & De Meester 2003, Petrusek et al. 2008b) and food level (Boersma & Vijverberg 1994a, b) has been considered as the most important factors affecting assemblage structure within the water body. However, the coexistence may be conditioned by wide range of additional ecological factors, such a parasitism (Wolinska et al. 2006, 2007), food quality (Seidendorf et al. 2007) or basic element limitation (Sterner & Essen 1994, Wacker & von Elert 2001).

Many laboratory studies investigated how do the environmental factors influence species composition in zooplankton (Boersma & Vijverberg 1994a, b; Spaak & Hoekstra 1997), but studies on natural populations still remain rare. The reason is mostly the impossibility to separate single factor from the others and to standardize field conditions. In addition, pelagic environment is usually very homogenous and does not offer habitats with sufficiently varying conditions. Impact of variation of environmental conditions can be therefore assessed by comparative study of several water-bodies (Keller et al. 2008) or by selection of localities with strong environmental gradients. Substantial environmental gradients exist in thermally stratified water bodies such a temperate lakes, but the distance formed by depth of the water- body usually constitutes no barrier for *Daphnia* vertical migration. Although the space for habitat differentiation is small, it has been shown that different depth-preference of certain *Daphnia* genotypes may exist (Seda et al. 2007a).

In our previous studies (Seda et al. 2007b, Petrusek et al. 2008b), canyon-shaped reservoirs appeared to be an appropriate model system for studying patterns of coexistence in the *Daphnia*. Canyon-shaped reservoirs are artificial water bodies characterized by elongated canyon-like morphology and by depth increasing along the longitudinal axis. In contrast to lakes, the river inflow located in upper part of the reservoir is a main resource of nutrients. Towards dam the accessibility of nutrients continuously decreases and horizontal gradient in nutrient distribution is formed. Density and distribution of phytoplankton community consequently follows nutrient availability. Due to significantly higher affinity of fish to upper location (Vašek & Kubečka 2004), strong horizontal gradient of fish density is observable in the same direction. At least two horizontal gradients crucial for *Daphnia* distribution are

therefore conspicuous – gradient of fish predation pressure and gradient of food availability. In time of summer stratification, similar situation may arise on the vertical profile. Whilst epilimnion is relatively rich for food and well lit, hypolimnion offers dark refuge against fish predation but low food concentrations.

It has been proposed that existence of such spatial heterogeneity may improve conditions for the coexistence of several *Daphnia* species (Seda et al. 2007b, Petrusek et al. 2008b) and may facilitate hybridization among them. As showed in study by Seda et al. (2007b), spatial distribution of species of the *D. longispina* complex is not random within the reservoir and species show clear preferences corresponding to their ecological characteristics. Furthermore, taxon distribution within the reservoirs seems to be stable over consequent seasons showing that spatially differentiated populations are re-formed after the winter bottleneck (Petrusek et al. 2008b). However, establishment of hybrid genotypes seems to more sensitive to fluctuations of environmental conditions than that one of parental species (Petrusek et al. 2008b), possibly because interspecific hybrids are characterised by lower hatching success (Schwenk et al. 2001, Keller et al. 2007).

Although the changes in species composition depending on particular environmental conditions and selective forces are well documented in *Daphnia*, our knowledge about detailed population structures of hybridizing species and about their intra-population relationships is limited. In this study we provide detailed structural study of *Daphnia* assemblages inhabiting three canyon-shaped reservoirs differing in morphology and strength of environmental gradients, including a single-species locality as well as those with three coexisting species of the *D. longispina* complex. Using several microsatellite loci (Brede et al. 2006), we assess species composition and spatial distribution of taxa within each reservoir, including a detailed identification of hybrid classes among recombinant genotypes. In contrast to our previous studies (Seda et al. 2007b, Petrusek et al. 2008b) we accentuate detailed taxon determination, which enables discussing of possible influence of horizontal gene flow on genetic variability of parental gene pools and estimation of genotypic richness within separate groups, particularly in hybrid populations. Relatively small genotypic richness within hybrid clusters would show rare hybridization events and spread of hybrids mostly mediated by asexual reproduction. High clonal richness, on the other hand, would show that hybridization is a frequent and repetitive process. Finally, we tested the hypothesis that environmental gradients may cause intra-population genotype diversification and spatial differentiation similarly as they cause it on the species level.

MATERIALS AND METHODS

Sample collection and locality selection

All samples used for the purpose of this study were sampled in summer 2004 and were analysed by two allozyme markers in previously published studies (Seda et al. 2007b, Petrusek et al. 2008b). Primary knowledge of taxon composition and its spatial variation within eleven Czech reservoirs allowed us to choose three localities appropriate for the present study (Table 1). The Vranov Reservoir, 18 km long, with maximum depth of 45 m and 134 days of theoretical retention time and the Vír Reservoir, 8 km long, with maximum depth of 58 m and 166 days of theoretical retention time offered good conditions for coexistence of three different parental species of the *D. longispina* species complex. Contact of species in the same time and space followed by interspecific hybridization was therefore expectable and was also confirmed by allozyme electrophoresis (Seda et al. 2007b). The third reservoir - the Stanovice Reservoir, 3.5 km long, 45 m deep and 555 days of theoretical retention time was inhabited by a single species, *D. galeata*. However, absence of other *Daphnia* species offered a good opportunity to study intraspecific genetic differentiation without the influence of interspecific competition with other *Daphnia* species.

Reservoir	Latitude [N]	Longitude [E]	Altitude [m asl]	Area [km2]	Maximum depth [m]	Length [km]	Theoretical retention time [days]	Taxa present 2004
Stanovice	50° 11´	12° 053´	518	1.4	45	3.5	555	G
Vír	49° 34´	16° 019´	469	2.1	58	8	166	C, G, L, G×L
Vranov	48° 54´	15° 049´	352	7.7	45	18	134	C, G, L, G×C, G×L

Table 1. Basic characteristics of the investigated reservoirs and their occupancy by taxa of the *D. longispina* complex in summer 2004 (Seda et al. 2007b). *D. cucullata*, *D. galeata* and *D. longispina* species are abbreviated by the first letters of their species names.

Zooplankton was colleted by vertical hauls of plankton nets (mesh size 170 μm). For each reservoir, set of five samples was obtained. Three samples were collected downstream at the deepest part of the reservoir covering vertical gradients: one from the hypolimnion, one from the metalimnion (both of these layers were sampled using a closing net) and one from the epilimnion. Extent of these layers within thermally stratified water column was previously estimated by measuring the temperature profile and oxygen concentration. Remaining two samples, one from the middle part of the reservoir and one from the upstream region near the

river inflow, completed sampling design by covering the longitudinal gradients (Figure 1). Each sample was immediately after haul frozen in liquid nitrogen and saved for consequent genetic analyses. For detailed description of sampling design see (Seda et al. 2007b).

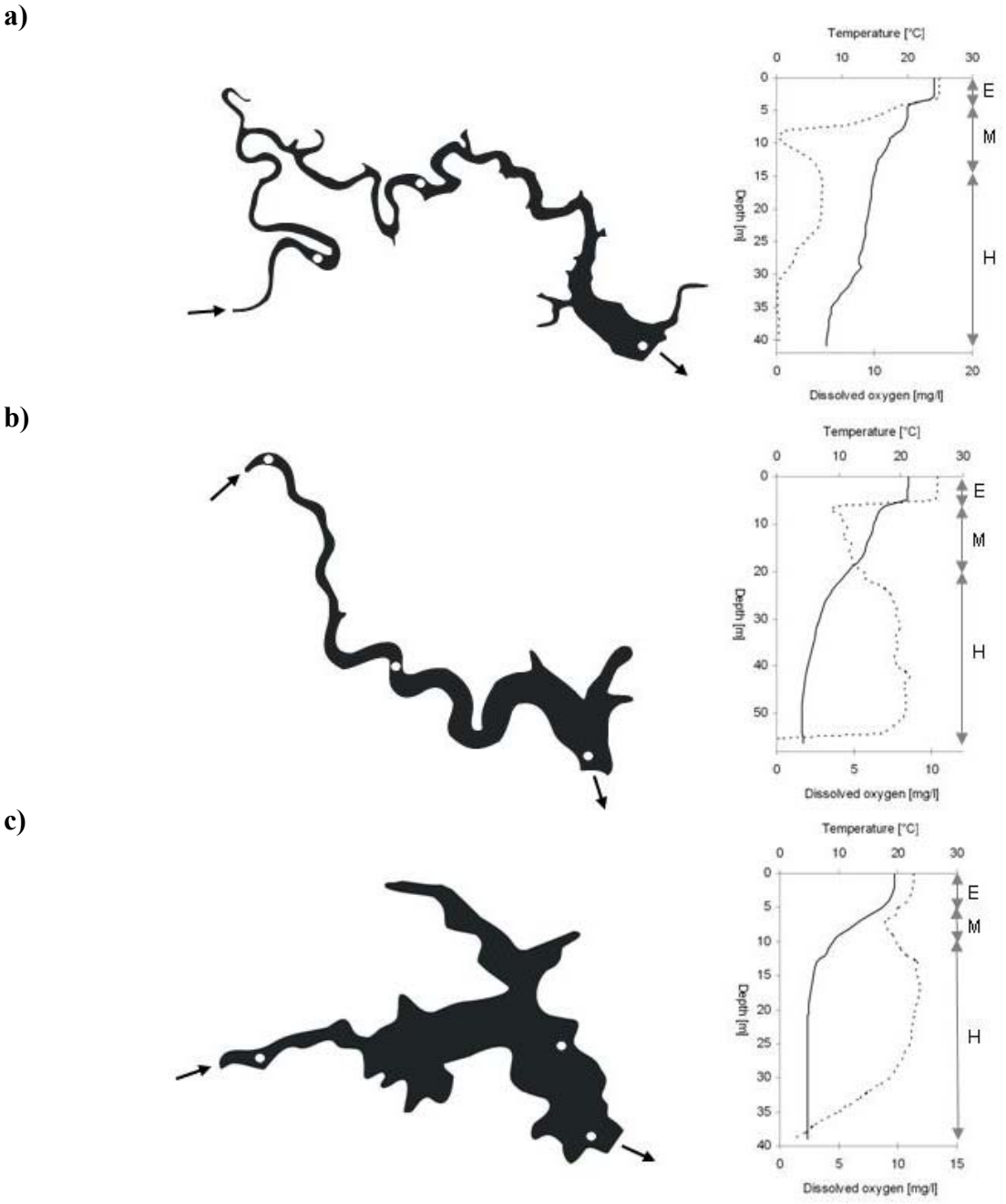

Figure 1. Sampling design in the Vranov **(a)**, Vír **(b)** and Stanovice Reservoirs **(c)**. White dots and arrows in reservoir outlines indicate our sampling stations and direction of the water flow, respectively. Extent of epi-, meta- and hypolimnion at the downstream sampling site, indicated in graphs by vertical bilateral arrows, was estimated by measuring the temperature profile (smooth line) and oxygen concentration (dashed line) immediately before sampling. Note that reservoir outlines are not to scale.

In August 2007, we collected additional samples of reservoir water in order to estimate concentrations of phosphorus and nitrogen. At each sampling site, 250 ml collected close to the water surface was directly filled into bottles, cooled and subsequently frozen; the same volume was then filtered through a net of 40-μm mesh size and stored in the same way. All samples were analysed in the laboratory of the Institute of Hydrobiology of the Czech Academy of Sciences in České Budějovice for nutrient concentration. Total phosphorus was estimated by spectrophotometry subsequently to the mineralization by HCl (Kopáček & Hejzlar 1993). Concentration of total nitrogen was measured by infrared CO_2 detector in Formacs TON Analyzer (Skalar) and by NO_x chemiluminicsent detector. We also roughly assessed in-vivo chlorophyll a concentrations for 3 to 6 locations situated along the longitudinal reservoir axis, using Aquafluor (Turner Designs).

DNA isolation and amplification

Approximately 40-50 adult females belonging to the *Daphnia longispina* species complex were randomly selected from each deep-frozen sample and homogenised. A 2.5 μL aliquot of the homogenate was transferred to 30-μL solution containing H3 buffer and proteinase K (Schwenk et al. 1998). DNA was isolated by incubation at 55 °C for 6-10 hours followed by inactivation of proteinase K by 95 °C for 10 min. DNA isolates were stored at –14 °C and de-frozen shortly before the usage.

Twelve microsatellite loci were chosen to obtain detailed genetic structure of *Daphnia* assemblages (Appendix, Table 1). Polymerase chain reactions (PCR) were performed in 0.2 ml Eppendorf tubes mostly followed protocols by Brede et al. (2006) and Thielsch et al. (in review). Fragments labelled with fluorescent dyes (Alexa 647 and Alexa 750 (Invitrogen); IRD (MWG)) were amplified in triads in four separate multiplex PCRs (MP1: Dp281NB, SwiD14, DaB10/14; MP2: DaB17/17, Dp196NB, Dp519; MP3: SwiD6, SwiD12, SwiD18; MP4: Dgm105, Dgm109, Dgm112). Each 10 μL reaction volume contained 2 μL of DNA isolate, 3 mM $MgCL_2$, 1× PCR buffer, 0.2 dNTPs, 0.2 mg/ml BSA (New England Biolabs), 1× DMSO (Roth) and 1 U *Taq* polymerase (Invitrogen). Primer concentrations in multiplex reactions were adjusted in order to obtain similar amounts of PCR products. A concentration of 0.1 μM was used for loci Dp281NB, Dgm112, Dp196NB and DaB17/17; 0.2 μM for loci SwiD6, SwiD12, SwiD18, SwiD14 and DaB10/14; 0.3 μM for loci Dgm105, Dgm109 and Dp519. Multiplex reactions MP1, MP2 and MP3 were performed in the thermal cycler PTC-225 DNA Engine Tetrad (MJ Research), MP4 in the thermal cycler PTC-100 (MJ Research). Cycling conditions for all reactions started with a 3 min denaturing step at 95 °C, continued

by 35 cycles of 1 min steps at 95 °C, 55 °C and 72 °C (MP1, MP3, MP4) or by 35 cycles of 1 min steps at 95 °C, 53 °C and 72 °C (MP2) and were completed by 7 min synthesis step at 72 °C. Amplicons were diluted according to their qualities and electrophoresed on a CEQ 2000 (Beckman Coulter; denaturation at 90°C for 2 min; injection at 2.0 kV for 30 sec; separation at 6.0 kV for 35 min) with a self-designed size standard based on Lambda phage DNA (Symonds & Lloyd 2004).

Data analysis

In order to visualize genetic structure of the assemblages and in order to distinct genetically separate groups of individuals, factorial correspondence analysis (FCA) of genotype frequencies performed by GENETIX 4.01 software (Belkhir et al. 1996-2004) was used. As missing data may significantly influence estimation of the genetic structure, we used only individuals characterised by at least six microsatellite loci. Although the visualization may clearly show genetic differences among and within separate taxa (parental species, interspecific hybrids), it was primarily performed to estimate number of separate clusters in the dataset.

We calculated proportion of distinct multilocus genotypes (G) in order to assess genotypic richness (R = [G-1] / [N-1]). It is strongly recommended to compute MGL with at least 10 loci (Thielsch et al., in review) and we therefore used information of all 12 microsatellite loci. In order to decrease a possibility that two different individuals would be recognised as one clone, we also added for each individual data on allele composition at four allozyme loci: sAAT and AO used in the previous studies (Seda et al. 2007b, Petrusek et al. 2008b) and PGI and PGM (Chapter 2), and combined it with the microsatellites. As it has been shown that genotypic richness of natural *Daphnia* assemblages is high (Thielsch et al., in review), we disregarded for the purpose of this study factors which may occasionally slightly overestimate the real clonal diversity, such PCR artefacts, somatic mutations and scoring errors. Genotypic richness was counted for each reservoir and for each taxon separately. In order to estimate differences in genotypic richness on the horizontal gradient of the Stanovice Reservoir, we computed both characteristic also for pooled dataset including all three sampling sites of the dam region. Proportion of the individuals from epi-, meta- and hypolimnion was corrected according to their real densities (Seda et al. 2007b). All individuals with missing data were excluded. Computations were performed by GENCLONE 2.0. software (Arnaud-Haond & Belkhir 2007).

Alleles of chosen mikrosatellites are not fixed and differ only in frequencies among *Daphnia* species. The determination of the species and hybrid classes couldn't be therefore performed directly. We used NEWHYBRIDS software (Anderson & Thomson 2002) using Markov chain Monte Carlo in a Bayesian setting to compute the posterior probabilities that individuals belong to certain taxa. As the software is able to work only with two parental species, we always counted the posterior probabilities just for one pair of them and for their recombinant genotypes. Previously to each step, we excluded all individuals possibly sharing characters specific for the third parental species – carrying species-specific allozyme alleles (known from Seda et al. (2007b)) or showing a position in the factorial correspondence analysis suggesting a likely introgression from the third species.

Finally we obtained three partly overlapping datasets – the first involving *D. galeata – D. longispina* individuals, the second involving *D. longispina – D. cucullata* individuals and the third involving *D. cucullata – D. galeata* individuals. In one separate run, we also added microsatellite alleles of two laboratory crossed *D. galeata* × *cucullata* F_1 hybrids (Thielsch et al., unpublished results) to the dataset, in order to verify results. Taxon origin was estimated in nine separate runs differing by used dataset and by the locality (individuals from all localities pooled, Vranov Reservoir only, Vír Reservoir only). Two random numbers defined starting position and at least 10^4 iterations were carried out after a burn-in period of 10^4 iterations.

Using this method we finally obtained 2-4 posterior probabilities computations for each parental species and 2 for hybrids. Where the posterior probability was equal or higher than 0.8 the estimated taxon was noted; in case of posterior probability < 0.8 the taxonomic status of individuals was considered undetermined. Results of different runs were consequently compared to each other. The taxon was considered unambiguously identified if posterior probabilities of all runs were over 0.8 and the results corresponded to each other. In other cases, results of differing NEWHYBRIDS runs were interpreted using the following set of rules: 1) when the mostly likely taxon estimated by all runs was identical and some but not all posterior probabilities were below 0.8, individual was identified as this "consensus taxon"; 2) in cases where posterior probabilities of all runs were over 0.8, but the results did not correspond to each other (in most cases, individuals determined as later generation hybrids and as pure species at the same time), individuals were marked as introgressants (i.e. hybrid of generation > F_2); 3) individuals with computed probabilities below 0.8 in all runs, or

individuals exhibiting discrepancies among results of particular runs together with posterior probabilities < 0.8 in several runs were considered undetermined.

In order to potential assess intraspecific genetic differentiation among sites within one locality, we computed F-statistics (F_{ST} estimates of Weir & Cockerham (1984)) for *D. galeata* at two localities, the Stanovice Reservoir (inhabited solely by this species), and the Vír Reservoir, in which *D. galeata* was present at all sampling sites. We did not compute F_{ST} for other taxa, as their proportion in samples was too low or they did not occur at all sampling sites. When the F_{ST} was computed for longitudinal gradients, dam region was considered as one sampling site (subpopulation) and number of *Daphnia* individuals from different layers in the dataset was adjusted according to their real densities (by random selection of adequate amount of animals from hypo- and metalimnion). Significance of computed divergence among subpopulations was verified by permutation test with 1000 permutations.

RESULTS

Nutrient and chlorophyll a concentrations along reservoir longitudinal axes

In general, nutrient concentrations in studied reservoirs decreased in the downstream direction from the inflow region towards the dam (Figure 2a, 2b; Table 2), which is in concordance with general characteristics of canyon-shaped reservoirs. Growth of the phytoplankton represented by chlorophyll a concentrations followed the changes of phosphorus concentration (Figure 2c). Intensity of these gradients (the difference in nutrient concentrations and chlorophyll a concentrations between upstream and downstream regions) was markedly higher in Vranov and Vír Reservoirs inhabited by all three parental species and by interspecific hybrids than in the single-species Stanovice Reservoir.

Species composition

In total, 651 individuals from three different reservoirs (190 from the Stanovice Reservoir, 280 from the Vranov Reservoir and 181 from the Vír Reservoir) were screened using 12 microsatellite markers. Factorial correspondence analysis (Figure 3) showed five distinct clusters representing three parental species and two groups of interspecific hybrids. Cluster representing *D. longispina* × *cucullata* hybrids was missing, in agreement with previous results based on allozymes. Whereas the Vranov and Vír Reservoirs were characteristic by occurrence of very numerous and variable genotypes, the cluster representing the Stanovice Reservoir included only genetically compact group of *D. galeata* individuals (Figure 3).

Based on posterior probabilities computations performed by NEWHYBRIDS (Anderson & Thomson 2002), we were able to ascertain taxonomic position of 637 individuals. 579 individuals were successfully distinguished on 95% threshold at least in one run, 552 of them exhibited posterior probabilities of being a particular species/hybrid class over 0.8 in all runs. Only 16 animals stayed undetermined. However, majority of the unidentified individuals seemed to exhibit recombinant genotype.

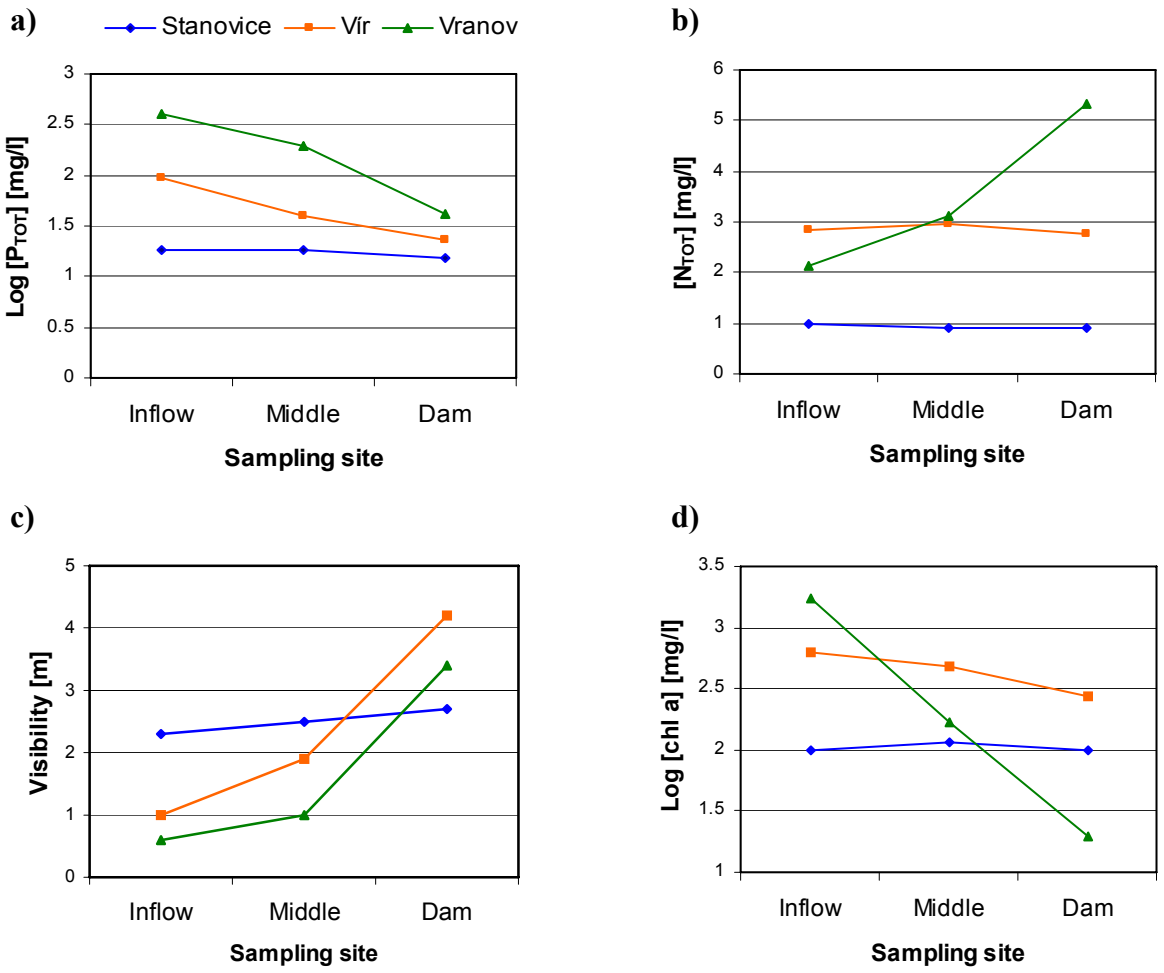

Figure 2. Gradients of total phosphorus **(a)**, total nitrogen **(b)**, Secchi depth **(c)** and chlorophyll a concentration **(d)** along the reservoir longitudinal profiles in the Stanovice, Vír and Vranov Reservoir. Increasing concentration of the total nitrogen (b) from upstream to downstream in the Vranov Reservoir suggests that nitrogen is not limiting element. Note that *y*-values for the total phosphorus and chlorophyll a are in logarithmic scales.

Reservoir	Sampling site	P_{TOT} [mg/l]	P_{TOT} <40 µm	P_{TOT} >40 µm	N_{TOT} [mg/l]	N_{TOT} <40 µm	N_{TOT} >40 µm	Chl a [mg/l]
Stanovice	Inflow	18.5	17.4	1.1	0.99	0.88	0.11	100
	Middle	18.0	14.2	3.8	0.91	0.89	0.02	116
	Dam	15.2	14.2	1.1	0.92	0.90	0.02	100
Vír	Inflow	93.0	58.0	35.0	2.84	2.54	0.30	633
	Middle	39.1	25.3	13.8	2.96	2.61	0.35	480
	Dam	23.0	21.9	1.1	2.75	2.72	0.03	273
Vranov	Inflow	399.0	263.0	136.0	2.15	1.23	0.92	1700
	Middle	193.0	69.0	124.0	3.11	2.41	0.70	170
	Dam	41.0	20.2	20.8	5.33	5.23	0.10	20

Table 2. Concentrations of phosphorus, nitrogen and chlorophyll a at three main sampling sites of the Stanovice, Vír and Vranov Reservoir. Phospohrus and nitrogen are shown for unfiltered surface water samples as well as for samples filtered through 40 µm mesh. Chlorophyll a concentrations were measured *in vivo* during the sampling in summer 2004, and therefore represent only rough estimates. The concentration of 1700 mg/l measured in inflow region of the Vranov Reservoir reflects cyanobacterial bloom. Water for laboratory measurements of phosphorus and nitrogen concentrations was sampled in August 2007.

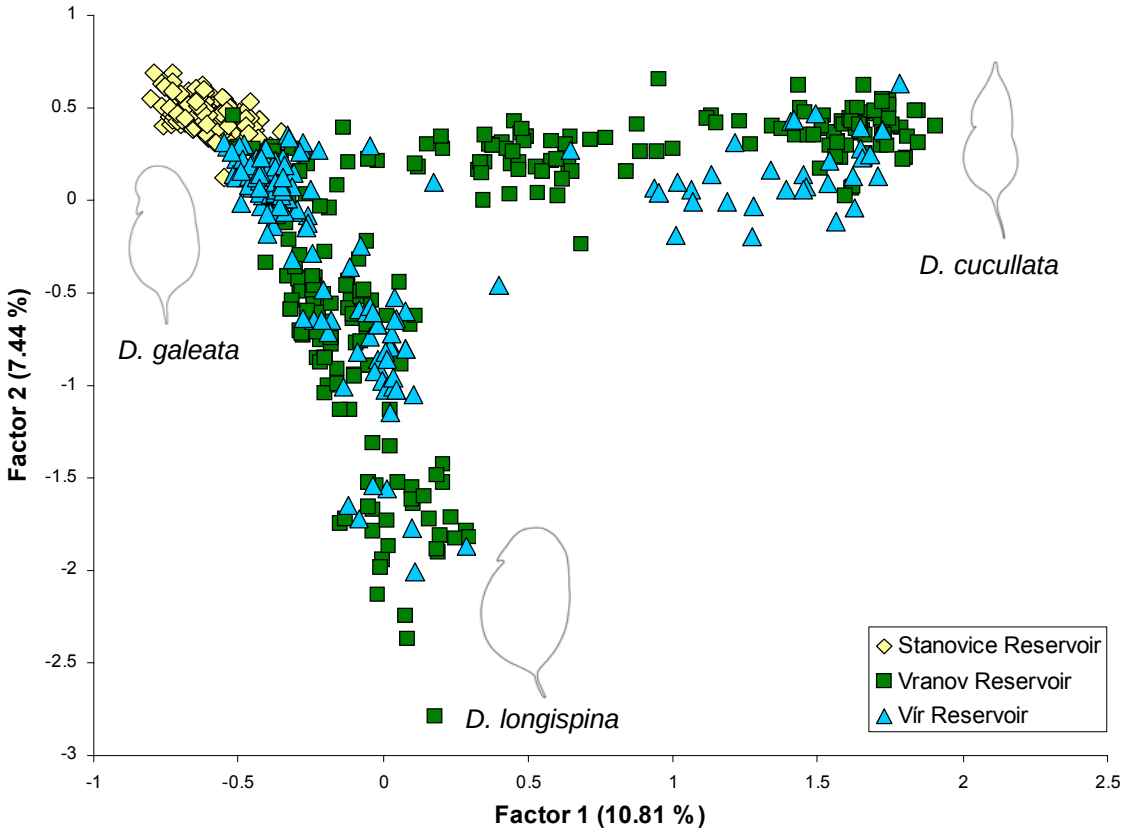

Figure 3. First two axes of the factorial correspondence analysis (GENETIX) based on 12 microsatellite markers, showing 651 individuals of the *Daphnia longispina* species complex from the three analysed reservoirs. Individuals sharing identical symbols originate from the same reservoir. Typical body shapes of individuals from the three marginal clusters (i.e., parental taxa) are indicated by outlines.

Microsatellite markers revealed presence of all three *Daphnia* species and interspecific hybrids (Figure 4, Table 3).

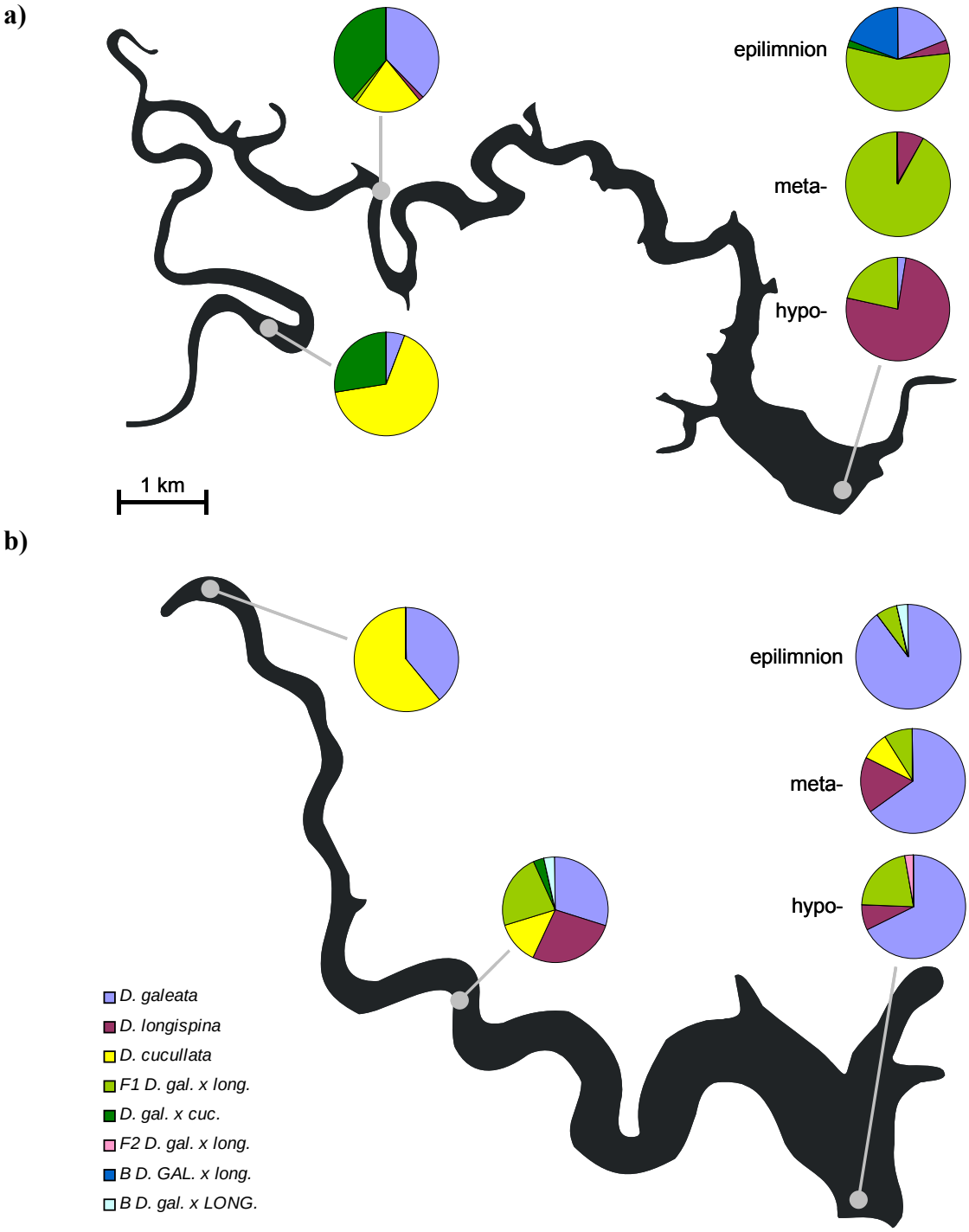

Figure 4. Spatial distribution of parental species and hybrids along horizontal and vertical gradients within the Vranov **(a)** and Vír **(b)** reservoirs. Pie charts show the relative abundance of the taxa at each sampling site. Taxon estimation was based on information from twelve microsatellite loci and was performed by Bayesian inference in NEWHYBRIDS (for details, see Methods).

Res.	Site		G	L	C	F1 GxL	GxC	F2 GxL	B GxI	B gxL	Total
Vranov	epilimnion	N	**9**	**2**	0	**26**	**1**	0	**9**	0	**47**
		%	**19.1%**	**4.3%**	0%	**55.3%**	**2.1%**	0%	**19.1%**	0%	**100%**
	metalimnon	N	0	**3**	0	**33**	0	0	0	0	**36**
		%	0%	**8.3%**	0%	**91.7%**	0%	0%	0%	0%	**100%**
	hypolimnion	N	**1**	**28**	0	**8**	0	0	0	0	**37**
		%	**2.7%**	**75.7%**	0%	**21.6%**	0%	0%	0%	0%	**100%**
	middle	N	**26**	**1**	**14**	**1**	**27**	0	0	0	**69**
		%	**37.7%**	**1.4%**	**20.3%**	**1.4%**	**39.1%**	0%	0%	0%	**100%**
	inflow	N	**5**	0	**57**	0	**24**	0	0	0	**86**
		%	**5.8%**	0%	**66.3%**	0%	**27.9%**	0%	0%	0%	**100%**
Vír	epilimnion	N	**27**	0	0	**2**	0	0	0	**1**	**30**
		%	**90.0%**	0%	0%	**6.7%**	0%	0%	0%	**3.3%**	**100%**
	metalimnon	N	**22**	**6**	**3**	**3**	0	0	0	0	**34**
		%	**64.7%**	**17.6%**	**8.8%**	**8.8%**	0%	0%	0%	0%	**100%**
	hypolimnion	N	**25**	**3**	0	**8**	0	**1**	0	0	**37**
		%	**67.6%**	**8.1%**	0%	**21.6%**	0%	**2.7%**	0%	0%	**100%**
	middle	N	**9**	**8**	**4**	**7**	**1**	0	0	**1**	**30**
		%	**30.0%**	**26.7%**	**13.3%**	**23.3%**	**3.3%**	0%	0%	**3.3%**	**100%**
	inflow	N	**16**	0	**25**	0	0	0	0	0	**41**
		%	**39.0%**	0%	**61.0%**	0	0	0	0	0	**100%**
Stanovice	epilimnion	N	**34**	0	0	0	0	0	0	0	**34**
		%	**100%**	0%	0%	0%	0%	0%	0%	0%	**100%**
	metalimnon	N	**40**	0	0	0	0	0	0	0	**40**
		%	**100%**	0%	0%	0%	0%	0%	0%	0%	**100%**
	hypolimnion	N	**39**	0	0	0	0	0	0	0	**39**
		%	**100%**	0%	0%	0%	0%	0%	0%	0%	**100%**
	middle	N	**40**	0	0	0	0	0	0	0	**40**
		%	**100%**	0%	0%	0%	0%	0%	0%	0%	**100%**
	inflow	N	**37**	0	0	0	0	0	0	0	**37**
		%	**100%**	0%	0%	0%	0%	0%	0%	0%	**100%**

Table 3. Numbers of individuals (N) identified as pure species and different hybrid classes sampled at various sites in the Vranov, Vír and Stanovice Reservoir. Taxon estimation based on information from 12 microsatellites loci was performed by Bayesian inference in NEWHYBRIDS (for details, see Methods). Percentages represent their relative abundances in the respective samples. Absolute abundances differed among sites (see Seda et al. 2007b). Species (*D. galeata*, *D. longispina and D. cucullata*) are abbreviated by the first letters of their species names. B indicates backcrosses; species coded in a capital letter indicates the more related parental taxon. Hybrid classes of *D. galeata* x *cucullata* are pooled (see Results).

F_1 hybrids were detected in both Vranov and Vír Reservoirs; however, only *D. galeata* × *longispina* hybrids occurred in Vír. Later generation hybrids were also present, but were rare. In the whole dataset, we found just one individual consistently identified as F_2 *D. galeata* × *longispina* hybrid, two *D. longispina* × *galeata* backcrosses with *D. longispina*, and nine *D. galeata* × *longispina* backcrosses with *D. galeata*. Ten individuals determined as *D. longispina* and one individual of *D. galeata*, both from the Vír Reservoir, carried signs of

introgression. In the Vranov Reservoir, the introgression seemed to be rarer, only a single *D. galeata* individual was found to possibly carry introgressed alleles of *D. cucullata*.

D. galeata × *cucullata* hybrids were present only in the Vranov Reservoir; recognition of the hybrid class was nevertheless complicated. In each separate run of Bayesian analysis in NEWHYBRIDS, all of these individuals were determined as F_2 hybrids or *D. galeata* × *cucullata* backcrosses. This trend stayed identical even if two laboratory-crossed hybrid clones were added to the dataset. Both of them were recognised as F_2 hybrids when compared to the genotypes of parental species from the Vranov Reservoir. We therefore marked all the 53 individuals sharing *D. galeata* and *D. cucullata* genes as *D. galeata* × *cucullata* hybrids, with their hybrid class remaining undetermined.

Detailed taxon composition at each sampling site in the Vranov and Vír Reservoirs (occupied by more than one *Daphnia* species) is demonstrated in Figure 4 and Table 4. In general, this pattern was almost identical to results published in Seda et al. (2007b), as the taxon determination performed by NEWHYBRIDS software agreed with the previous determination provided by allozyme electrophoreses nearly completely (>97% if all hybrid classes were pooled together as "hybrids"). The species composition changed with both horizontal and vertical ecological gradients. *D. longispina* and its hybrids preferred deep water-layers close to dam, whilst *D. cucullata* and its hybrids dominated in the upstream parts. *D. galeata* was a ubiquitous species. However, new analyses improved our understanding of spatial differentiation of the taxa by obtaining information on the distribution of individuals with introgressed genotypes. Their spatial distribution was in general similar to the distribution of the closest parental species. For instance, all *D. galeata* × *longispina* backcrosses from the Vranov Reservoir inhabited upper water layers in the downstream together with pure *D. galeata*. In the Vír Reservoir, *D. longispina* × *galeata* backcrosses and *D. longispina* individuals carrying introgressed alleles were found in samples from deep-water layers at the downstream sampling site, and from the central part of the reservoir, similarly to pure *D. longispina*.

Genotypic richness among different reservoirs and among different taxa

Out of 351 animals for which data from all 4 allozyme and 12 microsatellite loci were available, we found 291 distinct multilocus genotypes. Identical genotypes occurred only within and never among different reservoirs. The average genotypic richness for all populations was 0.829; this value stayed nearly the same (0.822) even if only microsatellite markers were used, suggesting that number of microsatellite loci was sufficient. Genotypic

richness (R) differed among taxa and among reservoirs (Table 4a). For *D. galeata*, R value was relatively high in the Vranov Reservoir (R=0.92; G=28) and in the Vír Reservoir (R=0.88; G=45), but lower in the single-species Stanovice Reservoir (R=0.69; G=90). Computations of G and R characteristics performed for each sample from the Stanovice Reservoir helped us to evaluate whether the lower genotypic richness of this *D. galeata* population was characteristic for the whole reservoir or whether it was caused by reduced genotypic richness (i.e., increase proportion of certain multilocus genotypes) at some sampling sites only. In fact, relatively low genotypic richness was characteristic only for the dam region (R=0.63; G=18) and especially for the epilimnion (R=0.61; G=15). Other sampling sites within the reservoirs were inhabited by more balanced mixtures of different genotypes; the respective R values were close to 0.9 (Table 4b).

The Vranov Reservoir was the only site where the G and R characteristics could be computed for all other taxa (Table 4a), as they were present in sufficient quantities. The estimated genotypic richness for both *D. longispina* and *D. cucullata* found in this reservoir was 1.0 (G=14 and 33, respectively). Similar value of R=0.97 (G=29) was characteristic for *D. galeata* × *cucullata* hybrids. *D. galeata* × *longispina* hybrids exhibited substantially smaller genotypic richness (R=0.76, G=30). This trend was nevertheless not observed in the Vír Reservoir, in which the R value for *D. galeata* × *longispina* hybrids was relatively high (R=0.94; G=16).

a)

Spec./Res.	Vranov		Vír		Stanovice	
	R	G	R	G	R	G
D. galeata	0,93	28	0,88	45	0,69	90
D. longispina	1,00	14	1,00	1	N/A	N/A
D. cucullata	1,00	33	1,00	2	N/A	N/A
GxC	0,97	29	N/A	N/A	N/A	N/A
GxL	0,76	30	0,94	16	N/A	N/A

b)

Sampling site	R	G
Inflow	0,89	26
Middle	0,81	27
Dam	0,63	18
Epilimnion	0,61	15
Metalimnion	0,92	24
Hypolimnion	0,88	16

Table 4 a) Number of distinct multilocus genotypes (G) and genotypic richness (R) calculated for parental species and hybrid genotypes from the three reservoirs. Individuals lacking data for one or more microsatellite loci were excluded from the computations. G×C and G×L indicate all classes of *D. galeata* × *cucullata* and *D. galeata* × *longispina* hybrid genotypes, including backcrosses and undetermined individuals showing patterns of introgression. N/A indicates that the respective taxon did not occur in the reservoir. **b)** Number of distinct MLGs and genotypic richness of *D. galeata* subpopulations inhabiting various sites of the Stanovice Reservoir. The dam region is represented by individuals randomly chosen from the epi-, meta- and hypolimnetic samples accordingly to their real densities.

Genetic differentiation within D. galeata *populations*

Genetic differentiation among subpopulations from different sampling sites could be calculated for the Stanovice and Vír Reservoirs, where *D. galeata* was present in all three regions sampled along the horizontal axis. However, significant, although relatively small, differentiation was observed only in the Stanovice Reservoir, where we observed the F_{ST} value of 0.033 ($p<0.001$) for the longitudinal gradient and $F_{ST} = 0.035$ ($p<0.001$) for the vertical profile in the dam region. A shift in allele composition of multilocus genotypes along the vertical profile is apparent from their distribution in the factorial correspondence analysis (Figure 5).

DISCUSSION

Our data show that canyon-shaped reservoirs may be used as an appropriate model system not only for studies of whole zooplankton communities, but also in investigations of intra-population dynamics. The presence of stable longitudinal gradients causing differentiation in habitat quality along the reservoir axis improves conditions for coexistence of several species of the *D. longispina* species complex and may also facilitate their hybridization (Seda et al. 2007b, Petrusek et al. 2008b). However, if only limited number of similar, directly competing species is present in the pelagic environment (e.g., related *Daphnia* species), effect of interspecific competition may be suppressed and the impact of gradients of environmental conditions may become evident in the intraspecific population structure.

Although it has been proposed that two factors affecting the spatial distribution of *Daphnia* species in the reservoirs – gradients of intensity of size-selective fish predation and of the food quality and quantity – are most crucial (Petrusek et al. 2008b), the reason why the reservoirs differ in number of present taxa stays unclear. As all sites are situated in a relatively small territory of the Czech Republic, distances among them are within a bird's flight (tens to few hundred kilometers), and the studied taxa are common throughout the country, the effect of geographical isolation of certain localities or propagule limitation is unlikely. Taxonomic composition of local assemblages thus seems to be influenced by internal conditions of the reservoirs. We assume that the number of coexisting species of the *Daphnia* longispina complex within canyon-shaped reservoirs could be substantially influenced by the gradient intensity (Appendix, Figure 1).

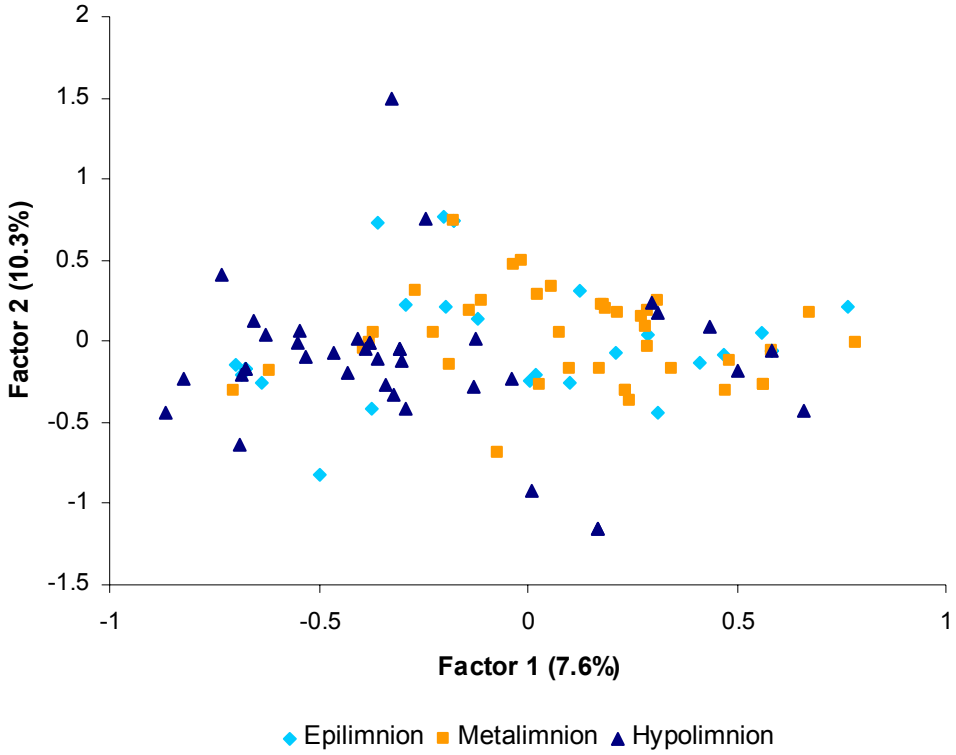

Figure 5. Visualization of genetic differentiation among 113 *D. galeata* individuals in the dam region of the Stanovice Reservoir provided by factorial correspondence analysis (GENETIX) based on 12 microsatellite markers. Individuals sharing identical symbols were sampled from the same water layer. Extent of intraspecific genetic differentiation was assessed by F-statistics (F_{ST}= 0.035), and is highly significant (permutation test, $p<0.001$).

Reservoirs with relatively high concentration of nutrients in the upper part rapidly decreasing towards dam probably offer more variable habitats allowing niche differentiation than reservoirs with weak gradients. This may suppress competitive exclusion and improve taxon coexistence. In contrast to this, species richness seems to be smaller, and subsequently the frequency of hybridization lower, in reservoirs where the gradients are not sufficiently developed (e.g. to short reservoirs, reservoirs with the nutrient-poor inflow).

In our previous studies (Seda et al. 2007b; Petrusek et al. 2008b), we showed that spatial distribution of certain taxa is non-random within canyon-shaped reservoirs. The taxon distribution patterns also differed among species and their hybrids, with hybrids showing tendency to prefer environmentally intermediate conditions. As shown in Figure 4, results of microsatellite analyses support these findings. Though representative set of backcrosses was found only in the Vranov Reservoir, so the results cannot be generalized, it seems that their habitat preferences are similar to those of closer-related parental species (Figure 4, Table 4).

Given the phenotype similarity between backcrosses and parental species (Jankowski & Straile 2004), this is not an unexpected result.

Determination of *Daphnia* taxa and their spatial distribution within the Vranov and Vír Reservoirs based on microsatellite analysis almost fully corresponded with results obtained by allozyme electrophoresis (Seda et al. 2007b), suggesting that even the latter method is reliable enough to be used for purpose of genetic identification of taxa within the *Daphnia longispina* complex (see also Chapter 2). Although the microsatellite analysis represents more powerful molecular method than the allozyme electrophoresis allowing detailed population studies and more reliable identification of hybrid classes, inconsistencies may occur. In our study, determination of all *D. galeata* × *cucullata* introgressed genotypes as later-generation hybrids in Bayesian analyses performed by NEWHYBRIDS seems to be unlikely. This pattern stayed identical even if only four allozyme markers were used for NEWHYBRIDS computations (Ruthova et al., unpublished results). Although natural populations composed entirely by backcrosses are known in *Daphnia* (Taylor & Hebert 1992), exclusive presence of later generation hybrids in the Vranov Reservoir seems to be unlikely, especially as the high genotypic richness of hybrid individuals suggests ongoing hybridisation.

Incorrect estimation of the hybrid class could be caused by shifts in allele frequencies which could arise if the real parents of the hybrids would not be present, or would be underrepresented in the analyzed samples. It has been shown that clonal composition within canyon shaped reservoirs differs among years as the population is restored by hatching from resting eggs after the winter bottleneck (Seda et al. 2007b). Clones of the parental species thus may completely disappear or change abundance when their hybrids start to hatch. Similar phenomenon may also occur if hybrids colonize new habitats from other locality as resting egg. Other studies nevertheless suggest that hybrids are more likely locally produced (Spaak 1997, Jankowski 2002). High genotypic richness of *D. galeata* × *cucullata* hybrids in the Vranov Reservoir and their presence in resting egg bank (Vaníčková et al., unpublished data) support the scenario that hybrids are produced within the reservoir.

Non-overlapping generations of parents and hybrids could influence misidentification of *D. galeata* × *cucullata* hybrids. However, it is intriguing that no such phenomenon occurs when identifying *D. galeata* × *longispina* hybrid classes, which could be well distinguished in both Vranov and Vír Reservoirs. Furthermore, misclassification of *D. galeata* × *cucullata* hybrids (but not *D. galeata* × *longispina* hybrids) seems to be an "all-European problem" affecting microsatellite (Kraus 2007, Chapter 2) and apparently also allozyme markers (Keller et al. 2008). Change of clonal composition among years thus can't fully explain problems

with the classification. However, absence (or shift in proportions) of real parental clones in the sample could be explained by other mechanism. Laboratory crossing experiments between *D. galeata* and *D. cucullata* reported by Schwenk et al. (2001) revealed an overall low reproductive success of interspecific crosses and showed that success of hybridization also depends on the direction of hybridization and on the choice of parental clones. This pattern indicates that complete or partial genetic incompatibility depends on the genotypic composition of the parental genomes. In a preliminary study of resting egg banks from canyon shaped reservoirs (Vaníčková et al., unpublished data), *D. galeata* × *cucullata* hybrids were frequently found also in reservoirs where such hybrids were apparently absent from the active population. This suggests that interspecific hybridization between *D. galeata* and *D. cucullata* could be common process, but the subsequent hybrid success is probably determined mainly by hatching of ephippial eggs, which seems to be very low in natural populations.

We therefore suppose that a substantial part of pure *D. galeata* and *D. cucullata* individuals could actually be reproductively incompatible, and would produce non-viable offspring. Relative rarity of genetically compatible crosses might result in shift in allele frequencies of real parental genotypes (in comparison with the whole populations of parental species), and subsequently could cause that all *D. galeata* × *cucullata* hybrids were considered as later generation hybrids in the statistical computations. In contrast to this, hatching success of *D. galeata* × *longispina* hybrids and their survival rate seems to be much higher (Vaníčková et al., unpublished data) Thus, hybridization between *D. galeata* and *D. longispina* could be less restricted and hybrid genotypes could combine equally the gene pools of both parental species. However, it would certainly be worth to test the proposed scenario by computer simulations.

Interspecific hybridization within *D. longispina* species complex is in general considered as a common process, where the fusion of species gene pools is inhibited by low fertility of interspecific hybrids. Our computations of genotypic richness within separate taxa also support this hypothesis. The proportion of distinct multilocus genotypes was in general very high in all groups and did not substantially differ among parental species and hybrids. Although *D. galeata* × *longispina* hybrids from the Vranov Reservoir exhibited the lowest genotypic richness from all present taxa (R=0.76), it was still higher than genotypic richness of pure *D. galeata* observed in the Stanovice Reservoir. This pattern signifies that sexual reproduction was common both within and also between species. Apparently, with very dense resting egg banks as found in reservoirs (Vaníčková et al, unpublished data.), even partial genetic incompatibility between parental species and reduced hatching success of some

hybrids would not be reflected by reduced genotypic richness of hybrid classes. The decrease of genotypic richness in some groups was more likely caused by temporal success and local multiplication of one or more clones than by lower success of sexual reproduction.

We assume that the decrease of genotypic richness in epilimnion of the downstream region of Stanovice reservoir was also an effect of clonal selection. Although the genotypic differentiation in *D. galeata* along the horizontal reservoir axis could be explained by isolation by distance, the same pattern on the vertical axis is more likely caused by different habitat preferences of certain clones, sharing some genotypic characteristics, who may be locally adapted and possibly partly reproductively isolated from other subpopulations (Seda et al. 2007a).

The Stanovice Reservoir is characteristic by much weaker trophic gradients than other two reservoirs, and dominance of *D. galeata* seems to be stable there (Petrusek et al. 2008b). Apparently, differences in habitat conditions between opposite ends of the reservoir do not promote coexistence of several *Daphnia* species, resulting in a competitive exclusion of other taxa than *D. galeata*. Long-time presence of the species could strengthen the effect of intraspecific competition and local adaptation, enabling genetic differentiation among subpopulations. On the contrary, we did not observe patterns of intraspecific differentiation in the Vír Reservoir, in which several species and hybrids co-occurred, and in which interspecific competition may have played more important role in structuring *Daphnia* assemblages.

CONCLUSION

Reservoirs with gradients along horizontal and vertical axes offer ideal conditions for ecological studies of whole zooplankton community as well as for population studies. As we showed in this study, gradients in canyon-shaped reservoirs may improve conditions not only for niche differentiation and taxon coexistence, but they may also facilitate clonal selection within particular populations. In case of stronger gradients, habitats with sufficiently different environment are available and species of the *D. longispina* complex may coexist. Thus, the assemblage structure is primarily formed by interactions among species and interspecific hybrids. In contrast to this, canyon-shaped reservoirs with poorly developed ecological gradients got occupied by no more than one or two species of the complex, impacts of intraspecific competition are local adaptation are stronger, which may result in genetic differentiation among subpopulations from different sites.

High genotypic richness in both *D. galeata* × *longispina* and *D. galeata* × *cucullata* hybrids suggests that hybridization is a common and repeated process in studied reservoirs. Despite this, occurrence of backcrosses and later-generation hybrids is rare. Backcrosses, if present, seem to exhibit similar patterns of spatial distribution as the more related parental species. Different trends in identification of hybrid classes between *D. galeata* × *longispina* and *D. galeata* × *cucullata* suggest that hybridization between the respective parental species may be uneven phenomenon; however, this warrants further detailed analysis of the available data.

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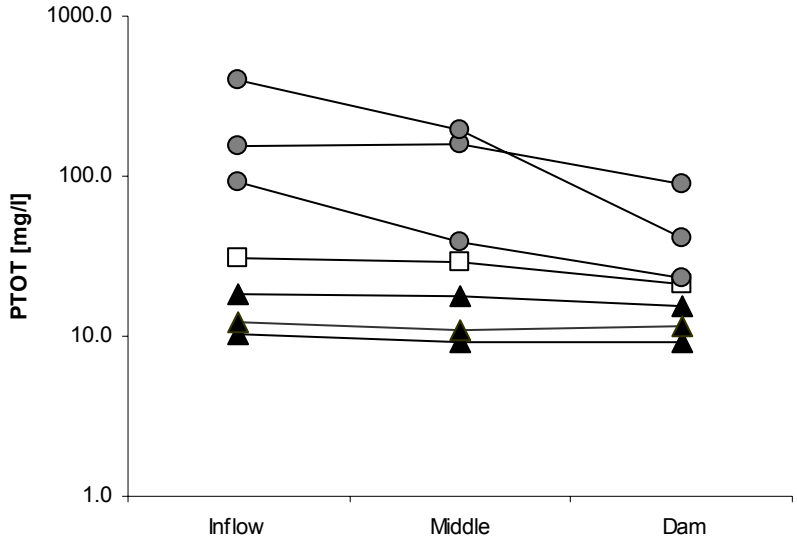
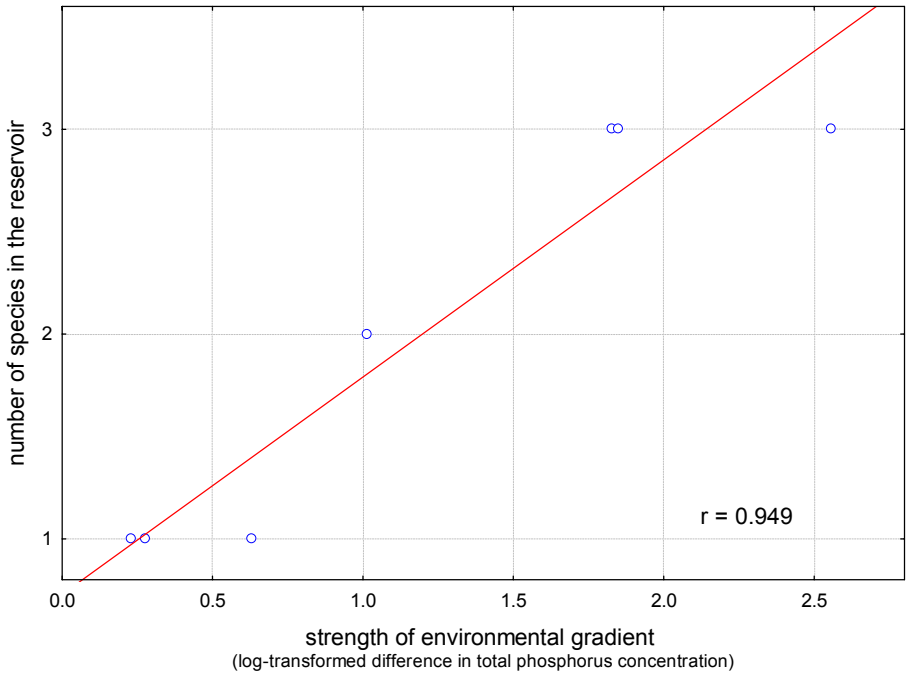
APPENDIX

a)



b)



Appendix, Figure 1. a) Gradients of total phosphorus (P_{TOT}) in seven canyon-shaped reservoirs (Czech Republic) along the reservoir longitudinal profiles. Lines with black triangles, line with white squares and lines with grey circles represent reservoirs inhabited by one, two and three species of the *D. longispina* species complex, respectively. Note that *y*-axis has a logarithmic scale. **b)** The strength of environmental gradients was estimated as the difference of total phosphorus concentration at the upstream and downstream site. Log-transformed values calculated as log (P_{inflow}–P_{dam}+1) were then correlated with the number of parental species of the *Daphnia longispina* complex present in reservoirs; correlation was strong (r=0.949) and highly significant (p=0.001).

Locus	Repeats	Primer sequence fw (5´–3´)	Size range [bp]	GenBank accession No.	PCR
DaB10/14	CAA_8	CTC TTA TAA CCA GCA CCT CG	222–234	U41402	MP1
Dp281NB	T_{10}	AAT AAC ACT CGT AGC ACG	69–78	WFms0000290	
SwiD14	$(GT)_{12}…(GT)_7$	AGA CGA TCG TTG GTT CAT CC	173–191		
DaB17/17	T_9	GAG AAC CTT TTA TCA GCT TCG	100–109	U41403	MP2
Dp196NB	AC_5	ATT TTC CGC CCT TAT TCT GC	115–130	WFms0000201	
Dp519	$(TG)_6(GA)_7$	AGT CGC GAC GAC ATA AAG C	144–160	AY057865	
SwiD6	$(CA)_{13}$	GAT CAG CAA GAT GAA ATA CAC	123–142		MP3
SwiD12	$(TC)_{14}TTA(TG)_{12}$	ATT CTT ATT GCC CCA AAT AAC C	105–127		
SwiD18	$(CA)_9$	GGA TGC CAA CTC TCT CCC CCT A	85–97		
Dgm105	$(CAG)_8AG$	ATG TGA GCG CGC GAG CAT TT	172–197	AY542269	MP4
Dgm109	$(ACC)_7AC$	CCA GCT GTT GAC CAC CTG	247–266	AY542272	
Dgm112	$(TGC)_6TGG$	GGA AAT AGG CCT AGA TGC TGT GT	109–130	AY542274	

Appendix, Table 1. Characteristics of the 12 amplified microsatellite loci (Brede et al. 2006): locus name, repeat motif, forward primer sequence, fragment size range, GenBank accession number (if available) and the combination of loci in multiplex PCR reactions.

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IDENTIFYING HYBRIDIZING TAXA WITHIN THE *DAPHNIA LONGISPINA* SPECIES COMPLEX: WHICH METHODS TO RELY ON?

(Chapter 2)

with

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ABSTRACT

Daphnia galeata, *D. longispina* and *D. cucullata* (Crustacea: Cladocera) are closely related species often producing interspecific hybrids in natural populations. Common inconsistencies among species-specific markers using for their determination were traditionally attributed to the complexity of their relationships and to the occasional introgression. In order to test this hypothesis, we used several different approaches for the taxon identification. Using allozyme electrophoresis and ITS-RFLP, we identified more than 1200 individuals from ten localities situated in the Czech Republic as parental species or hybrids. 444 animals were additionally analyzed and identified by analysis of 12 microsatellite loci. The data set was further extended by samples from 19 sites across the whole Europe, in which the taxon was estimated using microsatellites and ITS-RFLP. Results of microsatellite analysis corresponded well with allozymes. However, two sites from the Czechia and three sites from other European countries exhibited consistent discrepancies between ITS-RFLP and other markers. Although some marker disagreement could have been caused by occasional introgression, more serious deviations observed in ITS-RFLP more likely suggest a long-term maintenance of introgressed alleles in genomes of parental species, in which evolutional mechanisms such a gene conversion and meotic drive could support the maintenance of "alien" alleles.

Finally, we compared data from molecular markers with identification based on phenotypic characteristics of photographed animals for a randomly selected set of 240 *Daphnia* individuals, and quantitatively evaluated the body shape variation by geometric

morphometrics. Morphological identification, at least when based on photographs, gave substantially worse results than any molecular method. The least successful was differentiation between *D. galeata*, pelagic *D. longispina*, and their hybrids; these taxa showed particularly high degree of overlap of their body shape.

Key words: microsatellite analysis, allozyme electrophoresis, ITS-RFLP, introgression, gene conversion

INTRODUCTION

Taxonomy of the cladoceran genus *Daphnia* was for many years complicated by presence of large morphological variability among individuals within species, which caused many discrepancies in identification of forms and their taxonomical grouping. In the literature, it is not rare to find changes of status of certain taxa from species to form and back several times in a short period (Flössner & Kraus 1986, Petrusek 2008a). Due to the occurrence of so many morphological forms and high similarity of certain species, the genus *Daphnia* has been considered as "…one of the taxonomically most difficult groups of the animal kingdom" (Flössner & Kraus 1986). Although members of the genus have been for many years commonly used as model organisms in various fields, from aquatic ecology to ecotoxicology, the discussion about number and validity of *Daphnia* species remains still open (Nilssen et al. 2007, Petrusek et al. 2008a).

Taxonomical problems are partly caused by high level of environmentally induced phenotypic plasticity as natural forms commonly produce various morphs in variable environmental conditions. Genetically identical individuals thus may exhibit different size or different carapace- and head-shape (Flössner & Kraus 1986) Environmental conditions may also cause development of specialized chitinous formations. For instance, the creation of head helmets in *Daphnia cucullata* can be induced by increased turbulence of water (Brooks 1947; Hrbáček 1959 Tollrian & Laforsch 2006) or as a response to presence of fish (Brooks 1965; Jacobs 1965) and invertebrate predators (Tollrian & Laforsch 2006).

However, environmentally induced phenotypic plasticity is not only source of morphological variability within species. Intraspecific variation in phenotypes may be also genetically-based (Gießler 1997, Petrusek 2008a), and substantial morphological changes may be attributed to interspecific hybridization. Based on morphology, this has long been suspected in the *D. longispina* species complex (Lieder 1956, Einsle 1966, Lieder 1983), where occurrence of morphologically intermediate forms is common. Later genetic studies

confirmed presence of interspecific hybridization within the group (e. g. Wolf & Mort 1986, Schwenk & Spaak 1997) and showed that hybrids commonly occur in syntopy with parental species. Mostly commonly hybridizing species are *D. galeata*, *D. longispina* and *D. cucullata*, hybrid swarms of which are mostly maintained by amictic parthenogenesis. Sexual reproduction of hybrids and backcrossing followed by nuclear introgression also occurs, but seems to be relatively rare (Spaak 1996, Jankowski & Straile 2004, Chapter 1). However, it could have crucial consequences for the evolution of this group. Gene flow among the parental species may support reticulate pattern of the evolution which could lead to the increasing variability in local gene pools (Schwenk 1997) and in morphology. Though certain studies suggest an important role of reticulate evolution for the *D. longispina* complex (Schwenk et al. 1995; Gießler et al. 1999), gene pools of parental species seem distinct, and may be well distinguished by various molecular methods (Keller et al. 2007).

In order to understand evolutionary processes in *D. longispina* group and to resolve identification difficulties caused by interspecific hybridization and by environmentally induced genotypic plasticity, various molecular marker systems have been used. Early studies using allozyme electrophoresis were limited by the small number of fixed diagnostic loci (Wolf & Mort 1986, Gießler 1997) and complicated by uncomfortable preservation of frozen samples. Despite that, allozyme electrophoresis remains to be used even in recent years (Seda et al. 2007, Petrusek et al. 2008b, Keller at al. 2008). ITS-RFLP, i.e., the restriction fragment length polymorphism (RFLP) of the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (rDNA) (Billiones et al. 2004; Skage et al. 2007), being a DNA-based method, allows using ethanol-preserved material but seems to lack at least in some populations sufficient discriminatory power (Skage et al. 2007). And finally, recently developed microsatellite markers (Brede et al. 2006) enable detailed genetic studies, though so far with substantially higher costs.

The choice of an appropriate molecular tool is an important step in each genetic study, which should guarantee correct and reproducible results. It is even more crucial in investigations of hybridizing species, where the mismatch of molecular methods is expectable (Schwenk & Spaak 1995) and different approaches may lead to very different results. For example, in our previous studies of the *D. longispina* complex (Skage et al. 2007; Petrusek et al. 2008b), we showed that unexpected intraspecific variation may substantially influence interpretation of restriction patterns of ITS-RFLP (Billiones et al. 2004). However, wide

comparison of available molecular methods developed for genetic identification of species and hybrids of the *D. longispina* complex is still missing.

In this study, we investigated discriminatory power of three commonly used molecular methods for identification of species and hybrids in *D. longispina* species complex – allozyme electrophoresis (according to Wolf & Mort 1986, Gießler 1997), ITS-RFLP (according to Skage et al. 2007) and an analysis based on 12 microsatellite markers (from Brede et al. 2006). We assessed limitations of certain molecular markers and hypothesised possible causations of observed inconsistencies. We also compared discriminatory power of all molecular markers to morphology-based determination provided by several experts in taxonomy of cladocerans. The phenotype of each individual was additionally analysed by geometric morphometrics, using elliptic Fourier transformation of the carapace outline (Ferson et al. 1985). Phenotypic variability was subsequently compared to genetic variability, in order to resolve, whether the common identification difficulties in *D. longispina* species complex are caused by human's insufficiency to see minor morphological differences or by real phenotypic similarity among genetically diverse individuals in *Daphnia*.

MATERIALS AND METHODS

Sampling and preservation of the samples

Daphnia individuals were collected using plankton net (mesh size 170 μm) in several localities across the European continent; among them also in ten sites of the Czech Republic (Figure 1, Table 1). Territories of *D. galeata*, *D. longispina* and *D. cucullata* overlap in this region and interspecific hybrids are therefore relatively common. In order to capture as variable samples as possible, habitats included in the study varied in number of environmental parameters such a size, depth and trophic level. In addition, canyon-shaped reservoirs included in this study are characteristic by substantial range of environmental conditions, and often harbour two or three parental species as well as their interspecific hybrids (Seda et al. 2007, Petrusek et al. 2008b).

Samples from the Czech Republic were frozen in liquid nitrogen shortly after collection and stored in a deep-freezer. 160 to 210 adult fertile females from the *Daphnia longispina* species complex were randomly selected from each sample, photographed from the lateral side and subsequently used for allozyme- and DNA-based identification, as well as for shape analysis and the subset of them for the determination by phenotype. All additional samples were preserved in ethanol, and were used for DNA-based methods only. From the

total number of 2352 animals, 444 individuals were simultaneously identified by all three molecular methods, which enabled direct comparison of the markers.

Each individual was determined as one of the parental species (*D. galeata*, *D. longispina*, *D. cucullata*) or as a hybrid genotype. More detailed determination of certain hybrid class was not done for purpose of comparison of the molecular methods, as it was not essential. In addition, correct assignment of hybrid class is impossible if the ITS-RFLP method is used, as the ITS, although present in high number of copies in the genome, essentially behaves as a single locus. Similarly, proportion of later-generation hybrids may be underestimated when only the two commonly used loci with species-specific alleles (sAAT and AO) (Gießler 1997) are employed. However, more detailed information about taxon composition obtained by analysis of 12 microsatellite markers was used for some additional calculations (see below) and as evidence supporting proposed hypotheses.

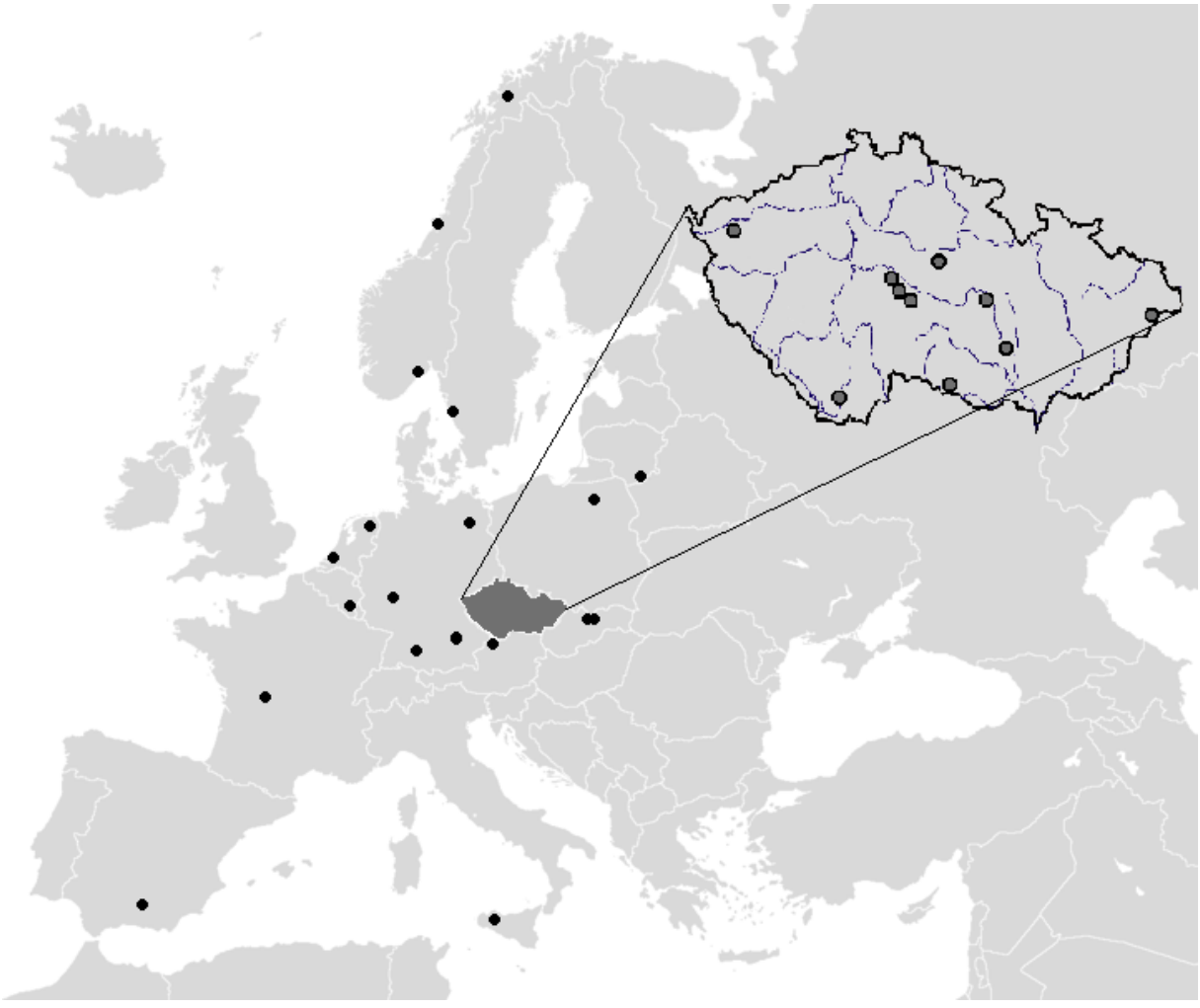

Figure 1. Location of the sampling sites analyzed in this study (dots). Canyon-shaped reservoirs sampled in the Czech Republic are indicated in the zoomed map, which also shows the major rivers within the country.

Sampling site	Country	Latitude	Longitude	Taxa present	Used methods
Kníničky	Czechia	49° 14´	16° 31´	g, l, c, gxl, gxc	allo, msats, ITS-RFLP, morph
Římov	Czechia	48° 50´	14° 30´	g, c, gxc	allo, msats, ITS-RFLP, morph
Seč	Czechia	49° 50´	15° 39´	g, c, gxc	allo, msats, ITS-RFLP, morph
Sedlice	Czechia	49° 31´	15° 12´	g, c	allo, msats, ITS-RFLP, morph
Stanovice	Czechia	50° 11´	12° 53´	g	allo, msats, ITS-RFLP, morph
Šance	Czechia	49° 31´	18° 25´	g, gxl	allo, msats, ITS-RFLP, morph
Trnávka	Czechia	49° 31´	15° 13´	g, c, gxc, gxl	allo, msats, ITS-RFLP, morph
Vír	Czechia	49° 34´	16° 19´	g, c, l, gxl	allo, msats, ITS-RFLP, morph
Vranov	Czechia	48° 54´	15° 49´	g, c, l, gxc, gxl	allo, msats, ITS-RFLP, morph
Želivka	Czechia	49° 43´	15° 06´	g, l, gxl	allo, msats, ITS-RFLP, morph
Mondsee	Austria	47° 51'	13° 23'	l	msats, ITS-RFLP
Etang de Bellebouche	France	46° 42'	01° 06'	g, c, gxc	msats, ITS-RFLP
Usingen, Hattstein Weiher	Germany	50° 20'	08° 30'	c	msats, ITS-RFLP
Ismaning	Germany	48° 13'	11° 46'	l, gxl, cxl	msats, ITS-RFLP
Stechlinsee	Germany	53° 09'	13° 01'	l, gxl	msats, ITS-RFLP
Bodensee	Germany	47° 34'	09° 31'	g, gxl	msats, ITS-RFLP
Piana Degli Albanesi	Italy	37° 58'	13° 18'	g	msats, ITS-RFLP
Drabuzis	Litvia	54° 34'	24° 39'	c, l, cxl	msats, ITS-RFLP
Groningen	Netherlands	53° 13'	06° 37'	g, gxl	msats, ITS-RFLP
Delftse Houd	Netherlands	51° 57'	04° 21'	g	msats, ITS-RFLP
Goksjo	Norway	59° 10	10° 09'	l, gxl	msats, ITS-RFLP
Nordfjordvatn	Norway	69° 16	19° 01'	l	msats, ITS-RFLP
Storveavatn	Norway	64° 50'	11° 22'	l, gxl	msats, ITS-RFLP
Mikolajkie	Poland	53° 46'	21° 35'	c	msats, ITS-RFLP
Nižné Jamnické	Slovakia	49° 12'	19° 46'	l	msats, ITS-RFLP
Satanie	Slovakia	49° 10'	20° 30'	l	msats, ITS-RFLP
Cogollo	Spain	27° 12'	-02° 50'	g, gxc	msats, ITS-RFLP
Göteborg	Sweden	57° 42'	12° 00'	l, gxl	msats, ITS-RFLP
St.Bernard	Switzerland	45° 52'	07° 10'	l	msats, ITS-RFLP

Table 1. List of sampling sites, their geographical location, occupancy by species and hybrids of the *D. longispina* species complex and list of methods, from which results of taxon identification are available: allozyme electrophoresis (allo), microsatellite analysis (msats), ITS-RFLP and morphology-based determination (morph). Lists of taxa present at the localities is based on results of the Bayesian analysis (NEWHYBRIDS software) using information of 12 microsatellite markers. Names of the species (*D. galeata*, *D. longispina*, *D. cucullata*) are abbreviated by the first letter of their species names.

Allozyme electrophoresis

Majority of individuals (from ten out of thirteen populations) was primary identified by allozyme electrophoresis on cellulose acetate gels (Hebert & Beaton 1989). Four allozyme loci were analysed: sAAT (amino aspartate transferase, EC 2.6.1.1), AO (aldehyde oxidase, EC 1.2.3.1), GPI (glucose-6-phosphtase isomerase, EC 5.3.1.9) and PGM (phosphoglucomutase, EC 2.7.5.1) (for details see Seda et al. 2007, Chapter 1). AO and sAAT loci are fixed for the species and could be therefore used for direct taxon identification. Individuals homozygous in both alleles of AO and sAAT loci were scored as pure species, whilst individuals sharing additive patterns were considered as hybrids. Small proportion of

the animals (2.2 %) exhibited patterns suggesting formation of backcrosses or later generation hybrids. However, they were pooled with other hybrids for the reasons described above.

ITS-RFLP

In order to obtain direct comparison of allozymes and DNA-based methods, we used homogenates from the allozyme electrophoresis as substrates for the DNA isolation. We transferred a 2.5 μL aliquot of the homogenate to 30-μL solution containing H3 buffer and proteinase K (Schwenk et al. 1998). DNA was isolated by incubation at 55 °C for 6-10 hours followed by inactivation of proteinase K by 95 °C for 10 min. Individuals from ethanol-preserved samples were primarily washed from ethanol and DNA was subsequently isolated in the same way. DNA isolates were used for both ITS-RFLP and microsatellite analysis.

Amplification and restriction of the nuclear ribosomal internal transcribed spacer (ITS) mostly followed the protocol by Skage et al. (2007). However, in order to clearly differentiate between *D. galeata* and possibly uncut products, we used an alternative forward primer ITS-NEW (Skage et al. 2007) producing ~190 bp band instead of ~75 bp band of the original protocol. Products of the amplification were restricted by overnight incubation with endonucleases Mbi I and Eco52I (Fermentas) and electrophoresed on agarose gel. The banding patterns were interpreted according to Skage et al. (2007) and Petrusek et al. (2008b). Individuals exhibiting clear additive patterns were scored as hybrids even if the bands were variously intense. However, very week and badly visible bands were not considered. We also occasionally used ITS-RFLP method designed by Billiones et al. (2004) in order to confirm results obtained by double digest; especially in populations where results of ITS-RFLP strongly differed from results obtained by other markers (see Results).

Microsatellite analysis

All samples except seven from the Czech Republic were analysed in details by set of twelve microsatellite markers. Amplifications and length assessment of the fragments mostly followed protocol by Brede et al. (2006). Fragments labelled with the same dye were nevertheless amplified in triads using multiplex PCRs, whereas primer concentrations were adjusted according to those conditions, in order to obtain similar amounts of PCR products (Thielsch et al., in review; for details see Chapter 1). After amplicons had been obtained, they were diluted adequately to their concentrations and electrophoresed on a CEQ 2000 (Beckman Coulter) with self-designed size standards based on Lambda virus DNA (Symonds & Lloyd 2004).

As no microsatellite locus has species-specific length, we used NEWHYBRIDS software (Anderson & Thomson 2002) to compute the posterior probabilities that individuals belong to certain taxa. Individuals were identified as the respective taxa if the computed posterior probability was ≥ 80 % threshold (for details see Chapter 1). Proportions of backcrosses and F_2 hybrids in the samples were also estimated. However, they were pooled with F_1 hybrids, for purpose of comparison with other molecular methods. Detailed taxon determination was used as supporting information when discussing inconsistencies between allozymes and microsatellites (see Results).

Morphological identification

In order to obtain relevant results of the morphological determination based on traditional morphological characters, we asked three experts with experiences in identification of zooplankton samples, in particular *Daphnia*, to determine individuals from our samples. 240 photographs of the lateral side of the carapace were chosen from the set of more than 2000 images for this purpose. Origin of the animals and taxon stayed unknown. All experts were instructed that each individual may belong to one of the following six taxa: *D. galeata*, *D. longispina*, *D. cucullata* or one of their hybrids. The results of identification by morphological traits were consequently added to the dataset and compared with results of genetic determination.

Elliptic Fourier transformation

Photographs of the animals were used as sources for the shape analysis. First of all, body shape of each individual was described by 55-65 coordinate points laying on the outline of the carapace and head shield; body extensions such as antennae and tailspine were ignored (Figure 2). The points were consequently subjected to an elliptic Fourier transformation (Ferson et al. 1985). Information of size, rotation and start position was used as invariants (Schwenk et al. 2004). Normalized coefficients of five harmonic functions (excluding first three parameters of the first function, which are constant under the selected analysis parameters) were arranged as input data for the principal component analysis (PCA) in the software package STATISTICA 6.1 (StaSoft, Inc. 2000).

Data comparison

Results of allozyme electrophoresis, ITS-RFLP and microsatellite analysis were compared with each other, the agreement between each two methods being always expressed

as a percentage. All individuals for which results of at least two different determination methods were available, were included for the comparisons; set of 444 individuals was identified by all three molecular methods. Agreement among molecular markers was calculated for the whole dataset, but also for each sampling site and for each taxon separately. If counted for separate taxa, taxon identification was provided by one marker of the pair – by allozymes, when allozymes / microsatellites and allozymes / ITS-RFLP compared and by microsatellites, when microsatellites / ITS-RFLP compared. As the disagreement between the ITS-RFLP and other two methods was excessively high in some cases (see Results), analyses were repeated or duplicated according to protocol by Billiones et al. (2004).

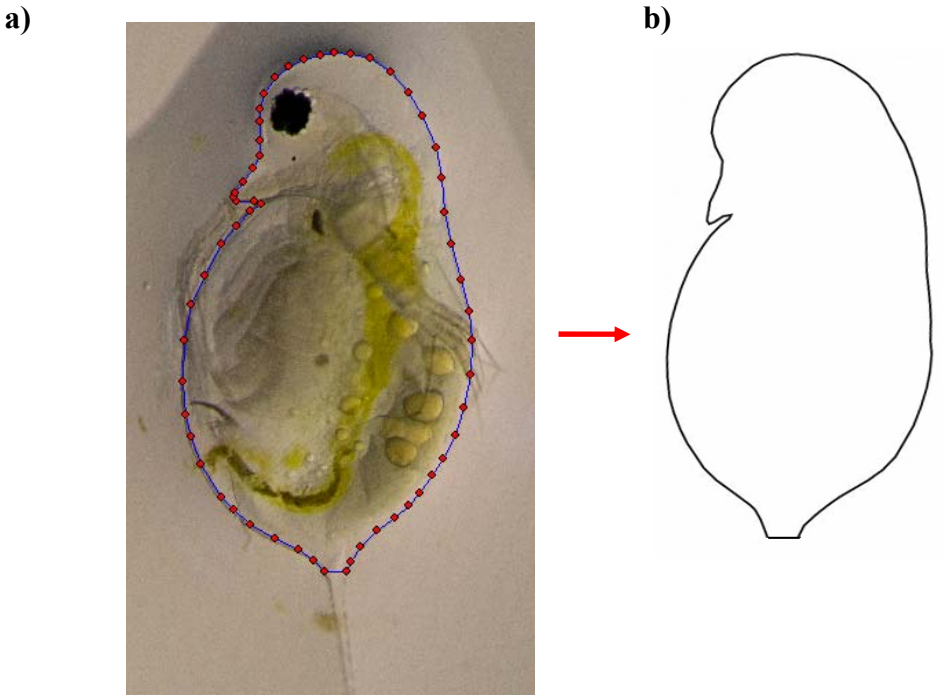

Figure 2. a) *Daphnia* body shape described by coordinate points positioned on the outline of the carapace and head shield (tpsDig2); the data were subsequently used for the elliptic Fourier analysis. **b)** Resulting outline.

RESULTS

Molecular markers

From the ten sampling sites in the Czech Republic, we analyzed altogether 1276 individuals by more than one molecular marker: allozymes and ITS-RFLP could be compared for 1275 individuals, allozymes and microsatellites for 636 individuals and microsatellites and ITS-RFLP for. 444 individuals were simultaneously determined by all three molecular methods, providing an opportunity to identify more likely result in cases where two methods

didn't correspond to each other. Allozymes, microsatellites, as well as ITS-RFLP analyses parted the dataset into five taxa – *D. galeata*, *D. longispina*, *D. cucullata*, *D. galeata* × *longispina* hybrids and *D. galeata* × *cucullata* hybrids. No individual representing *D. longispina* × *cucullata* hybrid was found in the dataset.

In general, the agreement of molecular markers was relatively high (Table 2). The highest fit (97.0%) was observed between results of the allozyme electrophoresis and the microsatellite analysis. Allozymes and ITS-RFLP corresponded to each other in 86.8% and microsatellites and ITS-RFLP in 82.7%. Majority of deviations from the fit among all three different molecular markers seems to occur non-randomly, under certain conditions only.

The level of agreement of molecular methods differed among various sampling sites. For the three reservoirs for which both allozymes and microsatellites were available, the agreement was 100 % in the Stanovice Reservoir, 98.8 % in the Vír Reservoir and 93.8 % in the Vranov Reservoir. This fits with our presumptions, as the Stanovice Reservoir was, in contrast to the Vír and Vranov Reservoirs, inhabited by single species *D. galeata* and mismatch of molecular markers thus was not expected.

Comparison of identification based on allozymes and ITS-RFLP, on the other hand, showed marked disagreement. Interestingly, in reservoirs where hybridization frequently occurred and all three *Daphnia* parental species were present, the mismatch of molecular methods did not markedly exceed that in reservoirs with less frequent hybridization (Table 3, Figure 3). Actually, the trend, if any, was opposite. The biggest mismatch occurred in the Trnávka Reservoir (agreement 54.8%) where the proportion of hybrids identified by allozyme markers was <3%, followed by the Stanovice Reservoir (agreement 81.0%) in which no hybrids were found. In the latter reservoir, the pattern was very similar (agreement 81.8%) when the results of ITS-RFLP were compared to those from the microsatellite analysis. In the Vranov and Vír Reservoirs, the agreement between these two methods was also slightly over 80% (81.2% and 86.0%, respectively).

In order to clarify why the fit among markers varied among different reservoirs, we also evaluated the success of all three molecular methods in identifying each parental species and their hybrids. The taxon, for which the determination by allozymes and microsatellites deviated most strongly, was *D. cucullata* (Table 4). Only 84.87% individuals identified as *D. cucullata* by allozyme markers were identified as such by microsatellite analyses, whereas the agreement of identification between these two marker systems was almost perfect in other taxa (99.7% for *D. galeata* and 100% for the rest). All of these „problematic" *D. cucullata* individuals originated from the Vranov Reservoir and were determined as pure species by

allozymes and by ITS-RFLP, but as hybrids by microsatellites. In a detailed analysis of hybrid classes, computed by the NEWHYBRIDS software (Anderson & Thomson 2002) from microsatellite data, all these individuals seemed to be backcrosses or F_2 hybrids.

Marker	Allozymes	ITS-RFLP	Microsatellites	Morphology
Alozymes	×	86.8%	97.0%	68.8%
ITS-RFLP	1107/1275	×	82.7%	N/A
Microsatelites	432/445	526/636	×	N/A
Morphology	165/240	N/A	N/A	×

Table 2. Agreement in determination of individuals belonging to the *Daphnia longispina* complex by allozyme electrophoresis, ITS-RFLP, microsatellite analysis and morphology (general phenotype form photographs). The whole dataset comprised 1276 individuals, 444 individuals were simultaneously determined by all three molecular methods. Data above diagonal indicate the agreement between the methods as the percentage of individuals identified identically by both methods, data below diagonal show respective absolute numbers (the first value represents number of individuals identically determined; the second value represents total number of individuals compared).

Site / Marker	Allo × Msats		Allo × ITS-RFLP		Msats × ITS-RFLP	
	N	Percentage	N	Percentage	N	Percentage
Kníničky (CZ)	×	×	121/134	90.3%	×	×
Římov (CZ)	×	×	131/134	97.8%	×	×
Šance (CZ)	×	×	118/131	90.1%	×	×
Seč (CZ)	×	×	115/124	92.7%	×	×
Sedlice (CZ)	×	×	24/25	96.0%	×	×
Stanovice (CZ)	190/190	100.0%	132/163	81.0%	130/165	81.8%
Trnávka (CZ)	×	×	68/124	54.8%	×	×
Vír (CZ)	169/171	98.8%	123/139	88.5%	104/121	86.0%
Vranov (CZ)	258/275	93.8%	165/172	95.9%	134/165	81.2%
Želivka (CZ)	×	×	110/129	85.3%	×	×
Usingen, Hattstein W.(GE)	×	×	×	×	3/33	9.1%
Etang de Bellebouche (FR)	×	×	×	×	9/17	52.9%
Mikolajkie (PL)	×	×	×	×	5/11	45.5%

Table 3. Agreement between identification of *Daphnia* individuals by allozyme electrophoresis (Allo), microsatellite analysis (Msats) and ITS-RFLP within various sampling sites in the Czech Republic (CZ), Germany (GE), France (FR) and Poland (PL). All Czech localities are shown, but only three selected European sites where marked disagreement between ITS-RFLP and microsatellites was observed. Relative agreement between two methods is shown as a percentage of individuals determined identically by both methods (first values in the column N) from the total number of compared individuals (second values). Results marked in red indicate disagreement of the methods caused by consistent trends for misclassification of certain taxon (see Results).

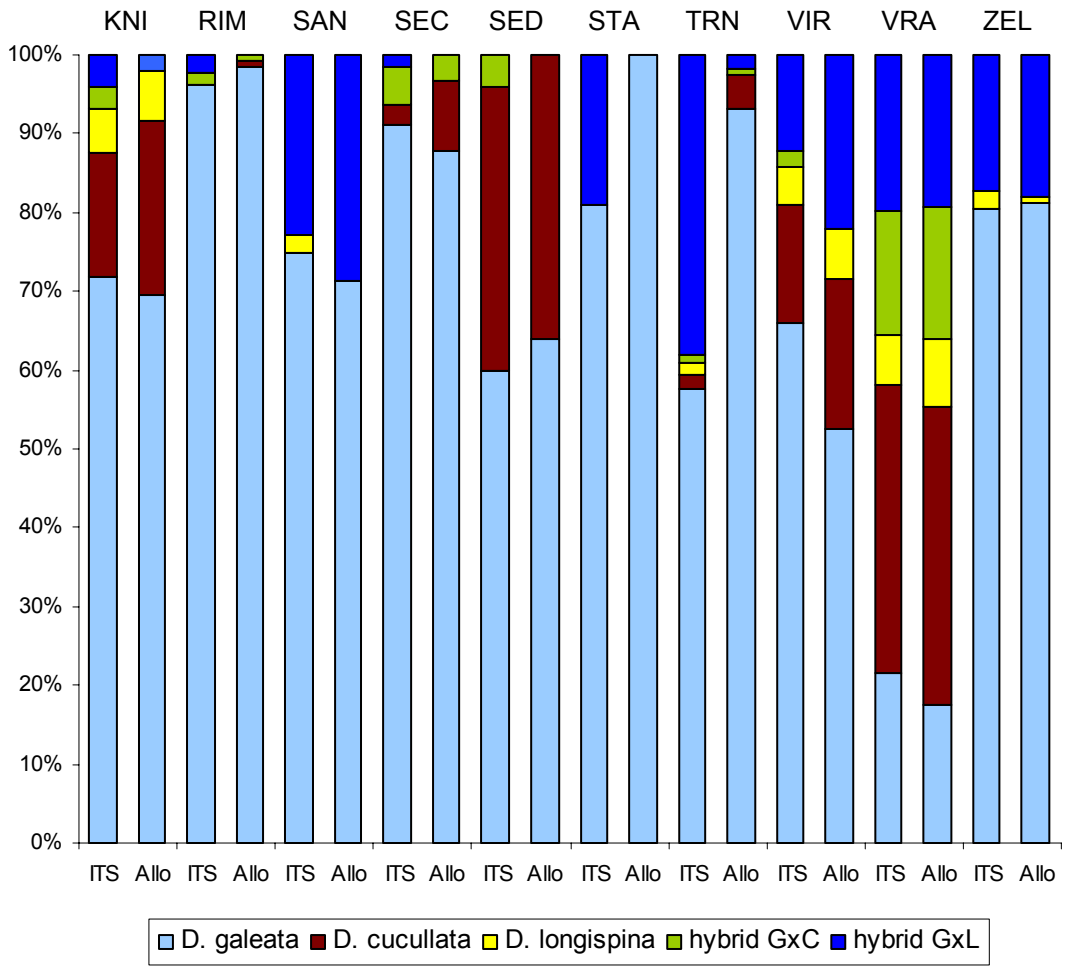

Figure 3. Taxon determination within ten Czech reservoirs comparing identification of the same individuals by ITS-RFLP (ITS columns) and allozyme electrophoresis (Allo columns). Name of the reservoir is abbreviated by first three letters and marked above the respective columns. Numbers of analyzed individuals from each sampling site are given in Table 3. Note the marked disagreement of the methods for individuals from Stanovice (STA) and Trnávka (TRN).

However, all individuals identified as *D. galeata* × *cucullata* hybrids by allozymes also appeared to be backcrosses or F_2 hybrids too based on microsatellites (Chapter 1), and no individual was classified as F_1 *D. galeata* × *cucullata* hybrid in the sample of 300 individuals form two reservoirs where all three *Daphnia* species co-occurred.

Agreement of ITS-RFLP with the other two markers, if counted for each taxon separately, ranged between 74.1% and 88.7% for ITS-RFLP/allozymes and between 60.0% and 88.5% for ITS-RFLP/microsatellites. In general, hybrids were the most problematic group where discrepancies between identification by ITS-RFLP and other markers were common (Table 4).

Species \ Marker	Allo × Msats		Allo × ITS-RFLP		Msats × ITS-RFLP	
	N	Percentage	N	Percentage	N	Percentage
D. galeata	329/330	99.7%	823/928	88.7%	223/252	88.5%
D. cucullata	101/119	84.9%	135/154	87.7%	68/77	88.3%
D. longispina	41/41	100%	25/30	83.3%	15/21	71.4%
hybrid *gal* x *cuc*	37/37	100%	27/32	84.4%	24/40	60.0%
hybrid *gal* x *lon*	109/109	100%	97/131	74.1%	38/55	69.1%

Table 4. Agreement between identification of *Daphnia* individuals by allozyme electrophoresis (Allo), microsatellite analysis (Msats) and ITS-RFLP within various species and interspecific hybrids. Relative agreement between two methods is shown as a percentage of individuals determined identically by both methods (first values in the column N) from the total number of compared individuals (second values). Results marked in red indicate disagreement of the methods caused by consistent trends for misclassification of certain taxon (see Results).

Identification of *D. galeata* was overall the most successful among all taxa, however, this pattern changed when the agreement among markers was calculated separately for taxa within each reservoir: two reservoirs, Stanovice and Trnávka, exhibited large proportion of inconsistent identification of *D. galeata* in particular, but not of other taxa.The only *Daphnia* species observed in Stanovice Reservoir in summer 2004 was *D. galeata.* This was independently confirmed by both allozyme and microsatellite analyses, and no other taxa were found. ITS-RFLP analysis of the same individuals nevertheless showed that over 19 % of animals also carried the allele ITS considered typical for *D. longispina*, and the resulting restriction pattern was thus the same as in *D. galeata* × *longispina* hybrids (Figure 4). Analogous situation was found also in the Trnávka Reservoir, where 37.9% of *D. galeata* individuals (determination based on allozymes) exhibited the same restriction pattern as observed in Stanovice. Although allozyme-based determination was not simultaneously confirmed by another molecular marker in this case, the morphology of those individuals corresponded more to *D. galeata* than to interspecific hybrids.

Subsequent investigation of additional samples from other European countries showed that problems with identification of ITS-RFLP cuts and presence of nonstandard restriction patterns are widespread. Three samples from a group of nineteen populations exhibited regular inconsistencies between results obtained by microsatellite analysis and ITS-RFLP In these three particular localities, fit between markers was very low: 9.9% in Usingen & Hattstein Weiher (Germany), 45.5% in the Lake Mikolajkie (Poland) and 52.9% in Etang de Bellebouche (France). All problematic individuals were determined as pure *D. cucullata* by morphology and microsatellites, according to ITS-RFLP patterns they would be identified as *D. galeata* × *cucullata* hybrids. In order to exclude possibility of an incomplete restriction,

which would falsely suggest the presence of a *D. galeata* allele, the results were verified by repeated amplification and restriction, using the new protocol according to Skage et al. (2007) with longer PCR products. The shortest band (ca 190 bp) confirming the restriction of a *D. galeata* allele was mostly visible, suggesting that the PCR-products were cut well.

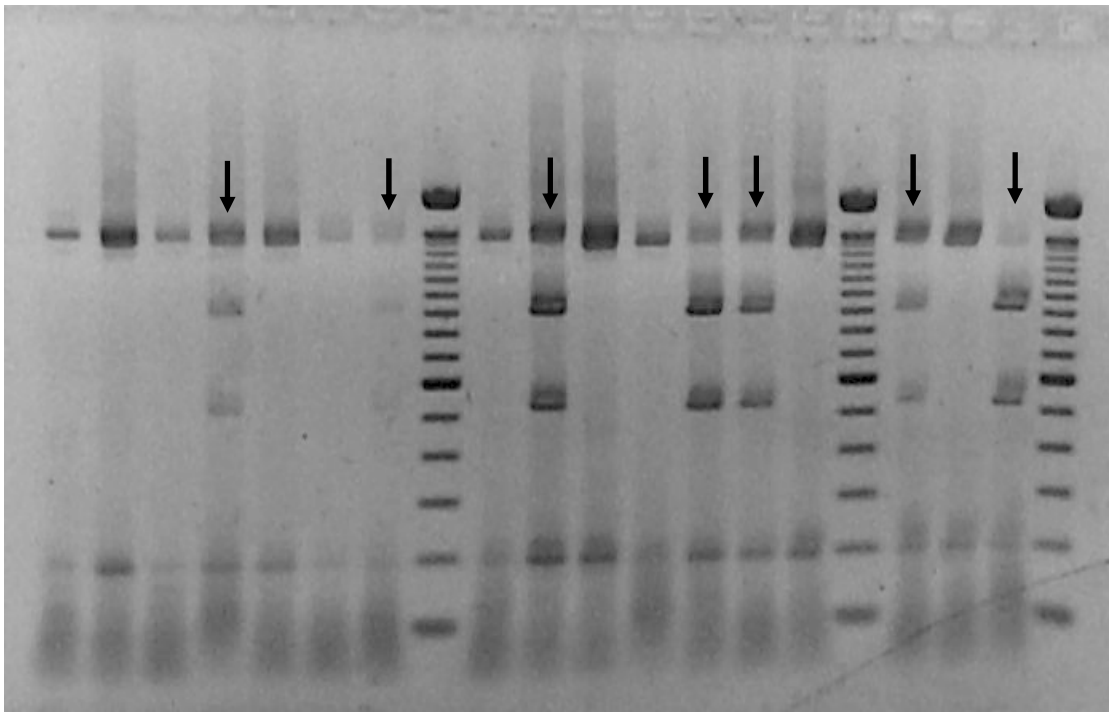

Figure 4. Restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) of 17 randomly chosen *Daphnia* females sampled in the Stanovice Reservoir (CZ), following the protocol by Skage et al. (2007); see Methods for details. Individuals labeled by arrows show clear additive patterns characteristic for *D. galeata* × *longispina* hybrids, although all of them were determined as *D. galeata* by 12 microsatellite and two allozyme markers. Remaining individuals were identified as *D. galeata* by all methods. 100 bp ladder was used as a size standard.

Morphology

Morphological determination provided by three experts was substantially less successful than molecular methods (Table 5). In average, it agreed with allozyme electrophoresis in only 68.8%. However, the most successful determination corresponded to allozymes in 82.9%, which was only slightly worse than the agreement between allozymes and ITS-RFLP (86.8%). All three persons were able to recognize pure species relatively successfully. The average success was the highest for *D. cucullata* (90.9%), followed by *D. longispina* (82.7%) and *D. galeata* (79.0%). Morphological determination of hybrids seemed to be more complicated: one expert was not able to differentiate them from the

parental species, other two persons were more likely to recognize *D. galeata* × *cucullata* hybrids (80% and 100%) than *D. galeata* × *longispina* hybrids (50% and 24%). *D. galeata* × *longispina* hybrids were apparently the most difficult to identify by morphological characters, with the overall success of identification only 24.7%.

	Expert 1		Expert 2		Expert 3		Average
Species	N	Percentage	N	Percentage	N	Percentage	success
all species	199/240	82.9%	156/240	65.0%	140/240	58.3%	68.8%
D. galeata	134/138	97.1%	99/138	71.7%	94/138	68.1%	79.0%
D. cucullata	18/22	81.8%	21/22	95.5%	21/22	95.5%	90.9%
D. longispina	18/25	72.0%	19/25	76.0%	25/25	100%	82.7%
hybrid *gal* x *cuc*	4/5	80.0%	5/5	100%	0/5	0%	60.0%
hybrid *gal* x *lon*	25/50	50.0%	12/50	24.0%	0/50	0%	24.7%

Table 5. Success of morphological determination provided by three experts experienced in cladocerans taxonomy or ecology, based on 240 photographs of the lateral view of the *Daphnia* body. Relative success of the determination is shown as a percentage of individuals determined identically by both morphology-based and genetic-based method (first values in the column N) from the total number of determined individuals (second values). Species and inferspecific hybrids were primarily identified by allozyme electrophoresis using two species-specific loci (sAAT, AO).

Geometric morphometric analysis of Daphnia *body outlines*

Morphological variability of *Daphnia* individuals, as summarized by principal component analysis of parameters from elliptic Fourier transformation of body outlines, was high. However, all individuals formed a relatively compact cluster in the PCA plot (Figure 5), and only *D. cucullata* could be separated from the others. When individuals were labeled by colors according to the identification by allozyme and microsatellite analyses, the cluster split into five compact regions, each representing different species or different hybrid group. Hybrid groups, apparently representing morphologically intermediate forms, formed clusters situated between and partly overlapping those of parental species. The overlap existed also between clusters of pure *D. galeata* and *D. longispina*; however, these did not overlap with pure *D. cucullata*. In general, *D. galeata*, *D. longispina,* and their hybrids were morphologically closer to each other than *D. galeata*, *D. cucullata*, and their hybrids.

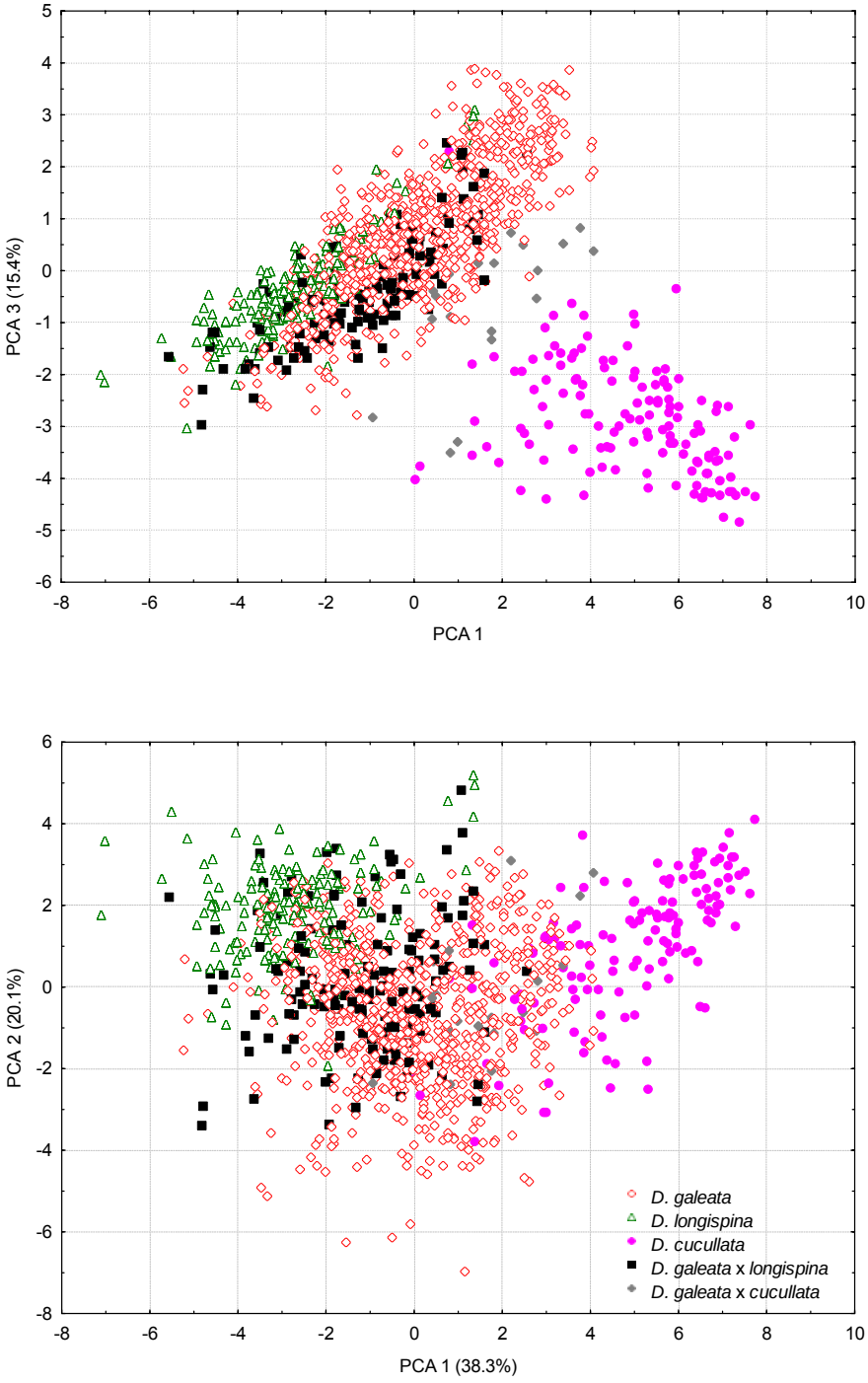

Figure 5. First three components of the principal component analysis (PCA) (STATISTICA 6.1) showing body shape variability of 1102 individuals of the *Daphnia longispina* species complex from eight canyon-shaped reservoirs (Czech Republic). Normalized coefficients of five harmonic functions from elliptic Fourier analysis of individual body outlines (altogether 17 variables) were used as input for PCA. Individuals are labeled by different colors accordingly to the taxon as identified by two allozyme markers. First three components explain 73.8% of variation in the input data.

DISCUSSION

Processes following interspecific hybridization, such as backcrossing and occasional gene-flow, may significantly influence correctness of genetic and morphological determination in hybridizing species (Schwenk et al. 1995, Billiones et al. 2004, Skage et al. 2007). Taxonomy in such groups is complicated by occurrence of various morphological forms. Thus, parental species can be undistinguishable from hybrids or even from other parental species (Flössner 1986). At the genetic level, backcrossing and gene-flow may lead to mismatch among different molecular methods (Harrison 1990, Arnold 1992), especially where only limited number of loci is used. In PCR-RFLP, for instance, recombinant genotypes may be easily associated to either interspecific hybrids or pure species (Bert et al. 1996; Boecklen & Howard 1997) as later generation hybrids exhibit a mosaic of parental gene-pools and single locus may give incomplete information. In the *D. longispina* species complex, such an underestimation of recombinant classes has been observed in commonly used molecular markers – in ITS-RFLP (Billiones et al. 2004) and also in allozymes (Seda et al. 2008), where only two loci in common use are species-specific (AO, sAAT). Observed inconsistencies among markers have been therefore mostly attributed to their insufficient discriminatory power, unable to reveal backcrossing and introgression (Billiones et al. 2004). Results of our study (Chapter 1) nevertheless showed that proportion of later generation hybrids and individuals exhibiting introgression was very low in all sampling sites, except the Vranov Reservoir. This finding is also in concordance with other published population studies (Spaak 1996, Jankowski & Straile 2004). Thus, it is necessary to look for the causes of observed discrepancies among molecular markers in other processes.

ITS-RFLP

The hypothesis that mismatch among ITS-RFLP and other two molecular markers in our samples was caused by high frequency of hybridization, by backcrossing or by gene-flow could be falsified by comparison of results from various sampling sites. If that was true, sites with more frequent hybridization should exhibit more discrepancies among different markers. However, the observed trend was not consistent with these assumptions – the biggest inconsistencies between ITS-RFLP and allozymes were actually observed in reservoirs with no or very low proportion of hybrids in the active population (Stanovice and Trnávka respectively).

We observed two common types of inconsistencies among ITS-RFLP and other, more proven markers. First, non-*galeata* individuals (as determined by allozymes or

microsatellites) exhibited restriction patterns typical for *D. galeata* or patterns typical for hybrids with *D. galeata*. Using double digestion according to the original protocol published by Skage et al. (2007), completely or partly uncut PCR product composed of alleles of other species might be mistaken for pure *D. galeata* or its hybrids. This problem, however, could not be the cause of inconsistencies observed in our study, as we used the primer pair producing longer amplicon, cutting ca 190 bp band from *D. galeata* allele during the restriction, which can be used for verification of the complete digestion. *D. cucullata* populations from Usingen & Hattstein Weiher, Lake Mikolajkie and Etang de Bellebouche exhibited extremely high proportion of such individuals, despite multiple verifications of the results. One possible cause of such problems could be a mutation in restriction site for endonucleases Mbi I / BsrB I. Cuts of ITS-amplicon in *D. cucullata* provided by Mbi I normally produce three bands – ~190 bp, 650 bp and 750 bp. If there would be a mutation in restriction site separating the 650 bp 750 bp band, individuals with mutation would exhibit restriction patterns typical for *D. galeata* × *cucullata* hybrids (if mutation in one allele) or patterns typical for *D. galeata* (mutation in both alleles), whereas the ca190 bp band would be still present and visible on gels. Analogous situation was described by Skage et al. (2007), where the point mutation in the restriction site for the endonuclease Mwo I (used by Billiones et al. 2004 for identification of *D. galeata*) caused misidentification of some *D. galeata* individuals in various populations. This hypothesis, though seems to be the most probable, needs further verification, preferably by sequencing of the respective ITS alleles from individuals showing deviations.

Secondly, a large proportion of *D. galeata* individuals exhibiting restriction patterns identical to *D. galeata* × *longispina* hybrids was observed in Trnávka and Stanovice Reservoirs. This type of restriction pattern is formed by Eco52I / Eag I endonucleases, which cuts the *D. longispina* ITS allele into two bands of approximately 900 and 540 bp in length, while the *D. galeata* allele should remain uncut. Independent origin of the restriction site for this endonuclease with 6-bp long recognition site in *D. galeata*, which would produce identical restriction pattern like in *D. longispina*, seems to be unlikely. In addition, presence of a point mutation resulting accidentally in convergent restriction pattern was excluded by the use of Mwo I restriction (Billiones et al. 2004), which also produced additive *D. galeata* × *longispina* hybrid-like restriction patterns.

Introgression of ITS alleles from *D. longispina* to *D. galeata* may result in the patterns described above. In past, hybrids could occur in the sampling sites and the horizontal gene flow between species could proceed. As the taxa were determined by two allozyme loci only,

the proportion of later-generation hybrids could be markedly underestimated. However, this could not be the case of the Stanovice Reservoir, in which the taxa composition was simultaneously estimated using 12 microsatellite loci (Brede et al. 2006). All individuals were determined as pure *D. galeata* (see Chapter one), exhibiting no signs of backcrossing or introgression. Introgression of the *longispina*-like ITS allele thus probably had to take place earlier, in more distant history. The question, nevertheless, remains: how was the *D. longispina* ITS allele maintained in the genome of *D. galeata*, and why was it not diluted by backcrossing with pure *D. galeata*?

ITS regions are segments of a ribosomal DNA occurring in multiple copies within a genome (multi-gene families). On the one hand, multi-copy character of the marker facilitates amplification, but on the other hand, may reveal inconsistent patterns due to processes such a concerted evolution (Arnheim 1983; Dover et al. 1993, Murti et al. 1994) and gene conversion. Gene conversion is an event in DNA genetic recombination removing sequence heterogeneity between two strands of different chromosomes. During this process, sequence of one of the chromosomes is re-written according to the template; so that both sequences are identical. This may lead to non-Mendelian inheritance and to increasing number of such copies in population (Stacey 1994). Concerted evolution and gene conversion have often been recorded in fungal crosses (Kull & Kalevi 2000); however, we are not aware of data available for animal hybrid genomes. Non-Mendelian inheritance may be also caused by other mechanisms favoring selfish genetic elements. It has been shown, for instance, that meiotic drive causes segregation distortions in mice (Futuyma 2005) or in the dipteran *Cyrtodiopsis* sp. (Wright et al. 2004). Same mechanism could be theoretically responsible for conservation of alien ITS rDNA sequences within *D. galeata* genome. However, verification of both proposed scenarios would be very complicated and would need further experiments.

Microsatellites

Fit between taxon determination from allozyme and microsatellite data was in general very high suggesting that both methods are reliable if used for basic determination of species and hybrids in the *D. longispina* species complex. High agreement between the two methods also suggests that discrepancies among any of these and ITS-RFLP can not be explained by increased level of introgression and high proportion of later-generation hybrids and backcrosses, as a relatively precise determination of hybrid class should be possible by using microsatellite and allozyme data.

Inconsistencies between allozymes and microsatellites nevertheless also occurred, significantly affecting individuals related to *D. cucullata*. As mentioned above, some apparently pure *D. cucullata* individuals, when determined by allozymes, were suggested to be *D. galeata* × *cucullata* hybrids by Bayesian inference calculated by NEWHYBRIDS, and all apparent *D. galeata* × *cucullata* hybrids as backcrosses or F_2 hybrids. The problems in hybrids persisted even if the same statistic was performed with four allozyme loci (Ruthová et al., unpublished results), suggesting that the inconsistencies were not caused by marker system, but were genetically-based. Difficulties with determination of *D. galeata* × *cucullata* hybrids by NEWHYBRIDS based on allozyme markers were also reported in Keller et al. (2008).

The observed patterns of disagreement between allozymes and microsatellites within the Vranov Reservoir suggest a certain level of horizontal gene flow among species of the *D. longispina* species complex, where backcrosses and later generation hybrids may serve as links among species. In such cases, gene pools of the parental species could be partly fused and inconsistencies among the species-specific markers may occur (Harrison 1990, Arnold 1992). Importance of this process has already been proposed in earlier studies (Schwenk et al. 1995, Gießler et al. 1999), which understood the *D. longispina* species complex as a group of taxa with incomplete reproductive isolation. Thus, species phylogeny could exhibit reticulate rather than hierarchical pattern of evolution. In the contrary, Keller et al. (2007) recently suggested that levels of effective gene flow within the complex are very low, and that parental species remain reproductively isolated despite hybridization, dismissing the concept of reticulate evolution as important process.

Results of our study support the hypothesis that backcrosses and later generation hybrids occasionally sexually produce viable offspring. However, inconsistencies of allozyme and microsatellite markers were observable only in *D. cucullata*. This suggested assortative mating between *D. cucullata* and *D. galeata*, as some of the individuals were probably genetically incompatible, combined with occasional horizontal gene flow. In contrast to this, substantial deviations of some ITS-RFLP patterns more likely suggested rarer introgression and long-term maintenance of introgressed alleles in genomes of parental species by non-Mendeliean inheritance.

Morphology

Comparison of body shapes of pure species and interspecific hybrids clearly suggested that all taxa produce morphologically variable forms. Although hypothetical “average

phenotypes" of each taxon would differ from the others, the variation within all groups was relatively high, and all clusters, except of parental *D. cucullata*, overlapped with each other. This suggests that individuals within the complex may exhibit nearly identical body shapes independently on their taxonomic origin. We expected that for pure species and their hybrids, as hybrids are known to exhibit various morphological forms, less or more similar to their parental species (Jankowski & Staraile 2004). However, we observed individuals exhibiting practically undistinguishable body shapes, which exhibited pure genotypes of different parental species (*D. galeata* and *D. longispina*).

Phenotypic similarity between individuals from different taxa apparently caused that morphological determination was general less successful than determination provided by molecular methods. General body shape seems to have been used as the most important character in the morphological determination of the photographed individuals by experts. Most incorrectly determined individuals, consistently with the shape analysis, were therefore among *D. galeata*, *D. longispina*, and their hybrids. Our results nevertheless illustrate that the human eye is capable to recognize hardly describable and non-specific differences in morphology and relatively well discriminate among species by "general habitus" even if limited number of species-specific characters is visible. Success of determination based on morphological characters thus could be relatively high when at least minor morphological differences among individuals of different taxa exist in natural populations. It is likely that addition of more characters, which could not be examined on *Daphnia* photographs (e.g. pigmented bands of antennae, shape of antennular mound) would further increase the identification success. However, no expert on morphological determination, however experienced, is likely to reach the success of molecular markers within the intricate *D. longispina* species complex.

CONCLUSION

Interspecific hybridization frequently occurs within the *D. longispina* species complex, in which markedly complicates ecological and evolutionary studies of the group. Occasional sexual reproduction of later generation hybrids probably enables gene flow, which may cause occurrence of individuals carrying "alien" DNA in their genomes. Thus, as we showed in microsatellites, relying on determination by only few species-specific markers can be misleading and inconsistencies among various markers may occur. The proportion of individuals possibly misidentified because of recent introgression nevertheless seemed to be relatively low in our study. More serious inconsistencies among molecular markers have

probably been caused by other processes, not directly related to hybridization. For example, mutations of restriction sites in the ITS may affect interpretation of restriction patterns, and lead to misleading results of ITS-RFLP in populations where affected alleles are common.

The most complicated was the interpretation of occurrence of additive restriction patterns in individuals, whose ancestors presumably have not been involved in hybridization for several generations. Presumably alien alleles in the gene-pool thus probably had to be maintained by non-Mendeliean inheritance. Possible mechanisms favoring "selfish alleles" include the gene conversion within a multi-gene ITS family and the meiotic drive. However, our knowledge of these processes in *Daphnia* is very limited, and would require further study.

Although the molecular methods had certain limitations, their discriminatory power was high in comparison to morphological determination by general phenotype, even in case of the worst performing marker, ITS-RFLP. The comparison of body shapes clearly demonstrated that such determination is complicated by phenotypic similarity of genetically distinct individuals. Not even experienced taxonomist or *Daphnia* ecologist thus can succeed when identifying animals in assemblages where hybridization frequently occurs.

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GENERAL CONCLUSIONS

Results of this thesis can be summarized in several points:

- canyon-shaped reservoirs may be used as an appropriate model not only in studies of species distribution and interspecific interactions, but also in investigations of intra-population dynamics and intraspecific genetic differentiation
- horizontal and vertical ecological gradients of reservoirs may improve conditions for niche differentiation and taxon coexistence, as well as they may facilitate clonal selection within particular populations
- intensity of environmental gradients may influence number of *Daphnia* species inhabiting the reservoir; reservoirs with weak gradients tend to be inhabited by one species only, localities with stronger gradients host more species as well as their hybrids
- high number of distinct hybrid genotypes and thus high genotypic richness in *D. galeata* × *longispina* and *D. galeata* × *cucullata* hybrids suggest that hybridization between *Daphnia* species in reservoirs is a common and repeated process
- despite frequent hybridization, later-generation hybrids and backcrosses were recorded only rarely
- spatial distribution of backcrosses on the reservoir gradients is similar to the distribution of the more related parental species
- individuals probably exhibiting introgressed alleles in their genomes were occasionally recorded, the horizontal gene flow among species nevertheless seems to be weak
- occasional incorrect estimation of the hybrid class in *D. galeata* × *longispina* could be explained by recent horizontal gene flow between *D. galeata* and *D. cucullata*, or by shifts in allele frequencies caused by non-random mating during the process of hybridization
- substantial deviations of some ITS-RFLP patterns more likely suggest a long-term maintenance of introgressed alleles in genomes of parental species; this can be facilitated by processes such as gene conversion and meiotic drive, resulting in non-Mendeliean inheritance
- despite certain limitations, allozyme electrophoresis and both evaluated DNA-based methods are more reliable for taxon identification within the *D. longispina* species complex than morphological determination by general phenotype, complicated by extreme phenotypic similarity of certain individuals belonging to different taxa