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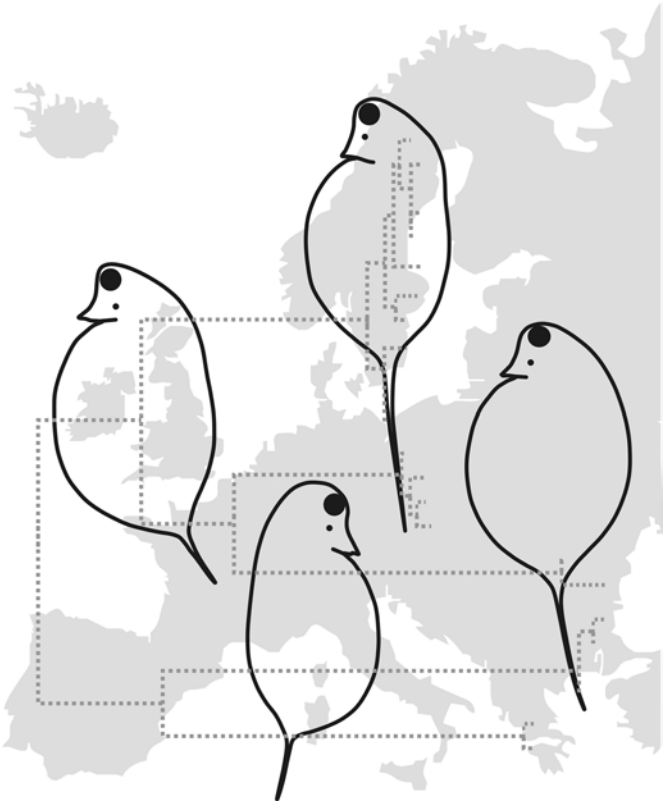
Diversity of European *Daphnia* on different scales: from cryptic species to within-lake differentiation

(PhD thesis)



Supervisor: RNDr. Martin Černý, PhD.

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Manuscripts and papers:

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1. **Petrusek A.**, Tollrian R., Schwenk K., Haas A., Laforsch C. *(unpublished MS)*: "Crown of thorns" protects *Daphnia* against an ancient predator: an exceptional inducible defense discovered by DNA barcoding.

Diversity of the European *Daphnia longispina* complex

2. **Petrusek A.**, Hobæk A., Nilssen J. P., Skage M., Černý M., Brede N., Schwenk K. *(unpublished MS)*: A taxonomic reappraisal of the European *Daphnia longispina* complex. [revised version in review for *Limnology and Oceanography*].
3. Nilssen J. P., Hobaek A., **Petrusek A.,** Skage M. (2007): Restoring *Daphnia lacustris* G.O. Sars, 1862 (Crustacea, Anomopoda) – a cryptic species in the *Daphnia longispina* group. *Hydrobiologia*. doi:10.1007/s10750-007-9076-3
4. **Petrusek A.**, Černý M., Mergeay J., Schwenk K. (2007): *Daphnia* in the Tatra Mountain lakes: multiple colonisation and hidden diversity revealed by molecular markers. *Fundamental and Applied Limnology / Archiv für Hydrobiologie*, 169, *in press*.

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5. Seďa J., **Petrusek A.**, Macháček J., Šmilauer P. (2007): Spatial distribution of the *Daphnia longispina* species complex and other planktonic crustaceans in the heterogeneous environment of canyon-shaped reservoirs. *Journal of Plankton Research*, 29(7): 619-628.
6. **Petrusek A.,** Seďa J., Macháček J., Ruthová Š., Šmilauer P. *(unpublished MS)*: *Daphnia* species and hybrids in reservoirs: patterns of hybridisation on ecological gradients in pelagic environment. [submitted to *Philosophical Transactions of the Royal Society of London, Series B – Biological Sciences*]
7. Seďa J., Kolářová K., **Petrusek A.**, Macháček J. (2007): *Daphnia galeata* in the deep hypolimnion: spatial differentiation of a "typical epilimnetic" species. *Hydrobiologia.* doi:10.1007/s10750-007-9075-4

As a general conclusion...

8. Forró L., Korovchinsky N. M., Kotov A. A., **Petrusek A.** (2007): Global diversity of cladocerans (Cladocera; Crustacea) in freshwater. *Hydrobiologia.* doi: 10.1007/s10750-007-9013-5

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This thesis would never exist without the help and contribution of many people. Some of them, such as co-authors of the chapters and those who are acknowledged in the papers, are obvious. Others, such as reviewers and journal editors, remain invisible but contribute directly to the final form of scientific publications. And then there are many others, who somehow influence the thread of life to get, at a certain stage, to the point of "thesis submission". I certainly cannot thank all of them here individually (but hey, all the friends, thanks for everything!), so I will restrict myself to some of those who somehow contributed to my *Daphnia* research (many others are mentioned in the respective chapters).

To start with the professional part of my life, although it necessarily merges with the private one, as many colleagues became friends: I would like to thank my supervisor Martin Černý for inviting me to the realm of cladoceran genetic analyses, and giving me freedom to roam there. Vladimír Kořínek and Jaroslav Hrbáček shared with me many ideas about *Daphnia*, and remain to be very inspiring both by their wide knowledge and by their rich professional activities, which never stopped with the formal retirement. The reservoir project is a fruitful collaboration with Mirek Seďa, Jiří Macháček and my bright student Štěpánka Ruthová. Special thanks go also to David Hardekopf, who carefully reviews the language of my manuscripts before they go to print, prunes all unnecessary articles and provides the missing ones, and gives the papers the final touch. One Czech person in particular attracted me to the aquatic research, and should not be left out: Radka, thanks a lot, and good luck in Oslo! (By which, we already move across the Czech border.)

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valuable field support I ever received, from western Spain to Israel, in the mountains as well as underwater, was from my then girlfriend and currently wife, Tereza. Biologist herself, she is not only the perfect partner for life but also in the field. To illustrate it: although studying bird behaviour, she learned incredibly fast how to sort different species of living *Daphnia* straight in the field! (Conversely, I learned, much slower, how to recognise tree pipit songs.) I should also appreciate that my three-month-old daughter Anna lets me sleep at night, and generally is a very well-behaved baby, otherwise the last days needed for writing the thesis could be much less productive. And last but not least, I thank my mother Helena Petrusková for bringing me up and allowing me to try all those things in the life to finally become a biologist...

And on this place, I would like to thank and apologise to all the aquatic animals which died in my hands – not only *Daphnia*, the target of this research, but also to all the victims of circumstances which happened to be in a wrong time at the wrong place (in my plankton net). I am sure no crustacean would exchange its life for being a hero of a scientific paper – but I am still glad at least some of them made it that far.

Finally a bit of the more formal stuff: My *Daphnia* research was in its various phases supported by the following funding sources: Grant Agency of the Charles University (GAUK 146/2001/B/Bio and 114807), Czech Science Foundation (GAČR 206/04/0190), Ministry of Education of the Czech Republic (MSM113100004 and MSM0021620828), German Academic Exchange Service (DAAD), Czech-Flemish bilateral cooperation in research and development (BIL/03/11), and Access to Research Infrastructure action of the Improving Human Potential Programme in Doñana Biological Station (ECODOCA).

I hereby declare that I never had any inclination to write this thesis alone, using solely the literature cited. That is completely impossible in the current team research, and makes no sense. Apart from the co-authors of the respective chapters, several reviewers (largely anonymous) and journal editors, as well as colleagues who read and commented earlier drafts, contributed to the current state of the papers and manuscripts.

No part of this thesis has been used to obtain any other academic degree.

In Prague, August 17, 2007

Introduction and conclusions

The water flea *Daphnia* (Crustacea: Cladocera: Anomopoda) belongs among the best-studied invertebrates, being used as a model organism in ecology, evolutionary biology as well as in applied research, such as ecotoxicology (Peters & De Bernardi 1987, Benzie 2005), and plays a central role in ecosystems of temperate ponds and lakes. Cladocerans in general and *Daphnia* in particular attained their popularity as models thanks to the fortunate (for researchers) combination of several factors – small size, short generation time, and relatively simple conditions required for mass culture, but especially due to their specific life cycle: cyclical parthenogenesis. Possibility to culture clonal lineages reproducing asexually without losing their ability of sexual reproduction allows experimental designs which disentangle effects of environment and genotype. The usefulness of *Daphnia* as a model should further increase with the recent first public release of the complete genome of American "*D. pulex*" (http://wfleabase.org), which will push cladocerology fully into the era of genomics.

Uncovering the cryptic diversity in *Daphnia*

Despite an intensive study, it is clear that the number of cladoceran taxa unknown for science exceeds the number of described species (Korovchinky 1996; Forró et al. 2007). Although *Daphnia* are probably the most intensively studied aquatic invertebrates, a number of species of this genus still remain either completely unknown or the available data are limited to one or a few sequences in phylogenetic datasets. The patterns of unravelled cryptic diversity more or less copy the geographic distribution of cladoceran researchers (especially those using molecular tools) or their regions of interest: *Daphnia* of North America (summarised in Hebert 1995) has been analysed particularly well, phylogeny of Australian *Daphnia* including a number of formally undescribed species has been recently published (Colbourne et al. 2006), new South American *Daphnia* are surfacing (Adamowicz et al. 2002, 2004; Kořínek 2003; Mergeay et al., unpublished), and the well-balanced fusion of traditional and molecular methods brings to light new species from Eastern Palaearctic (Ishida et al. 2006; Kotov et al. 2006).

However, there are still extensive geographic regions in which we may expect to find, with a relatively low effort, tens of undescribed *Daphnia* lineages. One of such areas is Subsaharan Africa, in which even very incomplete regional coverage already revealed a number of endemic taxa, including old, locally diversified species groups (Mergeay, Petrusek & Kořínek, unpublished). Temperate or high-altitude regions of Central and South-East Asia also include some highly interesting taxa, at this moment largely untouched by phylogeneticists (e.g., various melanic high-mountain *Ctenodaphnia* populations, weirdly shaped *D. turbinata* G. O. Sars of an unclear status, or *D. triquetra semilunaris* from Mongolia – a peculiar large-lake pelagic member of the *D. atkinsoni* complex).

In this comparison, Europe looked like a reasonably well-explored region. Although relatively little molecular work has been published until recently on European *Daphnia* populations, the

continent itself had been intensively studied by generations of cladocerologists, so the level of uncovered cryptic diversity might have been lower than in other, more exotic locations. At least, that was what we thought... Such an idea was supported by the evidence that some of the very widely distributed European species, in which a cryptic diversity could be expected, are surprisingly uniform over the whole continent or even among more biogeographic regions – e.g., *Daphnia magna* (De Gelas & De Meester 2005) or *D. curvirostris* (my unpublished data).

In my work, I originally attempted to evaluate the diversity of some cladocerans from temporary waters. An evidence existed that some populations of *D. obtusa* from Slovakia are genetically divergent from the common Central European *D. obtusa* lineage (Černý 1995), and had already been described almost a century ago as *D. tatrensis* Litynski, 1913 (Kořínek et al. 2003). This seemed to be a relatively simple case of forgotten but valid taxon, and we had little expectations to find many other doubtful or clearly undescribed taxa. However, during an accidental sampling in a military ground in Munich, I collected usual *Ctenodaphnia* – morphologically undistinguishable from *D. similis* (which would be itself quite unexpected in Germany) but, as mitochondrial DNA sequencing revealed, highly divergent from other European populations (Petrusek 2003; see also Fig. 1). My first sampling trip to Israel, from which *D. similis* was described, confirmed the distinctness of the "real" *D. similis* and seemingly resolved this riddle, although the question of the real origin of the German lineage remained unanswered.

Apart from *D. similis*, however, I collected in Israel additional cryptic lineages of *Ctenodaphnia*, which could not be linked to any existing name (Fig. 1). Approximately at the same time, we also found out that a third, most likely undescribed member of the *D. obtusa* lineage is common in Western Europe, and additional ones from the Mediterranean did not let us wait long. At the moment when I hoped to compile this knowledge into a catalogue of European *Daphnia* which would include also the cryptic lineages, the first apparently undescribed *"Hyalodaphnia"* (i.e., the member of the *D. longispina* group) surfaced in my samples (chapter 2). In every major group of *Daphnia* present in the Western Palaearctic, we had at least one but often more cryptic species, and that was not the end: the "snowball of diversity" only started rolling...

DNA Barcoding of Western Palaearctic *Daphnia*

The DNA barcoding, characterisation of taxa by short sequences of a selected unified gene, is a controversial but apparently successful approach to cataloguing biodiversity. Only a few years ago, Hebert et al. (2003a, b) suggested that 648 bp long fragment of the cytochrome c oxidase subunit I (COI) should be useful for identification to species level in all kinds of metazoan groups. Although the suggestion to collect such "COI barcodes" for the whole animal kingdom stirred a heated discussion and encountered a strong opposition (e.g., Will & Rubinoff 2004; Ebach & Holdrege 2005; Will et al. 2005), the Barcode of Life Initiative managed since then to collect COI barcodes for a huge number of species from various animal groups from fish and birds to collembolans and tardigrades (according to

http://www.bolinfonet.org, there were over 200,000 barcodes of about 25,000 species already available in summer 2007). Apart from a "simple" (but much needed) cataloguing and evaluating biodiversity, DNA barcoding (or equivalent approaches) found many useful applications, from identification of animal developmental stages (Webb et al. 2006; Pfenninger et al. 2007), invasive (Armstrong & Ball 2006; Scheffer et al. 2006; see also Fig. 1) or medically important species (Kumar et al. 2007), or even snake venom (Pook & McEwing 2005) to assessment of the mitochondrial genome composition (Min & Hickey 2007).

Apparently, the DNA barcoding is neither the feared end nor panacea for modern taxonomy, and not all animal groups may be as fit for characterization by a single gene as originally envisaged (e.g., Vences et al. 2005; Meyer & Paulay 2005). For me, nevertheless, an important fact is that mtDNA sequences (both of COI and of the genes for ribosomal subunits) seem to be very useful for identification of *Daphnia*. So far, the available data suggest that selective sweeps indeed regularly "clean up" mitochondrial genomes of *Daphnia* species, and interspecific divergences in this genus are in most cases substantially deeper than intraspecific ones (my unpublished data; see also Adamowicz et al. 2004). Additionally, barcoding may reveal patterns that inspire ecologically or evolutionary oriented questions (see chapter 1). We therefore attempted to collect DNA barcodes for all known *Daphnia* lineages living in Western Palaearctic, not only to facilitate their identification in cases when we lack species-specific identification characters but also to obtain data for phylogenetic analyses.

Our aim was to obtain several individuals, if possible from geographically distant locations, for each taxon known from the region, and sequence their two mitochondrial genes – the "standard barcoding" COI and the gene for the small ribosomal subunit (12S rRNA). The latter gene has been used in most papers dealing with *Daphnia* phylogeny (although some of the latest publications focus on a quite promising fragment of the protein-coding NADH dehydrogenase 2; e.g., Ishida et al. 2006; Kotov et al. 2007), and is therefore readily available for comparison not only for various European but especially many non-European taxa.

Figure 1 (next page): Neighbour-joining tree of COI barcodes of available Western Palaearctic *Daphnia* lineages. Nomenclature in use already follows the suggested revision of the *D. longispina* complex (chapter 2).

Three lineages out of those known positively from the region are missing: *D. cristata*, *D. lumholtzi*, and *D. triquetra* (see text). Dotted parts of the tree indicate apparently undescribed lineages, grey parts of the tree known but nomenclaturally problematic taxa (those with commonly but incorrectly used names). In the *D. chevreuxi* complex (grey dot-and-dash lines), it is at the moment impossible to decide which of the two lineage represents the nominal species.

Coloured vertical bars on the right indicate species complexes in which new lineages were recorded, black bars on the left denote major species groups (often designated as subgenera but see Ishida et al. 2006). Species covered by different chapters of this thesis are shown by coloured arrows; white arrows point out to other interesting, yet unpublished stories (see „Prospects for future work" below).

Although the marked species complexes are most likely monophyletic, the tree does not represent any phylogenetic hypotheses, and higher-level relationships are probably incorrect. Joining of unrelated *D. longiremis* and *D. hispanica*, which belong to different subgenera, is certainly an artefact.

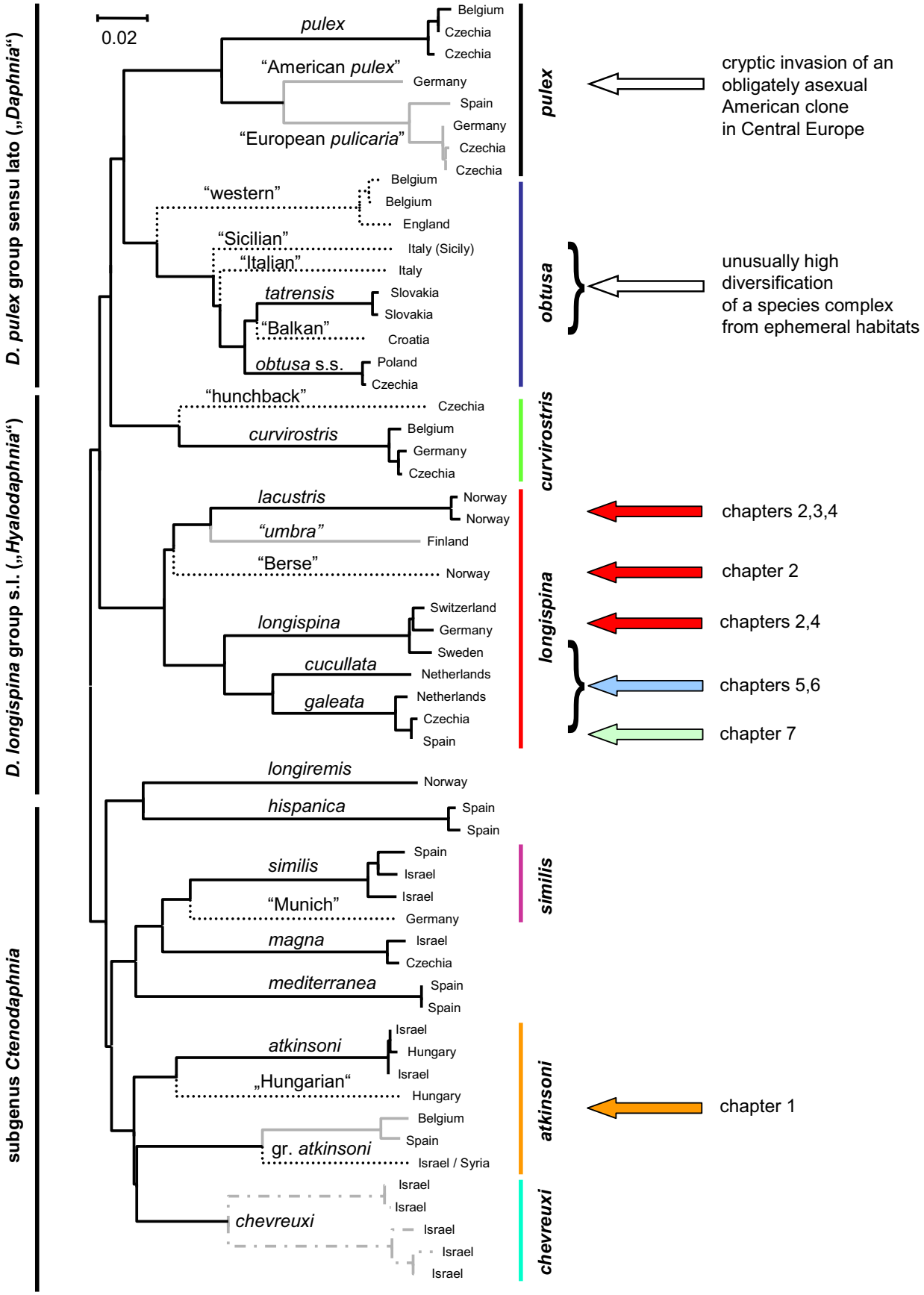

Figure 1. Neighbour-joining tree of COI barcodes of available Western Palaearctic *Daphnia* lineages. See previous page for details.

Figure 1 (previous page) shows the current state of our barcoding effort, using the COI fragment (results for 12S are essentially the same). We managed to obtain most of the lineages positively known from this region, except of two *Ctenodaphnia* species: *D. lumholtzi* (rare in Israel, relatively common in the Pontocaspian region and North Africa) and *D. triquetra* Sars (most likely a distinct member of the *D. atkinsoni* complex with the core of its distribution in Central Asia; but see Glagolev 1995 for the European record). Sequences of the former are available from other biogeographic regions, the latter has probably never been genetically analysed. Of course, we included also all cryptic lineages which were discovered during the data collection. Additionally, *D. cristata* (sister species of *D. longiremis*) is missing from the tree in Fig. 1. This is not due to the material unavailability but due to consistent failures to amplify the COI fragment using available primers (probably because of a mutation at the primer site); 12S sequences of this species are available.

From the frequency of dotted branches on the tree, it is clear that the level of cryptic diversity in European *Daphnia* is quite high, comparable to other regions where genetic tools have been applied to study this genus. Another aspect worth noticing is a relatively common presence of grey branches – these represent lineages which are nomenclaturally problematic, despite the fact that we consider the *D. longispina* complex mostly resolved (chapter 2). Many inconsistencies nevertheless still remain. Especially in the *D. pulex* complex, it is common to label only distantly related lineages from different continents by the same names. Although focusing on nomenclatural issues might look like a nitpicking, indiscriminate use of incorrect names may cause substantial confusion, lead to incorrect assumptions or conclusions, and hamper comparative analyses and use of literature data. That is why we invested a substantial effort into untying the taxonomical knot of the *D. longispina* complex.

Coloured arrows in the "barcode tree" indicate taxa which are discussed in individual chapters of my thesis. So far, most of them are concentrated on a single group. Hopefully, if I continue to put arrows to completed tasks, their distribution will soon become more even.

Outline of the papers and manuscripts included in the thesis

This thesis presents results of the research on *Daphnia* diversity in different spatial scales I contributed to during my studies. It consists of eight chapters composed as independent manuscripts, some of them already published or accepted for publication. Most of these chapters are directly or indirectly linked to the "barcode tree" shown above, and deal with cryptic species, lineage identification, or taxon diversity in general. Three chapters focus on a diversity in narrower scale – distribution of *Daphnia* species or intrapopulation diversity within individual water bodies, and differ from the rest also by the methodology in use (primarily allozyme electrophoresis instead of DNA methods).

The first chapter of the thesis contains the first case study on the little analysed but apparently diverse species complex of Palaearctic *Ctenodaphnia*, *D. atkinsoni*. Presented as an unpublished short note (which will nevertheless require expansion to a full paper), it demonstrates how DNA barcoding,

as a tool principally used for characterising taxa and cataloguing diversity, unravelled a plasticity of a spectacular morphological feature, the antipredator function of which we hypothesized from ecological characteristics of the respective taxa, and subsequently demonstrated by laboratory induction experiment.

Next three closely related chapters focus on diversity of the European *Daphnia longispina* complex. Members of this group are ecologically very important inhabitants of pelagic environment of temperate lakes and ponds, and have been targets of many ecological and evolutionary studies. Unfortunately, taxonomy of the *D. longispina* complex has been quite chaotic since centuries, as different lineages of the complex have few taxon-specific morphological characteristics, are phenotypically plastic and often indulge in interspecific hybridisation. Our analyses, using primarily sequence data from a large-scale pan-European survey, attempted to resolve at least some of the prevailing controversies. The three chapters, each more restricted in the topic than the previous one, focus respectively: on the whole complex in the Western Palaearctic (chapter 2), on the "taxonomic resurrection" of a single lineage on the complex (chapter 3) and on interesting *Daphnia* fauna of one Central European mountain range (chapter 4).

After the discovery that one of the Fennoscandian lineages of the complex, which used to be incorrectly labelled "*D. longispina*" is some recent studies, is undoubtedly identical with *D. lacustris* described by the great Norwegian carcinologist G. O. Sars (chapter 3; Nilssen et al. 2007), we launched, in an international collaboration, an attempt to comprehensively revise the taxonomy and nomenclature of all European members of the complex. By genetically characterising material from type localities of the problematic taxa (in one case including decades-old resting egg bank), we came to the conclusion that even some of the old and traditional *Daphnia* names, such as *D. hyalina* and *D. rosea*, do not designate biological species (in a sense of evolutionary independent entities with relatively restricted gene flow) but should be regarded as synonyms of the type species of the genus, *D. longispina* (chapter 2). The suggested taxonomical revision may be regarded as controversial, as it touches some of the "sacred cows" frequently used in the contemporary cladocerological research. Nevertheless, I believe the evidence provided (together with as yet unpublished detailed microsatellite analysis by Anne Thielsch; Thielsch 2005) will eventually convince the scientific community that the current practice of the taxon name use is incorrect, and both the nomenclatural stability and priority rules call for a change. I also hope that revisions of other taxonomically chaotic groups, especially the European and North American *pulex/pulicaria* complex, will eventually follow this example.

The last chapter of the "*D. longispina* section" is regionally much more restricted – it focuses on a single region on the border of Slovakia and Poland, the Tatra Mountains, which has been intensively studied at our department for a long time. Lakes in this mountain range host an interesting array of *Daphnia* morphotypes and lineages, not only of the *D. longispina* complex but also of the *D. pulex* group (e.g., Černý 1995; Kořínek et al. 2003; Marková et al. 2007). In my contribution (chapter 4; Petrusek et al. 2007), I focused on the diversity of *D. longispina*, *D. galeata*, and possibly

relict *D. lacustris* populations, and showed that the colonisation of the mountain lakes by these species was a complex process, in which played a role not only the quaternary climatic change but also anthropogenic alterations of the lake environment.

The next section, composed of three chapters as the previous one, focuses on the effects of local, intra-lake heterogeneity of environmental conditions on *Daphnia* taxon composition as well as intraspecific diversity. This is a result of my cooperation with colleagues from the Institute of Hydrobiology in České Budějovice, whose traditional objects of study are deep canyon-shaped reservoirs. Unlike most other standing waters, such reservoirs are characteristic by longitudinal gradients of various factors affecting directly or indirectly zooplankton populations, including *Daphnia*. Among these factors, nutrient contents and food (algal) concentration on one hand, and transparency and fish predation pressure on the other, strongly influence not only the interspecific competition but also hybridisation between coexisting related species. The first results of our three-year study of several Czech reservoirs are shown here. In one published paper (chapter 5; Seďa et al. 2007b) and a subsequent manuscript (chapter 6), we first summarised the patterns of spatial distribution of zooplankton (especially *Daphnia* species and interspecific hybrids) in reservoirs, and subsequently tried to evaluate the most important ecological factors affecting the *Daphnia* hybridisation and the success of different species and hybrid genotypes.

The last chapter of the "reservoir section" (chapter 7; Seďa et al. 2007a), to which I contributed especially with data analysis (diversity estimates) and interpretation of results, shows that the vertical gradients in a deep stratified reservoir may also have profound effect on intraspecific differentiation. We demonstrated an existence of genetically divergent subpopulation of pelagic *D. galeata* living in relatively hostile conditions of the deep hypolimnion; repetition of this apparently non-random pattern suggests that these daphnids were at least partially reproductively separated from the "mainstream" epilimnetic population. Our unpublished results from other reservoirs suggest that such a significant intraspecific differentiation in the vertically stratified waterbody is not an uncommon phenomenon.

The last chapter of the thesis (chapter 8; Forró et al. 2007), part of the special *Hydrobiologia* issue "A global assessment of animal diversity in freshwater", attempts to summarise the currently known diversity of cladocerans in inland waters. The author team consisted primarily of classical taxonomists; in my contribution to this paper I attempted to mirror my experience with discovery of cladoceran cryptic species by molecular methods, *Daphnia* regional diversity, effect of increased sampling effort on the rate of lineage discovery, and some ideas about patterns and processes affecting the cladoceran speciation. This chapter demonstrates well the recent advance in the taxonomic knowledge: between the proof stage and actual publication, the list of existing higher-level cladoceran taxa has become already incomplete by the description of a new cladoceran family from Australia, Nototrichidae (Van Damme et al. 2007).

Prospects for future work

The research presented in this thesis is far from finished. As usual in science, almost every answered question brings others, and newly emerging patterns call for interpretation or "just" require writing up into the manuscript form. The DNA barcode dataset shown in Figure 1 encompasses many topics and stories, which could, and should, be published. Some examples may be:

- providing to the scientific public the molecular barcodes of *Daphnia* lineages in this region
- describing in detail the rich lineage diversity of the European *Daphnia obtusa* group and its geographic distribution
- evaluating the phylogeny of Western Palaearctic *Ctenodaphnia*
- providing evidence of a cryptic invasion of an asexual clone from the American "*D. pulex*" complex in Central Europe, independent of the one recently discovered in Africa (Mergeay et al. 2005, 2006).

A great challenge for my near future is finishing the digital catalogue of the Western Palaearctic *Daphnia* diversity, which we started to produce about four years ago. This work was inspired by the CD-ROM on *Daphnia* of North America (Hebert 1995), which is a very useful addition to classical monographs, as well as a valuable educational tool. Seemingly a simple task – listing the lineages known to be present in Europe and adjacent Mediterranean areas, documenting their phenotype, and providing identification keys at least to the level of species complexes – has unexpectedly grown and became more and more complicated as additional lineages started to pop up all over the continent, from Italy to Scandinavia. However, knowing that a flock of undescribed species, which substantially reduces the reliability of any available identification key, can be found in various European regions, I am sure such a tool would be useful for non-specialists who routinely identify zooplankton. The process of new lineage discovery in Europe will probably not cease completely in the near future; nevertheless, we should try to complete the catalogue at least with the current level of knowledge.

So far, I pointed out only some of my own "publication debts". The tree in Figure 1 provides various additional challenges for the future research, which are open for everyone. One of the interesting questions is the high incidence of cryptic species among inhabitants of temporary or ephemeral waters – especially the *D. obtusa* complex but also various *Ctenodaphnia*. While the existence of unrecognised lineages might be partially explained by less intensive research of temporary habitats in comparison with lake environments in Europe, the high diversification of some of these lineages must have interesting evolutionary mechanisms behind. Less intensive long-range dispersal, higher habitat fragmentation and lower habitat stability probably played an important role in speciation of temporary-water *Daphnia*, and studying in more details these models might be very promising.

Yet completely different aspect emerging from this work is the opportunity for formal description of at least some of the apparently undescribed European *Daphnia* lineages. This is a real challenge, as it requires a tight collaboration with one or more morphologists, who have time and skills to invest into such a task. With some luck, we may be able to train an interested student, so the tradition of the classical cladoceran taxonomy does not disappear from Czechia.

As the last (but not least), I will discuss the potential future of our research on reservoir *Daphnia* species and hybrids, which got its own momentum and is going on well. Thanks to collaborating students and the Klaus Schwenk's group in Frankfurt, we already have large data on local intraspecific diversification on the ecological gradients, including microsatellite data describing some of the most interesting hybridising *Daphnia* populations. We focus also on the effects of hybridisation and introgression of *Daphnia* phenotype (body shape), using the tools of geometric morphometry. Another interesting question, posed by the nature itself, is the effect of catastrophic floods on zooplankton populations: unfortunately for the local people, a river on which one of our study reservoirs is located was hit by extreme floods twice during a single season (2006). The reservoir was probably completely flushed by the floods, and even the resting egg banks were covered by a thick layer of clay sediment. We therefore got a rare opportunity to collect samples documenting the population recovery and potential genotype and taxon replacement in such a locality.

When I started as an undergraduate student to study aquatic biology over 10 years ago, I was convinced that I will never smash *Daphnia* to pulp, to isolate some *molecules* for doing a work suitable only for white-coated laboratory rats. I am glad that I changed my mind. The trips from the field ecology to evolutionary biology and back are very inspiring, I met many interesting people and found several good friends among the *Daphnia* folks, zooplankton sampling brought me to amazing parts of our world... And last but not least, *Daphnia* are really *pretty* (Figure 2). Ending by this statement might not be the most scientific conclusion – however, the last fact is one of those I became really persuaded of during my studies. And unlike many scientific hypotheses and theories, it is unlikely to be falsified.

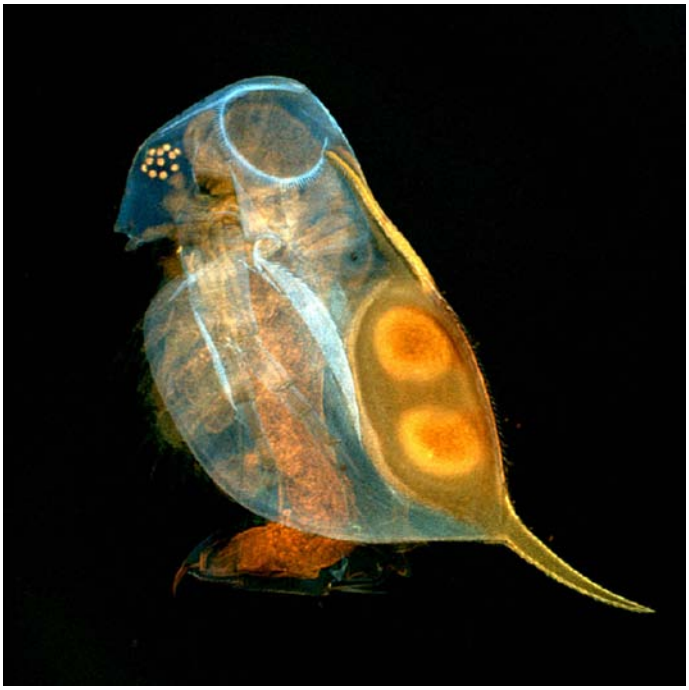

Figure 2. Spined morph of a member of the *Daphnia atkinsoni* species complex (compound autofluorescence image from a confocal microscope; foto J. Reischig). See chapter 1 for more details.

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CHAPTER 1

Petrusek A., Tollrian R., Schwenk K., Haas A., Laforsch C.:

"Crown of thorns" protects *Daphnia* against an ancient predator: an exceptional inducible defense discovered by DNA barcoding

unpublished manuscript

It certainly must be a coincidence that the first crowns of thorns in my pilot induction experiment occurred on juvenile *Daphnia atkinsoni* on Good Friday of 2004. (*Daphnia* photo: J. Reischig)

"Crown of thorns" protects Daphnia against an ancient predator: an exceptional inducible defense discovered by DNA barcoding

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The initiative of the "barcoding of life" attempts to create the database of cytochrome oxidase I (COI) sequences of metazoans for sequence-based species identification (Hebert et al. 2003). The utility of molecular barcodes for the identification of various animal taxa and cryptic diversity has been well demonstrated (Hebert et al. 2004; Blaxter et al. 2004). However, the added value of the barcoding activities may be in pointing to ecological and evolutionary mechanisms behind the animal variability. Here we show a remarkable example.

Head spines in the *Daphnia atkinsoni* species complex (Fig. 1B,C) have been used to distinguish among species (Alonso 1996). During the collection of barcoding data for European *Daphnia* species, we have obtained COI and 12S mtDNA sequences of several populations of this complex from Europe and the Mediterranean area. The phylogenetic analyses of both genes failed to support the systematic utility of this trait for distinguishing between the putative *atkinsoni* and *bolivari* species. Instead, both spined and unspined morphs have been assigned together into different evolutionary lineages (Fig. 1D). Thus, we predicted that the spiny structures are phenotypically plastic, a well-known strategy to cope with variable environmental conditions such as predation impact (Tollrian & Harvell 1999).

In freshwater habitats, prey organisms often sense predator-released chemical cues (kairomones). Those signals, which provide reliable information on the presence of predators, induce behavioral, life history or morphological changes in the prey leading to increased fitness under predation pressure (Tollrian & Harvell 1999). Water fleas belonging to the *Daphnia atkinsoni* species complex often occur in temporary fishless ponds in which tadpole shrimps (Notostraca), such as *Triops cancriformis* (Fig. 1A) are frequently the dominant predators. These omnivores are evolutionary very old, surviving with unchanged moprhology for at least 220 million years (Kellber 1999). As we show here, daphnids of the *D. atkinsoni* species complex adapted to this strong selective force by developing an "armor", consisting of rigid cuticular shields, armed with long spikes, to protect the most vulnerable body parts, the head and the base of the swimming antennae.

We tested the induction of this "crown of thorns" by incubating undefended females of both *Daphnia* lineages in net cages within vessels containing *Triops*. The next parthenogenetic generation indeed developed the typical wide, heavily spined lobe (nested ANOVA, both species, $F_{1,8.5} = 146.1$, $p < 0.001$; Fig. 1B,C). Our predation trials revealed a significantly higher survival of the induced morphs in the presence of medium-sized *Triops* (paired Wilcoxon test for related samples, N=10, $Z = -3.8$, $p < 0.001$). This defensive structure differs strongly from other known defenses, suggesting that the "crown of thorns" evolved specifically in coexistence with this effective predator. Our results show the potential of barcoding data to reveal a number of phenomena with an impact transcending the borders of taxonomy or identification.

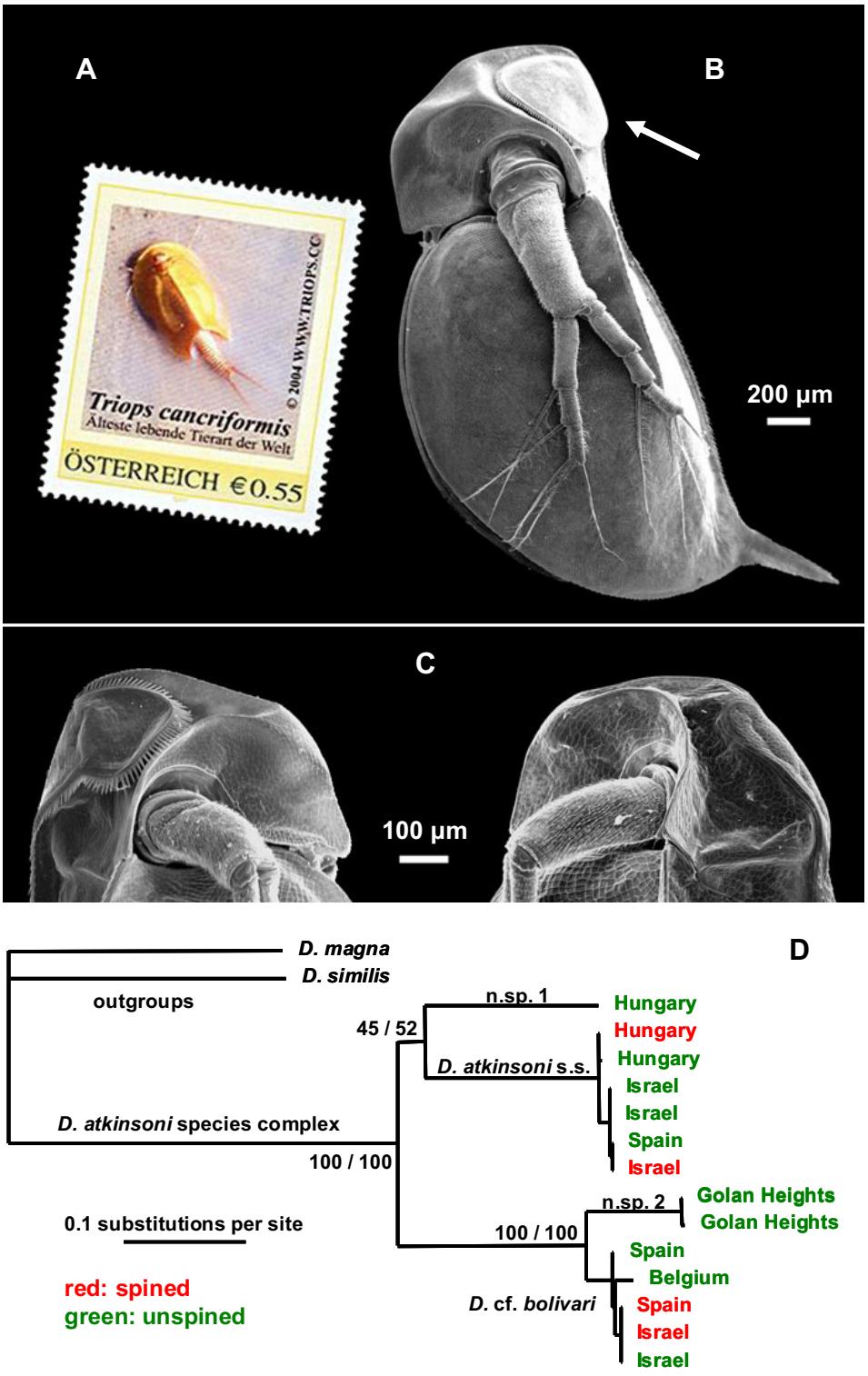

Figure 1. Induction of the "crown of thorns" in the *Daphnia atkinsoni* species complex exposed to chemical cues released by *Triops* (**A**), here portrayed on an Austrian stamp as "the most ancient extant animal species" (*7*). Such *Daphnia* show a distinctly enlarged carapace extension into the head shield, forming heart-shaped lobes lined with strong spines (**B**: *D. bolivari*; whole body SEM image, **C *left***: *D. atkinsoni* head). Uninduced individuals exhibit inconspicuous lobes without thorns (**C *right***: same clone of *D. atkinsoni*). (**D**) The phylogenetic relationship among analyzed lineages of the complex, proving that this trait cannot be used for taxon classification. Countries marked in red denote individuals possessing the "crown of thorns" collected in the wild, unspined *Daphnia* are in green. (Maximum likelihood tree based on mitochondrial genes for 12S ribosomal DNA and cytochrome oxidase I, GenBank accession numbers: DQ116589-603, DQ166842-9; numbers at selected branches indicate bootstrap values for maximum likelihood analysis / posterior probability values from the Bayesian inference of phylogeny.)

Methods (supplementary information)

Molecular analyses

DNA barcoding: The two genes used to identify species and subsequently to analyze the phylogenetic relationship among the lineages were selected according to their suitability for DNA-based identification within the genus: cytochrome *c* oxidase subunit I (COI) represents the standard locus for DNA barcodes (Hebert et al. 2003), while partial sequences of the small ribosomal subunit (12S rRNA) are available for many *Daphnia* species and have been used in several previous phylogenetic studies (e.g., Colbourne et al. 1996; Schwenk et al. 2000). The within- and among-species variation in these two genes is largely non-overlapping, allowing unambiguous species identification in most *Daphnia* groups.

DNA extraction and amplification: DNA was extracted from individuals preserved in ethanol or originating from laboratory cultures by proteinase K digestion (Schwenk et al. 1998). Fragment of 12S and COI genes were amplified using standard protocols (Schwenk et al. 2000), the PCR product was purified by column chromatography and sequenced on ABI automatic capillary sequencers (series 377 and 3700). Sequences were aligned using ClustalW (Thompson et al. 1994) and the alignment subsequently checked manually in MEGA version 3.1 (Kumar et al. 2004).

Phylogenetic analyses: Identification of lineages was first performed using similarity-based, i.e. phenetic method (neighbor-joining tree, results not shown). As more divergent lineages were found within the complex, their phylogenetic relationships were analyzed using the total evidence approach based on both COI and 12S gene regions. The test for homogeneity (Farris et al. 1995) confirmed the combinability of the two genes for the analysis. We used Modeltest 3.7 (Posada & Crandall 1998) to select the best model of nucleotide substitution, using the Akaike Information Criterion to choose among 56 different models of sequence evolution. The phylogenetic tree was constructed by the Bayesian inference of phylogeny (BI) in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). The parameters for both genes were estimated separately (using the "unlink" option). The Monte Carlo Markov chain (MCMC) analysis was run for 3 million generations, with 2 parallel runs of 4 chains run simultaneously and trees sampled every 100 generations. The first 20 % of the trees, including the burn-in phase, were discarded. Remaining 24,000 trees were used to construct the phylogram (node support values indicate the proportion of sampled trees sharing that particular node, and represent the posterior probability of the existence of the clade based on the available data and the model of evolution). Additional phylogenetic analyses, including maximum parsimony (MP) and maximum likelihood (ML) methods, were carried out in PAUP* 4.0b10 (Swofford 2002). Heuristic searches were conducted with tree bisection-reconnection branch swapping and 10 random sequence taxon additions; branch support was estimated by nonparametric bootstrapping with 1000 (MP) and 100 (ML) pseudoreplicates, respectively.

Induction and predation experiments

We used a laboratory-cultured clonal line of *D. atkinsoni* (sensu stricto) and another distinct lineage labeled as *D. bolivari* for our experiments. *D. bolivari* was isolated from a flooded field south of Tel Ashdod (Israel; 31°45'06.0"N, 34°39'06.6"E), *D. atkinsoni* was hatched from resting eggs from temporary puddles on a meadow in Hungarian plains northwest of Hajdúböszörmény (Hungary; 47°43'06.9"N, 21°23'17.6"E). *Triops cancriformis* was provided by Dr. E. Eder, Zoological Institute, University of Vienna and cultured in a temperature-controlled room at 20 ± 1°C.

Predator-cue induction experiments were carried out in 12 l glass-aquaria. The bottom of each aquarium was covered with sand, which has been sterilized prior to the experiments. The aquaria were filled with 10 l artificial medium (Jeschke & Tollrian 2000). One third of the medium was exchanged weekly. Both induction experiments were conducted at 20 ± 1°C in a temperature-controlled room under fluorescent light at a constant day-night rhythm (16h:8h). We cultured age-synchronized cohorts of both *Daphnia* species in 30 l plastic buckets. We started the predator-cue induction experiments by randomly placing 20 ovigerous daphnids of a single clone originating from the third clutch of those cohorts into each aquarium. Three juvenile *T. cancriformis* (400-500 µm), which body size was too small to feed on even neonate daphnids were introduced into the aquaria serving as the induction treatment. After reaching the size of approximately 800 µm, *T. cancriformis* were replaced by smaller animals to prevent strong predation effects on the daphnids but still to guarantee a sufficient amount of predator-released chemicals. Fish food (1 g), tested to be ineffective in inducing morphological changes of the daphnids in preliminary experiments, was used as food source for the omnivorous *T. cancriformis*. The same amount of fish food was also placed into the control-treatment aquaria. Each aquarium was cleaned from exuviae, feces and remaining fish food every single day. The daphnids were fed daily by adding *Scenedesmus obliquus* at a concentration of 1.5 mg C/l into each aquarium.

Each experiment was replicated five times in the experiment with *D. atkinsoni* and six times in the experiment with *D. bolivari*. Mothers of both the F1 and the F2 generation were removed after releasing their clutch. Matured daphnids (1200-2000 µm) of the F3 generation were then used for analysis to account for possible transgenerational effects (Agrawal et al. 1999). Morphological parameters recorded from both *Daphnia* species using a digital image-analysis system (Soft Imaging System, Analysis Pro, Münster, Germany) were the body length, the tail spine length and the lobe width. Additionally, the “shoulder”-shield width was recorded in *D. atkinsoni.* Statistics were calculated using the software package SPSS V12.0 (SPSS Inc.). To compensate for size-dependent changes, a relative value was calculated for each trait. Arcsin-square-root-transformed data (Sokal & Rohlf 1995) were then tested for normal distribution and a nested ANOVA, with the replicates as random factor, was performed for both experiments to analyze for treatment effects between control *Daphnia* and daphnids exposed to chemical cues released by *T. cancriformis*.

Predation trials with *T. cancriformis* hunting on *D. atkinsoni* were conducted in a temperature-controlled room at 20 ± 1°C in 500 ml glass beakers under daylight conditions. The body length of *T. cancriformis* used for predation trials, measured from the top of the carapace to the caudal part of the body, was 27-30 mm. Matured daphnids (1200-2000 µm) of both morphs, *Triops*-induced and non-induced (ten each) were introduced into each beaker. The experiment started at the time when a single predator was placed into the beaker and launched its first attack on the daphnids. After 30 minutes the number of killed and surviving animals was recorded and the surviving *Daphnia* were classified as predator-induced or non-induced using a stereo-microscope. The predation trial was replicated ten times and a paired Wilcoxon test for related samples was used to analyze this data set.

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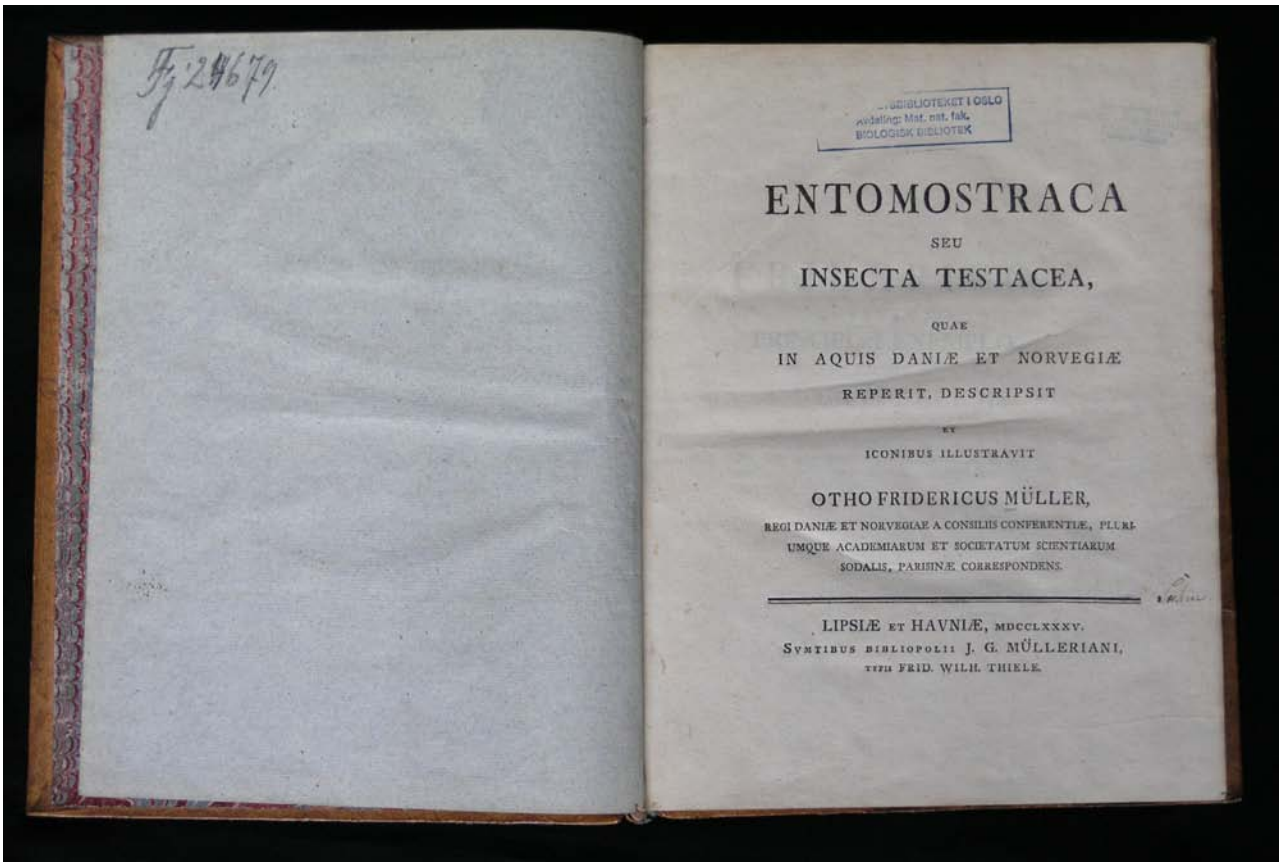
CHAPTER 2

Petrusek A., Hobæk A., Nilssen J. P., Skage M., Černý M., Brede N., Schwenk K.:

A taxonomic reappraisal of the European *Daphnia longispina* complex

revised version in review for *Limnology and Oceanography*



Title page of the book containing the original image of *Daphnia longispina* (O. F. Müller 1785).

A taxonomic reappraisal of the European *Daphnia longispina* complex

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Running head: Taxonomy of *Daphnia longispina* complex

Abstract

Systematics and nomenclature of the *Daphnia longispina* complex, which contains some of the most common species of the genus in the Palaearctic, including taxa widely used in ecological and evolutionary studies, have been in flux for the last 150 years, resulting in misinterpretations and erroneous use of species names. We revise the systematics of this species complex based on mitochondrial sequence variation (12S rDNA and COI) of representative populations across Europe, with a special focus on samples from type localities of the respective taxa; we also include data from a subfossil resting egg bank from a type locality altered by human activities. Combining genetic evidence and morphological assignments of analyzed individuals, we propose a comprehensive revision of the European members of the *D. longispina* complex. *D. hyalina* and *D. rosea* morphotypes have evolved several times independently, and we find no evidence to maintain these morphotypes as distinct biological species. Alpine individuals described as *D. zschokkei* are conspecific with the above-mentioned lineage. We suggest that this morphologically and ecologically plastic but genetically uniform *hyalina-rosea-zschokkei* clade should be identified as *Daphnia longispina* (O. F. Müller, 1776). The valid name of Fennoscandian individuals labeled *D. longispina* sensu stricto in the recent literature is *D. lacustris* G. O. Sars, 1862. Additionally, we discovered another divergent lineage of this group, likely an undescribed species, in southern Norway. Our results present a solution for several prevailing taxonomic problems in the genus *Daphnia*, and have broad implications for interpretation of biogeographical patterns, and ecological and evolutionary studies.

Introduction

The genus *Daphnia* includes some of the most frequently studied aquatic invertebrates, and constitutes a model organism in a number of fields, including ecotoxicology, ecology, biogeography, and evolutionary biology (Peters and de Bernardi 1987, Benzie 2005). Nonetheless, the taxonomy and nomenclature of several species groups within *Daphnia* remain unresolved (Benzie 2005). Nomenclatural inconsistencies, for instance in the use of taxon names in different geographical regions, continue to complicate comparative analyses, and limit the use of the rich information accumulated in the literature or public databases (such as NCBI GenBank). The inappropriate delineation of species boundaries may also hamper our understanding of ecological and evolutionary processes in this important genus. Therefore, a consensus on *Daphnia* nomenclature and systematics has implications reaching far beyond the field of taxonomy itself.

During the past decade, molecular markers have provided a new basis for delimiting species and analyses of cryptic lineages, which has led to significant progress in understanding cladoceran diversity, though research has been restricted to certain regions only (Forró et al., in press). Although we have been able to recognize different evolutionary lineages, we often lack information allowing us to reliably link them to existing species names. This confusion is often caused by the fact that nomenclature from one continent has readily been applied to populations elsewhere, while in fact most of the cladoceran fauna likely has much more restricted distributions than formerly assumed (Frey 1986; Forró et al., in press). For example, one of the most abundant and most intensively studied *Daphnia* species, the North American *Daphnia "pulex"*, represents a lineage different from the European nominal species (Colbourne et al. 1998), and requires its own name as well as an adequate formal description. Similar cases are known among various other members of the *D. pulex* group, as well as in the subgenus *Ctenodaphnia*.

Another ecologically important group frequently used as a model in evolutionary biology, the *Daphnia longispina* complex, also has a long record of taxonomic confusion. As defined here, the complex in a wide sense includes *D. longispina* (O. F. Müller, 1776); *D. hyalina* Leydig, 1860; *D. rosea* G. O. Sars, 1862; *D. zschokkei* Stingelin, 1894; *D. cucullata* G. O. Sars, 1862; *D. galeata* G. O. Sars, 1863; and "*D. umbra"* in Europe, and also the mostly Nearctic taxa *D. mendotae* Birge, 1918; *D. dentifera* Forbes, 1893; and *D. thorata* Forbes, 1893. Members of this complex are especially difficult to identify due to the lack of fixed qualitative identification characters, high intraspecific morphological variation, phenotypic plasticity in response to environmental factors, and also frequent interspecific hybridization (Flößner 2000; Benzie 2005). Since the first species descriptions, this group has been subjected to multiple taxonomic revisions which led to a series of alternative groupings of morphological variants (see Hrbáček 1987). Johnson (1952) claimed that nearly 100 published designations (species and varieties of more or less obscure status) belonged within his concept of *D. longispina* (O. F. Müller, 1776). Almost 150 years ago, Leydig (1860) and Sars (1863) expressed their frustration due to the great difficulties in deciding between the species or variety status of the different observed forms; and we are still confronted with similar problems (Hrbáček 1987; Benzie 2005).

The difficulties related to European members of the *D. longispina* complex can be divided into several categories: *1) Identification difficulties.* Although the genetic data suggest the presence of several evolutionary lineages, not all of these can be reliably separated by morphological traits. Additionally, widely used monographs providing identification keys (e.g., Amoros 1984; Flößner 2000; Benzie 2005) differ even in species-specific characters of the supposedly most common forms (e.g., for separation of *D. longispina* and *D.*

rosea). *2) Interspecific hybridization and introgression*. Several species of the group frequently form interspecific hybrids (Schwenk and Spaak 1995; Hobæk et al. 2004) and may further backcross with parental species (Keller and Spaak 2004; Jankowski and Straile 2004). Most available identification keys (apart from Flößner 2000) ignore interspecific hybrids, thus recombinant taxa are pooled with parental or sister species. Substantial introgression may further blur species boundaries between hybridizing taxa. *3) Nomenclatural problem*s. The use of the name *D. longispina* itself has often been problematic. Recent molecular genetic studies (Taylor et al. 1996; Schwenk et al. 2000, 2004) have based their standard for this species on an individual from a lake in Tatra Mountains, Poland. However, this population belongs in fact to a different species (*D. lacustris* G.O. Sars 1862; Nilssen et al. 2007). Further, a distinction between *D. longispina*, *D. rosea*, and *D. hyalina* has been upheld by most authors since Flößner (1972), but there is no consensus on how to delimit them. Previous records of *D. longispina* may include any of the above-mentioned taxa. Moreover, in Fennoscandia, the designation *D. longispina* has included an additional species, conspecific with North American populations named "*D. umbra"* (Schwenk et al. 2004). Several early designations from Fennoscandia can be nevertheless unequivocally attributed to this taxon (A. Hobæk and J. P. Nilssen, unpubl. data), and its nomenclature is in need of revision. Finally, a large number of additional names occur in the early literature, which today are considered synonyms or varieties of obscure status within the recognized taxa (see e.g., Flößner 2000). *4) Taxonomic problems.* A marked example of the unresolved taxonomy of a widely studied model taxon is the unclear relationship of *D. hyalina* and its alleged sister species *D. rosea*. These taxa differ morphologically (especially in height and shape of the head) both in field samples and under laboratory conditions (Gießler 2001), as well as ecologically (inhabiting lakes or ponds) (Flößner 2000, Benzie 2005). Despite apparent morphological divergence, however, no genetic marker consistently separating these two taxa has been found; either on the mitochondrial level (Taylor et al. 1996, Schwenk et al. 2000, Taylor et al. 2005) or with allozymes (Gießler et al. 1999). Billiones et al. (2004) recently suggested that a restriction analysis of the ribosomal internal transcribed spacer (ITS) might provide a species-specific marker for the separation of these two forms, however, this marker did not agree with the phenotypic variation of individuals selected from several European populations (A. Petrusek et al., unpubl. data). Another taxon with unclear taxonomical position is the alpine form *D. zschokkei* Stingelin, 1894, which is recognized as a valid species by some authors (e.g., Margaritora 1985, Flößner 2000), but not by others (e.g., Benzie 2005).

The aim of our study was to test the species status and validity of designations of members of the European *D. longispina* species complex using phylogenetic analyses. To be able to draw taxonomically sound conclusions, we included samples from type localities of the relevant taxa, in one case including sub-fossil material (resting eggs) isolated from lake sediment. Our motivation was to solve long-standing and prevailing controversies in the taxonomy of this group, and thereby facilitate comparative ecological studies in the future, as well as to increase the usefulness of the vast historical literature on European *Daphnia*.

Material and methods

Selection of populations and morphological identification

We assembled a representative set of populations covering the main morphological forms and phylogenetic lineages of the *Daphnia longispina* complex across Europe (Table 1). Additionally, we included two non-European populations representing *D. hyalina* and *D. rosea*, the former from Ethiopia and the latter from Israel. Related species not belonging to the *D. longispina* complex, *D. longiremis*, *D. cristata*, and *D. curvirostris*, were used as outgroups in the phylogenetic analyses. The *hyalina* morphotype was identified according to criteria given by Flößner (2000), namely by the shape of the head and presence of a crest. We did not attempt to differentiate between *rosea* and *longispina* morphotypes, as there are no generally accepted diagnostic characters, and both of them would be identified as *D. rosea* according to the current nomenclature based on genetic markers (Schwenk et al. 2000, Billiones et al. 2004).

To ensure that our results are relevant from the taxonomical point of view, we sampled type localities or type regions of taxonomically problematic taxa: *D. longispina* (O. F. Müller, 1776): Frederiksdal and the surroundings of Copenhagen on Zealand (Sjælland), Denmark; *D. hyalina* Leydig, 1860: Lake Constance (Bodensee), Germany; *D. rosea* G. O. Sars, 1862: lake Trollvann, Norway; *D. lacustris* G. O. Sars, 1862: lake Maridalsvann, Norway; *D. zschokkei* Stingelin, 1894: ponds above the Great St. Bernard pass, Switzerland). No type locality has ever been designated for *D. longispina*, but the region where O. F. Müller worked and where his *Daphnia* were sampled is known (Müller 1867, Hrbáček 1987). However, many lakes and ponds in this region, as well as their zooplankton species composition, may have been significantly affected by human activities, especially eutrophication. We therefore selected a *Daphnia* population from the region which morphologically resembled the first published drawing of the species (Müller 1785: pl. 12), the morphotype denoted *D. longispina* var. *mülleri* by P. E. Müller (1867). The individuals from other type localities agreed in their morphology and pigmentation level with the original descriptions of the respective taxa.

In order to rule out any taxon replacement since the initial species description in the type locality of *D. hyalina*, Lake Constance, we compared genetic information from subfossil resting eggs and currently occurring individuals. We used this approach because local *Daphnia* species composition has changed due to the introduction of *D. galeata* and subsequent interspecific hybridization with the indigenous *D. hyalina* during a phase of anthropogenic eutrophication (Einsle 1978; Jankowski and Straile 2003). We included a DNA sequence derived from a resting egg deposited in the lake sediment during the pre-eutrophication period (approximately the 1930s).

Genetic analysis

Partial sequences of two mitochondrial genes, a 526-531 bp segment of the small ribosomal subunit (12S rDNA), and a 657 bp segment of the cytochrome *c* oxidase subunit I (COI), were used to evaluate the phylogenetic relationship among taxa and to assess the haplotype diversity within taxa (12s rDNA). Additional 12S sequences were used to assign individuals from type localities to the respective mitochondrial lineages and to evaluate the relationship between genotypes and morphotypes.

DNA was extracted from individuals preserved in ethanol or originating from laboratory cultures by proteinase K digestion following the protocol in Schwenk et al. (1998) or by Chelex extraction as described in Hobæk et al. (2004). Both mitochondrial genes were amplified using previously described protocols (Schwenk et

Table 1.

List of analyzed *Daphnia* individuals and the sequence accession numbers. Type localities of the problematic taxa are marked in bold, numerical codes before the locality refer to individuals in Figs. 1 and 3. Individuals labeled as "*rosea*" would be identified either as *D. rosea* or *D. longispina,* depending on the identification key used. Countries of origin are indicated by their international license plate codes (A – Austria, B – Belgium, CH – Switzerland, CZ – Czechia, D – Germany, DK – Denmark, ETH – Ethiopia, FIN – Finland, IL – Israel, N – Norway, NL – Netherlands, PL – Poland, RUS – Russia, S – Sweden, SK – Slovakia, SP – Spain). Sequences from other studies are indicated as follows: S2000, S2004 – Schwenk et al. (2000, 2004), N2007 – Nilssen et al. (2007), P2007 – Petrusek et al., in press. 12S rDNA sequences used only for the total evidence phylogenetic analysis (Fig. 4) are marked by asterisks.

				GenBank acc. no.		
taxon/morph	**locality; region**	**country**	**12S**	**COI**	**note**
hyalina	1 Mondsee; Oberösterreich	A	EF375827		
hyalina	2 Lake Tana	ETH	EF375828		
hyalina	3 **Lake Constance**	D,A,CH	EF375829	EF375860	*hyalina* type locality
hyalina	4 **Lake Constance**, resting egg, 1930s sediment	D,A,CH	EF375830		*hyalina* type locality
hyalina	5 Stechlinsee; Brandenburg	D	EF375831		
hyalina	6 Goksjø; Vestfold	N	EF375832		
hyalina	7 Lake Glubokoje; Moscow region	RUS	EF375833		
rosea	8 Droužkovice - pond Žiďák; north Bohemia	CZ	EF375834		
rosea	9 Žďárské jezero, Bohemian Forest; west Bohemia	CZ	EF375835		
rosea	10 Brededam; Zealand	DK	EF375836		*longispina* type region
rosea	11 **Midtre Kobberdam**, Zealand	DK	DQ536400		*longispina* type region, N2007
rosea	12 Pernillesø; Zealand	DK	EF375837		
rosea	13 Ismaning - Ismaninger Fischteiche; Bayern	D	EF375838		
rosea	14 Frankfurt am Main - botanical garden; Hessen	D	EF375839		
rosea	15 Lake Hula; north Israel	IL	EF375840		
rosea	16 Mildevatn; Hordaland	N	EF375841		
rosea	17 **Trollvann**; Oslo	N	EF375842		*rosea* type locality
rosea	18 Nižné Jamnícke Lake, West Tatra Mts.	SK	DQ337937		P2007
rosea	19 Zahillo, Doñana National Park; Andalucía	SP	EF375843		
rosea	20 Villar del Rey reservoir; Badajoz	SP	EF375844		
rosea	21 Göteborg, pond in Laerjeholm; west Sweden	S	EF375845	EF375861	
rosea	22 Unterer Arosasee, Arosa; Graubünden	CH	EF375846		
zschokkei	23 **Ponds above Great St. Bernard pass**; Valais	CH	EF375847	EF375862	*zschokkei* type locality
lacustris	**Maridalsvann**; Oslo	N	DQ337943	DQ871251	N2007
lacustris	Alpine pond at Finse; Hordaland	N	AF277279*	EF375863	12S: S2000
lacustris	Myrdalsvatn; Hordaland	N	DQ337945		N2007
lacustris	Niżni Toporowy staw, High Tatra Mts.	PL	DQ337940		N2007
n.sp. A	Lake Berse; Aust-Agder	N	EF375848	EF375864	two individuals
"umbra"	Mallalampi A; Finnish Lapland	FIN	EF375849	EF375865	S2004
"umbra"	Sarsvatn; Svalbard, high Arctic	N	EF375850		
"umbra"	Bjornesfjord (alpine); Buskerud	N	DQ864520		N2007
"umbra"	Alpine lake in Jotunheimen, Oppland	N	AF277276*	EF375866	S2000
galeata	Tjeukemeer; Friesland	NL	EF375851	EF375867	
galeata	Torkelvatn; Nord-Trøndelag	N	EF375852		
galeata	Morskie Oko; High Tatra Mts.	PL	DQ337927		P2007
galeata	Lake Norrviken; east Sweden	S	EF375853*	EF375868	
cucullata	Tjeukemeer; Friesland	NL	AF277271	EF375869	12S: S2000
cucullata	Akersvann; Vestfold	N	EF375854		
cucullata	Medlov; central Moravia	CZ	AF277270	EF375870	S2000
longiremis	Lake Berse; Aust-Agder	N	EF375855	EF375871	
longiremis	Lake Longum; Aust-Agder	N	EF375856		
cristata	Maseh; Finnish Lapland	FIN	EF375857		
cristata	Vassbotten; west Sweden	S	EF375858		
curvirostris	Grosse Stienitzsee; Brandenburg	D	EF375859	EF375872	

al. 2000). The PCR products were purified by spin-column separation (GFX PCR DNA or Gel Band Purification Kit, Amersham Biosciences) either directly or after excision from the agarose gel. Purified products were subsequently sequenced on ABI automatic capillary sequencers (series 377, 3130, and 3700) using the dideoxynucleotide termination method. Additionally, we utilized several sequences from previous and concurrent studies (Schwenk et al. 2000, 2004; Nilssen et al. 2007). The GenBank accession numbers of all sequences are listed in Table 1. Sequences were aligned using ClustalW (Thompson et al. 1994) and the alignment was subsequently edited manually in MEGA version 3.1 (Kumar et al. 2004).

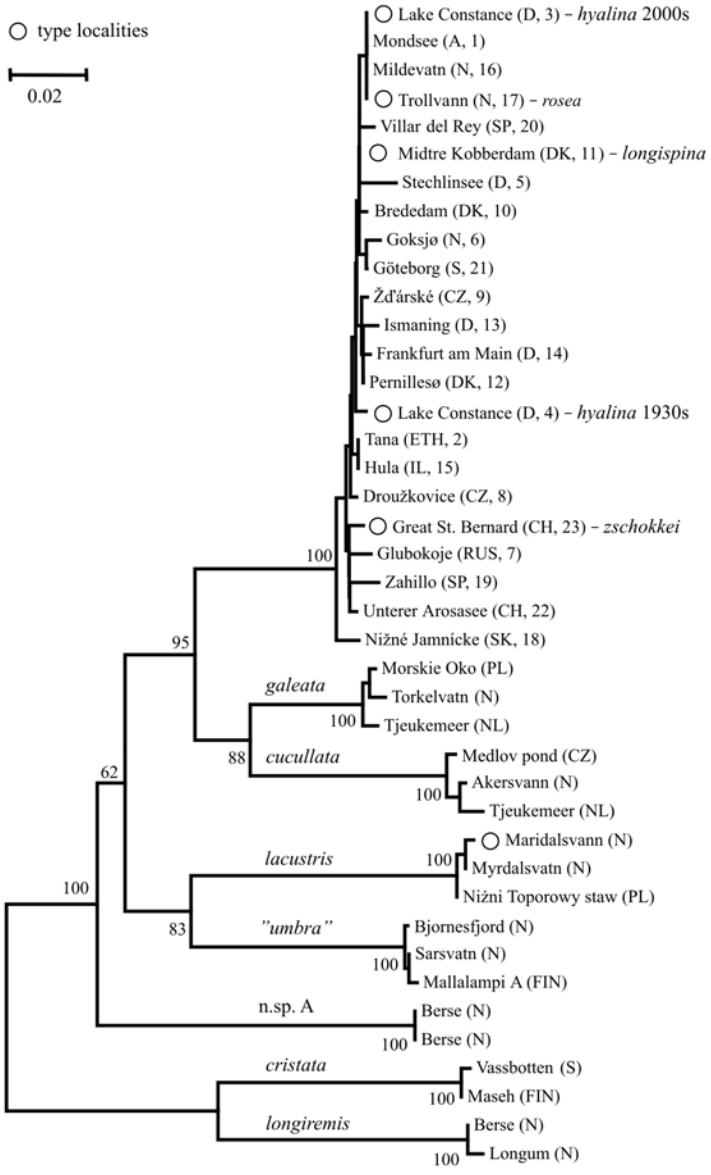
The phylogenetic relationships among individuals were evaluated by several approaches. Firstly, a neighbor-joining tree of 12S rDNA sequences based on the Kimura 2-parameter distance was constructed in MEGA 3.1 (with pairwise deletion of gaps and 1000 bootstrap pseudoreplicates). Secondly, a statistical parsimony network with a 95% parsimony limit of 12S rDNA sequences from all individuals belonging to the "*rosea/hyalina*" clade was generated with TCS version 1.21 (Clement et al. 2000).

Additionally, we analyzed the phylogenetic relationships of various lineages of the *D. longispina* complex, simultaneously using information from both the COI and 12S rDNA genes (1191 bp). *D. curvirostris* was used as one outgroup in this analysis, as COI of *D. cristata* could not be amplified using universal primers. A test for homogeneity of partitioned data (Farris et al. 1995) allowed us to subject both genes to a joint analysis ($p = 0.96$). At least two individuals per clade within the *D. longispina* complex for which both COI and 12S rDNA sequences were available were used (see Table 1). We used Modeltest 3.7 (Posada and Crandall 1998) to choose the best-fit model of nucleotide substitution from 56 different models of sequence evolution. The phylogenetic tree was constructed by Bayesian inference of phylogeny using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). A Markov chain Monte Carlo (MCMC) analysis was run for two million generations, with two parallel runs of four chains run simultaneously and trees sampled every 100 generations. The first 20% of the trees, including the burn-in phase, were discarded. The remaining 3.2×10^4 trees were used to construct the phylogram; branch support values indicate the posterior probability of the existence of the clade based on the available data and the selected model of evolution (calculated as the proportion of sampled trees sharing that particular branching pattern). The topologies of resulting phylograms did not differ whether the parameters for both genes were linked, or estimated independently (using the "unlink" option). Additional phylogenetic analyses included maximum parsimony (MP) and maximum likelihood (ML) methods, carried out on the same dataset in PAUP* 4.0b10 (Swofford 2002). Heuristic searches were conducted with tree bisection-reconnection branch swapping and ten random sequence taxon additions; branch support was estimated by nonparametric bootstrapping with 1000 pseudoreplicates.

Results

Our 12S rDNA dataset consisted altogether of six clearly differentiated, well-supported lineages within the *D. longispina* complex (Fig. 1). The sequence from the type locality of *Daphnia lacustris* (Lake Maridalsvann, Norway) was grouped together with sequences from populations labeled in previous phylogenetic studies as "*D. longispina*". The name *D. lacustris* will therefore be adopted for this lineage in the remaining text (for more details see Nilssen et al. 2007). In addition to already-known taxa, an unknown lineage not belonging to any currently recognized species (indicated as *Daphnia* n.sp. A) was discovered in a single locality, Lake Berse in southern Norway. This apparently undescribed taxon is distinct both at the mitochondrial (12S and COI

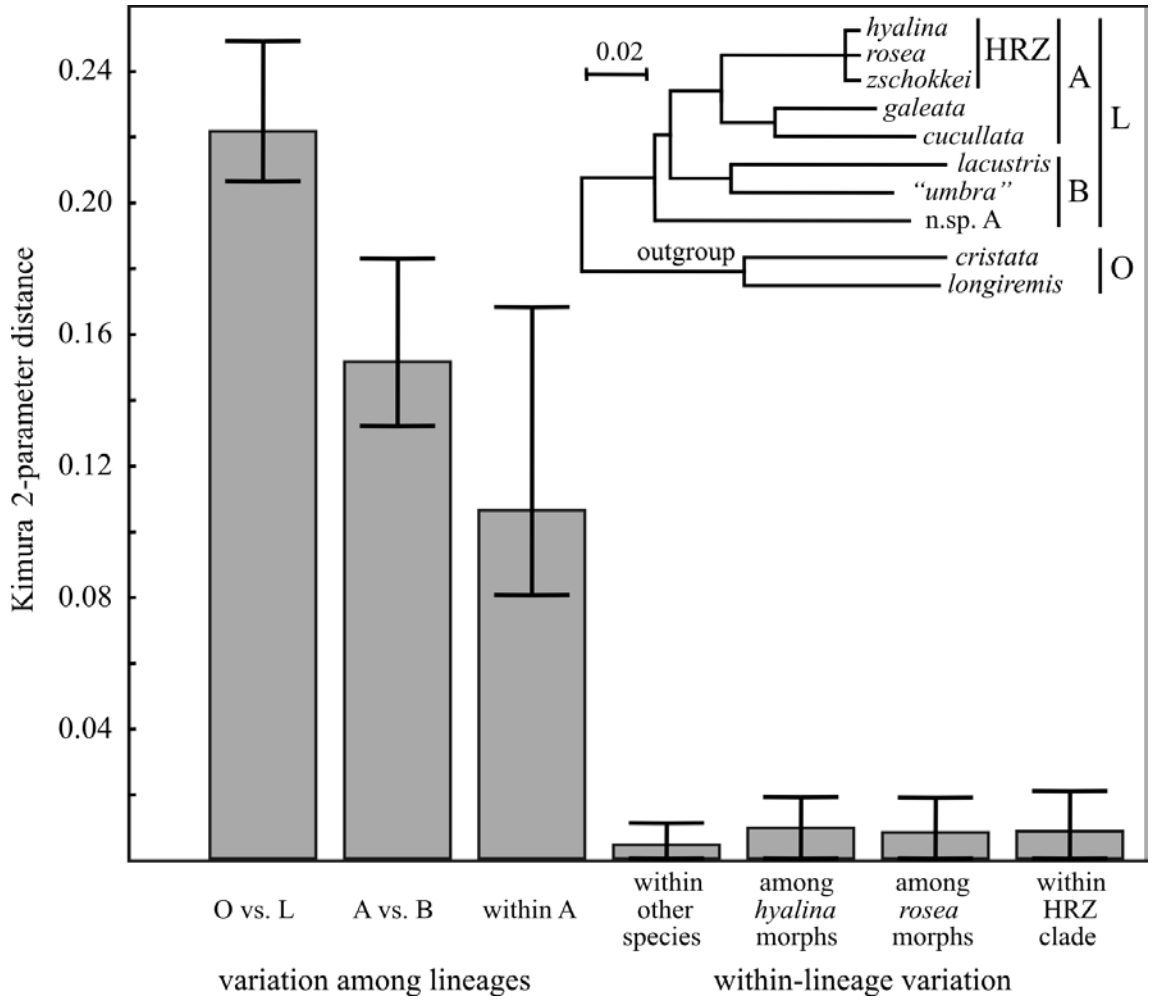
Figure 1.
Neighbor-joining tree showing the sequence variation of 12S rDNA in the *Daphnia longispina* complex (Kimura-2 parameter distance, pairwise deletion of gaps; bootstrap support is shown for selected branches). The country of origin of each individual is indicated by abbreviations in parentheses; numbers indicating individuals are identical with those in Table 1 and Fig. 3. Individuals from type localities are marked by circles. *D. longiremis* and *D. cristata* were used as outgroups.



sequences) and nuclear (restriction patterns of the ribosomal internal transcribed spacer; Billiones et al. 2004) level from other taxa of the group, though some ITS variants of *D. lacustris* are very similar (Skage et al. 2007; Nilssen et al. 2007).

Individuals from type localities (or a type region) of the following taxa clustered together in one clade: *D. rosea, D. hyalina*, *D. zschokkei,* and *D. longispina*. This clade also included other individuals of *hyalina* and *rosea* phenotypes*,* and the DNA sequence obtained from a resting egg representing "original" Lake Constance *D. hyalina* from the pre-eutrophication period. The maximum 12S rDNA sequence divergence (Kimura 2-parameter distance) within this cluster was 2.1%. The sequence variation of this gene at different hierarchical levels within European members of the *D. longispina* complex is shown in Fig. 2. The average pairwise species

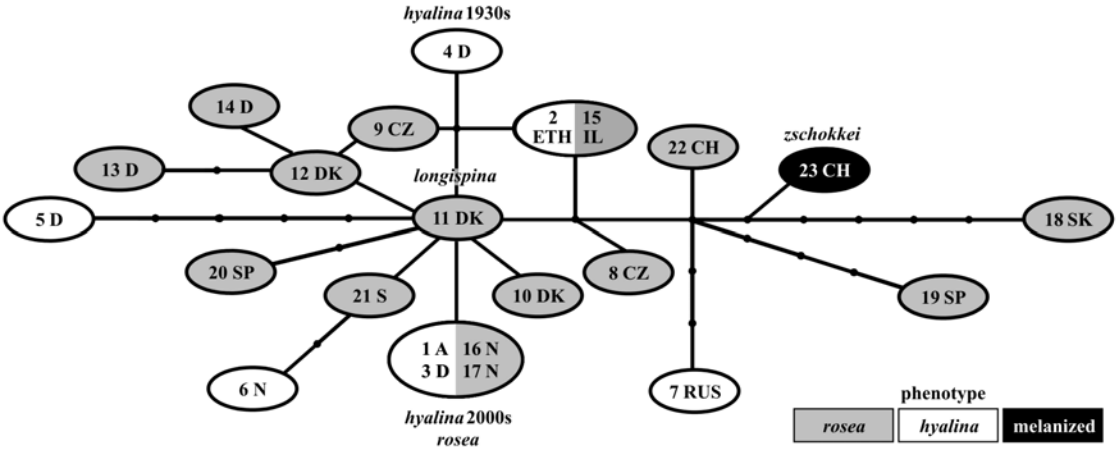
Figure 2.
Pairwise 12S rDNA sequence divergence within the European *Daphnia longispina* complex. Average genetic distances (Kimura 2-parameter) are compared among and within different clades as depicted in a schematic neighbor-joining tree based on the analysis presented in Fig. 1. Columns indicate mean values, bars indicate range (min – max). Letters denote clades: L = *D. longispina* complex, O = outgroup (*cristata* + *longiremis*), HRZ = *hyalina*/*rosea*/*zschokkei* clade, A = HRZ + *galeata* + *cucullata*, B = northern ("boreal") lineages of the *D. longispina* complex (*lacustris* + *"umbra"* + n.sp. A from Lake Berse); "other species" = all analyzed species excluding HRZ.



divergence within the complex (excluding the *rosea-hyalina-zschokkei-longispina* clade) was 15.6% (range 8.1 to 19.4%). The within-species divergence (based on geographically distant European populations of the following taxa: *D. galeata*, *D. cucullata*, *"D. umbra"*, and *D. lacustris*) was 0.4% on average (but the intraspecific maxima ranged from 0.7 to 2.0%). The mean sequence divergence was 1.0% (max. 1.9%) for *hyalina* morphotypes, 0.8% (max. 1.9%) for *rosea* morphotypes, and 0.9% (max. 2.1%) when all populations of the *hyalina-rosea-zschokkei* clade ("HRZ" in Fig. 2) were pooled together.

The statistical parsimony network of individual 12S rDNA haplotypes within the HRZ clade (Fig. 3) did not reveal any structure supporting traditional species assignments, as no link between matrilines and morphology could be observed. Haplotypes of individuals with a *hyalina* morphotype were scattered in several non-adjacent parts of the network, suggesting no recent common ancestry for this morph. On the contrary, the most common haplotype in the network was shared among morphologically divergent individuals from four

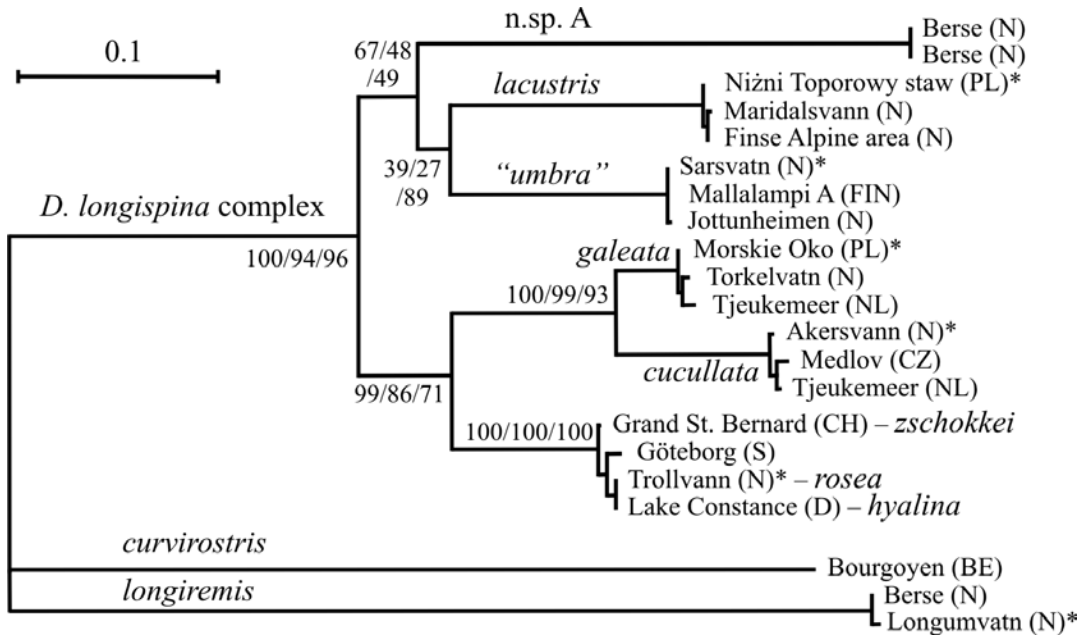
Figure 3.
Association of phenotypes and haplotypes within the clade consisting of *D. longispina, D. rosea*, *D. hyalina*, and *D. zschokkei*. Each node within the parsimony network of 12S rDNA represents a single point mutation; the size of ovals corresponds to the number of individuals sharing the particular haplotype. Individuals carrying the respective haplotype are marked by the locality number (Table 1, Fig. 1) and the abbreviation for the country of origin. Type localities are marked by the respective taxon names, haplotypes from Lake Constance (*D. hyalina* type locality) also by the decade in which the resting egg was produced.



different populations (Fig. 3; haplotype marked 1A/3D/16N/17N). Two of them belonged to the *hyalina* morphotype (Mondsee and Lake Constance, *D. hyalina* type locality), and the two others to the *rosea* morphotype (Mildevatn and Trollvann, *D. rosea* type locality). Similarly, another haplotype was shared also among individuals representing two non-European populations of differing morphology, *hyalina* from Lake Tana, Ethiopia, and *rosea* from Lake Hula, Israel. The haplotype from the pre-eutrophication period of Lake Constance differed by three nucleotide substitutions from the one representing the recent population in the lake.

Results of the phylogenetic analyses using the total evidence approach (12S rDNA and COI sequences) are shown in Fig. 4. The optimal model selected by Modeltest using either the hierarchical likelihood ratio tests or the Akaike information criterion was the transversional model with Gamma distribution (TVM+G). All phylogenetic methods (Bayesian inference of phylogeny, maximum likelihood, and maximum parsimony) strongly supported the monophyly of the *D. longispina* complex, a sister relationship between *D. galeata* and *D. cucullata*, and monophyly of the clade consisting of *D. galeata*, *D. cucullata*, and the lineage containing representatives of the putative species *D. hyalina*, *D. rosea*, and *D. zschokkei*. A sister relationship between *D. lacustris* and *"D. umbra"* was supported as well but with lower branch support values. This topology is in agreement with the NJ tree based on 12S variation (Fig. 1). On the other hand, the position of the new lineage (Lake Berse) differed, as it was placed in one branch together with the other two northern European taxa – *D. lacustris* and "*D. umbra*". The support values within this branch were, however, generally lower than for other clades.

Figure 4
Phylogenetic relationship among European members of the *Daphnia longispina* complex, based on maximum likelihood analysis of the COI and 12S rDNA genes. Numbers at branches indicate the posterior probability values from Bayesian inference of phylogeny, and bootstrap support values of the maximum likelihood and maximum parsimony analyses. Asterisks indicate individuals where only 12S rDNA sequence was available.



Discussion

The observed patterns of genetic divergence among individuals of the *Daphnia longispina* complex have profound implications for systematics of the group. The number of basal lineages in the complex, all of them occurring almost exclusively in Fennoscandia, has increased to three. The new Lake Berse lineage (*Daphnia* n. sp. A) is apparently undescribed. Its mtDNA clearly shows the affinity of this lineage to the other two previously known northern European species (*D. lacustris* and *"D. umbra"*), although their exact phylogenetic relationship needs to be further elucidated. The highest species diversity within the complex so far is found in northern Europe (where all remaining lineages are present as well). On the other hand, it is not unlikely that other genetically distinct but morphologically uniform lineages will also be found in low frequencies elsewhere in Europe if a more detailed genetic screening of various populations is undertaken, especially in alpine regions where divergent *Daphnia* forms have long been observed (e.g., Burckhardt 1899).

The most important finding of our study is the lack of any significant separation among the alleged species *D. rosea*, *D. hyalina*, *D. longispina*, and *D. zschokkei*. The 12S rDNA variation within this clade only slightly exceeded values of intraspecific variation in other *Daphnia* species (Fig. 2), and some individuals of *hyalina* and *rosea* morphotypes (including those from the type localities) actually shared identical haplotypes. The diversification within this clade might represent a very recent split of lineages, which would not yet be reflected in the analyzed mitochondrial gene. Nevertheless, the distribution of different morphotypes across the haplotype parsimony network (Fig. 3) does not support this hypothesis. The maximum divergence between two *D. hyalina* individuals (Lake Glubokoje, Russia – Stechlinsee, Germany; 1.9%) was identical to the divergence

between the two most divergent *rosea* populations (Zahillo pond, Spain – Nižné Jamnícke Lake, Slovakia), and neither of the morphotype groups shared a fixed trait which would differentiate them.

The observed haplotype distribution might also reflect an ancestral polymorphism in the maternal lineages, which would be maintained despite reproductive isolation if the morphotypes represented biological species. In such a case, however, we would expect a genetic differentiation of the polymorphic nuclear-encoded markers. The available evidence does not support this hypothesis. The two morphotypes could not be separated by allozymes (Gießler et al. 1999) or by RFLP patterns of the internal transcribed spacer (A. Petrusek et al., unpubl. data). More importantly, a detailed analysis of the genetic variation of 14 populations of *D. hyalina*, *D. rosea* and *D. zschokkei* all over Europe (Thielsch 2005), based on 13 unlinked microsatellite loci (Brede et al. 2006) for 20-40 individuals per population, did not provide any support for separating these putative taxa. Hierarchical partitioning of genetic variation showed that the differentiation among populations was higher than differentiation among phenotypes (i.e., putative species), and neither phylogenetic nor factorial correspondence analysis identified an association of phenotypes and clusters of individuals based on microsatellite data. On the contrary, genetic differentiation in nuclear DNA followed very similar patterns to mtDNA, despite the large difference in mutation rates and mode of inheritance of these marker systems. No genetic evidence, therefore, suggests reproductive barriers or restricted gene flow among the morphs; local processes limiting the gene flow among populations, such as local adaptation and strong monopolization of resources (De Meester et al. 2000), seem to be more important in shaping the observed pattern of genetic variation.

Although there are marked morphological differences between typical *hyalina* and *rosea* morphotypes, which have been used as support for their species status (Gießler et al. 1999), it is more likely that these differences are habitat- and predation-dependent, especially as populations with intermediate morphology are frequently found. Our results are therefore consistent with the hypothesis of a single, morphologically, and ecologically plastic species, which has independently switched habitats several times and adapted to pelagic or pond/littoral conditions, with consequent gradual changes towards *hyalina*- or *rosea*-like morphology, respectively. The occurrence of intermediate forms between *D. rosea* and *D. hyalina* morphotypes and the lack of genetic divergence suggest that both forms are insufficiently isolated to form independent evolutionary lineages. Sustained divergent selection on the pond- and lake-adapted forms, with a selective disadvantage for transitional forms, may eventually lead to ecological speciation. However, so far no signs of reproductive isolation have been detected and nuclear loci, including microsatellites, suggest high levels of gene flow among morphotypes. Similarly, we found no significant genetic divergence between the melanic alpine animals from the type locality of *D. zschokkei* and other members of the *hyalina-rosea* clade. Thus, even conspicuous phenotypic differences, such as melanization, head size and shape, or body size and shell spine length, are not associated with strong genetic differentiation. Much greater differentiation at the mitochondrial DNA level, well over 10% at 12S rDNA gene, was detected among morphologically much more similar lineages of the *D. longispina* complex, such as *D. lacustris, "D. umbra"* and the one from Lake Berse (Fig. 1).

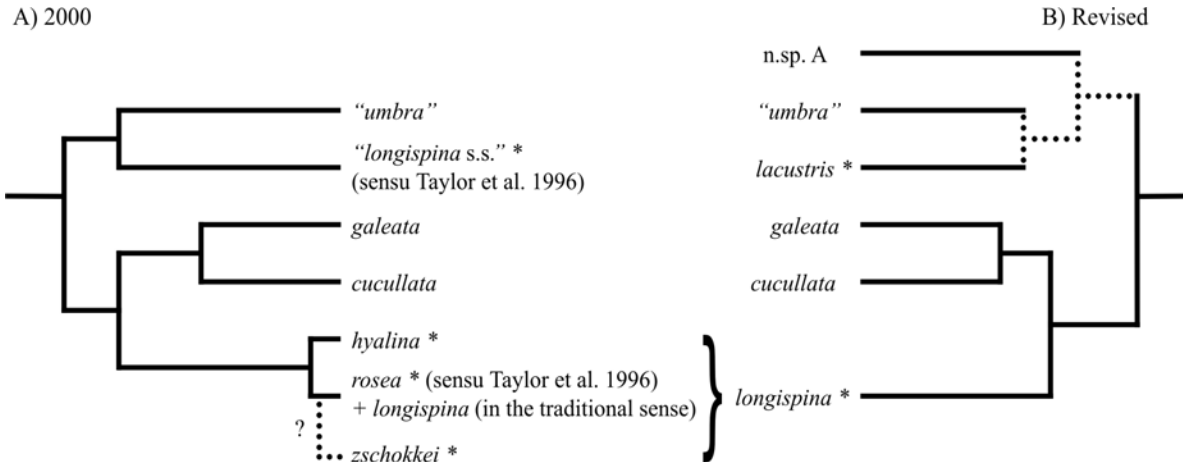
A strikingly parallel pattern to the *D. longispina* / *D. hyalina* confusion in Europe occurs in American taxa of the complex. The species pair *D. dentifera* / *D. thorata* forms a distinct group, being the closest relative of the *hyalina-rosea-zschokkei* clade (Taylor et al. 1996, 2005). Interestingly, the differences in morphology and habitat preferences between these two mostly Nearctic taxa (Taylor & Hebert 1994) follow a very similar pattern as in the alleged species pair *D. rosea* / *D. hyalina*. Similarly, there is little evidence for their genetic

diversification: *D. thorata* has 12S rDNA haplotypes similar or even identical to *D. dentifera* (Taylor et al. 1996, 2005), and no nuclear markers allowing clear differentiation of these forms have been found, either using allozymes (Taylor and Hebert 1994) or ITS sequence analysis (Taylor et al. 2005). We predict that the observed differences between these alleged sibling species might actually represent a similar case of intraspecific variation (local adaptation to pelagic environment associated with helmet formation) as in their Old World counterparts *D. rosea* and *D. hyalina*. In fact, Ishida and Taylor (2007) no longer distinguished between any of these "species" pairs, and treated them all as members of a broad cluster labeled "*D. rosea* s.l.". This Holarctic clade showed clear geographic structure with three lineages possibly representing different species (Nearctic, Siberian, European) but without any internal structure consistent with morphotypes. In accordance with our results, one of the subclades identified by Ishida & Taylor (2007) included European *D. hyalina* and *D. rosea* morphotypes.

Suggested nomenclatural revision of the European D. longispina *complex*—Our systematic findings, namely the lack of any evidence for reproductive isolation of several putative species, together with the analysis of individuals originating from type localities of several species of the complex, allow us to propose a comprehensive revision of the nomenclature of European members of the *D. longispina* complex (summarized in Fig. 5). With this revision, we intend to rectify misleading name assignments in some recent publications, and make the nomenclature consistent with the traditional use of taxon names in the substantial body of European literature. We reflect the re-evaluation of the species boundaries and the principle of priority, at the same time respecting the goals of the International Code of Zoological Nomenclature to minimize confusion and maximize stability.

A major problem with recent literature based on genetic markers has been the arbitrary selection of populations to represent established taxa. When resolving taxonomic problems, it is critical that evidence from the type localities is considered. In this study, we have made every effort to include representatives from type

Figure 5
Comparison of hypotheses on the phylogenetic relationships, species boundaries, and nomenclature of European members of the *Daphnia longispina* complex. (A) Current state – phylogeny after Schwenk et al. (2000) and Taylor et al. (2005), hypothetical position of *D. zschokkei* according to Flößner (2000). (B) Our results and suggestions for nomenclatural revisions (taxon names marked by asterisk are affected). Topology indicated by a dotted line requires further corroboration.



localities, to assure that our evaluation of species boundaries and nomenclatural suggestions are well founded. In particular, it was important to elucidate the identity of O. F. Müller's original *D. longispina*. We succeeded in this respect by examining ponds in the vicinity of Müller's residence on Zealand, and analyzing morphologically most resembling phenotypes. Further, in the case of Lake Constance (the type locality of *D. hyalina*), we examined sub-fossil *Daphnia* resting eggs to minimize potential problems with known environmental changes and taxon replacement. These resting eggs were produced during the early 1930s and have a higher likelihood of resembling the type material from the mid-19th century than currently occurring individuals, which are the result of introgressive hybridization (Jankowski and Straile 2003). Although this approach remains restricted to taxa that produce dormant stages and localities with suitable sediment records, we demonstrate here that the genetic analysis of subfossil material represents a powerful tool for taxonomy.

The three distinct lineages confined to northern Europe (*D. lacustris*, "*D. umbra*" and the as-yet undescribed species) are all problematic from a nomenclatural point of view. The new lineage from Lake Berse needs further study, including a taxonomic description as well as a name. While there is no doubt about the distinction of *"D. umbra"*, this designation lacks a formal description as well as a type (Benzie 2005), and the possible identity of this species with older European taxa needs to be examined. The third northern lineage of the complex was certainly in need of a taxonomic revision: Nilssen et al. (2007) showed that individuals labeled *D. longispina* sensu stricto in several recent genetic studies (e.g., Taylor et al. 1996; Schwenk et al. 2000; Billiones et al. 2004) should correctly be called *Daphnia lacustris* G. O. Sars, 1862..This lineage is absent from the region of the original description of *D. longispina* (Denmark); outside of Fennoscandia, only two extant populations are known from two adjacent lakes in the Polish Tatra Mountains (Petrusek et al. in press). Retaining the incorrectly assigned label "*D. longispina*" for this taxon would therefore be in disagreement not only with the majority of previously published European literature, but also with all important monographs on European cladocerans (e.g., Margaritora 1985; Alonso 1996; Flößner 2000) including the latest monograph focusing on the genus *Daphnia* worldwide (Benzie 2005).

The most important finding with nomenclatural consequences, however, is the lack of differentiation among phenotypes identified as *D. longispina* O. F. Müller, 1776, *D. hyalina* Leydig, 1860, *D. rosea* G. O. Sars, 1862, and *D. zschokkei* Stingelin, 1894, all of which apparently represent morphological variation within a single biological species. All four names appear in both old and recent literature, and the former two especially have a long history of continuous use.

Of the above-mentioned names, *Daphnia longispina* is clearly the oldest designation, which, as we argue, should have priority over the other three. The name has been in continuous use for over 200 years, and it has (correctly) been used to designate many populations of this species all over Europe. This species was described from Zealand (Sjælland), Denmark, and although there has been some uncertainty about the identity of the animals actually sampled by O. F. Müller (P.E. Müller 1867; Hrbáček 1987), we show here that a very similar morphotype, still occurring in the region where O.F. Müller worked at the time (J. P. Nilssen, unpubl. data), belongs to the same clade as representatives of the other three putative species of the complex (Figs. 1 and 3).

The taxon *D. hyalina* Leydig would be next in priority. While it could be argued that this name is based on a more complete description than O. F. Müller's *D. longispina*, and the type locality if this taxon is known, the designation *D. hyalina* has been applied much less frequently than *D. longispina* across Europe, both in

terms of number of localities and number of published papers. Favoring Leydig's over Müller's designation (i.e., *D. hyalina* over *D. longispina*) could therefore cause widespread confusion, whereas the reverse solution causes fewer problems, especially when the whole distribution area of the taxon in the Western Palaearctic is considered.

D. longispina (O. F. Müller, 1776) is also generally considered to be the type species of the entire genus (e.g., Hrbáček 1987), and suppressing this name is clearly undesirable. Finally, the difficulties of distinguishing between *D. longispina, D. hyalina* and *D. rosea* have long been recognized (e.g., Flößner 2000), and the latter two have been treated as subspecies or merely variants of the former (e.g., Herbst 1962). However, *D. longispina* has to our knowledge never been considered a form of any of the others, which illustrates that *D. longispina* has generally been perceived as the basic name. We conclude that not only priority, but also nomenclatural stability requires that the name *D. longispina* takes precedence.

Another alternative approach, the use of the designation *D. rosea* s.l. for the whole clade, as recently done by Ishida and Taylor (2007), can hardly be justified either, as it suppresses the previously described and well-established taxa *D. longispina* and *D. hyalina*, disregarding both nomenclatural stability and priority.

On the other hand, evidence that *D. zschokkei* is not a separate species is uncontroversial. The fact that the population from the type locality of this taxon was not genetically divergent from other *D. longispina* populations confirms previous doubts about its validity. Although some authors have treated *D. zschokkei* as a distinct species (Flößner 2000, Margaritora 1985), Hrbáček (1969) did not find consistent differences from *D. longispina* in his redescription of *D. zschokkei* based on Stingelin's type material, and the latest monograph on the genus *Daphnia* (Benzie 2005) does not recognize its specific status. Our findings, however, do not necessarily imply that all European alpine populations previously reported as "*D. zschokkei*" (e.g., Margaritora and Ferrara 1979; Flößner 2000) are conspecific with those from the type locality. The isolated occurrence of *D. lacustris* populations in southern Poland (Nilssen et al. 2007; Petrusek et al., in press) suggests that the Central European mountain ranges might harbor additional "relict" species, such as *"D. umbra"*. This hypothesis is supported by Flößner (2000) who considered alpine *"zschokkei"* populations to be possibly conspecific with those in Swedish Lapland, which certainly belong to *"D. umbra"* (A. Hobæk and M. Skage, unpubl. data). Whatever the taxonomic position of other populations designated as *D. zschokkei* may be, the name itself should be regarded as a junior synonym of *D. longispina* and is therefore not applicable.

To properly conclude the suggested systematic revision of the *D. longispina* complex, a formal redescription of *D. longispina* including the designation of neotype and a type locality is warranted. We believe this would contribute significantly to the nomenclatural stabilization of the entire *Daphnia longispina* complex, and it is our intention to complete this task in the near future, based on the already analyzed Danish material.

Conclusions—The nomenclatural confusion among members of the *D. longispina* complex, which originated from improperly selected standards for genetic characterization of the taxa, clearly stresses the need for using generally accepted reference material, preferably specimens from type localities or regions. This is especially true for taxa with little morphological divergence and problematic identification characters. The *D. longispina* complex is no exception within the genus *Daphnia* – similar taxonomic ambiguities prevail in most of the *D. pulex* complex, the target of many ecological and genetic studies (including the *Daphnia* genomics consortium).

The implications of the proposed revision of the *D. longispina* group reach far beyond the fields of taxonomy and systematics. The outcome of many projects in limnology, ecology, and biogeography depend on the correct identification of species and a universally accepted nomenclature, which is consistently applied across entire species' ranges. Comparative studies among different habitats and geographical regions will only be feasible if we overcome regional differences in nomenclatural practice. Another consequence of the proposed revision is related to our understanding of the origin of species within *Daphnia*. For example, our data reject the belief that large-lake *D. hyalina* populations represent an evolutionary lineage distinct from *D. longispina* or *D. rosea*, which usually inhabit smaller water bodies (Flößner 1973, 2000; Gießler 2001).

The discovery that a single biological species seems to encompass such a wide ecological and phenotypic range opens new lines of research, and raises the question of which processes might have caused and maintained the morphological divergence despite ongoing gene flow. This finding is especially interesting in contrast with the current trend of discovering cryptic species in most groups of organisms. In general, *Daphnia* is not an exception to this trend, and undoubtedly many undescribed lineages still remain to be discovered. However, our analysis of the European *D. longispina* complex demonstrates that molecular methods may simultaneously unravel both cryptic diversity and phenotypic polymorphism. Last, but not least, phylogeographic studies of "arctic" lineages with disjunct distributional patterns may be useful in unraveling the role of past glaciations and the effect of processes such as long distance dispersal and climatic changes on arctic and alpine populations of planktonic species.

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CHAPTER 3

Nilssen J. P., Hobaek A., Petrusek A., Skage M.:

Restoring *Daphnia lacustris* G.O. Sars, 1862 (Crustacea, Anomopoda) – a cryptic species in the *Daphnia longispina* group

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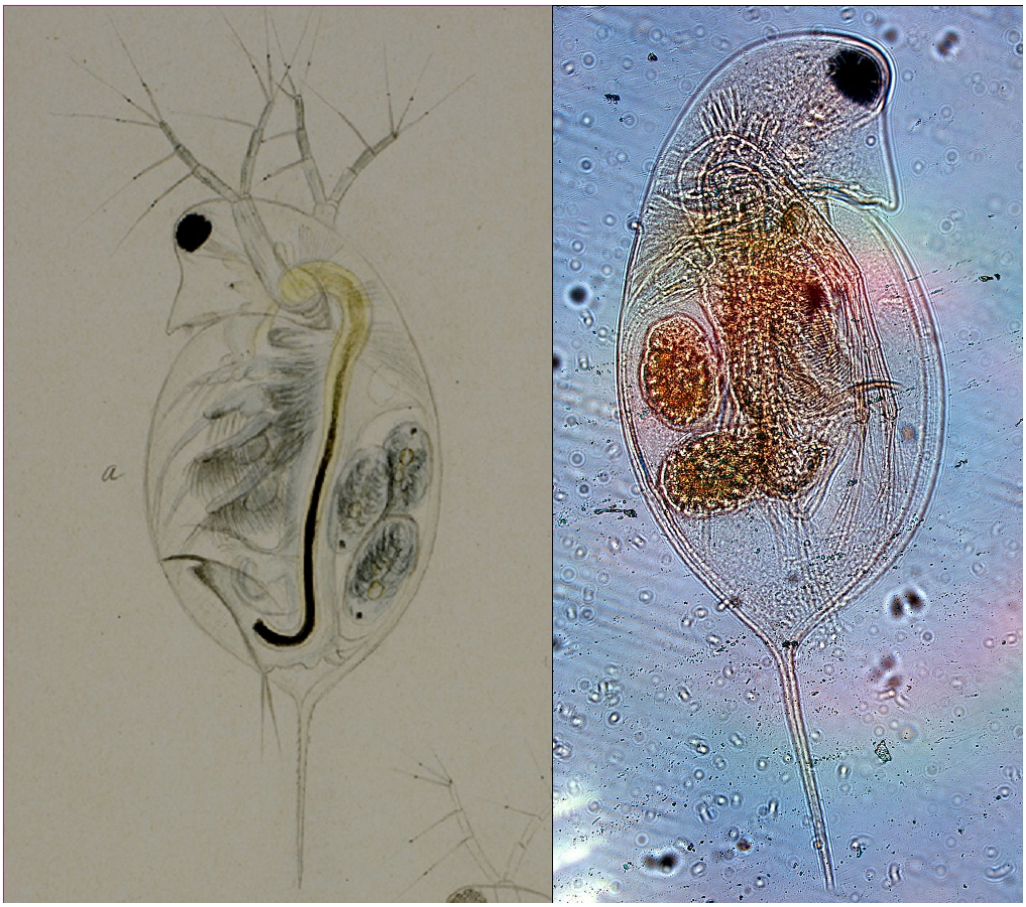

Original colour drawing and permanent slide of *Daphnia lacustris* by Georg Ossian Sars.

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CLADOCERA

Restoring *Daphnia lacustris* G.O. Sars, 1862 (Crustacea, Anomopoda): a cryptic species in the *Daphnia longispina* group

Jens Petter Nilssen · Anders Hobæk · Adam Petrusek · Morten Skage

Abstract While molecular markers have revealed several distinct species within the *Daphnia longispina* group, there is a need to reconcile these species with traditional nomenclature. Here we show that one such species, called *D. longispina* in recent literature based on molecular markers, can reliably be associated with the described taxon *Daphnia lacustris* G.O. Sars, 1862. Both mitochondrial and nuclear molecular markers readily distinguish this species from others in the *D. longispina* group. *D. lacustris* is absent in the region from which *D. longispina* was first described (Denmark), and the designation *D. longispina* must be reserved for another widespread species represented by Danish lineages. While the diagnosis of *D. lacustris* (and other species of the *D. longispina* group) by molecular markers is unequivocal, distinguishing it morphologically from other species is still problematic. The presently known distribution range of *D. lacustris* includes most of Norway, northern Finland and a single lake in the Polish Tatra Mountains. Its typical habitat is oligotrophic lakes without intense fish predation.

Keywords Nomenclature · Genetic markers · 12S mitochondrial gene · ITS · Biogeography · Pelagic predation

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Introduction

Daphnia longispina (O.F. Müller, 1776) is considered the type species of the genus *Daphnia* (Hrbáček, 1987). However, even 220 years after its illustration by O.F. Müller (1785), confusion over the delimitation of the species still prevails (Hrbáček, 1987; Flößner, 2000; Benzie, 2005). A group of related species (usually called the *D. longispina* group or complex) are dominant across the Holarctic as key species of lake ecosystems. This group is notorious among both ecologists and taxonomists for the morphological plasticity of its members. A bewildering plethora of names (species, subspecies, varieties and forms) occur in the literature, and regional

traditions differ as to how these designations are applied. For instance, following Flößner (1972, 2000) in continental Europe, *D. rosea* G.O. Sars, 1862 has been recognized as distinct from *D. longispina*, while in Scandinavia, the former taxon has rarely been reported (in spite of its original description being derived from this region).

Studies on the *D. longispina* group applying genetic markers (e.g. Hobæk & Wolf, 1991; Taylor et al., 1996; Schwenk et al., 2000) have confirmed the distinctness of several lineages within the *D. longispina* complex in Europe. However, while these lineages can be recognized as separate species (notwithstanding widespread hybridization), some of them are difficult to affiliate with morphologically defined species. Their nomenclature, is therefore, obscure. In particular, the designations *D. longispina*, *D. hyalina* Leydig, 1860, and *D. rosea* remain problematic (Benzie, 2005). Hence, there is a need for a nomenclatural revision of valid species within this problematic group. In the present article, we aim to show that a genetically distinct species recently called "*D. longispina*" (sensu Taylor et al., 1996; Schwenk et al., 2000, 2004; Billiones et al., 2004; Hobæk et al., 2004) can validly be classified as *Daphnia lacustris* G.O. Sars, 1862. We also provide basic information on its morphological variation, ecology and distribution range.

Historical background

Georg Ossian Sars (1837–1927) first described the taxon *D. lacustris* in his 1861 thesis, and subsequently published a Latin diagnosis with comments in Norwegian (Sars, 1862). His thesis with its accompanying figures was finally published in 1993 in English translation (Sars, 1861/1993). While Sars modified his views on species delimitation in *Daphnia* during the following decades, he seems to have maintained *D. lacustris* as a distinct species throughout his career (Frey, 1982). This was also reflected in the major revision by Richard (1896), who followed Sars' opinions on the taxonomy of this group. Sars donated all his material to the scientific community, which is now available at the Zoological Museum at the University of Oslo and the Norwegian National Library in Oslo (Nordgaard, 1918; Frey, 1982; Christiansen, 1993). Examination of his posthumous artworks, notebooks, diaries and scientific material confirms Sars' persistence in maintaining *D. lacustris* as a separate species.

Around 1900, the only available illustrations which undoubtedly depict the taxon described by Sars were two outline drawings by Richard (1896) and Brady (1898). The species was difficult to distinguish from a series of varieties (including vars. *caudata* and *rectispina*) assigned to *D. longispina*. The lack of a clear diagnosis added to the general confusion concerning this group. Lilljeborg's (1901) erroneous lumping of *D. galeata* G.O. Sars, 1863 and *D. lacustris* into *D. hyalina* appears to have been a decisive event. Although Wesenberg-Lund (1904) (among others) expressed doubts whether *D. hyalina lacustris* really represented Sars' conception of the taxon, most scientists swiftly adopted this classification (e.g. C. Wesenberg-Lund, D.J. Scourfield, D.S. Johnson, D. Flößner, R. Šrámek-Hušek), or even more elaborate nomenclature involving the *lacustris* designation (T. Stingelin, G. Burckhardt, R. Woltereck, E. Wagler, K. Berger). Subsequently, it seems to have been gradually forgotten, surviving mainly as the designation for low-crested *D. hyalina* (and probably also round headed *D. galeata*) in British literature (Johnson, 1952; Scourfield & Harding, 1966; Christie, 1983; Fryer, 1985), and in some taxonomical literature (Šrámek-Hušek, 1962; Flössner, 1972).

Materials and methods

Historical material and museum collections

All relevant samples and data on the *D. longispina* group in the posthumous material of G.O. Sars, covering a period from 1860 to 1905, have been investigated in the Norwegian National Library and in the Zoological Museum of the University of Oslo. This includes artworks, notebooks, diaries and scientific material, such as tubes and slides. Further, we have examined most of the posthumous material classified as *D. longispina* by P.E. Müller, L. Lund, C. Wesenberg-Lund, K. Berg and U. Røen in the Zoological Museum at the University of Copenhagen.

Extant material

We examined extant Danish populations in the type region of *D. longispina* (O.F. Müller, 1776). We chose animals that closely resembled the general body shape of *D. longispina* as illustrated in Müller

(1785) and Müller (1868), as representing the original *D. longispina*. These were collected from a tarn called Midtre Kobberdam in Frederiksdal, adjacent to the domicile where Müller resided in the 1760s during his most active period in collecting freshwater cladocerans (Müller, 1868). By mitochondrial (12S sequence) and nuclear (ITS RFLP) markers, this population represents a species widespread in Europe (Petrusek et al., unpublished data), here designated *D. longispina*.

In Norway, we collected material in the lakes Maridalsvann and Sognsvann (Oslo, south-east Norway), from which the original description of *D. lacustris* was made (Sars 1861/1993; 1862). Animals conforming to this description are still present in both lakes. In addition, we have also included varieties that Sars (1890) ascribed to this species (i.e. *angustifrons*, *aquilina*, *alpina*) or to *D. longispina* (i.e. *caudata*), collected from lakes or regions where Sars himself applied these designations. Finally, we have established a preliminary overview of the geographic distribution of *D. lacustris* in Norway by applying genetic markers (see below) to about 90 populations of the *D. longispina* group from all over the country. So far, 23 of these populations could be identified as *D. lacustris* by molecular markers.

One population with genetic markers corresponding to *D. lacustris* is known from the lake Niżni Toporowy Staw in the Polish Tatra Mountains (Taylor et al., 1996). Animals sharing these markers have been designated as *D. longispina* sensu stricto in recent literature, as outlined above. We included animals from this locality in our analysis as well.

Molecular markers

DNA was extracted from ethanol-preserved individuals following a proteinase K protocol (Schwenk et al., 1998) or a Chelex protocol (Hobæk et al., 2004). Part of the mitochondrial 12S rRNA gene was amplified following standard methods (e.g. Schwenk et al., 2000), and subsequently purified and cycle sequenced on ABI automatic capillary sequencers (series 377, 3130 and 3700) using the Big Dye terminator sequencing kit (Applied Biosystems). For a few selected isolates from Lake Maridalsvann, sequences of the mitochondrial gene for cytochrome c oxidase subunit I (COI) were also obtained in a similar manner (protocol details in Schwenk et al., 2000). The 12S sequences were compared with those from Norwegian and Finnish populations available from previous studies (Schwenk et al. 2000, 2004), and a new sequence was obtained from the same Polish locality (Niżni Toporowy Staw) as analysed by Taylor et al. (1996). In addition to *D. lacustris*, we included representatives of four related *Daphnia* species as outgroups. All sequences used in this study with their GenBank accession numbers are listed in Table 1. The 12S sequences were aligned using ClustalW (Thompson et al., 1994), checked manually, trimmed to a fragment length available for all individuals (528–531 base pairs (bp), depending on species), and further analysed in MEGA version 3.1 (Kumar et al., 2004). Altogether, we analysed sequences representing 12 Norwegian, 11 Finnish (data from Schwenk et al., 2004) and 1 Polish population of *D. lacustris* (1–3 animals from each; only 1 sequence per population is shown except for Finnish locality Tsahkallampi A where two different haplotypes were detected).

The nuclear ribosomal internal transcribed spacer (ITS) region was amplified and subjected to restriction fragment analysis after digestion with endonucleases following the methods described by Billiones et al. (2004). For *D. lacustris*, digestion by *Mwo*I produced unique restriction patterns that permits its reliable diagnosis. ITS restriction profiles are available from ca. 150 individuals, representing 24 populations. In addition, an alternative endonuclease applied to the ITS fragment yielded unique restriction profiles for *D. lacustris* as well. Animals from the *D. lacustris* type population were included when testing the alternative protocol (see Skage et al. this volume for details).

Results

Genetic markers

The 12S mitochondrial marker clearly delimited *D. lacustris* (25 sequences) from other species of the *D. longispina* complex (Fig. 1), forming a very tight cluster. Within-species variation was very low, with Kimura 2-parameter sequence divergence (calculated in MEGA 3.1) ranging from 0 to 1.0%. A sequence from the isolated Polish population in the Tatra Mountains (Niżni Toporowy Staw) differed

Table 1 Sequences of the mitochondrial gene for 12S rRNA used in this study

Locality name	Country	GenBank Acc. No.	Sequence source
Aurland alpine pond	Norway	DQ337941	This study
Finse Alpine area	Norway	AF277279	Schwenk et al. (2000)
Gåvålivatn (var. *angustifrons*)	Norway	DQ864521	This study
Haukelandsvatn	Norway	DQ337942	This study
Maridalsvann (type locality)	Norway	DQ337943	This study
Langavatn	Norway	DQ337944	This study
Litl-Jonsvatn (var. *caudata*)	Norway	DQ864522	This study
Myrdalsvatn	Norway	DQ337945	This study
Sipletjønn	Norway	DQ337946	This study
Store Tryvann	Norway	DQ337947	This study
Ubergsvatn	Norway	DQ337948	This study
Væleren (var. *aquilina*)	Norway	DQ337949	This study
Hillalampi	Finland	DQ337950	Schwenk et al. (2004)
Kevo 16	Finland	DQ337951	Schwenk et al. (2004)
Laassavaara	Finland	DQ371482	Schwenk et al. (2004)
Löysinpä	Finland	DQ337952	Schwenk et al. (2004)
Muotkantaka	Finland	DQ337953	Schwenk et al. (2004)
Salmivaaralampi	Finland	DQ337954	Schwenk et al. (2004)
Sierkisvaara	Finland	DQ337955	Schwenk et al. (2004)
Siilasvuo	Finland	DQ337956	Schwenk et al. (2004)
Tielammikko	Finland	DQ337957	Schwenk et al. (2004)
Tsahkallampi A	Finland	DQ337958-9	Schwenk et al. (2004)
Tsahkallampi B	Finland	DQ337960	Schwenk et al. (2004)
Niżni Toporowy Staw	Poland	DQ337940	This study
Outgroups			
D. cucullata—Medlov pond	Czechia	AF277270	Schwenk et al. (2000)
D. galeata—Obiger See	Germany	AF277268	Schwenk et al. (2000)
"*D. umbra*"—Bjornesfjord	Norway	DQ864520	This study
D. longispina—Midtre Kobberdam	Denmark	DQ536400	This study

The type locality of *D. lacustris* (Lake Maridalsvann), and the different varieties (*angustifrons*, *caudata* and *aquilina*) of Sars (1890) are indicated

only by one or two point mutations from the most common Fennoscandian haplotypes. By contrast, the corresponding distance to sequences assigned to other species of the complex ranged between 12 and 18%, with "*D. umbra*" as the species closest to *D. lacustris* (Fig. 1).

Amplification of the nuclear ITS marker yielded products of variable lengths. In most cases, products of ca. 2,000 bp were obtained, but in some cases the products were shorter (ca. 1,500 bp). The long ITS products are by themselves diagnostic (Billiones et al., 2004), since all other members of the *D. longispina* group known so far yield shorter fragments (ca. 1,400 bp). Characteristic MwoI restriction patterns of *D. lacustris* populations are shown in Fig. 2. A pair of bands at 260–280 bp is always present in *D. lacustris*, as well as one or more bands between 600 – 1000 bp. These latter bands vary considerably in size, while the shorter pair is a constant feature in this species. Animals from Niżni Toporowy Staw showed the same ITS characteristics as Norwegian and Finnish populations (PCR product size, restriction fragment patterns).

Included in the above analyses were representatives of the most important varieties from Norwegian lakes designated by Sars: var. *aquilina* (Lake Væleren; the taxon was originally described as a separate species by

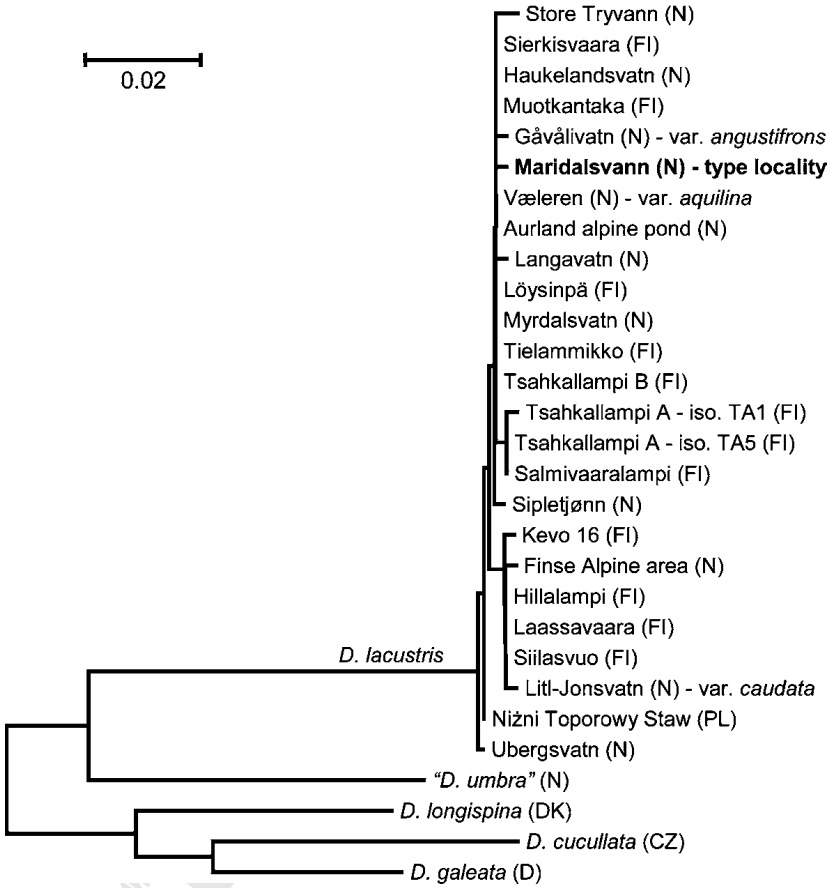

Fig. 1 Neighbour-joining tree representing sequence variation of a 528–531 bp long fragment of the mitochondrial 12S rRNA gene (Kimura 2-parameter distance) among all available *D. lacustris* sequences. Four additional species of the *D. longispina* group are represented in the tree by one individual each to show the phylogenetic topology within the group. The letters in parentheses indicate the countries of origin (N—Norway, FI—Finland, CZ—Czechia, PL—Poland, DK—Denmark, D—Germany). The type locality of *D. lacustris* (Lake Maridalsvann) is in bold font, and the varieties designated by Sars (*angustifrons*, *caudata*, *aquilina*) are indicated. Sequence information is given in Table 1

Sars (1863)), var. *angustifrons* (Lake Gåvålivatn: G.O. Sars, unpublished illustrations and notes). *D. longispina* var. *caudata* is represented by the Lake Litl-Jonsvatn population, from the same micro region (Trondheim) where it was first described as a separate species by Sars (1863). Using both 12S and ITS molecular markers, the above varieties were indistinguishable from *D. lacustris*. Finally, Sars' *D. lacustris* var. *alpina* (>30 localities: Hobæk & Skage, unpublished data) is identical with *"D. umbra"*, which itself requires a nomenclatural revision (see Benzie, 2005).

Taxonomy

Daphnia lacustris G.O. Sars, 1862, p. 267.

The species was described by Sars (1862) in Latin and Norwegian without drawings, whereas illustrations were first presented by Richard (1896), based on material sent by Sars himself to Richard (Sars, unpubl. data, National Library Manuscript Department, Ms Fol. 1109, Item 467: Diaries). The original drawings by Sars were finally published in 1993 (Sars, 1861/1993: Plate 35), which also includes an English translation of the Norwegian description. Note that the valid diagnosis is the 1862 version, which was slightly amended from the 1861 thesis version.

In order to facilitate DNA-based identification of *D. lacustris*, a COI sequence from specimens originating from the type locality was deposited in the Barcoding of Life database (www.barcodinglife.org) under the Barcode ID DAPWP001-06 and in GenBank (accession number DQ871251).

Synonyms:

D. lacustris: Sars, 1890, Richard, 1896, Huitfeldt-Kaas, 1906;
D. aquilina: Sars, 1863, *non D. aquilina*: Sars, 1864;
D. caudata: Sars, 1863, Wierzejski 1882;
D. caudata (*?in partim*): Daday, 1897;
D. lacustris var. *lacustris*, var. *angustifrons*, var. *aquilina*: Sars, 1890;
non D. lacustris var. *alpina*: Sars, 1890;
D. longispina var. *caudata*: Sars, 1890, Minkiewicz, 1911, Wagler, 1937;

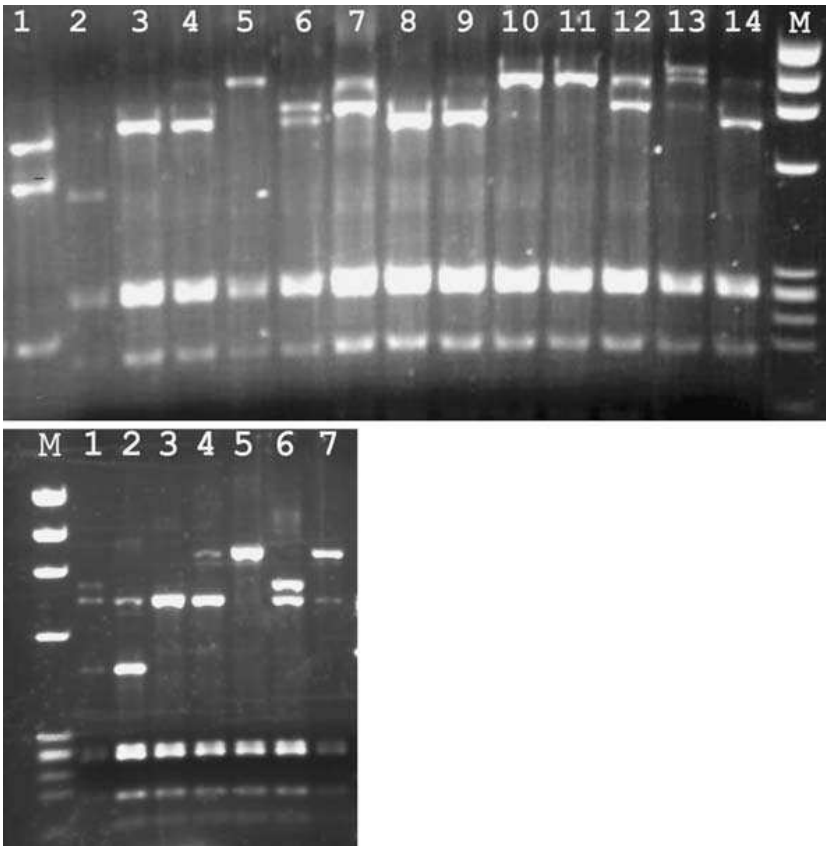

Fig. 2 Fragment separation following digestion by *Mwo*I of ITS PCR products on agarose gels. Lanes are labelled as follows: Left panel: M—standard size marker. 1—*D. longispina* (Midtre Kobberdam, Denmark), 2–14—*D. lacustris*: 2–3: Lake Sognsvann; 4–6: Lake Maridalsvann (type locality); 7–9: Lake Store Tryvann; 10–12: Lake Skjennungen; 13–14: Lake Væleren. Right panel: M—standard size marker; 1–3—Lake Sognsvann; 4–7: Lake Maridalsvann. All *D. lacustris* on these gels are from lakes in Eastern Norway, where we have found the greatest variation in ITS product lengths and MwoI restriction patterns. The size marker was ΦX174 DNA digested with *Hae*III (Promega), resulting in fragment lengths (top to bottom) of: 1353, 1078, 872, 603, 320, 281 + 271, 234, 194 and 118 bp

D. longispina var. *rectispina* (*?in partim*): Sars, 1890;
D. variabilis var. *caudata-cavifrons*: Lityński, 1913;
non D. lacustris: Scott, 1899, Gurney, 1923;
non D. hyalina lacustris: Lilljeborg, 1901, Wesenberg-Lund, 1904, 1926, Scourfield & Harding, 1941, 1966, Johnson, 1952, Šrámek-Hušek, 1962, Flössner, 1972, Christie, 1983, Fryer, 1985;
D. longispina: Taylor et al., 1996, Flössner, 2000 (in partim), Schwenk et al., 2000, 2004, Billiones et al., 2004, Hobæk et al., 2004, Taylor et al., 2005.

Type locality

Sars (1862) originally recorded *D. lacustris* from both Lake Maridalsvann and the neighbouring Lake Sognsvann. We selected Lake Maridalsvann (59.986°N, 10.777°E; A_0 = 3.9 km^2, Z_{max} = 46 m) as the type locality. The lake is oligotrophic and almost undisturbed (NIVA, 1961), being protected as a drinking water reservoir for the city of Oslo. It has relatively intense fish predation (Brabrand & Saltveit, 1983), especially in the epilimnion, by resident planktivorous fishes, such as roach (*Rutilus rutilus* L.), smelt (*Osmerus eperlanus* L.), and Eurasian perch (*Perca fluviatilis* L.). *D. lacustris* is not a dominant species in the lake (Brabrand & Saltveit, 1983; Nilssen, unpubl. data), as observed also by Sars in the 19th century (Sars, unpubl. data, National Library Manuscript Department, Ms Fol. 1109, Item 468: Notebooks).

Neotype designation

G.O. Sars preferred to make his drawings from live specimens and then paint them to capture their live

appearance. The specimens used seem not to have been saved; in such cases his unpublished drawings and paintings are the closest one can get to a holotype. We have found no trace of samples or slides of *D. lacustris* dating from the 1860s in the archives. We designate a neotype from slide F8642 in the G.O. Sars collection, deposited at the Zoological Museum, University of Oslo (Fig. 4). The slide was made and labelled by Sars around 1885 from animals collected in Lake Maridalsvann. The selected specimen shows the typical body shape of the Maridalsvann population.

Morphology and intraspecific variation

Sars (1890) demoted many of his previously described species to varieties. The 1890 list includes three morphological varieties of *D. lacustris: aquilina*, *angustifrons* and *alpina.* The former two clearly belong within *D. lacustris*, as verified by molecular markers, while the third (var. *alpina*) represents a distinct species (referred to as "*D. umbra"*, see Benzie, 2005). Further, Sars' (1890) list includes a variety *caudata* ascribed to *D. longispina* (briefly described as a separate species by Sars (1863). All animals of this morphotype analysed so far (including typical animals from the region Sars originally described it) clearly belong within *D. lacustris* by the 12S and ITS markers. Sars' concepts of two of the *caudata* and *angustifrons* morphotypes are shown in Fig. 5, together with an individual of the typical form. Hybrids between *D. galeata* and *D. lacustris* are known occasionally from Western Norway (Hobæk et al., 2004), but no other interspecific hybrids involving *D. lacustris* have been detected until present.

The typical morph from Lake Maridalsvann (Figs. 3, 4) is characterised by the shape of the head, the morphology of adult males, and the large head of juveniles compared with the rest of the body. The morphotype *caudata* (Fig. 5) is characterised by its extremely prolonged caudal spine (Richard, 1896, Sars, 1903), and the *angustifrons* morphotype by its shortened rostrum, reminiscent of the genus *Simocephalus* (Fig. 5). *D. lacustris* var. *aquilina* is characterised by a fairly long and beak-like rostrum (Sars, 1863). Intermediates between morphotypes are common in the *D. longispina* group, however, and some *D. lacustris* populations are at present almost

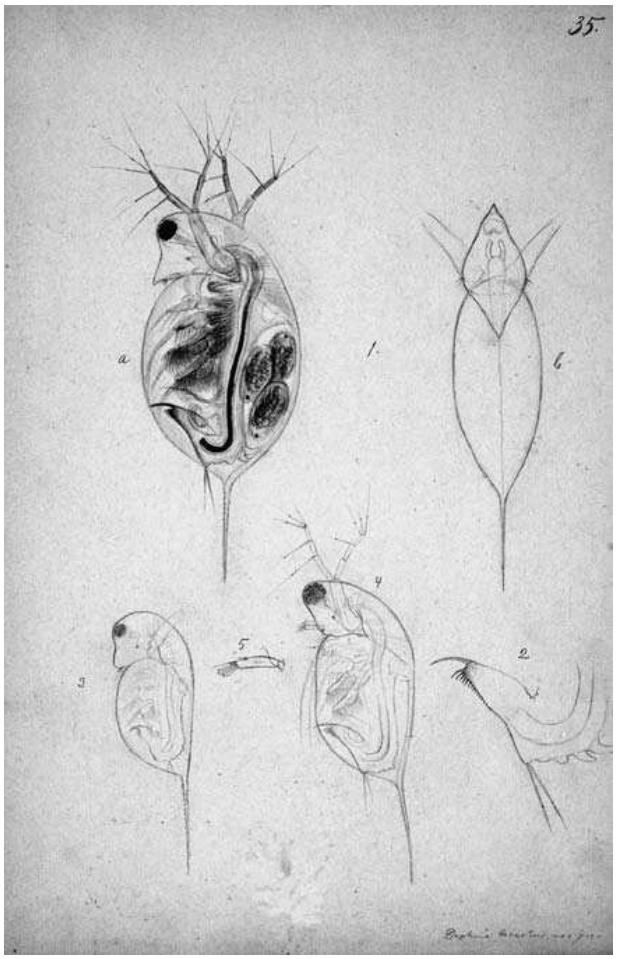

Fig. 3 Original illustration of *Daphnia lacustris* G.O. Sars, 1862, from Sars 1861/1993, with original plate numbering. (**a**, **b**) Adult parthenogenetic female; 2: Postabdomen of adult parthenogenetic female; 3: Juvenile female; 4: Adult male; 5: First pair of adult male antennae. Scanned from original in Ms-Fol. 1109 Item 279 in the G.O. Sars collection, deposited at the Norwegian National Library, Oslo

impossible to distinguish from *D. longispina* to lowland ponds and lakes with relaxed fish predation (Tveten & Hobæk, 2002; Hobæk & Nilssen, unpubl. data).

Alpine, fishless pond populations of *D. lacustris* often harbour large (>2.5 mm) and plump individuals, which differ markedly from the type morph (Hobæk & Nilssen, unpubl. data). They usually have relatively smaller heads than the typical form, and may be very similar to *D. rosea*, as described by Flössner (2000). They are also very similar in habitus to what Sars (1890) designated, as *D. longispina* var. *rectispina*, which is why we have tentatively included this designation among the synonyms.

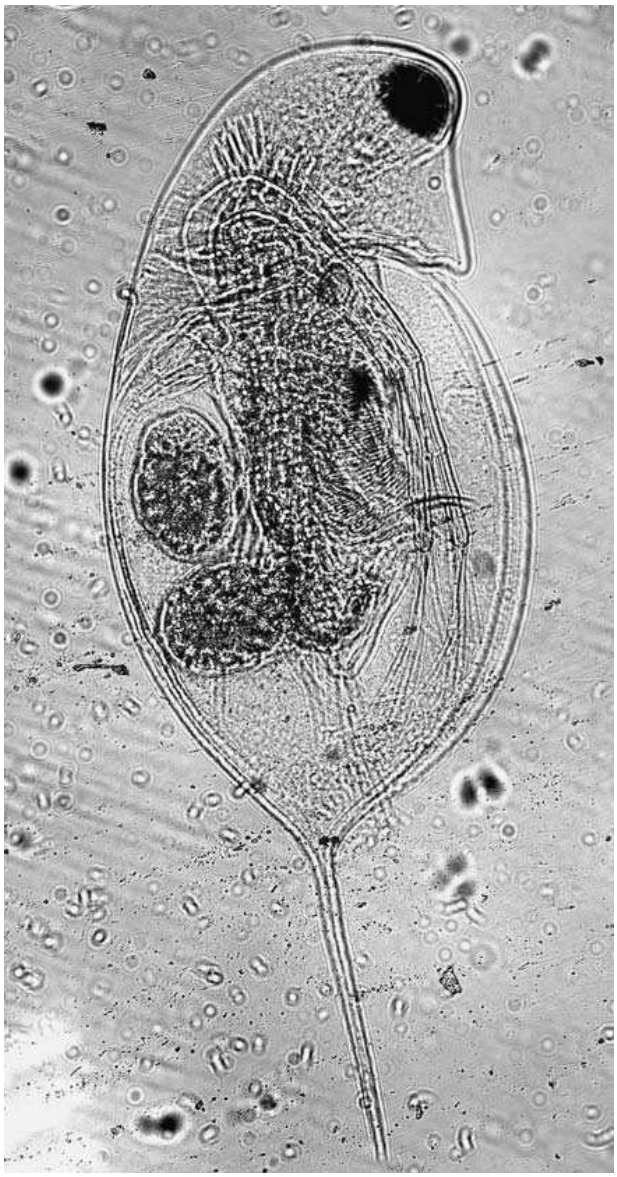

Fig. 4 Designated neotype of *D. lacustris* G.O. Sars, 1862 from Lake Maridalsvann. From slide by G.O. Sars (item F 8642 in the G.O. Sars collection, deposited at the Zoological Museum, University of Oslo)

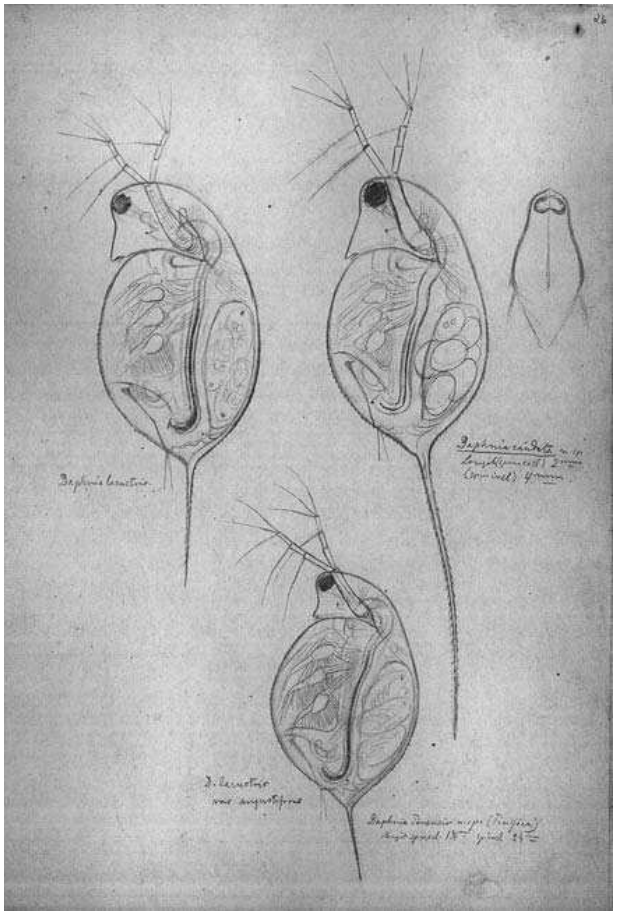

Fig. 5 Most common Norwegian varieties of *D. lacustris*: var. *caudata* (top right) and var. *angustifrons* (bottom) in Sars' outline drawings together with a typical specimen from the type locality (top left). Scanned from unpublished original in Ms-Fol. 1109 Item 279 in the G.O. Sars collection, deposited at the Norwegian National Library, Oslo

Distribution and ecology

Genetic analyses from many additional populations confirm the presence of *D. lacustris* in virtually all parts of Norway (Fig. 6), making it one of the most widespread *Daphnia* species in the country. It is also known from Northern Finland, and one lake in the Polish Tatra Mountains. Although our knowledge is incomplete, the species appears to be absent from most or all of lowland Europe, and thus has a northern distribution.

D. lacustris is typically found in oligotrophic lakes and tarns with relaxed fish predation. However, it may occur in lakes with fairly intense predation if a depth refuge is present (as exemplified by the type locality). It is primarily a boreal species, but found close to the coastline in areas where brown trout (*Salmo trutta* L.) is the sole fish species present. It is also known from alpine environments, and has been recorded up to 1,320 m a.s.l. in Norway (Hobæk et al., unpubl.). At altitudes above 1,000 m a.s.l. in southern Norway, it occurs only in shallow and slightly humic ponds. In the alpine zone it often alternates in microgeographical distribution with the melanic taxon "*D. umbra*" (which dominates in clear-water lakes and ponds) but their syntopic

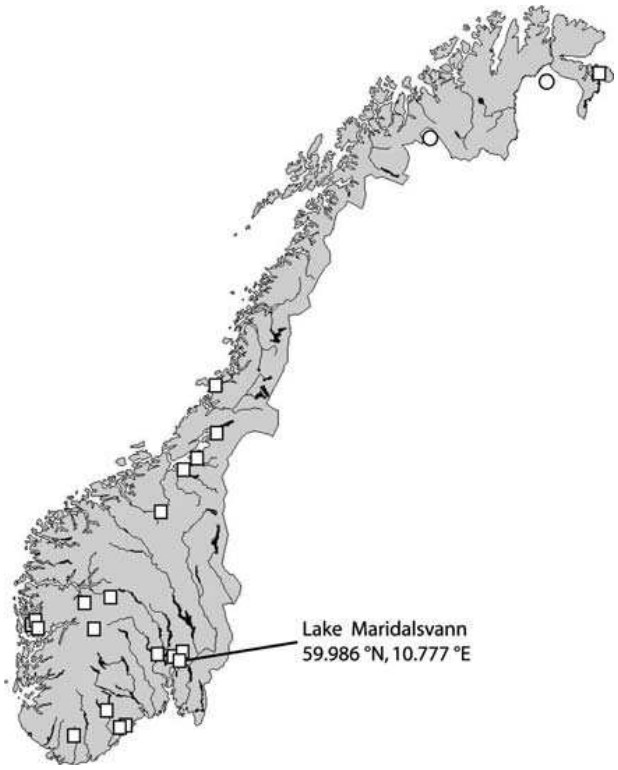

Fig. 6 The geographical distribution of *D. lacustris* populations as confirmed by molecular markers in Norway (open squares). Approximate locations of confirmed Finnish records (after Schwenk et al. 2004) are given by open circles. The type locality Lake Maridalsvann is indicated. Outside this range, one population is known in the Polish Tatra mountains (see text)

co-occurrence is highly unusual (Hobæk & Wolf, 1991; Schwenk et al., 2004).

Discussion

Delimitation of *D. lacustris* and affiliation within the *D. longispina* group

Both mitochondrial and nuclear markers unequivocally confirmed the identity of *D. lacustris* with *D. longispina* sensu stricto as this designation has been recently applied (Taylor et al., 1996, 2005; Schwenk et al., 2000, 2004; Billiones et al., 2004; Hobæk et al., 2004). Furthermore, the genetic markers strongly support *D. lacustris* as a coherent species well delimited from other species of the *D. longispina* group. The topology given in Fig. 1 is also in agreement with recent phylogenetic analyses (Schwenk et al. 2000, 2004). The available evidence indicates that "*D. umbra*" and *D. lacustris* are sister species, constituting a northern clade of the *D. longipspina* group.

It seems very unlikely that any description predating 1862 can be referred to the present taxon with certainty, especially considering its northern distribution. *D. longispina* (O.F. Müller, 1776) was originally described from Denmark, i.e. outside of the presently known distribution of *D. lacustris*, and the original Müllerian name must be reserved for the species represented by this Danish lineage (Petrusek et al., unpubl. data).

On the nuclear ribosomal ITS marker, *D. lacustris* populations and individuals show a remarkable variability in total fragment length (PCR product), as well as in fragment sizes resulting from restriction digestions. These patterns are caused by the occurrence of several large indels, which seem to be unique for *D. lacustris* (Skage et al., this volume; Skage & Hobæk, unpublished sequence data; Taylor et al., 2005).

The first genetic analyses to trace the existence of this species were by Wolf & Hobæk (1986) and Hobæk & Wolf (1991), who pointed out its distinctness from a melanic lineage (later ascribed to "*D. umbra*") found in the same alpine area, and also from a third, lowland lineage (*D. longispina* morphotype *rosea* in the present taxonomy). Although the authors noted that the different genetic lineages likely represented distinct species, the data (based on allozyme polymorphism) were not considered sufficient to justify such a conclusion. In a subsequent analysis of the North American *D. longispina* group, Taylor et al. (1996) selected an isolate from the Polish Tatra Mountains as a representative of the European *D. longispina*. However, this population (Niżni Toporowy Staw) is in fact the only known occurrence of *D. lacustris* outside Fennoscandia. This remarkable coincidence led to the designation *D. longispina* being used for *D. lacustris* genotypes also by European researchers, who recognised its distinction from the more widespread species designated as *D. rosea* (Gießler et al., 1999; Schwenk et al., 2000, 2004; Billiones et al., 2004; Hobæk et al., 2004).

The new sequence from the lake Niżni Toporowy Staw in Poland differed significantly from the

sequence obtained from this population by Taylor et al. (1996; GenBank accession number U34738), which has also been used in subsequent studies (Schwenk et al., 2000, 2004; Taylor et al., 2005). The nature of this difference (unique point mutations and a deletion in the terminal part of the latter sequence) suggests potential sequencing artefacts in the former study. The mitochondrial variation among *D. lacustris* populations may therefore be even lower and show less geographical structure than indicated by Schwenk et al. (2004).

Taxonomy and nomenclature

By far, the most serious taxonomical and nomenclatural problems derive from the inclusion of *D. lacustris* in the taxon *D. hyalina* Leydig, 1860 (e.g. Flößner & Kraus, 1986), as the subspecies *D. hyalina lacustris* (Lilljeborg, 1901; Wesenberg-Lund, 1904, 1926; Scourfield & Harding, 1941, 1966; Johnson, 1952; Flößner, 1972). Animals so designated can most likely be assigned to *D. galeata* and/or some of its frequent interspecific hybrids. We suggest this may be the case also for populations designated as *D. hyalina lacustris* in the UK, as well as several Danish populations (Wesenberg-Lund, 1904, 1926; Scourfield & Harding, 1941, 1966; Johnson, 1952), and possibly also certain Swedish populations (Lilljeborg, 1901). Further studies are needed to clarify the real affinity of populations ascribed to *D. hyalina lacustris* in the past, and the designation certainly should not be used in the future.

Another unresolved issue concerns the designation *D. longispina* var. *caudata*, used by several taxonomists (e.g. Sars, 1890; Richard, 1896; Rylov, 1935; Herbst, 1962). All populations of this morphotype available to our genetic analysis (including Niżni Toporowy Staw, PL) were assigned to *D. lacustris*. However, it remains to be seen, whether this results holds for other regions, where this name has been applied (e.g. Russia). Further, Sars (1890) applied the designation *D. longispina* var. *rectispina* Krøyer, 1838 to certain Norwegian alpine populations. We consider it likely that those populations belong within *D. lacustris* (being quite similar in habitus to alpine pond populations that we have studied previously (Hobæk & Wolf, 1991)), but this assertion needs to be verified by molecular markers.

Distribution and ecology

Due to historical taxonomical problems, little information has accumulated concerning the distribution and ecology of *D. lacustris*. Until now, populations of this species were pooled with other taxa under the label *D. longispina*. This was also true in Norway (Aagaard & Dolmen, 1996). The following paragraphs are mainly based on our experience with populations verified by genetic markers, as well as some additional occurrences which conform morphologically to the known morphotypes (*aquilina*, *angustifrons*, *caudata*).

In Norway, the available data suggest that *D. lacustris* is quite common across most of the country (Fig. 6). Schwenk et al. (2004) confirmed its presence in northern Finland. Although no verified records are available from Sweden, we consider it likely that *D. lacustris* occurs at least in the northern parts of the country. This may also apply to north-western Russia. In general, we expect its occurrence also in these regions to be largely controlled by fish predation (see below). The occurrence of *D. lacustris* in the Polish Tatra Mountains is of particular interest, suggesting that it may potentially be more widely distributed in the alpine ranges of Central Europe. Reports of animals with a general body shape and shell spine reminiscent of the *caudata* or *rectispina* morphs do occur in the literature (e.g. Stingelin 1910), suggesting that renewed biogeographic efforts in these mountain ranges may be warranted. So far, however, all populations of this group that we have examined from the alpine ranges belong to *D. longispina* (*D. rosea* morph).

Daphnia lacustris co-occurs with *D. longispina* (*rosea* morph) in several Norwegian lakes, but only occasionally with "*D. umbra*" (Hobæk & Wolf, 1991). No hybrids between these species have been detected. The only known hybrid involving *D. lacustris* is with *D. galeata* (Hobæk et al., 2004).

Daphnia lacustris is rarely encountered during winter, in contrast to the related species "*D. umbra*" and *D. longispina*, which may both persist under ice-cover through the winter (Nilssen, unpubl. data). *D. lacustris* is a preferred prey for planktivorous fish (Brabrand & Saltveit, 1983; Nøst & Langeland, 1994). It is, therefore, rare or absent in lakes harbouring efficient fish predators (Brabrand & Saltveit, 1983; Løvik, 1984; Nøst & Langeland,

1994), or is mainly recorded at considerable depths below which fish do not regularly hunt (Løvik, 1984). *D. lacustris* occurs sympatrically (i.e. in the same region, although not necessarily in the same habitats) with most *Daphnia* species in Norway, but tends to be dominant mainly in inland boreal and alpine humic sites. With increased eutrophication it is replaced by *D. longispina* (*D. rosea* morph), and where there is intensive fish predation the species *D. longispina* (*D. hyalina* morph), *D. galeata*, *D. cristata* G.O. Sars, 1861, *D. longiremis* G.O. Sars, 1861 and *D. cucullata* G.O. Sars, 1862 seem to be much more successful.

As outlined above, several morphotypes of *D. lacustris* are known (Fig. 5), and several morphs may co-occur, as illustrated by Sars several times in his unpublished artwork (Sars, unpubl. data, National Library Manuscript Department, Ms Fol. 1109: Items 258, 294). No geographical patterns in the occurrence of these morphotypes can be discerned, which suggests that they represent phenotypic plasticity related to invertebrate and fish predation (Tollrian & Harvell, 1999) rather than intraspecific differentiation. *D. lacustris* does not seem to possess "neck-teeth" in juveniles and small males (cf. Wagler, 1913), which are so frequently observed in *D. longispina* (*D. rosea*) exposed to invertebrate predation (Nilssen, unpubl. data). The elongated caudal spine (var. *caudata*) may serve the same purpose (cf. Balseiro & Vega, 1994; Lysebo, 1995), as suggested by the predominance of long-spined morphs associated with high abundance of invertebrate predators such as *Chaoborus* larvae. In contrast to Norwegian populations of "*D. umbra*", we have not observed animals with deeply melanic head shield and carapace, although a faint touch of pigment may be present along the fornices, carapace edges and antennae of alpine animals. However, Finnish populations of *D. lacustris* are reported to possess melanin (Schwenk et al., 2004).

Concluding remarks

We maintain that molecular markers presently provide the only feasible avenue to a sound and stable taxonomy in this problematic group, after at least 150 years of failing to reach this goal via morphological analysis. By re-establishing the species *D. lacustris* G.O. Sars, 1862, designating a type locality, and providing the tools to identify the species, we hope to have paved the way for a formal redescription of the species and its morphological variation. A similar procedure for other problematic species of the *D. longispina* group (particularly *D. longispina* and "*D. umbra*") should facilitate taxonomic consensus and stable nomenclature for this group, thereby allowing comparative analyses of morphological variation, ecological relations and evolutionary adaptations to be made with an improved level of confidence.

Acknowledgements This work constitutes part of a collaborate effort to elucidate species delimitation and nomenclature of the European *D. longispina* group, which has involved an open exchange of samples and data as well as joint analyses. A number of persons and institutions have unselfishly supported this initiative in various ways. A sample from the Polish locality was obtained from Martin Černý, and 12S sequences of Finnish populations from Klaus Schwenk. During a stay at the Zoological Museum in Copenhagen, JPN was especially supported by Jørgen Olesen. At the Zoological Museum in Oslo we were kindly supported by Marit E. Christiansen, who also shared her great knowledge on Georg Ossian Sars with us, and by Åse Wilhelmsen at the G.O. Sars' collection. We are grateful to Svein Birger Wærvågen, Wenche Emely Nessler, Dag Klaveness, Jan Ivar Koksvik and Jørn Enerud for field assistance and/or discussions, and to the staff at the Norwegian National Library, Oslo, for their help with the Sars collection deposited there. JPN acknowledges economical support for biogeographical studies on Norwegian zooplankton from the counties of Aust-Agder, Oslo and Akershus, Buskerud, Telemark, Vestfold and Oppland. AH was supported by the Norwegian Research Council (Grant 121181/720) and by internal funding from the Norwegian Institute for Water Research. AP was supported by the Czech Ministry of Education (project MSM0021620828). His work has been partially carried out during stays at Johann Wolfgang Goethe Universität in Frankfurt am Main (financed by the German Academic Exchange Service DAAD) and Katholieke Universiteit in Leuven (financed by Czech-Flemish bilateral cooperation in research and development). The support by Klaus Schwenk, Nora Brede, Luc De Meester and Joachim Mergeay during these stays is gratefully acknowledged. We thank three anonymous referees, whose comments helped improve the article. Finally, thanks are due to Lawrence Kirkendall for linguistic corrections.

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CHAPTER 4

Petrusek A., Černý M., Mergeay J., Schwenk K.:

Daphnia in the Tatra Mountain lakes: multiple colonisation and hidden diversity revealed by molecular markers

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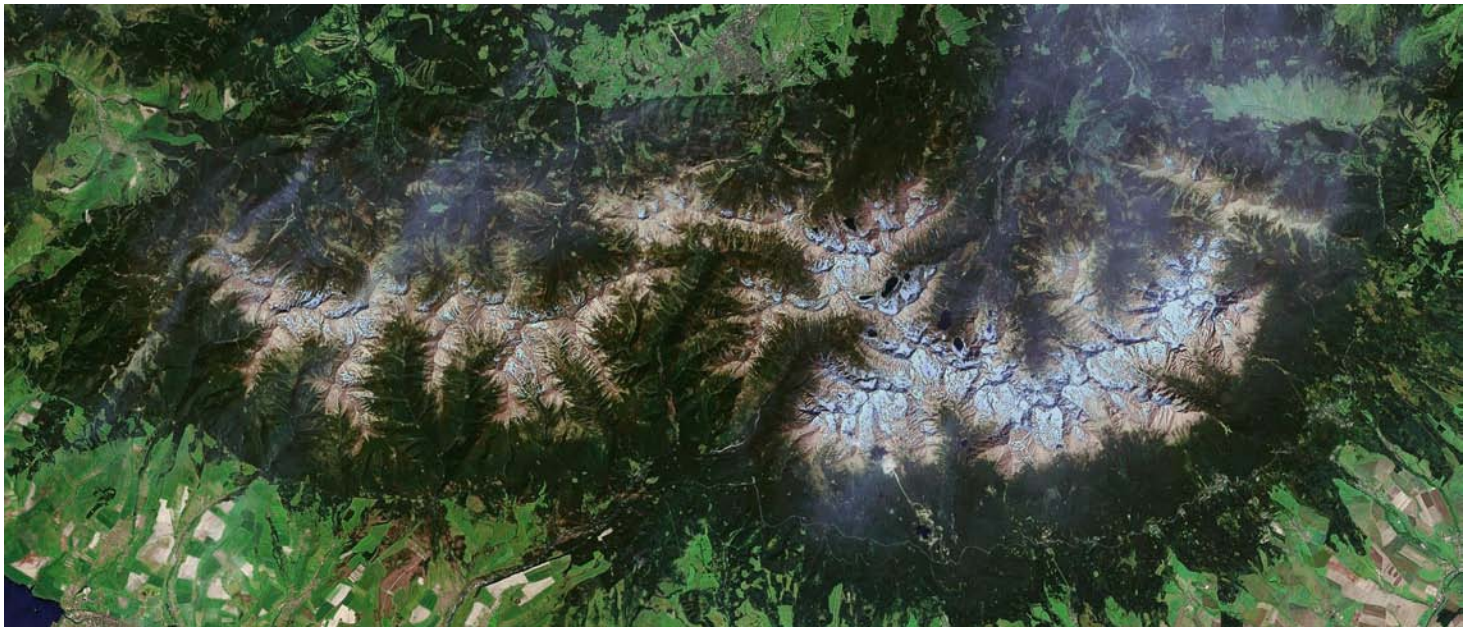

Landsat 7 satellite image of the Tatra Mountain range – the stage for this story.

Daphnia in the Tatra Mountain lakes: multiple colonisation and hidden species diversity revealed by molecular markers

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Abstract

Ecosystems of European mountain lakes may harbour relict populations of boreal aquatic species, including cryptic lineages not easily recognised using traditional taxonomic methods. As a previous genetic study revealed the presence of the cryptic cladoceran species *Daphnia lacustris* in the area, we explored the species diversity of the *Daphnia longispina* group (Crustacea: Cladocera: Anomopoda) in lakes of the Tatra Mountains (Central Europe: Slovakia – Poland). *Daphnia* populations representing various morphotypes from sixteen mountain lakes were analyzed by DNA methods, including restriction fragment length polymorphism of the nuclear ribosomal internal transcribed spacer region (ITS-RFLP) and sequencing of the mitochondrial 12S rDNA gene. Altogether, three *Daphnia* species of the *D. longispina* group were found in the region: *D. longispina*, *D. galeata*, and *D. lacustris*; we detected neither their syntopic occurrence nor interspecific hybrids. *D. lacustris* was found in two neighbouring lakes in the Polish High Tatras (Niżni Toporowy Staw and Wyżni Toporowy Staw); these may represent relict populations, since the closest known extant populations of this species are found in Fennoscandia. Morphologically highly variable populations of *D. longispina* formed the majority (69%) of the analysed populations. Relatively high divergence of 12S rDNA haplotypes from various lakes suggests multiple colonisations of the Tatra Mountain region by this species. Similarly, each of the three recorded *D. galeata* populations is probably of different origin. In addition, we found that the species replacement in one lake, from either *D. lacustris* or *D. longispina* to *D. galeata*, was associated with anthropogenically mediated environmental changes (fish stock increase, eutrophication).

Key words: alpine lakes, *Daphnia longispina* complex, cryptic species, monopolisation hypothesis, haplotype variation

Running title: *Daphnia* in the Tatra Mountains

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Introduction

Quaternary "ice ages" strongly influenced the distribution of European biota. Numerous studies have focused on the patterns of recolonisation of various temperate species after glaciation periods as well as on the genetic structure of these species (see e.g., reviews of HEWITT 1999, 2004a, b). European mountain ranges not only affected the migration of organisms to and from southern refugia (HEWITT 1999), but may also have provided suitable environments for a number of cold-adapted species during warmer periods, including the Holocene. Not surprisingly, many European plant and animal species, both terrestrial and aquatic, have a boreo-alpine distribution. Postglacial colonisation of glacial lakes by passively dispersed species could have different sources. For example, a number of low-altitude Scandinavian lakes are inhabited by various invertebrate species which are thought to have dispersed primarily along periglacial regions during the retreat of glaciers (SÄRKKÄ et al. 1990). On the other hand, species from alpine lakes are widely distributed, and their occurrence in continental highlands may be explained by numerous scenarios, such as colonisation from local refugia or long-range dispersal from more distant refugia, other mountain ranges or even, much more recently, from subarctic regions.

Among zooplankton, the phylogeography of circumarctic members of the cladoceran *Daphnia pulex* species complex (Crustacea: Anomopoda) in particular has been thoroughly analysed (WEIDER et al. 1999a, b; HOBÆK & WEIDER 1999). These studies have revealed the presence of several clades with different periglacial refugia and complex recolonisation patterns of formerly glaciated regions. Much less is known, however, about the recent history of other planktonic species present in the European Arctic, such as species of the *Daphnia longispina* complex, which are commonly found across the Palaearctic region. The lack of reliable diagnostic traits for some species, extensive phenotypic plasticity, the presence of cryptic species, as well as frequent interspecific hybridization, are responsible for many taxonomic problems within this clade. The application of genetic methods among populations identified as *"D. longispina"* in Europe revealed the presence of two additional species (*D. lacustris* and *D. "umbra"*) which show a distinct boreal distribution, with most localities known from Fennoscandia (SCHWENK et al. 2004, NILSSEN et al. 2007). In addition, a third cryptic lineage of this group was recently discovered in Norway (PETRUSEK et al., unpublished data; SKAGE et al. 2007). Given these findings, we may speculate that Central European mountain lakes could also harbour cryptic lineages with links to boreal fauna. Such an assumption is additionally supported by observations of unusually high morphological variation of the *Daphnia longispina* group in various mountainous regions (e.g., BURCKHARDT 1899, STINGELIN 1910, LITYŃSKI 1913, PLJAKIC 1961). The Tatra Mountains on the Slovak-Polish border, which harbour a variety of boreo-alpine plant and animal species including aquatic taxa, provide a good opportunity to explore this phenomenon.

The morphological variability of populations of the *Daphnia longispina* group from lakes in the Tatra Mountains already attracted the attention of early authors studying the regional zooplankton. Both LITYŃSKI (1913, 1917) and MINKIEWICZ (1914, 1917) noted the high morphological variation among local populations, and assigned the animals to four or five different varieties and forms of "*Daphnia variabilis*". Among the most varying morphological characters (see Figures 2 and 4) are the relative head size (ranging from unusually low-headed morphs to helmeted ones), head shape (rounded, skewed forward, with a strongly concave frontal margin, etc.), relative body width and spine length (ranging from <10% to over 95% of carapace length). As it became clear that the morphological variation in *Daphnia* species was often due to phenotypic plasticity, such detailed classification of *Daphnia* morphs was no longer applied later in the 20th century (see HRBÁČEK 1987),

and all Tatra populations were identified as either two species (*D. longispina* and *D. rosea*) or just one (*D. longispina*; HRBÁČEK et al. 1974, KNESLOVÁ et al. 1997, HOŘICKÁ et al. 2006).

What lies behind the morphological variation observed among Tatra Mountain *Daphnia* populations? Part of it may be explained by the coexistence of several unrecognised species. A phylogenetic study (TAYLOR et al. 1996) incorporating specimens from two High Tatra localities as European representatives of the morphotypes "*D. longispina*" (Niżni Toporowy Staw) and "*D. rosea*" (Nižné Furkotské Lake) confirmed their genetic distinctness. Only a subsequent comparison with other European populations showed that the presumed "*Daphnia longispina*" from the Polish lake Niżni Toporowy Staw (TAYLOR et al. 1996) belongs to a cryptic species with otherwise exclusively boreal distribution, *Daphnia lacustris* Sars, 1862 (NILSSEN et al. 2007); the original classification of the Tatra populations influenced several subsequent publications, which have mislabelled this taxon as *D. longispina* (see NILSSEN et al. 2007). So far, *D. lacustris* has been known only from lakes in Norway, Finland, and a single Tatra lake (SCHWENK et al. 2004, NILSSEN et al. 2007); the distribution of this species in the Tatra Mountains is therefore interesting from both a biogeographic and conservational point of view. Apart from the above-mentioned study (TAYLOR et al. 1996), however, no data on the genetic composition of Tatra Mountain populations of the *D. longispina* complex were available. Recently, another study incorporated samples from a few additional Tatra populations (ISHIDA & TAYLOR 2007), though without any particular focus on this region.

Part of the morphological variation of Tatra Mountain *Daphnia* may have been caused also by interspecific hybridization between the resident taxa. Hybridization among Central European species of the complex is widespread (SCHWENK & SPAAK 1995, SCHWENK et al. 1998), and even genetically distant *D. lacustris* is known to hybridise with *D. galeata* (HOBÆK et al. 2004). The hybrid genotypes are often phenotypically intermediate between the parental taxa (FLÖSSNER 2000, SCHWENK et al. 2001, HOBÆK et al. 2004), increasing the overall morphological variability of *Daphnia* populations. Lastly, the intraspecific genetic variation among populations (e.g., due to local adaptation) or phenotypic plasticity in response to environmental factors that differ among the lakes, could contribute to the among-lake morphological variation of *Daphnia*.

In this study we wanted to explore the sources of variation of the Tatra Mountain populations of the *Daphnia longispina* complex, namely to 1) unambiguously identify the species present in the area by genetic methods, 2) check for the potential presence of hybrid genotypes, 3) assess biogeographical patterns, and 4) evaluate the distribution and threats for the relict taxon *D. lacustris*.

Methods

Study sites

The Tatra Mountains, located along the border between Slovakia and Poland (20° 05' E, 49° 10' N; Figure 1) and reaching an elevation of 2655 m above sea level (a.s.l.), are the highest part of the Carpathian mountain range. The Tatra Mountains harbour over 250 lakes of glacial origin of various sizes (138 large lakes, i.e., perennial and over 0.01 ha in area, and over 120 smaller and/or temporary ones), ranging in altitude between 973 and 2189 m a.s.l. (KOPÁČEK et al. 2000, GREGOR & PACL 2005). The majority of Tatra lakes (c. 70%) are located in the alpine region above 1800 m a.s.l. (KOPÁČEK et al. 2000, KOPÁČEK et al. 2006). About 15% of the lakes are located in the western part of the mountain range, called the West Tatras, all remaining are situated in the central part of the range, the High Tatras. Probably all of them, including those at the lowest elevations, were

covered by glaciers during the last (Weichselian) glaciation (LINDNER et al. 2003).

Many lakes in the region have been severely impacted by anthropogenic acidification in the second half of the 20th century, accompanied by the subsequent loss of a number of zooplankton species (FOTT et al. 1994, SACHEROVÁ et al. 2006, HOŘICKÁ et al. 2006). Recovery from acidification in the 1990s (KOPÁČEK et al. 1998) was followed by the slow return of littoral planktonic species (SACHEROVÁ et al. 2006) and also of daphnids to some lakes.

Populations of the *Daphnia longispina* group have historically been recorded from at least 28 Tatra Mountain lakes: 7 in the West Tatras and the rest in the High Tatras (HOŘICKÁ et al. 2006 and unpublished data). In some of them, however, daphnids became extinct during the acidification period (HOŘICKÁ et al. 2006), or occurred only occasionally and in very low densities. We analyzed *Daphnia* samples from 15 Tatra Mountain lakes (four located in the West Tatras and 11 in the High Tatras) and from an additional lake located in the neighbouring Spišská Magura range (Figure 1, Table 1). The selected Tatra lakes include most localities where populations of this group occur regularly, and cover a wide altitudinal gradient (1089-1894 m a.s.l.). Moreover, analysed populations adequately represent the local morphological diversity of the *D. longispina* group. The list of localities, including their basic morphometric data and additional information about environmental conditions, is given in Table 1; more details can be found elsewhere (PARYSKA & PARYSKI 2004, GREGOR & PACL 2005, KOPÁČEK et al. 2006). The single lake known to harbour a population of *Daphnia lacustris* (Niżni Toporowy Staw) is the second lowest lake in the whole Tatra Mountain range (1089 m a.s.l.); we therefore also included in our study a small natural lower-altitude lake with similar environmental conditions (Dankovo lake, 874 m a.s.l.), located in the forested watershed in the Spišská Magura hills eastwards of the Tatra Mountains (Figure 1, d).

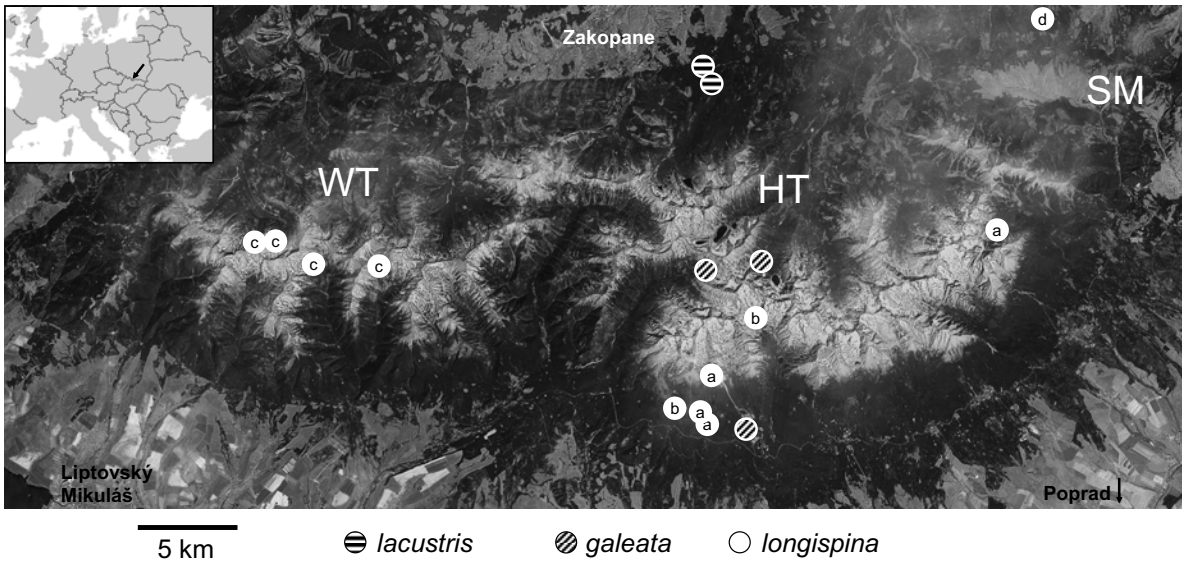
Table 1

Basic morphometric and environmental data of the sampled lakes. Abbreviations indicating the mountain ranges: HT – High Tatras, WT – West Tatras, SM – Spišská Magura; abbreviations of the watershed character: F – forest, DP – mostly dwarf pine, M – alpine meadow and dwarf pine, MR – meadow and rocks, H – human settlement. Fish stock in Štrbské pleso is currently a mixture of several species, especially whitefish *Coregonus maranea*, perch *Perca fluviatilis* and roach *Rutilus rutilus* (MUŽÍK et al. 2004).

Lake name	Abbre-viation	Mountain range	Latitude (N)	Longitude (E)	Altitude (m)	Area (ha)	Max. depth	Timberline	Watershed	Fish
Nizny Toporowy Staw	NiTo	HT	49.283	20.031	1089	0.62	5.7	below	F	no
Wyżni Toporowy Staw	WyTo	HT	49.279	20.029	1131	<0.05	1.5-2	below	F	no
Morskie Oko	Mor	HT	49.198	20.072	1395	34.5	50.8	below	MR, DP	brown trout
Nižné Temnosmrečinské	NiTe	HT	49.193	20.031	1677	11.7	38.1	above	M	no
Štrbské	Str	HT	49.123	20.058	1346	19.7	6.6	below	F, H	mixed fish stock
Štvrté Roháčske	Ro4	WT	49.206	19.736	1719	1.44	8.2	above	MR	no
Prvé Roháčské	Ro1	WT	49.206	19.744	1562	2.23	7.7	above	M	no
Nižné Jamnícke	NiJa	WT	49.203	19.772	1732	1.13	8.2	above	M	no
Vyšné Račkové	VyRac	WT	49.200	19.807	1697	0.74	12.3	above	MR	Alpine bullhead
Jamské	Jam	HT	49.132	20.013	1448	0.68	4.3	below	F	no
Nižné Rakytovské	NiRak	HT	49.126	20.025	1307	0.13	2.1	below	F	no
Vyšné Rakytovské	VyRak	HT	49.125	20.027	1307	0.22	2.3	below	F	no
Vyšné Furkotské	VyFu	HT	49.144	20.031	1698	0.41	2.4	above	DP	no
Vyšné Satanie	Sat	HT	49.171	20.064	1894	0.20	3.5	above	MR	no
Malé Čierné	MaCe	HT	49.208	20.225	1566	0.07	2	below	F	no
Dankovo	Dan	SM	49.318	20.254	874	ca 0.5	1.5-2	below	F	no

Figure 1

Geographic location of the sampled lakes. Inset: location of the Tatra Mountains in Europe. Different species are indicated by pattern, haplotype groups of *D. longispina* (Figure 2) are marked by the letters a-d in white circles. Abbreviations indicate mountain ranges (HT – High Tatras, WT – West Tatras, SM – Spišská Magura), location of the nearest major towns is indicated by their names. The satellite photo allows the assessment of the character of lake watersheds: forests and dwarf pine stands are dark, alpine meadows and rocks grey (image source: orthorectified Landsat 7 data, U.S. Geological Survey).



Sample collection and analysis

Zooplankton samples were collected by tows of a plankton net (mesh size 40 or 200 µm) from the lake shore (in the case of small lakes), or by several vertical hauls at the deepest area of the lake from an inflatable rubber boat (large lakes), and preserved in 96% ethanol. Sampling dates are provided in Table 2. Samples were initially visually screened for the potential coexistence of different taxa of the *Daphnia longispina* group under a stereomicroscope. In particular, we focused on the intra-lake variation of the head and tailspine size (relative to the carapace size), presence or absence of helmets, size of the antennular mound, and shape of the rostrum and frontal margin of the head. If available, approximately 50 individuals per sample were examined; in cases of less numerous samples, all available *Daphnia* were screened. Several individuals per sample, covering the morphologically most divergent individuals to represent the within-lake variation, were then selected for DNA extraction by proteinase K digestion (SCHWENK et al. 1998) in 50-100 µl volumes. Some of the plankton samples collected in autumn did not contain any (or in the case of Wyżni Toporowy Staw, only very few) *Daphnia* individuals. Freshly shed ephippia (chitinous structures containing sexually produced resting eggs), however, were found in these lakes. In such cases, individual resting eggs from different ephippia were separately subjected to DNA extraction.

Two genetic methods were used for taxon identification. A restriction fragment length polymorphism analysis (RFLP) of the nuclear ribosomal internal transcribed spacer (ITS) was used for species identification, as well as to check for the potential presence of interspecific hybrids (BILLIONES et al. 2004). This marker consists of a short part of ITS1, the 5.8S ribosomal RNA gene, and a large fraction of ITS2, and yields an approx. 1400 bp long PCR product for most species of the *Daphnia longispina* complex. The exception is *D. lacustris* with

longer PCR products (up to 2000 bp), often varying in length (NILSSEN et al. 2007, SKAGE et al. 2007). The RFLP analysis of this fragment provides species-specific fragment lengths in most cases, which also allows the identification of hybrid genotypes. The amplification and RFLP of the ITS followed the methods described in BILLIONES et al. (2004); restriction patterns were interpreted as provided in PETRUSEK et al. (2005). As the preliminary morphological examination did not suggest the occurrence of hybridising populations (see Results), usually three to seven individuals per population were analyzed for their ITS restriction patterns (Table 2). For one population (Morskie Oko), in which the ITS-RFLP patterns did not agree with other markers, we additionally applied an alternative, recently developed ITS-RFLP protocol for detection of species and hybrids, which is less susceptible to intraspecific restriction site variation in *Daphnia galeata* (SKAGE et al. 2007).

The identification of maternal lineages was confirmed by sequencing the mitochondrial DNA (mtDNA) of a representative individual from each population of each taxon (as determined by ITS-RFLP); resulting sequences were then compared to known mtDNA sequences of European *Daphnia* species (the species nomenclature follows PETRUSEK et al. 2005 and NILSSEN et al. 2007; for sequence accession numbers, see below). This approach is similar to "DNA-barcoding" as coined by HEBERT et al. (2003); however, instead of using sequences of the gene for the cytochrome c oxidase subunit I (COI), we analysed the gene for 12S rRNA, as it was previously used in phylogenetic studies of the group (TAYLOR et al. 1996, SCHWENK et al. 2000) and is therefore the most readily available mitochondrial gene for European *Daphnia* identification. Part of this gene was amplified using the protocol modified from TAYLOR et al. (1996) and SCHWENK et al. (2000). The 25 µl PCR reaction contained 1-3 µl of the template (*Daphnia* DNA extract), 1x PCR buffer, 0.15 mM deoxynucleotides, 1.5 mM $MgCl_2$, 0.4 µM primers (5' ATGCACTTTCCAGTACATCTAC 3' and 5' AAATCGTGCCAGCCGTCGC 3'; TAYLOR et al. 1996), 1% dimethyl sulfoxide (DMSO), and 0.5 U Taq polymerase. The following cycle settings were used: denaturation at 97 °C for 3 minutes, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1.5 min, elongation at 72 °C for 45 s; and the final elongation step at 72 °C for 3 minutes. The PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and subsequently cycle sequenced on ABI automatic capillary sequencers (series 377 or 3130) using the Big Dye terminator sequencing kit (Applied Biosystems, Foster City, USA). All 12S sequences have been deposited in GenBank (see Table 2 for accession numbers).

Sequences were aligned using ClustalW (THOMPSON et al. 1994), the alignment was checked manually, trimmed to a fragment length available for all individuals (528 or 529 bp, depending on the species), and further analysed using the software package MEGA version 3.1 (KUMAR et al. 2004). Positions with gaps in the 531 bp long alignment were excluded from further analyses. As we did not focus on the phylogenetic relationship among species but rather on lineage identification, we applied the Neighbour-Joining method using Kimura 2-parameter distances to build a tree; branch support was estimated with 500 bootstrap replicates. Species assignments of Tatra Mountain populations were confirmed by comparison with sequences from related species and conspecific populations from other European regions (sequences taken from Schwenk et al. 2000, 2004; Nilssen et al. 2007; Petrusek et al., unpublished). The reference sequences originated from two populations of *D. lacustris* from Norway (Myrdalsvatn, GenBank accession number DQ337945) and Finland (Tsahkallampi A, DQ337958), one population of *D. galeata* from the Netherlands (Tjeukemeer, EF375851), two populations of *D. "umbra"* from Fennoscandia (lake in Jotunheimen, Norway, AF277276 and pond Mallalampi A, Finnish

Lapland, EF375849) and two populations of *D. cucullata,* one from the Netherlands (Tjeukemeer, AF277271) and one from the Czech Republic (pond in Medlov, AF277270).

In order to assess the patterns of local haplotype diversity in *Daphnia longispina*, the most common taxon of the group in Tatra Mountain lakes, we compared Tatra haplotypes with various others representing the species' European diversity: one from Sweden (pond in Laerjeholm, Göteborg, EF375845), one from Norway (Trollvann, Oslo, EF375842), three from Denmark (Zealand, 1: Brededam, acc. no. EF375836; 2: Midtre Kobberdam, DQ536400; 3: Pernillesø, EF375837), two from the Czech Republic (1: pond in Droužkovice, north Bohemia, EF375834; 2: dam Žďárské jezero, Bohemian Forest, EF375835), one from Germany (pond in Frankfurt am Main, EF375839), one from Switzerland (Unterer Arosasee, EF375846), and two from Spain (1: reservoir Villar del Rey, Badajoz, EF375844; 2: pond Zahillo, Doñana, EF375843). The first mentioned reference sequence (Göteborg) was included in the species tree (Figure 2); all others were used in the statistical parsimony network of 12S rDNA haplotypes of *D. longispina*, which was constructed using the program TCS v. 1.21 (CLEMENT et al. 2000).

Results

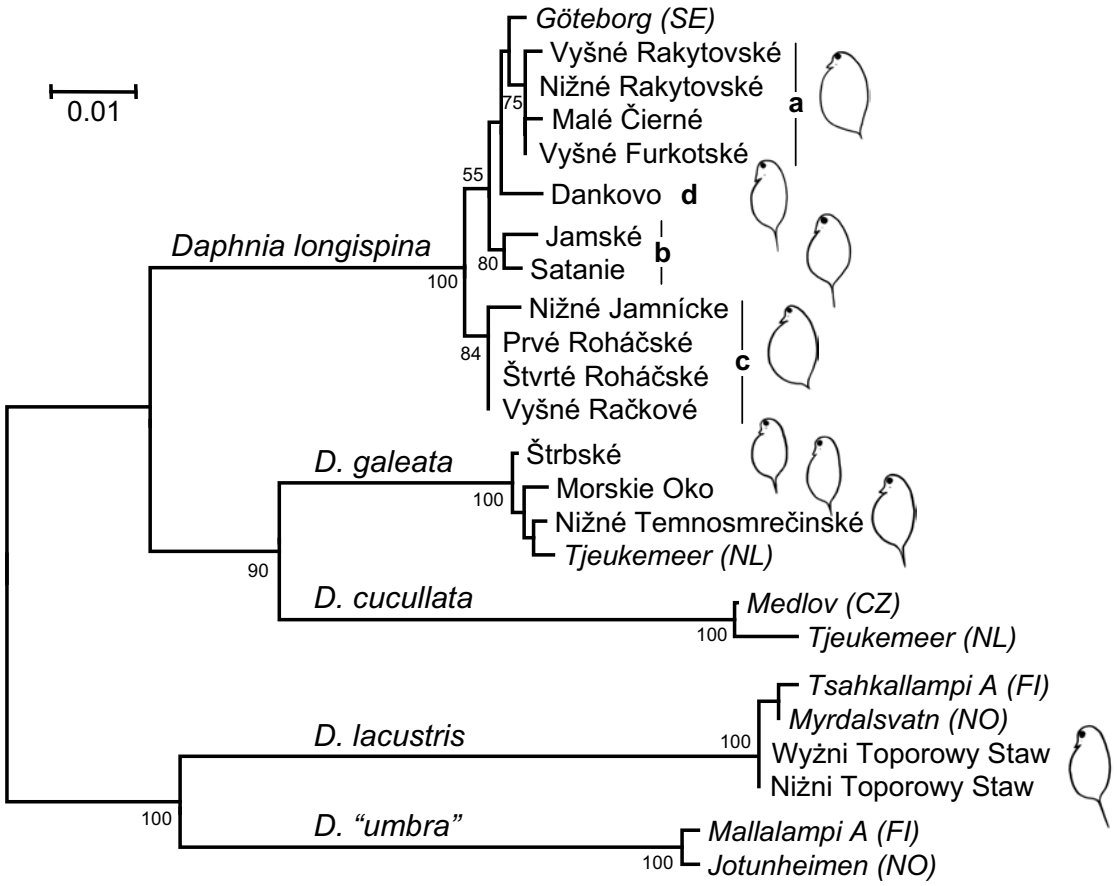
Initial screening of within-population morphological characters suggested that none of the lakes was inhabited by more than one taxon of the *D. longispina* group. Although significant variation in body shape, head size and shape, and spine length was observed among lakes (Figure 2), within-lake variation in the examined characters was much lower, and none of the populations contained *Daphnia* individuals with "intermediate" body shapes or other morphological characteristics which would suggest interspecific hybridization.

Both ITS-RFLP and 12S rDNA sequencing confirmed the occurrence of altogether three different lineages (species) of the *Daphnia longispina* complex in studied populations (Table 2, Figure 2). *D. longispina* was the most common species, occurring in 11 out of 16 studied lakes. Apart from the already known locality of *D. lacustris*, Niżni Toporowy Staw (see NILSSEN et al. 2007), the presence of this species was confirmed in an additional nearby locality, the small tarn Wyżni Toporowy Staw. Individuals of *D. lacustris* from the Tatra Mountains were morphologically similar to their conspecific populations from Norway (see NILSSEN et al. 2007). Amplified ITS fragments from *D. lacustris* individuals were substantially longer (approx. 2000 bp) than those from other species of the *D. longispina* complex (1400 bp).

D. galeata was recorded in three relatively large lakes (two of them with significant fish predation pressure). Although their general phenotype differed among them (Figure 2), individuals from all three *D. galeata* populations shared a prominent antennular mound, which is a morphological trait characteristic for this species (FLÖSSNER 2000). However, ITS-RFLP patterns according to the protocol by BILLIONES et al. (2004) disagreed with the identification by 12S sequences and morphology in one of these populations, from the lake Morskie Oko. Individuals from this lake showed an atypical restriction pattern lacking bands characteristic for *D. galeata* after digestion by the endonuclease Mwo I (BILLIONES et al. 2004), and would be identified as *D. cucullata*. It has been shown, however, that a loss of the presumably species-specific restriction site, caused by a single point mutation, is common in *D. galeata* populations across Europe (SKAGE et al. 2007). An alternative ITS-RFLP protocol (SKAGE et al. 2007) identified these individuals as *D. galeata* in accordance with mtDNA and morphology.

Figure 2

Neighbour-joining tree representing sequence variation of the 531 bp long alignment of a fragment of the mitochondrial gene for 12S rRNA (Kimura 2-parameter distance) among sequences from all sampled *Daphnia* populations and reference haplotypes of related species of the complex. Samples from other geographic areas used as a reference are listed in italics, with ISO country abbreviations in parentheses. The scale bar represents genetic distance; letters **a-d** and vertical lines indicate corresponding groups of *D. longispina* haplotypes from the Tatra Mountain region; numbers show bootstrap support for selected branches (species, haplotype groups). Schematic outlines of body shapes of representative individuals are shown next to each clade (from top to bottom: *D. longispina* – Vyšné Furkotské, Dankovo, Vyšné Satanie, Prvé Roháčské; *D. galeata* – Štrbské, Morskie Oko, Nižné Temnosmrečinské; *D. lacustris* – Niżni Toporowy Staw).

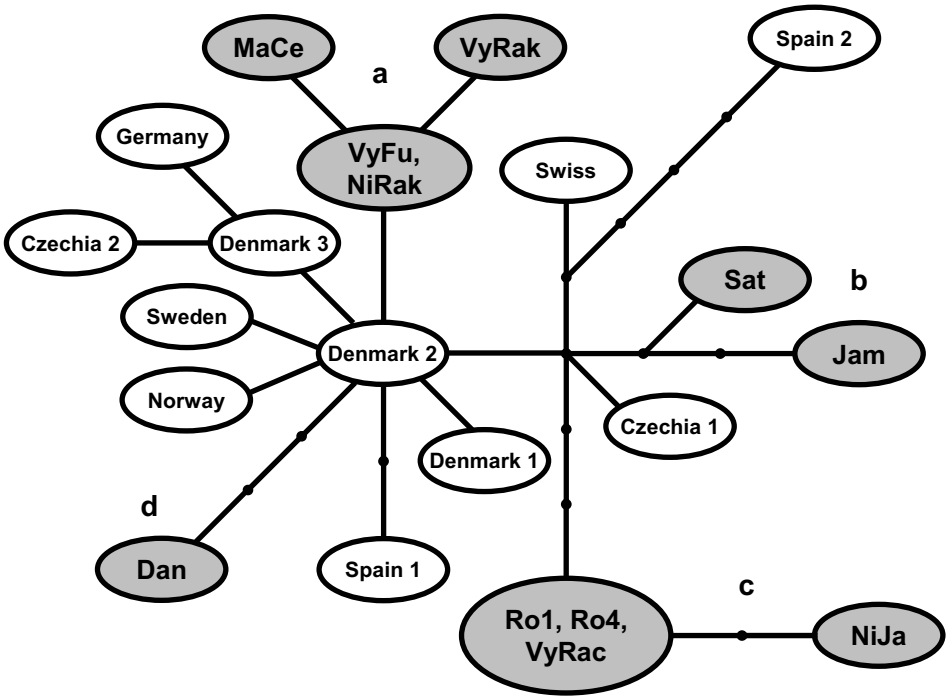


Both molecular markers consistently identified *D. longispina* individuals, although they showed significant among-lake variation in body shape, relative head height, and spine length (representative outlines of body shapes are shown in Figure 2). The most unusual morphotypes, characterised by very low heads, were recorded in all analyzed West Tatra lakes and similar morphotypes were also found in Nižné Furkotské Lake in the High Tatras. No intraspecific hybrids were detected by ITS-RFLP in any studied populations.

12S rDNA haplotypes originating from eleven *D. longispina* populations were highly differentiated, showing a certain geographic structure (Figures 1-3). Altogether, four distinct haplotype groups were recognised in the Tatra Mountain region (marked **a-d** in all figures), with between-group pairwise sequence divergence ranging from 0.8 to 1.4%, and maximal divergence (between haplotypes from Nižné Jamnícke and Dankovo lakes) reaching up to 1.7%. This represents a sequence divergence comparable to the haplotype variation of this species at a much larger geographic scale in Europe (Figure 3). The West Tatra lakes (haplotype group **c**) and the

Figure 3

Parsimony network of 12S haplotypes from eleven analysed populations of *Daphnia longispina* s.s. (grey ovals) together with reference haplotypes illustrating the continental variation within the species (white ovals). Each node represents one nucleotide substitution. Lake abbreviations are listed in Table 1. Letters **a-d** indicate haplotype groups from the Tatra Mountain region as in Figures 1 and 2.



isolated Dankovo lake (**d**) were geographically most distant, and their populations were well separated genetically. The remaining two groups of 12S rDNA haplotypes (**a** and **b**) were neither geographically nor ecologically well defined. For example, although very close to each other, three small forest lakes from the western High Tatras (Jamské, Vyšné Rakytovské, Nižné Rakytovské) contained individuals belonging to two different haplotype groups.

Discussion

Species composition and genetic variation

Our results revealed two major factors explaining the high morphological variability among populations of the *Daphnia longispina* complex in the Tatra Mountains: 1) the occurrence of three distinct species (*D. longispina*, *D. galeata*, and *D. lacustris*), commonly pooled together or mislabelled in recent studies (HRBÁČEK et al. 1974, KNESLOVÁ et al. 1997, GLIWICZ et al. 2001, SKÁLA 2003, HOŘICKÁ et al. 2006), and 2) high intraspecific, morphological as well as genetic, variation among populations of *D. longispina* in a strict sense.

Although three species of the group were detected in the region, the majority of the studied lakes were inhabited by populations of *D. longispina*. The haplotype divergence observed among the Tatra Mountain populations (up to 1.7% for 12S rDNA) was unusually high, comparable to the variation observed on continental scale in Europe (Figure 3). This substantial genetic divergence can hardly be explained by local postglacial diversification of a relatively slowly evolving mitochondrial gene. Instead, this pattern suggests that lakes of the Tatra Mountain region were colonised from genetically divergent source populations. Although we have not

measured the genetic variation within populations to assess the extent of gene flow among lakes, differences in morphological characteristics of various populations together with the genetic differentiation of analysed haplotypes suggest that founder effects and subsequent local adaptation (BOILEAU et al. 1992, DE MEESTER et al. 2002; ISHIDA & TAYLOR 2007) may have played an important role in the apparent divergence of *D. longispina* populations. For example, *Daphnia* from the examined West Tatra Mountain lakes (group **c**) are morphologically very similar. These populations form a geographically separate cluster (Figure 1) and share identical or related haplotypes (Figures 2, 3). It is likely that such a pattern is the result of a single colonization event, followed by local dispersal to suitable neighbouring lakes. Four lakes in the haplotype group **a** may represent another independent colonization of the mountain range. The haplotype representing a morphologically distinct population from Vyšné Satanie Lake (haplotype group **b**), where *Daphnia* recently reappeared after the acidification period, was also genetically divergent from all other haplotypes in the region.

In contrast to the 12S rDNA haplotype of *D. lacustris*, which differed by only a single point mutation (0.2 %) from a common haplotype found in several Norwegian and Finnish populations (NILSSEN et al. 2007), *D. longispina* haplotypes recorded in our study were usually more divergent, only the haplotype group **a** was closely similar (<0.4% divergence) to some of the haplotypes isolated from over forty European localities (A. Petrusek, unpublished data). The West Tatra haplotypes (especially the one recorded in Nižné Jamnícke Lake) actually represent one of the most divergent groups within the whole *D. longispina* lineage (Figure 3 and unpublished data).

Similarly as in *D. longispina*, the occurrence of different haplotypes in three *Daphnia galeata* populations, together with the atypical ITS-RFLP pattern observed in Morskie Oko, suggest that these populations, although they are located close to each other, could have been founded from different sources. This hypothesis is further supported by the fact that the three *D. galeata* populations occur in environmental conditions promoting differently adapted genotypes: Nižné Temnosmrečinské Lake and Morskie Oko are deep oligotrophic lakes located at or just above timberline, the first is fishless, the second has a large population of brown trout; Štrbské Lake is much shallower, has a largely forested watershed, an increased trophic level and a mixed fish stock exerting strong predation pressure on the zooplankton (Table 1).

Faunistic, biogeographic and conservational implications

Although the contemporary understanding of species boundaries as well as *Daphnia* nomenclature differs from that used at the beginning of the 20th century, we are able to link the results of our genetic analysis with the original identifications of *Daphnia* in the first comprehensive studies of zooplankton from the Tatra Mountains.

LITYŃSKI (1913, 1917) and MINKIEWICZ (1914, 1917) recognised four or five different morphological forms of the so-called *Daphnia variabilis*. Out of these, var. *caudata-cavifrons* can be associated with *D. lacustris* (the summer morph of the Tatra population is morphologically close to Norwegian populations of *D. lacustris* var. *caudata* described by G. O. Sars). The form *lacustris* of MINKIEWICZ (1914, 1917) and LITYŃSKI (1913) refer to *D. galeata* (following the classification of LILLJEBORG (1900), who misconceived the taxon *D. lacustris*). The same is true for f. *obtusifrons* in LITYŃSKI (1917). The latter labelling is in agreement with the taxon perception of G. O. Sars, who introduced the name *obtusifrons* for a form of *D. galeata*. Two more forms

reported from the Tatra Mountains, var. *longispina-rosea* and f. *frigidolimnetica,* refer to different morphotypes of *D. longispina* s.s.

Our results, however, differ markedly from those of the above-mentioned authors in the identification of individuals from Štrbské Lake (Szczyrbskie or Csorber See in the historical literature). While we detected only *D. galeata* in that lake, earlier studies report the *longispina-rosea* form. This discrepancy almost certainly documents a recent species replacement rather than a misidentification. The drawings of *Daphnia* from Štrbské Lake in LITYŃSKI (1913) or KUBÍČEK (1958) differ substantially from any *D. galeata* morphs (including those currently occurring in the lake), and morphologically resemble some populations of *D. lacustris* or *D. longispina* (Figure 4). The morphotype from the Štrbské Lake population from the beginning of the 20th century with an extremely long-spine (Figure 4B) is most similar to the Fennoscandian morph *caudata* of *D. lacustris* or to *D. lacustris* population from Niżni Toporowy Staw. The hypothesis that *D. lacustris* may have lived in the lake 100 years ago is also supported by very similar patterns of *Daphnia* cyclomorphosis and phenology between lakes Štrbské and Niżni Toporowy Staw at that time (LITYŃSKI 1913, 1917). A drawing based on specimens collected in June 1954 also showed *Daphnia* which are clearly different from the contemporary population (cf. Figures 4C and 4D). Interestingly, as KUBÍČEK (1958) explicitly stated, the mid-1950s morphotype seems to have differed in some morphological characteristics from those listed by LITYŃSKI (1913) over four decades earlier. The history of *Daphnia* in Štrbské Lake during the recent period of anthropogenic influence is therefore confusing, and only paleogenetic analyses of the resting egg bank from the lake sediment may give an unambiguous answer about the identity of the original population.

Two main factors, both related to human activities, may have played a role in the species replacement in Štrbské Lake. Firstly, there had been a significant increase in the fish predation pressure. Apart from brown trout (*Salmo trutta* m. *fario*), which was already present in the lake in the 19th century, other salmonids (e.g. whitefish

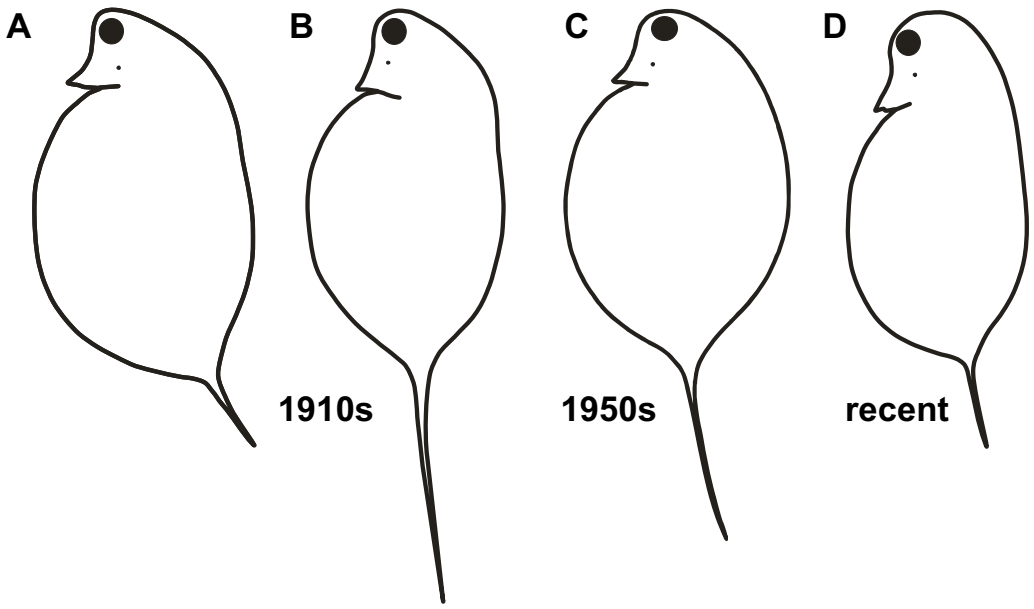
Table 2

Information about *Daphnia* samples, and current and historical species identification in the sampled lakes. The number of individuals analyzed by the ITS-RFLP is indicated in the corresponding column. "E" denotes analysis of resting eggs from ephippia. Question marks next to the taxon name indicate records where the historical sources may refer to similar water bodies in the close vicinity, asterisks mark apparent species replacement since the beginning of the 20th century, and "N/A" indicates lakes from which historical data on *Daphnia* were unavailable. Historical two-word taxon names designate various morphotypes (varieties, morphs) and do not assume the presence of interspecific hybrids.

		Our analysis			Historical records	
Lake name	**sampling date**	**specimens analyzed**	***Daphnia* species**	**12S Genbank accession no.**	**Minkiewicz, 1917**	**Lityński, 1917**
Niżni Toporowy Staw	25.9.2006	2	*lacustris*	DQ337940	*caudata-cavifrons*	*caudata-cavifrons*
Wyżni Toporowy Staw	25.9.2006	5 (E)	*lacustris*	as above	*caudata-cavifrons*	*caudata-cavifrons*
Morskie Oko	26.9.2000	7	*galeata*	DQ337927	*hyalina lacustris*	*obtusifrons*
Nižné Temnosmrečinské	26.9.2000	7	*galeata*	DQ337926	*hyalina lacustris*	*obtusifrons*
Štrbské	26.9.2004	4	*galeata**	DQ337928	*longispina-rosea**	*longispina-rosea**
Štvrté Roháčske	16.9.2000	3	*longispina*	DQ337936	N/A	N/A
Prvé Roháčské	30.9.2002	3	*longispina*	DQ337935	N/A	N/A
Nižné Jamnícke	24.9.2000	5	*longispina*	DQ337937	N/A	N/A
Vyšné Račkové	17.10.2000	3	*longispina*	DQ337934	N/A	*frigidolimnetica*
Jamské	26.9.2004	1 (E)	*longispina*	DQ337932	*longispina-longispina*	*longispina-longispina*
Nižné Rakytovské	26.9.2004	1 (E)	*longispina*	DQ337931	*longispina-longispina* (?)	*longispina-longispina* (?)
Vyšné Rakytovské	26.9.2004	2 (E)	*longispina*	DQ337930	*longispina-longispina* (?)	*longispina-longispina* (?)
Vyšné Furkotské	25.9.2000	10	*longispina*	DQ337929	N/A	N/A
Vyšné Satanie	27.9.2003	3	*longispina*	DQ337939	N/A	N/A
Malé Čierné	24.9.2004	1 (E)	*longispina*	DQ337933	N/A	*longispina-longispina* (?)
Dankovo	7.7.2004	2	*longispina*	DQ337938	N/A	N/A

Figure 4

Schematic outlines of body shape of *Daphnia* from Štrbské Lake, documenting the species replacement in the lake during the 20th century: **A, B:** beginning of the 20th century, summer (A) and spring (B) morphotypes (after LITYŃSKI 1914); **C:** the mid-1950s, June (after KUBÍČEK 1958); **D:** recent samples (1990s - 2000s).



Coregonus maraena) and more recently other planktivorous fish (especially perch *Perca fluciatilis* and roach *Rutilus rutilus*) were stocked into the lake (HOLČÍK & NAGY 1986, MUŽÍK et al. 2004). Increased fish predation pressure favours *D. galeata*, which often occurs in habitats with planktivorous fish, over *D. longispina* and other larger *Daphnia* species (NILSSON & PEJLER 1973). Fish predation was probably the key factor that caused the complete disappearance of *Daphnia* from the lake in the 1980s (E. STUCHLÍK, pers. communication). Our results suggest that although the *Daphnia* population later re-appeared in the lake, it did not recover from the resting egg bank in the sediment but rather invaded the lake from elsewhere, and that the surrounding mountain lakes were not the source of the colonising specimen. Apart from fish predation, changes in the trophic level of the lake, which increased due to the development of recreation infrastructure in the watershed, may have contributed to the success of *D. galeata* in Štrbské Lake. The successful invasion of *D. galeata* to an oligotrophic lake in the course of eutrophication has also been documented from other localities, such as Lake Constance (EINSLE 1978, 1983, JANKOWSKI & STRAILE 2003).

The persistent occurrence of *D. galeata* in a pristine fishless alpine lake above timberline (Nižné Temnosmrečinské) is remarkable. Unlike the papers from the beginning of the 20th century (LITYŃSKI 1917, MINKIEWICZ 1917), which recognised its distinctness, recent studies have referred to this population as *D. longispina* (KNESLOVÁ et al. 1997, SKÁLA 2003, HOŘICKÁ 2006). Similarly, in Morskie Oko, a lake with lower elevation and populated by brown trout, the *Daphnia* was recently misidentified as *D. longispina* (GLIWICZ et al. 2001). Especially in case of Nižné Temnosmrečinské Lake, such misidentification is not particularly surprising, as the morphological characteristics (e.g., helmet height) of local individuals differ from lowland *D. galeata* populations, and it was presumed that *D. galeata* does not occur in such an alpine habitat. However, this population seems not to be unique in the High Tatras – one more Tatra lake at a similar elevation (Nižné Žabie

Bielovodské; 1675 m) harbours a morphologically very similar population which, although not analysed genetically, most likely belongs to the same species (A. PETRUSEK, unpublished results).

In southern Norway, *D. galeata* may occur above timberline but preferably in habitats with intense fish predation (HUIFELDT-KAAS 1906, J. P. NILSSEN, pers. comm.). Records of this species from lakes above the timberline in other European mountain ranges are lacking, possibly due to the use of unsuitable morphological identification characters. Nevertheless, even in a study of zooplankton composition in 26 high-altitude lakes in the Swiss Alps which used allozyme electrophoresis for species identification (WINDER et al. 2001), *D. galeata* was found only in two lakes, both widely influenced by human activities, stocked by fish, and located below the timberline. No *D. galeata* was reported in another study of 15 alpine lakes in the Italian Central-Southern Alps, although *D. longispina* was commonly found there (MANCA & ARMIRAGLIO 2002). Comparisons with older studies of mountain lake zooplankton based solely on morphological identification are nevertheless problematic, as misidentifications of similar species cannot be ruled out. A recent study on alpine plankton diversity (TOLOTTI et al. 2006) actually no longer attempted to differentiate among various taxa of the *D. longispina* group, apparently due to unreliable identification to species level. The use of genetic tools for species identification will likely facilitate the discovery of more truly alpine *D. galeata* populations elsewhere in Europe, as well as other overlooked *Daphnia* species. It can also be expected that molecular approaches will uncover substantial cryptic diversity in other alpine zooplankton taxa.

Apart from the above-mentioned extinct population in Štrbské Lake with unknown taxonomic affiliation, the observation of *D. lacustris* in only two localities, in Niżni and Wyżni Toporowy Staw, agrees with the historical data. The *caudata* morph was not reported from any other lake (LITYŃSKI 1913, 1917, MICKIEWITZ 1914, 1917). Genetic analyses point out how unique these *Daphnia* populations actually are. So far, the nearest extant populations of this boreal species are known from southern Norway, over 1300 km away (NILSSEN et al. 2007). Based on the existence of isolated Tatra Mountain populations, *D. lacustris* may be expected to occur in other Central European alpine lakes. However, although several other lakes in the Tatra Mountains (as well as in the neighbouring Spišská Magura range) have similar environmental conditions (dystrophic fishless lakes with a forested watershed) as those currently inhabited by *D. lacustris*, all seem to be occupied solely by *D. longispina*. This pattern is likely due to the low chance of effective dispersal to a suitable environment already inhabited by a closely related taxon, due to strong monopolisation of the resources by priority effects, and the numerical advantage of resident populations (JENKINS & BUIKEMA 1998, DE MEESTER et al. 2002).

The presence of this isolated population of *D. lacustris* is similar to another boreal element of the aquatic fauna of the Tatra Mountains, the anostracan *Branchinecta paludosa* (O. F. Müller, 1784). This fairy shrimp has a circumpolar distribution, and only a few isolated populations are known from lower latitudes. The Tatra Mountains are the southernmost European locality of the species. Historically, it occurred in two Tatra lakes (WIERZEJSKI 1882, GAJL 1934, HRABĚ 1934) but one population went extinct at the end of the 1960s (KOWNACKI 2004). In North America, this species is present at high elevations in southern Wyoming, northern Colorado (SAUNDERS et al. 1993), and Utah (STERN & BELK 1999), and it was speculated that its persistence in southern Canada and in mountainous areas of the USA might be maintained by the migration of Arctic-nesting waterfowl (SAUNDERS et al. 1993).

Although a rare case of a recent long-range dispersal of *B. paludosa* or *D. lacustris* from their current boreal distribution area to the Tatra Mountains cannot be ruled out, we rather speculate that their presence in the region is the result of much earlier postglacial colonisation. Clearly, neither the Tatra Mountain region nor Fennoscandia could harbour populations of these planktonic species during the Weichselian glaciation, as *in-situ* survival of planktonic species in ice-free areas above the ice shield is quite unlikely, and the glaciers in the Tatra Mountains during this glaciation period extended over the present location of lakes (LINDNER et al. 2003). On the other hand, it is likely that the refugia of aquatic species with present boreo-alpine distribution may not have been restricted to immediate periglacial regions.

While the potential locations of ice age refugia for terrestrial plants and animals as well as fish have been intensively studied, much less is known about the Pleistocene history of passively dispersed boreal zooplankton, with the notable exception of the circumarctic members of the *Daphnia pulex* group (which, nevertheless, have very different distributional patterns than *D. lacustris*; WEIDER et al. 1999a, b). Interestingly, it has been recently shown that Carpathian glacial refugia existed even for small woodland mammal species (KOTLÍK et al. 2006); we may therefore presume that local survival of zooplankton in low-altitude waterbodies was not impossible. If their distribution at the end of the glaciation period reached lower elevations of Slovakia and/or Poland, it is conceivable that during the climate warming such species could retreat both to higher latitudes and higher elevations where newly formed lakes offered suitable environmental conditions. The colonisation of lakes in the Tatras and surrounding mountain ranges by zooplankton species certainly must have happened only recently, sometime between eight and twenty thousand years ago, depending on their elevation.

The dispersal of aquatic invertebrates with a durable diapausing stage is in many aspects similar to that of plants. It is therefore worth noting that Tatra Mountain populations of a characteristic boreo-alpine plant species, *Dryas octopetala*, are closely related to populations which probably survived in Siberian or Eastern European refugia (SKREDE et al. 2006). Although this plant is primarily wind-dispersed, we cannot entirely exclude re-colonisation scenarios from the east for Tatra Mountain aquatic biota, especially as the glacial refugium of *D. lacustris* remains unknown. The *Daphnia* fauna of Siberia, Central Asia and the Far East is still underexplored, new species are being discovered in the Eastern Palaearctic (e.g., KOTOV et al. 2006), and it is not unlikely that populations of *D. lacustris* will be eventually found in Siberia, beyond the extent of Weichselian ice shield.

Yet another unique element of the aquatic biota of the Tatra Mountains is the narrow-leaved bur-reed, *Sparganium angustifolium* Michaux, 1803. It is a circumpolar plant species that has its centre of European distribution in the northern part of the continent. However, isolated populations are known from various southerly-located European mountain ranges as well (MEUSEL et al. 1965, MÜLLER-DOBLIES & MÜLLER-DOBLIES 1980). Five lakes with records of this species in the Tatra Mountains (including Niżni and Wyżni Toporowy Staw) represent the only occurrences in the Carpathian range (DÍTĚ et al. 2004). The population in Niżni Toporowy Staw had disappeared by 1976, and the same seems to have happened recently in Wyżni Toporowy Staw (DÍTĚ et al. 2004), which is in the final stage of succession and almost filled with peat. The original presence of *Sparganium angustifolium* and *Daphnia lacustris* in these Polish lakes suggests that local conditions were favourable for the survival of populations of boreal aquatic species.

Given the absence of *D. lacustris* in other forest lakes in the region, conservation of the pristine habitat of both Niżni and Wyżni Toporowy Staw seems to be the only means of preserving this species in the region.

Fortunately, these dystrophic lakes have not been affected by acidification or fish stocking, and their location within the boundaries of the national park prevents any strong direct human impact on the lake environment. However, natural processes may soon lead to the complete filling of Wyżni Toporowy Staw, and therefore to the loss of the *Daphnia* population there. The distribution pattern of this species suggests that in other mountainous areas that have been more adversely affected by human activities, relict aquatic species or unusual divergent genotypes may have disappeared even before having been recorded for science.

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CHAPTER 5

Seďa J., Petrusek A., Macháček J., Šmilauer P.:

Spatial distribution of the *Daphnia longispina* species complex and other planktonic crustaceans in the heterogeneous environment of canyon-shaped reservoirs

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July 2007 issue of the Journal of Plankton Research, featuring aerial view of Římov, one of our study sites, with clearly visible longitudinal gradient of chlorophyll content.

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Spatial distribution of the *Daphnia longispina* species complex and other planktonic crustaceans in the heterogeneous environment of canyon-shaped reservoirs

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Canyon-shaped reservoirs are often characterised by longitudinal gradients of environmental factors (including trophic level, phytoplankton and zooplankton biomass and abundance of planktivorous fish) affecting the taxonomic composition of the pelagic community. We tested the hypothesis that the spatial distribution of different species and interspecific hybrids of the Daphnia longispina species complex is non-random under such conditions. During the summer stratification, we sampled crustacean zooplankton from 11 reservoirs, covering both longitudinal (upstream, middle, dam) and vertical (epi-, meta- and hypolimnion) environmental gradients. Allozyme electrophoresis was used to discriminate among different Daphnia taxa. All three frequently hybridizing European species of the complex (galeata, cucullata, longispina = hyalina) and hybrids with Daphnia galeata were commonly recorded. Smaller-bodied Daphnia cucullata and its hybrids, when present, preferred mostly the nutrient- and food-rich upstream regions; D. longispina and its hybrids were more commonly found in the downstream part, and often dominated in the meta- or hypolimnion. Redundancy analyses confirmed significant differences in the Daphnia taxon composition, as well as in spatial distribution of other crustacean species, along both gradients. For the first time, we demonstrate regular patterns in the horizontal distribution of Daphnia species and hybrids within a water body, thus accepting our hypothesis. Such spatial distributional patterns may strongly impact local hybridization processes.

INTRODUCTION

Artificial reservoirs constructed by damming rivers may represent good model systems for studying ecological and evolutionary factors affecting the composition of pelagic communities. Canyon-shaped reservoirs, with their typical elongate morphometry and a single main river inflow, are especially suitable, as they are characterised by a longitudinal gradient involving gradual changes of a number of interlinked environmental factors (Straškraba, 1998). Similarly to complex gradients in terrestrial systems, e.g. altitudinal variation, such strong environmental gradients in an aquatic environment should significantly affect the distribution of organisms at various trophic levels.

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The primary cause of spatial heterogeneity within reservoirs is the decreasing trophic level along the reservoir main axis. Increased concentrations of nutrients in the upstream parts, sustained by the river inflow, positively stimulate algal growth, so the algal biomass and chlorophyll *a* concentration typically decreases towards the dam (Fernandes-Rosado *et al.*, 1994; Desortová, 1998; Hejzlar and Vyhnálek, 1998). The distribution of zooplankton usually reflects this food source gradient (Urabe and Murano, 1986; Dohet and Hoffmann, 1995; Thys *et al.*, 1998), although both patterns may be disrupted by water flow (Hayward and Van Den Avyle, 1986; Pont and Amrani, 1990). A similar general distribution can be observed also for fish, which are reported to be more abundant in shallow sites near headwaters (Brosse *et al.*, 1999; Gido *et al.*, 2002; Vašek *et al.*, 2004).

Various species of crustacean zooplankton often seem to have different spatial preferences for the occurrence in long and narrow reservoirs. Generally, large-sized zooplankton favours downstream regions with a lower trophic level, while small-sized species are more abundant in the usually more eutrophic upstream parts. This is in agreement not only with the abundance of planktivorous fish and therefore the strength of predation pressure, but also with the size-efficiency hypothesis (Brooks and Dodson, 1965; Gliwicz, 1990), which predicts that larger filtrators are more efficient at lower food levels. The downstream reservoir regions also offer an additional advantage for more vulnerable large-bodied crustaceans, as the deep strata devoid of fish may serve as a refuge against predation (Flik and Vijverberg, 2003; Hembre and Megard, 2003).

Such a longitudinal distribution pattern of zooplankton, explained primarily by fish predation, was reported for example from Ogochi Reservoir, Tokyo (Urabe, 1990). Relatively large-sized *Daphnia galeata* predominated near the dam site and its abundance declined towards the upstream region, while the abundance of other species showed the opposite pattern. The small cladoceran *Bosmina longirostris* was found especially abundantly at the site near the river inflow. Similarly, Pont and Amrani (Pont and Amrani, 1990) demonstrated the importance of fish predation for spatial distribution of differently sized cladocerans in Sainte-Croix Reservoir (S.E. France). While the densities of small *Ceriodaphnia pulchella* were similar all along the reservoir, larger-bodied *Daphnia* and *Diaphanosoma* species were significantly more abundant near the dam.

In our study, we focused on the spatial distribution of crustacean zooplankton in Czech canyon-shaped reservoirs with a special emphasis on interspecific differences within a single cladoceran genus, *Daphnia*. Species of this genus are among the most important grazers in temperate lakes and reservoirs, and have served as models in many ecological and evolutionary studies. The most common *Daphnia* inhabiting large European reservoirs are members of the *Daphnia longispina* group (Flössner, 2000): *Daphnia galeata* Sars, *Daphnia cucullata* Sars and *D. longispina* (O. F. Müller) (the genetic evidence suggests that both the pelagic *Daphnia hyalina* and pond and littoral *Daphnia rosea* should be considered only the morphs of the last taxon; Petrusek *et al.*, submitted). Members of the complex often coexist (e.g., Glagolev, 1986; Spaak *et al.*, 2000) and occasionally all three species may be found in the same water body. These taxa may differ in size (*D. cucullata* is in general the smallest and *D. longispina* may become the largest), susceptibility to fish predation (*D. cucullata* and its hybrids with *D. galeata* being least susceptible) (Spaak and Hoekstra, 1997; Spaak and Boersma, 2006), reaction to predator kairomones (Spaak *et al.*, 2000; Spaak and Boersma, 2006), as well as responses to varying food quantity and quality (Boersma and Vijverberg, 1994a, b; Repka, 1996; Seidendorf *et al.*, 2007). However, extensive interspecific size variation and phenotypic plasticity, as well as hybridisation and introgression, may cause substantial overlap in most of ecologically relevant traits.

Interspecific hybridisation within the *D. longispina* species complex, and especially among the three abovementioned species, is a common phenomenon (Schwenk and Spaak, 1995; Schwenk *et al.*, 1998). As hybrids may advantageously combine parental traits, under certain levels of fish predation they may become more efficient than parental taxa because of combination of relatively small size but high growth rate (Spaak and Hoekstra, 1995; Declerck and De Meester, 2003; Spaak and Boersma, 2006). It may therefore be presumed that different taxa of the species complex will dominate in environments differing in the food supply and predation pressure. Indeed, this is the case when different water bodies are compared – such as in the extensive survey of the taxonomic composition of the *D. longispina* complex in 31 Dutch lakes (Schwenk, 1997).

We tested the hypothesis that species and hybrids of the *D. longispina* species complex will also show different spatial distribution within a single water body along an environmental gradient. Distribution of food as well as predation pressure, together with the respective *Daphnia* species characteristics, would predict that *D. cucullata* should be more favoured in the upstream region, and *D. longispina* (*hyalina* morph) at the deep lacustrine region. In 11 long and deep dammed valley reservoirs, we therefore compared the taxonomic composition of *Daphnia* at three sites along the longitudinal reservoir axis, as well as in different layers of the vertically

Table I: Basic characteristics of investigated reservoirs and the sampling dates

Reservoir	Latitude	Longitude	Altitude (m asl)	Area (km^2)	Max. depth (m)	Length (km)	Year of construction	Sampling date
Horka	50°11′	12°30′	507	1.3	40	5	1970	8.7.2004
Knínički (Brno)	49°14′	16°31′	231	2.3	19	5	1940	20.7.2004
Římov	48°50′	14°30′	471	2.1	44	9	1978	10.7.2004
Šance	49°31′	18°25′	507	3	45	4	1971	21.7.2004
Seč	49°50′	15°39′	491	1.9	29	4	1934	14.7.2004
Stanovice	50°11′	12°53′	518	1.4	45	3.5	1978	9.7.2004
Trnávka (Želiv)	49°31′	15°13′	415	0.8	17	3.5	1982	12.7.2004
Vír	49°34′	16°19′	469	2.1	58	8	1959	15.7.2004
Vranov	48°54′	15°49′	352	7.7	45	18	1939	19.7.2004
Želivka (Švihov)	49°43′	15°06′	379	14	49	29	1975	13.7.2004
Žlutice	50°05′	13°08′	509	1.5	20	3.8	1968	7.7.2004

Alternative names under which some reservoirs are known are noted in parentheses.

stratified lacustrine part of the reservoir. The extent of *Daphnia* differentiation was also compared to the taxonomic composition of the whole community of planktonic crustaceans.

METHOD

Zooplankton samples for the analyses of longitudinal and vertical distribution patterns were collected from 11 Czech reservoirs between July 7 and July 21, 2004 (Table I, Fig. 1). The investigated reservoirs were selected to fulfil the following criteria: (i) canyon-shaped morphology (Fig. 1), i.e. reservoir length significantly longer than the reservoir width, and the depth increasing towards the dam and (ii) position on a watercourse ensuring that the zooplankton composition observed in the inflow region is shaped primarily by local processes and not by import from upstream water bodies.

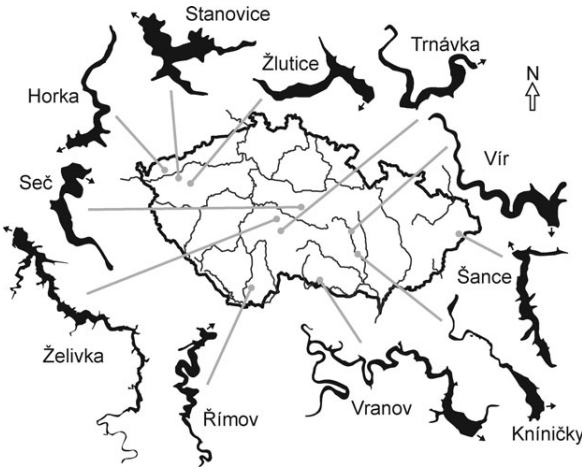

Fig. 1. Location of the studied reservoirs in the Czech Republic (principal rivers are shown) and schematic outlines of their morphology (outlines not to scale; see Table I for reservoir lengths). A small arrow indicates position of the dam and outflow of each reservoir.

From each reservoir, we collected five samples: upstream near the river inflow, in the centre of the reservoir and from the epilimnion, metalimnion and hypolimnion of the deepest area near the dam. The extent of the epi-, meta- and hypolimnion was determined by measurement of temperature and oxygen profiles immediately prior to sampling. Samples from the upstream end of the reservoir, central part of the reservoir and epilimnion near the reservoir dam were collected by vertical hauling using a plankton net of 170 μm mesh size. Samples from the metalimnion and hypolimnion were collected with a similar closing net.

Two types of zooplankton samples were collected at each station: firstly, a quantitative sample for the analysis of species composition of the whole crustacean community, which was preserved by 4% formaldehyde; secondly, a sample for the discrimination of species and hybrids within the *D. longispina* group, preserved on site by deep-freezing in liquid nitrogen. In total, 55 samples (five samples per reservoir, 11 reservoirs) were available for the analysis of crustacean communities. Hypolimnetic samples from three reservoirs (Horka, Knínički, Šance) contained no *Daphnia* (or negligible densities), so altogether 52 samples were available for the analysis of *Daphnia* spatial distribution.

Allozyme electrophoresis on cellulose acetate gels (Hebert and Beaton, 1989) was used for identification of *Daphnia* taxa within the *D. longispina* group. Approximately 40 randomly selected adult females were used from each sample. Two species-specific allozyme loci were scored: sAAT – amino aspartate transferase (EC 2.6.1.1) and AO – aldehyde oxidase (EC 1.2.3.1). It has been shown that sAAT and AO can be used as diagnostic markers to discriminate among *D. galeata*, *D. longispina* and *D. cucullata* and to identify their hybrids (Wolf and Mort, 1986; Gießler, 1997). *Daphnia galeata* is fixed for F (fast) alleles whereas *D. longispina* is fixed for S (slow) at both loci. *Daphnia cucullata* is fixed for F and S^{-} (very slow) alleles at AO and sAAT loci, respectively.

Using these markers, we distinguished eight genealogical classes present in our samples: three parental species (two homozygous species-specific alleles at each loci), two F1 generations of hybrids for *D. galeata* × *cucullata* and *D. galeata* × *longispina*, respectively (both loci heterozygous), two potential backcrosses of *D. galeata* × *longispina* hybrids (one locus homozygous for one species and the other heterozygous; these include also some F2 hybrids) and an F2 generation of hybrids for *D. galeata* × *longispina* (both loci homozygous, but one characteristic for the first species and the other for the second species). As the relative frequencies of backcrosses and the F2 generation of *D. galeata* × *longispina* hybrids were very low (only 2.05% of all individuals; Table II) we pooled them with F1 hybrids for the purpose of this study.

Multivariate analyses of the spatial distribution were performed with the software package Canoco for Windows 4.5 (ter Braak and Šmilauer, 2002). Linear multivariate methods (principal components analysis (PCA) and redundancy analysis (RDA)) were chosen based on the relatively low heterogeneity of the compositional data (Lepš and Šmilauer, 2003). Original counts were log-transformed and standardization by samples was used to focus the analyses on the differences in relative proportion of individual taxa. Centroids representing individual reservoirs or particular positions along the horizontal or vertical profiles were projected into ordination diagrams to aid in their interpretation.

Variation in the relative occurrence of *Daphnia* taxa or of other crustacean species was summarized using PCA. To visualize and test for the differences in community composition along the horizontal or vertical spatial profiles, partial RDA (using reservoir identity as a covariate) was performed, separately for each of the three sampling stations along the longitudinal gradient and for the three downstream samples along the vertical gradient. We pooled the data from the epi-, meta- and hypolimnion at the downstream sampling station for analysis of horizontal distribution. The mean density of individual taxa at this region was calculated as the weighted average of respective densities in all three vertical layers, with the weight of each layer determined by its volume (see Table III for results). All analyses were performed separately for the members of the *D. longispina* complex and for the remaining crustacean species. The significance of the relationship between species composition data and the selected spatial gradient was tested using a model-based type of Monte Carlo permutation test (ter Braak and Šmilauer, 2002).

RESULTS

Altogether 2103 *Daphnia* analysed from 52 samples were assigned to eight taxa of the *D. longispina* species complex based on their electrophoretic patterns. Table II summarises the distribution of these taxa in reservoirs and analyzed samples, and their proportion among all analysed individuals; densities and detailed spatial distribution in individual reservoirs is shown in Table III. The most common parental species from the complex was *D. galeata*, which occurred in 10 out of 11 reservoirs, and formed over 66% of all analysed individuals. The other two parental species and their hybrids were less frequent, but their distribution among reservoirs seemed to be relatively balanced: each of *D. longispina*, *D. cucullata*, *D. galeata* × *longispina*, as well as its backcrosses, was found in six reservoirs, *D. galeata* × *cucullata* in four reservoirs.

The distribution of taxa in individual samples and their frequency among all analysed individuals, however, followed a different pattern (Table II): *D. galeata* × *longispina* F1 hybrids were the second most common taxon after *D. galeata* both in the number of samples with their occurrence (19, i.e. 37%) and in the

Table II: Distribution of taxa (species and hybrids) of the D. longispina complex in the investigated samples

Distribution of the taxon	In reservoirs (n = 11)		In samples (n = 52)		No. of individuals (n = 2103)	
D. galeata	10	91%	47	90.4%	1400	66.6%
D. longispina	6	55%	15	28.8%	208	9.9%
D. cucullata	6	55%	13	25.0%	160	7.6%
F1 *D. galeata* × *longispina*	6	55%	19	36.5%	272	12.9%
F1 *D. galeata* × *cucullata*	4	36%	9	17.3%	21	1.0%
backcross *gal.* × *long.* × *long.**	4	36%	8	15.4%	33	1.6%
backcross *gal.* × *long.* × *gal.**	5	45%	7	13.5%	9	0.4%
F2 *D. galeata* × *longispina*	1	9%	1	1.9%	1	0.05%

*classes "backcross" encompass also certain proportion of potential F2 hybrids.
A taxon was considered present in a reservoir or sample if at least one *Daphnia* individual exhibited the corresponding combination of allozyme alleles (see methods). Details of the taxon composition in individual samples are listed in Table III.

Table III: Density and taxonomic composition of the D. longispina complex at the sampled sites

	Upstream		Middle		Dam: Epilimnion		Dam: Metalimnion		Dam: Hypolimnion		Dam
Reservoir	Density	Taxa	Density	Taxa	Density	Taxa	Density	Taxa	Density	Taxa	overall density
Horka	190 7	l 83%; gll 17%	1680 8	l 88%; gll 12%	2040 34	l 92%; gll 8%	600 15	l 78%; gll 22%	0	N/A	2640 10
Knínički	3650 120	g 32%; c 66% gl 2%	1230 22	g 73%; c 27%	420 21	g 95%; l 5%	240 3	g 58%; l 33%; gl 5%; gll 2%;	0	N/A	660 5
Římov	1410 32	g 100%	790 3	g 100%	3360 84	g 100%	60 1	glg 2% g 93%; c 2%; gc 5%	60 0.3	g 100%	3480 12
Šance	20 1	g 98%; gl 2%	740 3	g 68%; gl 32%	450 9	g 97%; gl 3%	490 7	g 17%; gl 83%	0	N/A	940 3
Seč	600 29	g 82%; c 13%; gc 5%	3780 38	g 60%; c 37%; gc 3%	2520 36	g 100%	140 2	g 94%; c 3%; gc 3%	320 4	g 92%; c 5%; gc 3%	2980 14
Stanovice	20 1	g 100%	3160 14	g 100%	1800 36	g 100%	80 2	g 100%	200 1	g 100%	2080 7
Trnávka	180 6	g 100%	2330 32	g 100%	320 8	g 100%	100 4	g 100%	40 1	g 77%; c 20%; gc 3%	460 4
Vír	100 3	g 35%; c 65%	2780 13	g 37%; c 13%; l 5%; gl 45%	550 11	g 90%; gl 10%	160 2	g 63%; c 10%; l 20%; gl 5%;	580 2	g 62%; gl 38%	1290 3
Vranov	250 6	g 2%; c 88%; gc 10%	2060 11	g 49%; c 20%; l 3%; gc 25%; gl 3%	30 1	g 24%; l 4%; gc 2%; gl 66%; gll 2%; glg 2%	600 6	F2gl 2% l 7%; gl 90%; gll 3%	190 1	g 3%; l 69%; gl 20%; gll 8%	820 3
Želivka	510 20	g 100%	1720 7	g 100%	960 32	g 84%; gl 10%; gll 2%; glg 4%	300 3	g 31%; l 15%; gl 54%	3 0.01	g 80%; gl 10%; gll10%	1260 3
Žlutice	800 32	g 96%; l 4%	2190 19	g 70%; gl 30%	3480 87	g 48%; gl 52%	400 10	g 15%; gl 85%	80 1	g 8%; gl 92%	3960 25

Density is expressed both per area (ind 0.01 m^{-2}; upper line) and volume (ind L^{-1}; bottom line); density at the dam is calculated for each vertical layer separately as well as integrated for the whole water column (last column). Taxa are abbreviated as follows: c – *D. cucullata*, g – *D. galeata*, l – *D. longispina*; gc, gl – F1 hybrids of the respective parental species; glg, gll – backcrosses (or F2 hybrids); F2gl – F2 hybrids.

number of analysed individuals (12.9%), being followed by both remaining parental species. *Daphnia galeata* × *cucullata* hybrids were found in nine samples (17%) but usually at relatively low densities, accounting for no more than 1% of all analysed individuals.

The longitudinal distribution of *D. cucullata* and the *D. galeata* × *cucullata* hybrids in all reservoirs with their presence is shown in Fig. 2a. Usually, these taxa were more common in upstream regions of the reservoir than at the dam – this trend was apparent in all reservoirs where the relative frequency of these taxa exceeded 5%, though it was reversed in two reservoirs, Trnávka and Římov, where their frequencies were low (below 4%). Vertical distributions of *D. cucullata* and its hybrid are not shown, as these taxa were encountered at the dam in only negligible densities. Interestingly, however, in four out of five reservoirs where either of them was found at the dam, they were recorded only in the meta- or hypolimnion. This affinity for deeper layers and a tendency to avoid the epilimnion at the dam is reflected in the ordination diagram in Fig. 3e.

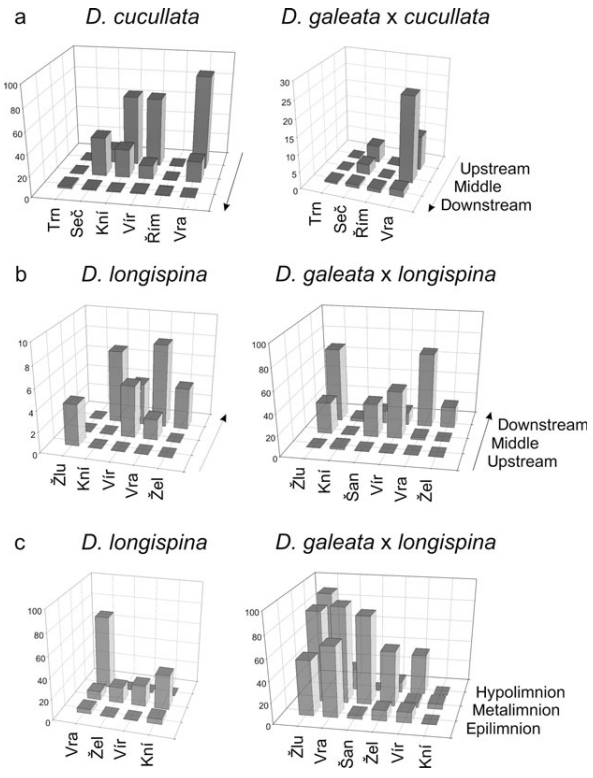

Fig. 2. Spatial distribution of *D. cucullata*, *D. longispina* and their interspecific hybrids with *D. galeata* in the studied reservoirs (only those with the presence of respective taxa are shown, reservoirs are indicated by the first three letters of their names). Arrows indicate direction of the water flow. (**a**) Longitudinal distribution of *D. cucullata* and *D. galeata* × *cucullata*; (**b**) Longitudinal distribution of *D. longispina* and *D. galeata* × *longispina;* (**c**) Vertical distribution of *D. longispina* and *D. galeata* × *longispina* hybrids at the dam (in the epi-, meta-, and hypolimnion). Y-axes indicate relative abundances in the respective samples; note that the graphs for *D. galeata* × *cucullata* and *D. longispina* differ in scale from the rest.

The longitudinal distribution of *D. longispina* and the *D. galeata* × *longispina* hybrid is shown in Fig. 2b. The trend for these two taxa was opposite to *D. cucullata* and *D. cucullata* × *galeata* hybrids: relative abundances of both *D. longispina* and *D. galeata* × *longispina* were higher at downstream stations and these taxa were either found in low densities or completely absent from upstream regions. The only exception to the trend in the *D. longispina* longitudinal distribution was found in the reservoir Žlutice. At this locality, hybrids *D. galeata* × *longispina* strongly dominated at the dam and middle sampling stations, while parental *D. longispina* was found at only a relatively small frequency (4 %) in the upstream part.

The vertical distribution of *D. longispina* and *D. galeata* × *longispina* in samples collected at the dam is summarized in Fig. 2c. In all reservoirs with the presence of these taxa, their relative frequency in at least one of the two deeper layers exceeded that in the epilimnion: relative frequencies in the metalimnion were higher than in the epilimnion in all but one case (the hybrid in Vír), and occasionally *D. longispina* or its hybrid dominated also in the hypolimnetic samples. This does not necessarily mean that these taxa always prefer meta- or hypolimnetic conditions, but certainly indicates that they are able to cope better with the environmental conditions in deeper strata than *D. galeata*. The absolute densities in the meta- or hypolimnion exceeded epilimnetic values in the following cases: *D. longispina* in Vír, Želivka and Vranov, and the *D. galeata* × *longispina* hybrid in Vranov and Šance.

Figure 2 indicates that *Daphnia* from the *D. longispina* species complex in deep canyon shaped reservoirs seem to be spatially structured on both longitudinal and vertical reservoir axes. The inter-reservoir comparisons in the previous three figures are also influenced by the fact that the zooplankton community structure in the five samples from each reservoir is not independent. In other words, the differences in the zooplankton communities among reservoirs are relatively higher than the differences among sampling stations. This can be seen from the PCA ordination diagrams in Fig. 3a and 3d where the scatter of reservoirs is larger than the scatter of locations within each reservoir.

The importance of the spatial structuring of the *D. longispina* species complex on both longitudinal and vertical reservoir axes was analysed by RDA (Fig. 3b and 3e). The differences in *Daphnia* taxa composition in longitudinal and vertical directions explained, respectively, 19 and 20% of the variability remaining after

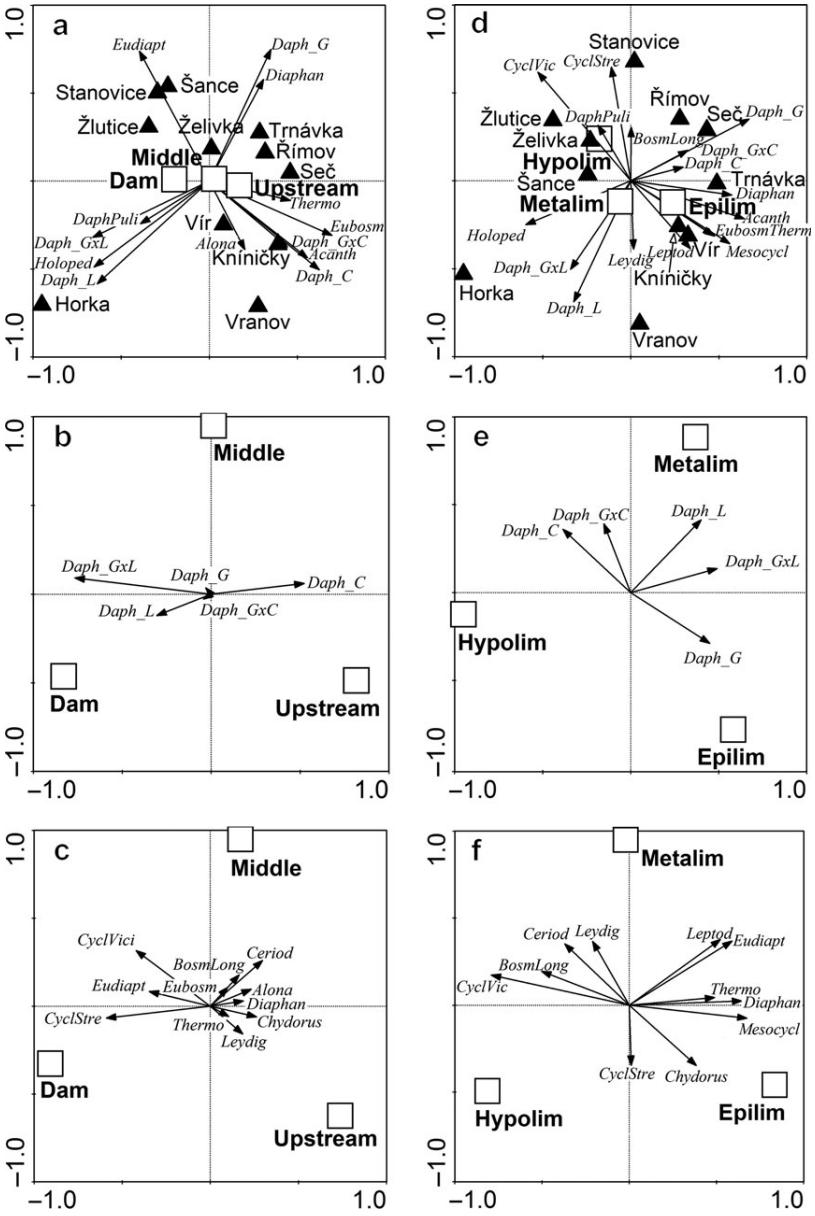

Fig. 3. Ordination diagrams of Principal Component (**a, d**) and Redundancy (**b, c, e, f**) Analyses of the zooplankton taxonomic composition along longitudinal (**a, b, c**) and vertical (**d, e, f**) gradients. Members of the *D. longispina* complex and other planktonic crustaceans are shown in separate plots; only the first two ordination axes are displayed. Position of reservoirs in plots (**a**) and (**d**) is shown by black triangles. Centroids of positions of samples collected from the dam, middle, and upstream stations, or from the epi-, meta-, and hypolimnion are shown by empty squares. Abbreviations: *Daph_G*: *Daphnia galeata; Daph_C*: *D. cucullata; Daph_L*: *D. longispina; Daph_GxL*: *D. galeata* × *longispina; Daph _GxC*: *D. galeata* × *cucullata; DaphPuli*: *D. pulicaria; Alona*: *Alona quadrangularis; BosmLong*: *Bosmina longirostris; Eubosm*: *Eubosmina coregoni; Chydorus*: *Chydorus sphaericus; Ceriod*: *Ceriodaphnia quadrangula; Diaphan*: *Diaphanosoma brachyurum; Holoped*: *Holopedium gibberum; Leptod*: *Leptodora kindtii; Leydig*: *Leydigia leydigi; Eudiapt*: *Eudiaptomus gracilis; Acanth*: *Acanthocyclops trajani; CyclStre*: *Cyclops strenuus; CyclVic*: *C. vicinus; Mesocycl*: *Mesocyclops leuckarti; Thermo*: *Thermocyclops crassus*.

correction for differences among reservoirs. These differences are statistically significant ($P = 0.04$ and 0.01, respectively).

Although the *D. longispina* complex is a very important component of reservoir zooplankton, the species richness of crustacean zooplankton is much higher. We therefore also tested for spatial structuring of all planktonic crustaceans except *Daphnia* using RDA (Fig. 3c and 3f). The differentiation of the crustacean community was highly significant ($P < 0.001$) in both the

longitudinal and vertical directions and explained 18 and 19%, respectively, of the variability remaining after correction for differences among reservoirs.

DISCUSSION

Our study clearly demonstrates that significant differences in the spatial distribution of species and interspecific hybrids from the *D. longispina* complex can be commonly observed in canyon-shaped reservoirs. The longitudinal gradients in the reservoirs apparently facilitate the coexistence of the different taxa within a single water body. However, their differing spatial distribution must also influence the local processes of interspecific hybridization and potential horizontal gene flow among parental species.

In general, the distribution of the respective taxa followed the expected pattern – the smallest species, *D. cucullata*, showed a preference for the upstream regions. The conditions there – increased planktivorous fish predation pressure, high food concentration and higher levels of phosphorus – certainly favour this small-bodied taxon (Gliwicz, 1990, 2003). Interestingly, the distribution of *D. galeata* × *cucullata* hybrids was much more variable, and less predictable. No such hybrids were found in the Knínički and Vír Reservoirs where both parents coexisted and longitudinal variation of *D. cucullata* abundance was observed (Fig. 2a). Nevertheless, the distribution of hybrids in Vranov Reservoir suggested that at least in some cases, regions in central parts of the reservoir may occur where hybrids are favoured.

This observation of an apparent spatially defined "hybrid zone" is in contrast with most observations of the co-occurrence of *Daphnia* hybrids and parental species in a single water body – little horizontal differentiation is to be expected in the relatively homogenous environment of natural lakes, so the changes in taxon dominance occur in a temporal rather than spatial scale. The temporal hybrid superiority model (Spaak and Hoekstra, 1995, 1997) explains the short-term success of hybrids by variation of factors favouring hybrids in time. However, trophic gradients in a reservoir provide a wide range of environmental conditions simultaneously, and such spatial variation may be of equal importance as the temporal one. We may therefore speculate that during most of the growing season, it might be possible to find a location along the gradient where hybrid genotypes could gain a competitive advantage.

In contrast to the previously mentioned taxa, shallow upstream regions of reservoirs seemed to be generally devoid of *D. longispina* or *D. galeata* × *longispina* hybrids, which found a more suitable environment further downstream (Fig. 2c). The pelagic form of *D. longispina* – *D. hyalina* – as well as its interspecific hybrids are known to be taxa that often exhibit depth-specific preferences or diel vertical migrations in vertically stratified environments (e.g., Pijanowska, 1992; Weider and Stich, 1992; King and Miracle, 1995; Sakwińska and Dawidowicz, 2005). The published affinity of *D. longispina* and *D. galeata* × *longispina* hybrids to the deeper layers largely corresponds with the pattern observed by us (Fig. 2c). Most likely, this vertical spatial distribution in reservoirs is also due to the potential presence of planktivorous fish. Unlike the usually non-migrating *D. galeata*, *D. longispina* may decrease the impact of visual predators by moving to deeper strata where fish are largely absent (Weider and Stich, 1992; King and Miracle, 1995). We do not know, though, whether the studied reservoir populations exhibited diel vertical migrations or whether animals encountered in the meta- and hypolimnion stayed in the deeper layers also overnight, as was the case for a genetically differentiated hypolimnetic *Daphnia galeata* subpopulation in Římov Reservoir (Seda *et al.*, in press).

The interesting pattern in the vertical distribution of *D. cucullata* and its hybrids at the downstream sites – an apparent strong preference for the deeper layers (Fig. 3e) – is unlikely to be explained by the presence of fish alone. These taxa are not typical migrating taxa, and their relatively smaller size would constitute a competitive advantage over other coexisting *Daphnia*, so they would most likely survive in the epilimnion even under fish predation pressure (Spaak and Boersma, 2006). It is probable that both *D. cucullata* and *D. galeata* × *cucullata* are mostly outcompeted by more efficient filtrators (Gliwicz, 1990; Boersma and Vijverberg, 1994a), such as larger *D. galeata* or *D. longispina*. This nevertheless does not explain their vertical distribution. Possibly, the observed pattern may be rather related to differences in the food quality between the epilimnion and deeper layers. For example, an important indicator of a food quality, C:P ratio, may affect the success of *Daphnia* species and clones, which respond differently to feeding on phosphorus-limited algae (Seidendorf *et al.*, 2007). Although certainly less abundant, the seston in deeper layers has higher phosphorus content than in the epilimnion (Garcia-Ruiz *et al.*, 1999). Similarly, differences in the content of other essential nutrients, such as fatty acids, may play a role. Under certain circumstances, the hypolimnetic seston is likely a more suitable food source than seston from the epilimnion. This is also supported by laboratory experiments from Římov Reservoir where *D. galeata* clones produced significantly larger clutches

(though partly outweighed by slightly longer post-embryonic development time) when grown in hypolimnetic rather than in meta- or epilimnetic water (Macháček and Seda, 2007).

On the other hand, both the horizontal and vertical distribution of the studied taxa within reservoirs may be affected by additional factors, such as parasitism. It has recently been shown that parasites may not only strongly influence the density of different taxa of the *Daphnia longispina* complex but even overturn the outcome of interspecific competition (Wolinska *et al.*, 2006). If the parasite densities differed among different reservoir parts or vertical layers, they could add to other, better known sources of environmental heterogeneity. Investigation of the parasite spatial distribution along environmental gradients may be a promising avenue of further research.

The common co-occurrence of different taxa of the *D. longispina* species complex in the studied reservoirs was to be expected. As far as we know, however, the horizontal variation in the frequency of hybrids and parents within a water body has not been reported before, apparently due to the little spatial variation of natural lakes, and possibly also due to the focus on inter-lake comparisons in previous studies rather than local spatial variation. Patterns in the *Daphnia* taxon composition observed by us may have important consequences especially in the period of sexual reproduction. The extent of interspecific hybridization or backcrossing should be dependent on the proportion of parental taxa; therefore, we may presume that differing proportions of hybrid resting eggs are produced in different parts of reservoirs. If the horizontal environmental gradient facilitates hybrid dominance at least locally in periods of sexual reproduction, we may presume that the extent of introgression or production of later-generation hybrids in some reservoirs may be higher than in more homogeneous lakes. Although our results so far do not suggest that F2 or backcrossed individuals form a significant proportion of the studied populations, detection of horizontal gene flow based on only two markers is rather crude. An analysis applying more variable markers, such as microsatellites already available for all members of the complex (Brede *et al.*, 2006), may reveal traces of more substantial gene flow.

Similarly as in *Daphnia*, the spatial distribution of other species of crustacean zooplankton in the studied reservoirs was heterogeneous, confirming the results of previous studies (Urabe and Murano, 1986; Dohet and Hoffmann, 1995; Thys *et al.*, 1998). The size structure of zooplankton communities followed expectations and conformed to the pattern observed in *Daphnia*, with upstream regions favoured by small-sized crustacean species (*Ceriodaphnia, Chydorus, Leydigia, Thermocyclops*) and downstream regions inhabited by larger crustaceans (cf. Fig. 3b and 3c). This is in accordance with presumed high fish predation pressure at these locations (Vašek *et al.*, 2003, 2004; Brosse *et al.*, 1999; Gido *et al.*, 2002) and corresponds to patterns first reported by Urabe (Urabe, 1990).

The spatial heterogeneity of zooplankton species composition in deep canyon-shaped reservoirs is apparently a general rule, and implies that an accurate picture of the pelagic community structure in these localities cannot be obtained without a balanced sampling design taking into account pronounced horizontal heterogeneity. Despite this heterogeneity, however, a number of species, including the most common daphnid *D. galeata*, were often found along the whole reservoir length. As the local subpopulations of such taxa are also influenced by the environmental gradient, we may presume that spatial structure could also be developed at the intraspecific level.

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CHAPTER 6

Petrusek A., Seďa J., Macháček J., Ruthová Š., Šmilauer P.:

Daphnia species and hybrids in reservoirs: patterns of hybridisation on ecological gradients in pelagic environment

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Solitary pine on the bank of Vír, one of the most beautiful (and when it comes to *Daphnia*, most interesting) reservoirs sampled in our project.

Daphnia species and hybrids in reservoirs: patterns of hybridisation on ecological gradients in pelagic environment

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Short title: ***Daphnia* hybrids in reservoirs**

Summary

Cyclical parthenogenetic mode of reproduction allows hybrids in the *Daphnia longispina* complex (Crustacea: Cladocera) to successfully compete with parental species in asexual phase of their life cycle, while still retaining ability of sexual reproduction. Hybridizing species *D. galeata, D. cucullata* and *D. longispina* (=*hyalina*) often coexist but differ in ecological requirements, which affects the frequency and directionality of hybridisation. We focused on the distribution of *Daphnia* species and hybrids along environmental gradients (particularly of food supply and size-selective predation) in eleven canyon-shaped reservoirs, and analysed patterns of carapace size and fecundity among coexisting taxa. Spatial distribution of species and hybrids agreed with their ecological characteristics; taxa showing different affinities along longitudinal profile differed in carapace size, which corresponded to the presumed gradient of fish predation. Only hybrids of *D. galeata* with other species were recorded; *D. cucullata* and *D. longispina* preferred opposite ends of gradients and never occurred in the same samples, this spatial segregation explaining absence of their hybrids. Patterns of taxon distribution were relatively stable in two consecutive summer seasons, although frequency of *galeata*×*cucullata* dropped substantially, even disappearing from some localities. We presume that spatial variation of environmental conditions in reservoirs may facilitate existence of local hybrid-dominated zones.

Keywords

canyon-shaped reservoirs, interspecific hybridisation, *Daphnia longispina* complex, ITS-RFLP, allozyme electrophoresis, cladocerans

Introduction

Reproductive mode of cladocerans, the cyclical parthenogenesis, makes this crustacean group an interesting model for ecological and evolutionary studies. Ability to switch between parthenogenetic reproduction during favourable conditions and sexual reproduction associated with formation of diapausing stages ("resting eggs"), and possibility to grow clonal lineages in the laboratory conditions, and therefore to disentangle the effects of environmental factors, genetic background and phenotypic variation makes cladocerans, particularly genus *Daphnia* (Crustacea: Anomopoda), favourite study organisms.

While little is known about interspecific hybridisation in other cladoceran genera, hybridisation between *Daphnia* species is a relatively common phenomenon. It has been documented in several species groups (Schwenk & Spaak 1995), however, the resulting reproductive modes of hybrids and subsequent evolutionary patterns differ substantially among them. Some hybrids are apparently sexually sterile and unable to form diapausing eggs (Hebert & Finston 1996); such genotypes may temporarily dominate their habitat while reproducing parthenogenetically but eventually get extinct. Others reproduce by obligate parthenogenesis with asexual formation of resting eggs, therefore escaping immediate extinction during unfavourable environmental conditions (e.g., Hebert et al. 1989a; Hebert & Finston 1996); this is often accompanied by polyploidy, especially in the Arctic (Dufresne & Hebert 1994).

Interspecific hybridisation within the *D. longispina* complex shows a different pattern: hybrids may produce resting eggs sexually, and therefore contribute to the gene flow among parental lineages. Hybridisation and potential introgression within this group has also ecological consequences, as hybridising members of this complex (*D. galeata* Sars*, D. cucullata* Sars*,* and the pelagic form of *D. longispina* (O. F. Müller) = *D. hyalina* Leydig) belong among the most common species of *Daphnia* inhabiting large European permanent water bodies. The occurrence of hybrids within the *D. longispina* complex was reported from many natural as well as artificial lakes (e.g. Hebert et al. 1989b; Wolinska et al. 2007; Seďa et al. 2007). In some localities, hybrids may be even more abundant than parental species (e.g., Spaak & Hoekstra 1993; Seďa et al. 2007).

Despite potential introgression and occasional hybrid dominance, parental species within the complex remain genetically distinct since interspecific hybrids exhibit various aspects of the hybrid breakdown. Hybrid F1 generations are apparently produced less frequently than would be predicted under random mating (Keller et al. 2007), and hybrids have lower hatching and survival rate than parental species (Schwenk et al. 2001; Keller et al. 2007) as well lower sexual reproductive success (Keller & Spaak 2004). It has been therefore proposed that reproductive isolation effectively exists among hybridizing species within the complex (Keller et al. 2007).

Hybrids are nevertheless fully competitive with parental species when reproducing parthenogenetically, and successful hybrid clones may at least temporarily avoid hybrid breakdown due to asexual reproduction. Experimental tests in the laboratory as well as in the field show that some *Daphnia* interspecific hybrids may exhibit superior population rates of increase during certain periods of time. This phenomenon has been attributed to heterosis (Repka et al. 1999) or a combination of advantageous traits of both parental species (Spaak & Hoekstra 1995; Declerck & De Meester 2003).

The temporal hybrid superiority hypothesis (Spaak & Hoekstra 1995) has been proposed to explain the often observed hybrid dominance in lakes by the temporal variation of environmental conditions at the localities, which may temporarily favour hybrid genotypes. The most important factors affecting the presence of species and hybrids of the *D. longispina* complex seem to be size-selective fish predation pressure and food level. *D.*

cucullata is known to be best adapted to fish predation, especially due to its small body size, but is competitively inferior to larger *Daphnia* species. Consequently, localities with high fish densities, especially eutrophic ones, are often inhabited by *D. cucullata*. *D. galeata* has very wide niche but is typical for lakes with moderate level of fish stock, and pelagic *D. longispina* morphs (*D. hyalina*) are most common in lakes with lower nutrient levels and predation pressure (Gliwicz 2003). However, other factors such as parasites (Wolinska et al. 2006, 2007) or food quality (Seidendorf et al. 2007) my also strongly affect the patterns of species coexistence.

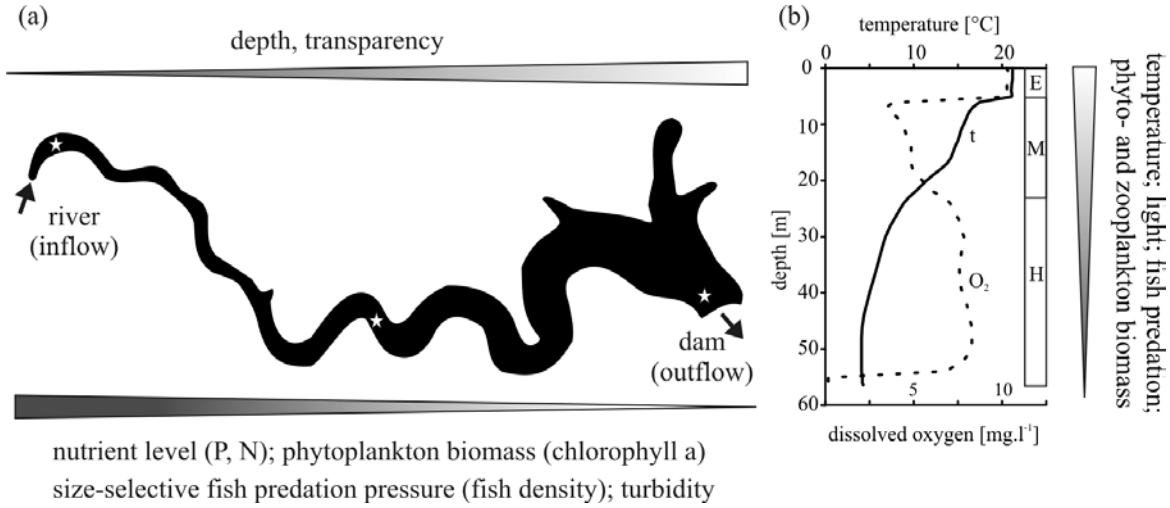
To study impact of variation of environmental factors affecting the success of the parental taxa and hybrids, three potential approaches are available. Firstly, zooplankton communities from various isolated waterbodies with differing ecological conditions may be compared (e.g., Keller 2007). Secondly, it might be possible to track long-term changes in community structure in localities with substantial temporal variation or gradual change of the key environmental factors (e.g., fish stock or trophic level). Recent development of paleogenetic methods allows for reconstructing past population genetic structure from resting egg banks (e.g., Weider et al. 1997; Jankowski & Straile 2003). Such an approach may nevertheless still require long-term data from particular localities, as hybrids may dominate the pelagic community without being adequately represented in the resting eggs, or parental taxa may exhibit strongly differing patterns in investment into sexual reproduction (Jankowski & Straile 2003; Keller & Spaak 2004; Keller et al. 2007).

Thirdly, we may examine variation in spatial distribution of the parental and hybrid taxa within water bodies in which substantial environmental gradients exist. For thermally stratified waterbodies such as temperate lakes, vertical gradients are characteristic (figure 1). Hybridizing *Daphnia* may show different spatial distribution on these gradients (Weider & Stich 1992; Seďa et al. 2007) but such differences are not substantial barriers for *Daphnia*, which often exhibit diel vertical migrations (e.g. King & Miracle 1995; Spaak et al. 2004). Standing waters with horizontal environmental gradients, on the other hand, are uncommon. Unlike terrestrial habitats, which often exhibit various gradients of factors affecting the distribution of taxa and often promoting the presence of interspecific hybrids in intermediate conditions, lakes and ponds tend to have relatively homogeneous conditions in horizontal direction (the only strong horizontal gradient being from the shallow littoral to the deeper pelagic zone) and ecologically different pelagic environments are usually isolated from each other in island-like nature (Schwenk & Spaak 1995). Nevertheless, water bodies with internal horizontal gradients may be particularly interesting, as dispersal of pelagic species is less limited by physical barriers, and uneven distribution of taxa in areas connected by water may suggest “barriers” of ecological nature, i.e. differential selection forces.

Our model systems, the deep canyon-shaped reservoirs, provide good conditions for studying hybridisation along environmental gradients in pelagic environment (figure 1). The well-defined main reservoir tributary as a point source of nutrient input, unidirectional flow of water down the reservoir, occasional turbidity stress from upstream sites, and significantly higher affinity of fish to upstream locations, are all significant environmental factors for the maintenance of a strong unhomogeneity of selective forces responsible for overall longitudinal heterogeneity of reservoir zooplankton (Urabe 1990; Seďa et al. 2007).

In our recent study (Seďa et al. 2007), we demonstrated significant differences in horizontal distribution of species and hybrids of the *D. longispina* complex within canyon-shaped reservoirs in Central Europe (Czech Republic). Presence of two or even three hybridizing species in a particular reservoir was common but each taxon showed different patterns. While *D. galeata* was ubiquitous species, *D. cucullata* was generally more

Figure 1
Schematic representation of key environmental gradients in canyon-shaped reservoirs affecting the zooplankton community. (a) horizontal gradients: water inflow brings in the limiting nutrients, which stimulate phytoplankton growth in the upstream reservoir regions. For grazers, food quantity (algal biomass, often expressed as chlorophyll a content) as well as quality (C:P ratio) is higher in the upstream part; however, this region is also more attractive for planktivorous fish, size-selective predators preferring larger zooplankton prey.
(b) vertical gradients in thermally stratified water bodies include, among other factors, changes of temperature, light intensity (i.e. visibility to predators), oxygen concentration, food quantity and predation pressure. While temperature and light always decrease with increasing depth, some parameters such as oxygen or chlorophyll concentration may show heterogeneous profiles. (Outline, temperature and oxygen profiles are of a typical canyon-shaped reservoir Vír, 8 km long and 58 m deep at the dam. Arrows and stars in (a) indicate direction of the water flow and our sampling stations; rectangles E, M and H in (b) indicate the extent of epi-, meta- and hypolimnetic layers, sampled separately.)



common in upstream regions of reservoirs (high-food and high-predation environment); *D. longispina* had an opposite tendency. Distribution of hybrids of *D. galeata* with the remaining two species partly overlapped with their non-*galeata* parents; the preference of *D. galeata×cucullata* hybrids for upstream regions was characteristic.

This data, however, were based on a single growing season. To test whether the observed patterns are highly dynamic or remain relatively stable over the winter (when *Daphnia* populations either disappear completely from the water column or at least experience severe bottlenecks), we followed the same localities in the next season. In this paper, we summarise our current knowledge on the *Daphnia* hybridisation in reservoirs. We discuss the patterns of taxon coexistence on environmental gradients and their temporal stability, describe a characteristic example of the canyon-shaped reservoir with hybridising *Daphnia* populations, and focus on the variation of the taxon body size and fecundity as population characteristics strongly influenced by the selection forces in reservoirs, which likely contribute to the hybrid success there.

Methods

Locality selection

To study spatial distribution of taxa and genotypes on environmental gradients within reservoirs, it is important to ensure that local processes rather than immigration or import of individuals are crucial for shaping the observed patterns. Therefore, we selected primarily localities in which import of zooplankton from the

Table 1.
Basic characteristics of investigated reservoirs, their occupancy by taxa of the *D. longispina* complex and number of analysed individuals (for allozymes, data for each year are shown separately; variation in numbers is caused by occasional absence of *Daphnia* in hypolimnion and/or scoring problems). Species (*D. galeata, cucullata, longispina*) are abbreviated by the first letter; taxa composing at least 5% in any sample from the particular reservoir are listed in uppercase, accessorial taxa recorded in lower relative abundances are in lowercase. Asterisk indicates locality where all "hybrid" individuals were probably backcrosses or later-generation hybrids; cases where one of the parental species of a hybrid was not detected in a particular reservoir are marked by "#". More details about reservoirs (geographic position, altitude, age) can be found in Seďa et al. (2007).

Reservoir	Area	Max. depth	Length	Taxa present		No. of individuals analysed by	
	(km^2)	(m)	(km)	2004	2005	allozymes	ITS-RFLP
Horka	1.3	40	5	L, G×L*#	L, G×L*#	160 + 220	N/A
Kníničky	2.3	19	5	C, G, G×L, L	C, G, L, g×l	177 + 218	145
Římov	2.1	44	9	G, c, g×c,	G, c	200 + 210	134
Šance	3	45	4	G, G×L#	G, G×L#	160 + 175	132
Seč	1.9	29	4	C, G, g×c	C, G, g×c	199 + 219	N/A
Stanovice	1.4	45	3.5	G	G, g×l#	199 + 200	163
Trnávka	0.8	17	3.5	C, G, g×c	C, G	200 + 219	N/A
Vír	2.1	58	8	C, G, G×L, L	C, G, G×L, L	200 + 258	141
Vranov	7.7	45	18	C, G×C, G, G×L, L	C, G, G×L, L	208 + 228	172
Želivka	14	49	29	G, G×L, L	G, G×L, L, c	200 + 199	130
Žlutice	1.5	20	3.8	G, G×L, l	G, G×L, L	200 + 215	N/A

watershed to inflow regions was unlikely to have strong effects, excluding especially those positioned in cascades immediately below other standing waters. The primary selection criterion was, however, the canyon-like morphology of reservoirs, i.e. elongated shape along the longitudinal axis, and increasing depth from the inflow regions towards the dam.

Altogether, we analysed longitudinal and vertical distribution of *Daphnia* species and hybrids in eleven reservoirs located in the Czech Republic (table 1), from which we collected samples in two consecutive summer seasons, in July 2004 and 2005. Schematic outlines and locations of the studied reservoirs are shown in Seďa et al. (2007), where also additional details are provided.

Sample collection and processing

The methods of sample collection and processing, identical for both years, were described in details in Seďa et al. (2007). At each sampling date, we collected zooplankton by vertical hauls of plankton nets (mesh size 170 μm) from three sampling stations along the reservoir main axis, which covered the longitudinal environmental gradients: upstream near the river inflow, in the centre of the reservoir, and at the deepest part of the reservoir near the dam (figure 1a, 2). Sampling at the downstream site focused also on the vertical spatial distribution in the stratified water body, therefore we collected separately zooplankton from epilimnion, metalimnion and hypolimnion, using the closing net for deeper layers. Prior to sampling, we measured vertical profiles of temperature and dissolved oxygen to evaluate the extent of stratification and determine the borders of epi-, meta-, and hypolimnion (figure 1b). From each site/layer, we collected a quantitative sample for the analysis of *Daphnia* abundance, which was preserved in 4% formaldehyde solution, and a sample for genetic analyses, deep-frozen on site in liquid nitrogen.

To determine the taxonomic composition of the *Daphnia longispina* complex at sampled sites, we analysed approximately 40-50 randomly selected adult *Daphnia* females from each sample (if available). Before genetic analyses, individuals were digitally photographed under the microscope for measuring the body size and evaluation of the general habitus, and their eggs were counted.

We selected two independent molecular methods for discrimination of species and hybrids within the complex. Primarily, we used allozyme electrophoresis on cellulose acetate gels (Hebert & Beaton 1989), which has been successfully applied in a number of studies. Two allozyme loci were scored: sAAT – amino aspartate transferase (EC 2.6.1.1) and AO – aldehyde oxidase (EC 1.2.3.1). It was shown that sAAT and AO can be used as diagnostic markers to discriminate among *D. galeata*, *D. longispina* and *D. cucullata* and to identify their hybrids (Wolf & Mort 1986; Gießler 1997). *D. galeata* is fixed for F (fast) and *D. longispina* for S (slow) allele at both loci; *D. cucullata* is fixed for S^- (very slow) allele at sAAT. The alleged potential for discrimination of all species using the locus AO (Gießler 1997), however, could not be fully used. Apparently, at least in some cases *D. cucullata* AO alleles may not be reliably differentiated from those of other species; in samples from the Czech Republic, bands of *D. cucullata* usually overlapped with those of *D. galeata*. Similarly, *D. cucullata* could not be well differentiated from *D. longispina* at AO in a North German lake (Spaak et al. 2004).

Using the allozyme markers, we classified *Daphnia* individuals as parental species or hybrid genotypes (Seďa et al. 2007). In case of the *D. galeata – D. longispina* hybridizing pair, individuals occasionally showed patterns clearly suggesting backcrossing or formation of later-generation hybrids. However, as the relative frequencies of such genotypes were usually very low (they formed no more than 2% of analysed individuals), we pooled them with apparent F1 hybrids for the purpose of this study. (Specific situation in one particular reservoir, Horka, is discussed below.)

Additionally, we used a DNA-based method – the restriction fragment length polymorphism (RFLP) of the nuclear ribosomal internal transcribed spacer (ITS) – to validate allozyme results on a subset of samples from 2004 (1017 individuals from seven out of eleven studied reservoirs; table 1). This method, originally developed by Billiones et al. (2004), uses the restriction of a short part of ITS1, the 5.8S ribosomal RNA gene, and a large part of ITS2 to obtain species-specific fragment patterns, additive in hybrid genotypes. As the original ITS-RFLP protocol commonly suffered from the point mutation in *D. galeata* alleles which caused its misidentification, we used a newly developed alternative double-digest protocol (Skage et al. 2007) to circumvent this problem.

To allow direct comparison of allozyme and ITS-RFLP patterns, DNA was prepared from *Daphnia* homogenates used for the allozyme electrophoresis. 2.5 µl aliquot of the homogenate was transferred to 30 µl of the solution containing H3 buffer and proteinase K (Schwenk et al. 1998) and incubated at 55°C for 6 to 10 hours; proteinase was subsequently inactivated by heating to 95°C for 10 minutes. The subsequent DNA amplification and restriction by overnight incubation with endonucleases Mbi I and Eco52I (Fermentas) mostly followed protocol by Skage et al. (2007). However, we used an alternative forward primer ITS-F-New (5'-GGT AAC CGC TGA ACC TCC TTC-3'; Skage et al. 2007), which provides longer amplified fragments to reliably differentiate between *D. galeata* pattern and potential uncut PCR products. The banding patterns were interpreted according to Skage et al. (2007); individuals combining fragments from two species were identified as hybrids even if bands of one species were more intense than those of another species. Occasional very weak bands were nevertheless not considered. Based on the fit between the two methods (see results) and the

morphology of studied individuals, allozyme data were used further for evaluating the distribution of species and hybrids.

Statistical analyses

Multivariate analyses of *Daphnia* spatial distribution were performed with the software package Canoco for Windows 4.5 (ter Braak & Šmilauer 2002). Original counts were log-transformed and standardisation by sample norm was used to focus the analyses on the differences in the relative proportion of individual taxa. To summarise occurrence patterns of *Daphnia* taxa, principal component analysis (PCA) was used. To test for the differences in the community composition along the horizontal profiles, partial redundancy analysis (RDA), using reservoir identity as a covariate, was performed. We pooled the data from epi-, meta- and hypolimnion at the downstream sampling station for analysis of horizontal distribution, weighting taxon abundances from different layers by the respective layer volume. The significance of the relationship between species composition and spatial gradient was tested using a model-based type of Monte Carlo permutation test (ter Braak & Šmilauer 2002).

Contribution of individual factors (unique effects of locality, of longitudinal or vertical position, and their interaction) was quantified using three different partial RDA models (Lepš & Šmilauer 2003). In two analyses, one of the two factors was used as the explanatory variable and the other as covariate, in the third analysis the interaction term was tested. The partial analyses were needed also for the two main effects due to the unbalanced nature of the sampling design.

Differences in the carapace size and fecundity of adult females among individual taxa were tested using a nested design ANOVA model. This model included, beside the effect of taxon, also the fixed effect of habitat (epilimnion, hypolimnion, metalimnion, inflow and middle) and the random effects of reservoir identity and sampling year (nested within reservoir identity). The size of carapace and fecundity of adult females were log-transformed to achieve homogeneity of variances.

Due to partially hierarchical nature of the ANOVA model, there is no straightforward way to perform multiple comparisons allowing to test differences among individual taxa. In addition, only some differences were of interest, as not all taxon pairs co-occurred in the same samples, neither they hybridised. Therefore, we tested the differences among *D. cucullata*, *D. galeata* and their hybrid, and among *D. longispina*, *D. galeata* and their hybrid separately, by performing six pairwise comparisons followed by Holm's correction for simultaneous tests (Holm 1979).

Results

Fit between taxon identification by allozymes and ITS-RFLP

Results of the two molecular methods used to identify taxa were generally in agreement, although they did not correspond to each other completely. Altogether, identification by sAAT allozyme pattern and ITS-RFLP agreed to each other for 86% of 1042 individuals analysed by both methods. About 13.7% of individuals would be classified differently by the two methods. Such misclassifications included most often: identifying a hybrid genotype on allozymes as a parental species by ITS-RFLP (53% of misclassified cases) or an apparently pure parental species (on allozymes) as a hybrid (37% of cases). However, slightly less than 10% of individuals showing disagreement between sAAT and ITS-RFLP (1.2% of the total) would be identified as pure but

different parental species (one of them always being *D. galeata*, the other either *D. cucullata* or *D. longispina*); typically, morphology of such animals was closer to the identification by sAAT. No case occurred in which *D. cucullata* individuals would be identified as *D. longispina* or vice versa, however, two individuals (from Kníničky and Vír reservoirs) homozygous for *D. cucullata* sAAT allele displayed ITS-RFLP banding patterns characteristic for *D. galeata×longispina* hybrids.

Disagreement of identification between allozyme and ITS-RFLP patterns were almost always observed at sites where species in question co-occurred and hybridisation was common. A notable exception was the reservoir Stanovice, in which only *D. galeata* was detected by allozymes in 2004 but 19% of individuals showed patterns characteristic for *D. galeata×longispina* hybrids; morphological characteristics of most of these individuals agreed with the allozyme identification. This reservoir was, however, the only one in which patterns of spatial distribution of parental species and hybrids would be substantially affected by the choice of the molecular method to identify the taxa. In other cases, the patterns obtained from both methods were roughly the same. Due to a better fit between morphology and allozyme patterns, we used allozyme data for subsequent analyses. However, occasional differences between the two marker systems may indicate that certain proportion of introgressed individuals remained undetected.

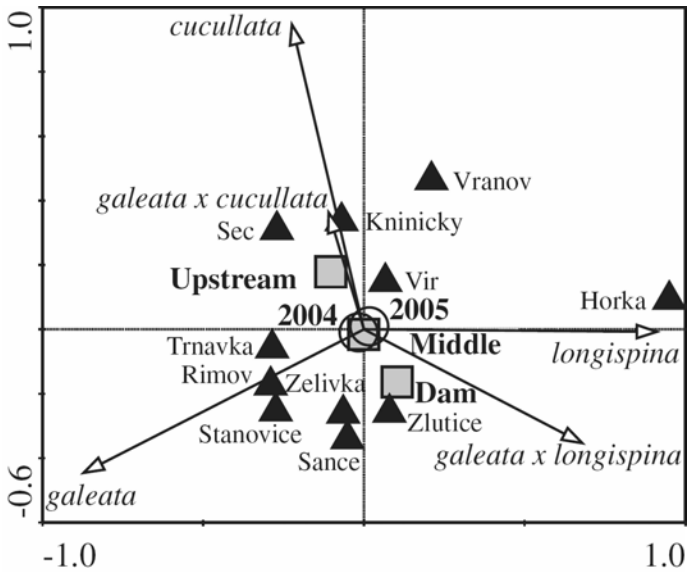
Patterns of taxon coexistence and their temporal stability

All three parental *Daphnia* species (*D. galeata*, *D. longispina*, *D. cucullata*) occurred commonly in the investigated set of reservoirs (table 1), and all three actually co-occurred in three of them. Out of three potential interspecific hybrids, however, we recorded only two; hybrids *D. longispina×cucullata* were never observed. *D. galeata×longispina* strongly dominated over *D. galeata×cucullata* in number of individuals as well as localities occupied (656 over 22 individuals out of 4464 analysed; seven vs. four localities). In three reservoirs with the presence of hybrid genotypes, we did not record one of their parental species (table 1). In one of these, reservoir Horka, we recorded only *D. longispina* and hybrid genotypes with *D. galeata*, the allozyme patterns however suggest that these individuals did not represent F1 hybrid generation but rather backcrosses towards *D. longispina*.

The taxon composition of the *D. longispina* complex significantly differed among localities (RDA, P=0.001, pseudo-F statistic 10.832). The results of principal component analysis (PCA) of the same data (figure 2) shows that the eleven samples reservoirs can be divided into three groups based on the presence of *Daphnia* taxa. Four reservoirs in the upper half of the ordination diagram are characterised by a significant occurrence of *D. cucullata*. Isolated position of Horka at the right edge reflects the fact that *D. galeata* was completely absent from this locality (the only case among investigated reservoirs), and only *D. longispina* and backcrossed *D. galeata×longispina* were present. Remaining six reservoirs contained a substantial proportion of *D. galeata*, *D. cucullata* was rare or absent. The positions of sampling sites within reservoirs in figure 2 (grey squares) indicates the general tendency of *D. cucullata* and its hybrids to occur more at upstream sites, and the opposite tendency of *D. longispina* and its hybrids. The *Daphnia* taxon composition within reservoirs was very similar in the two seasons 2004 and 2005 (table 1, figure 2).

To compare the extent of compositional variability along both horizontal and vertical gradients with the variation among the localities, we decomposed the total compositional variance in an ANOVA style, using partial RDA methods (table 2). In both cases, about two thirds of the variation could be explained by the

Figure 2.
Principal component analysis of the *Daphnia* taxonomic composition in 11 analyzed reservoirs during 2004 and 2005, based on data from the three sampling stations along the longitudinal reservoir axes. Reservoirs are shown by black triangles, sampling stations by grey squares, and different seasons by open circles. Taxa of the *D. longispina* complex are indicated by arrows. The first two principal axes, shown in this diagram, explain 82% of the total variation.



difference among localities. The differences in *Daphnia* taxon composition along longitudinal and vertical directions explained, respectively, 22% and 16% of the variability remaining after correction for differences among reservoirs; the effects of position along the gradient as well as the interactions "sampling station – locality" were always statistically significant. The residual variance, which includes the effect of season, was substantially lower for the longitudinal gradient, showing that the horizontal differentiation in the taxon composition was more stable than the vertical structure at the dam.

Apart from summarizing variability over all taxa and localities, we tested whether individual taxa exhibit significant differences in their relative abundance along both gradients. In longitudinal direction, the strongest response was observed for *D. galeata×longispina* hybrids occurring significantly more often in the downstream locations ($F=9.267$, $p<0.001$); other two significantly responding taxa were *D. longispina* ($F=4.951$,

Table 2. Hierarchical decomposition of the variance in taxon composition within the *D. longispina* complex along longitudinal and vertical gradients in the reservoirs. Numbers in parentheses give percentages after correcting for the differences among reservoirs.

	longitudinal gradient (upstream, middle, dam)			vertical gradient (epi-, meta-, hypolimnio		
effect	% variability	df	P	% variability	df	P
locality	66.3	10	0.001	64.2	10	0.0
position along the gradient	7.4 (22.0)	2	0.001	5.6 (15.6)	2	0.0
overlap locality - position	17.2 (51.0)	20	0.002	14.2 (39.7)	20	0.0
residuals (including the effect of year)	9.1 (27.0)	33	-	16.0 (44.7)	33	-

p=0.012) and *D. cucullata* (F=4.703, p=0.014), the former apparently preferring the downstream and the latter the upstream sites. Interestingly, these two species never co-occurred in investigated samples, at least not in proportions allowing their detection in analysed samples. Out of the three taxa commonly occurring at the downstream locations (*D. galeata*, *D. longispina*, and their hybrids), significant response to vertical differentiation was found for both parental species, *D. galeata* typically dominating in the epilimnion and *D. longispina* being more common in the deeper layers (F=12.284, p<0.001 and F=7.405, p=0.002, respectively).

Hybridisation in the Vranov Reservoir

The Vranov Reservoir, 18 km long and up to 45 m deep, is the second longest locality in our dataset, and it is the only one in which we found all three parental species and the two hybrids. The patterns observed at this locality may be used as a characteristic example of the effect of both longitudinal and vertical environmental gradients on *Daphnia* taxon composition, and on the body size and fecundity of the respective taxa. The distribution pattern of all taxa in the two seasons, shown in figure 3, illustrates the differing characteristics of taxon spatial distribution but also the temporal dynamics of such patterns. In 2004, the interspecific hybrids occupied intermediate position between their parental species. However, we observed remarkable changes in the occurrence of hybrids between the two seasons. *D. galeata×cucullata*, which was common in upstream and especially central part of the reservoir in 2004 (being actually the most common taxon at the middle sampling station) completely disappeared in 2005; on the other hand, the relative abundance of *D. galeata×longispina* increased in the second year.

The patterns in *Daphnia* size and fecundity along the horizontal axis of the Vranov Reservoir (figure 4) reflected the environmental gradients. We observed a gradual decrease in the carapace size and increase of *Daphnia* brood size towards the upstream region, this pattern being consistent in both years; both the sampling station and the taxon had significant effect on size and fecundity (ANOVA, $p<10^{-5}$ in all cases). *D. galeata×cucullata* hybrids were intermediate and significantly differed from their parents in size (adj. p <0.002)

Figure 3
Spatial distribution of parental species and hybrids along horizontal and vertical gradients within the Vranov Reservoir in two consecutive seasons. Pie charts show the relative abundance of the taxa at each sampling station (absolute *Daphnia* abundances differed among sites; see also Seďa et al. 2007).

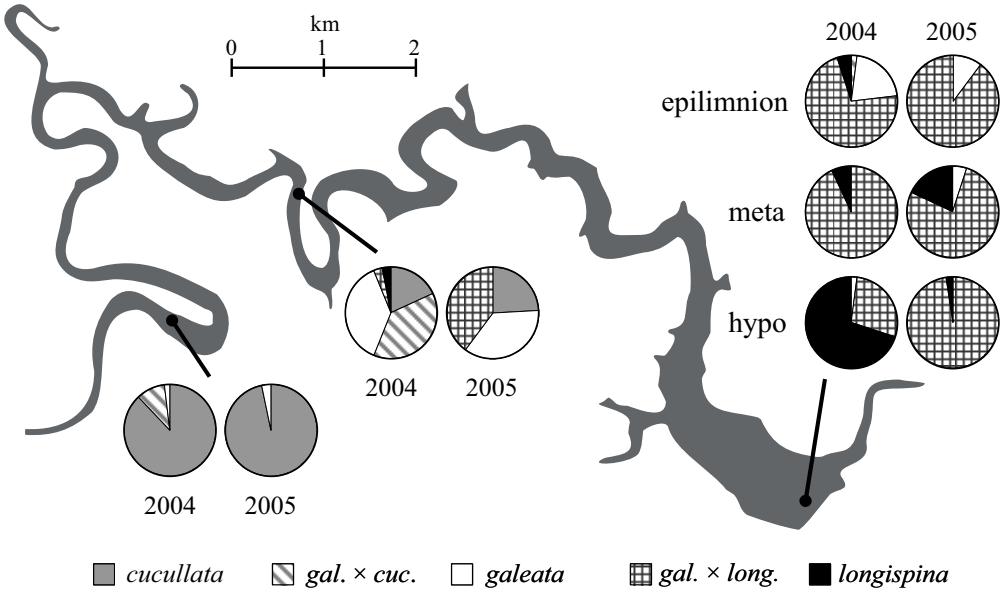
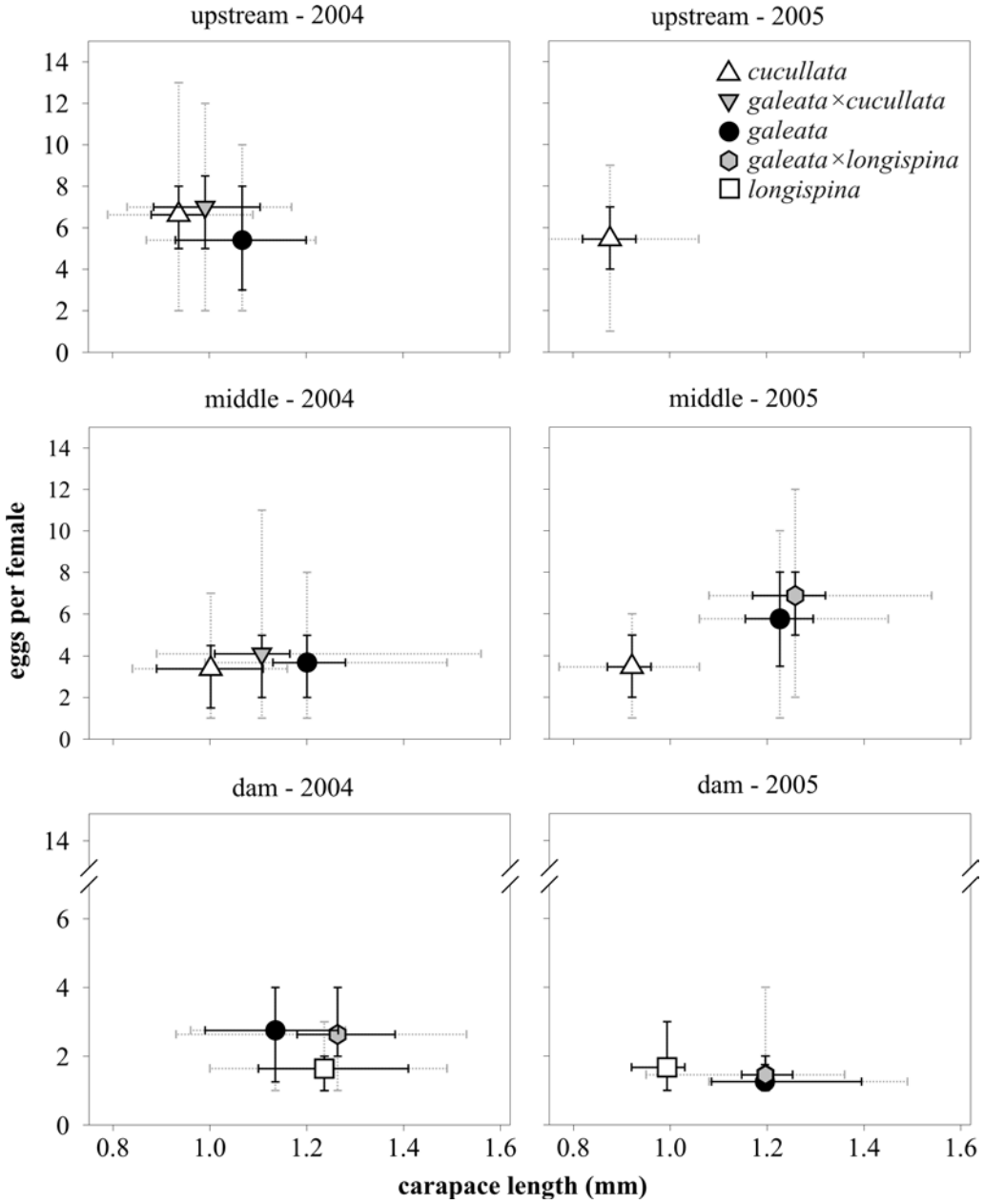


Figure 4
Relationship between carapace length and fecundity (egg number) of coexisting species and hybrids of the *D. longispina* complex in the Vranov Reservoir along its longitudinal gradient. Data from both seasons are compared. Symbols indicate mean values, solid black lines interquartile ranges and grey dotted lines minimal and maximal values. Note that the Y-axis for the dam samples differs from the rest.



but not in fecundity. The relationship among *D. galeata×longispina* hybrids and their parents was different. Size of the three taxa did not differ significantly, and the patterns of fecundity varied depending on the sampling station and date. However, in overall comparison, coexisting *galeata* and *longispina* did not differ in brood size, and hybrids had actually slightly but significantly higher fecundity (adj. $p<0.05$) than either of the parents.

Taxon-specific differences in body size and fecundity

To check whether such patterns of body size and fecundity are consistent in other localities as well, we analyzed differences in carapace and brood size between co-existing and spatially overlapping taxa in the three reservoirs where all three parental species occurred (Kníničky, Vranov, and Vír). Comparison of animals from

the same samples demonstrated highly significant effect of taxon identity on body size (ANOVA, $p<10^{-15}$) as well as on fecundity ($p<10^{-4}$).

We observed highly significant differences in each pairwise comparison among *D. cucullata*, *D. galeata* and their hybrid: the carapace size increased from *cucullata* to hybrid and to *galeata,* with corresponding adjusted p-values 1.32×10^{-15} and 0.015, respectively. However, there was no significant difference in the fecundity between the hybrids and parental species, and the pure species themselves differed only marginally, *D. cucullata* having slightly larger brood sizes than *D. galeata* co-occurring at the same sampling site (adj. p=0.041).

The ordering of average carapace size in the second hybridizing pair was *longispina* < *galeata×longispina* < *galeata*; however, only the difference between the two parental species was significant (adj. p=0.005), differences between either parent and hybrids were less strong (adj. p=0.073). The fecundity of all three taxa was very similar; the parental species did not differ in fecundity significantly. In contrast to the Vranov Reservoir alone, the hybrid had slightly lower brood sizes than either of the parents but this difference was significant only when compared with *D. galeata* (adj. p=0.01).

Discussion

The taxon distribution patterns on longitudinal profiles observed in the studied reservoirs in 2004 (Seďa et al. 2007) remained relatively stable over two consecutive seasons, showing that spatially differentiated *Daphnia* communities re-formed after the winter bottleneck. Hybrids mostly coexisted with the parental taxa, suggesting that they are formed locally within reservoirs, as has been shown for lakes (Spaak 1997). This is also in agreement with a common presence of hybrid eggs resting egg banks in studied localities (Vaníčková et al., unpublished). In reservoirs where all three parental species coexisted, differences in selection forces along horizontal gradients resulted in complete spatial separation of two potentially hybridizing parents (*D. cucullata* and *D. longispina*). This segregation, acting as ecological reproductive barrier, is the most likely cause of absence of *D. cucullata×longispina* hybrids in our samples. In general, the coexistence of these two species, which prefer opposite ends of predation and trophic gradients, is less likely than coexistence of either of them with *D. galeata*; this may explain the relative scarcity or absence of *cucullata×longispina* hybrids in lakes as well (e.g., Wolf & Mort 1987; Hebert et al. 1989b, Spaak 1997).

The numerical dominance of *D. galeata×longispina* over *D. galeata×cucullata* in our samples, however, is unlikely to be explained by different degree of coexistence of the two taxa. Both parental species pairs co-occur, and while species of the former show different preferences on the vertical profile (figure 3; see also Seďa et al. 2007), this certainly does not prevent successful hybridisation – on the contrary, *D. galeata×longispina* hybrids were common and apparently successful in reservoirs. Hybridisation between *D. galeata* and *D. cucullata* is common as well – in a preliminary analysis of reservoir resting egg banks (Vaníčková et al., unpublished), hybrids with *D. cucullata* were actually found more frequently than those with *D. longispina*, and *D. galeata×cucullata* hybrid eggs were common in resting egg bank of the Kníničky Reservoir, where such hybrids were absent from the active population.

A reduced hatching success of hybrids (Schwenk et al. 2001, Keller et al. 2007) combined with the competition with parental species may partly explain this absence of hybrids in the water column. However, our long-term data on clonal composition from Římov Reservoir (Seďa et al., unpublished) as well as the temporal

pattern of taxon dominance in the Vranov reservoir (figure 3) suggest another factor contributing to the scarcity of *galeata×cucullata* hybrids: apparently, they are less likely to overwinter than *galeata×longispina*, and new hybrid genotypes have to be recruited from resting egg banks. Higher sensitivity of *D. galeata×cucullata* in winter may be due to their smaller size (and therefore lower efficiency under low-food winter conditions) or because shallower parts of reservoirs, where they occur more often, provide less suitable environment in winter and the bottlenecks in *Daphnia* populations are much more severe there than in the deep parts (Seďa et al., unpublished). On the other hand, at least some successful *D. galeata×longispina* clones likely survive in the deep lacustrine parts of reservoirs and contribute to the next season's community. A long-term survival of such hybrid genotypes has been documented in various European lakes (e.g., Spaak & Hoekstra 1993, Jankowski & Straile 2004).

Of all factors affecting the spatial heterogeneity of *Daphnia* taxonomic composition, we presume the intensity of size-selective fish predation and different susceptibility of different taxa to it are crucial (Spaak & Hoekstra, 1997; Spaak & Boersma, 2006). Fish predation pressure physically limits the size of *Daphnia* occurring in different parts of the water body, and only when the predator pressure is relaxed under certain threshold, interspecific competition among larger taxa come into play. Fish are preferentially present in upstream reservoir regions (Vašek et al. 2004); this fact is reflected in the small size of *Daphnia* present there (figure 4). The tendencies in longitudinal arrangement of the taxa from the hybridizing pair *D. galeata* / *cucullata* agree well with significant differences in carapace size of the respective taxa, favouring the smallest *Daphnia* upstream.

The dominance of larger *Daphnia* further downstream corresponds with their higher filtering efficiency (Gliwicz 2003), small *D. cucullata* or its hybrids being probably outcompeted by larger taxa. Predation pressure may contribute not only to the absence of *D. longispina* and its hybrids in the downstream regions of eutrophic reservoirs but also to the vertical distribution of these taxa at the dam. Although the differences in size of coexisting taxa are rather low, hardly giving one taxon selective advantage against predators, species and hybrids differ in the predator susceptibility due to their differing vertical distribution in the stratified water column. Additionally, differences in life-history traits certainly contribute to changes in community composition. Responses to food quality and quantity, however, highly depend on individual genotypes (Seidendort et al. 2007). Fecundity of at least some hybrid clones may certainly exceed their coexisting parental species (figure 4); nevertheless, the brood size variation seems to be locality-dependent – in Vranov, *galeata×longispina* hybrid exhibited higher fecundity than parents but overall the pattern was opposite. This may due to different genetic background of hybrids in different reservoirs, or due to their response to temporally or spatially varying environmental conditions (Spaak & Hoekstra 1995).

The existence of longitudinal environmental gradients in reservoirs may actually improve conditions for hybrid presence. Intermediate characteristics of interspecific hybrids may promote their intermediate spatial distribution in comparison with the parental species, in a way observed in the Vranov Reservoir. This pattern of "hybrid-dominated zone" in the pelagic environment suggests that in the presence of environmental gradients, hybrid dominance may be not only a temporal phenomenon but may have also a specific spatial aspect. We hypothesise that somewhere along reservoir axes, environment favouring intermediate phenotypes may exist over most of the growing season. Such areas would be certainly spatially restricted (i.e., limited to only a certain section of the reservoir longitudinal profile) and possibly shifting along the reservoir axis, depending on the

stability of environmental gradients. Three sampling stations in our project only very crudely covered the longitudinal heterogeneity, so it is possible that denser sampling would reveal more areas where relative abundance of hybrid genotypes exceed those of the parental species. The long-term hybrid presence in reservoirs may have important consequences on the gene flow among parental species during induction of sexual reproduction, as zones of hybrid dominance may increase likelihood of the production of later-generation hybrid or backcrossed genotypes.

Our results show that the two molecular methods currently used for studying hybridisation in the *D. longispina* complex – allozyme electrophoresis and ITS-RFLP – while generally in agreement, are not completely complementary. The choice of the marker system would not substantially affect the general patterns of taxon spatial distribution; however, a more detailed analysis focusing on the sources of variation is clearly needed. It is worth noting that in the studied Czech reservoirs, the degree of disagreement between the new ITS-RFLP protocols and other markers is higher than in other localities, such as mountain or boreal lakes (Skage et al. 2007).

It is not unlikely that the reservoir environment, which offers suitable conditions to species adapted to strikingly different environment, is also suitable for introgression among species to a higher degree than in other, more homogeneous lakes. This is supported by the fact that individuals from localities with more coexisting taxa showed more often a disagreement between the two molecular markers. However, patterns of introgression and maintenance of polymorphism may be complicated by various specifics of the ITS rDNA region (multiple copies in the genome, concerted evolution). In at least one reservoir (Stanovice), it is likely that the "hybrid" patterns detected by ITS-RFLP were not because of contemporary local hybridisation and the alleged *longispina* allele had been introgressed into parental genotypes earlier. Apparently, a detailed comparison of different marker systems, and use of a wide array of markers, preferably microsatellites (Brede et al. 2006), is needed to reveal the fine-scale patterns of potential introgression.

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CHAPTER 7

Seďa J., Kolářová K., Petrusek A., Macháček J.:

Daphnia galeata in the deep hypolimnion: spatial differentiation of a "typical epilimnetic" species

Hydrobiologia, doi:10.1007/s10750-007-9075-4

Although it is possible to find such a pretty helmeted *Daphnia galeata* in Czechia, they are rare. This individual comes from an oligotrophic sandpit, those from reservoirs are less attractive (photo: J. Fott).

note: occasional problem with typesetting of multilocus genotypes will be corrected in the final version

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CLADOCERA

Daphnia galeata in the deep hypolimnion: spatial differentiation of a "typical epilimnetic" species

Jaromir Seda · Katerina Kolarova · Adam Petrusek · Jiri Machacek

Abstract *Daphnia galeata* is traditionally regarded to be a non-migratory species, which lives in warm epilimnetic waters. Depth segregation or vertical migration is usually attributed to other *Daphnia* species such as *D. hyalina* or *D. longispina.* In a two-year study, we found that in a deep, dammed-valley reservoir (Římov Reservoir, Czechia) the majority of the population of *D. galeata* lives in the warm epilimnetic waters during the summer months, but some specimens of this species could be always found in the deep strata as well. This hypolimnetic subpopulation stays in the cold hypolimnetic water and does not migrate. The abundance of hypolimnetic *D. galeata* does not exceed one specimen per litre and usually shows seasonal variation (minimal densities in early spring, maximal in late summer). Using allozyme electrophoresis, we found that the subpopulation from the deep hypolimnion was clearly genetically differentiated from the population in the epilimnion. We found significant differences in both allele and multilocus genotype frequencies; the F_{ST} values at most sampling dates exceeded 0.05. However, the spatial segregation between the epilimnetic and hypolimnetic subpopulations is not permanent. The reservoir is dimictic and hence, at least twice per year, all vertically segregated parts of the population are mixed together. Our results suggest that the deep hypolimnetic subpopulation is repeatedly re-established in spring by deepwater "colonists", at least some of which seem to be ecologically specialised for the hypolimnetic conditions, and dominate the hypolimnion by the end of the season. The genetic differentiation is likely the result of both the different depth preferences of various *D. galeata* clones and different selective pressures in the epilimnion and hypolimnion.

Keywords Vertical distribution · Clonal structure · Hypolimnion · Reservoirs

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Introduction

Since the classic studies of Stich & Lampert (1981, 1984), *Daphnia galeata* has generally been considered to be a non-migratory species which more or less constantly inhabits warm epilimnetic waters. Depth segregation or vertical migration is usually attributed to other pelagic *Daphnia* species such as *D. hyalina*/

D. longispina, or to their hybrids (Weider & Stich, 1992; King & Miracle, 1995; Lampert et al., 2003). This ecological concept seems to be widely accepted, and there are only sporadic indications contradicting its general validity in the literature.

The introduction of species-specific molecular markers, such as allozymes, has improved the reliability of species diagnostics within the *D. longispina* species complex in field surveys (Wolf & Mort, 1986). An allozymic survey of the *D. galeata-hyalina* species complex in Lake Constance has confirmed that in the daytime samples, *D. galeata* was the dominant taxon in the upper 20 m of the water column, while *D. hyalina* and *D. galeata* × *hyalina* hybrids were most abundant below 30 m during summer (Weider & Stich, 1992). The data supporting this pattern, however, were not without exception. On at least two sampling occasions (June, August) some *D. galeata* were found in the depths between 30 and 60 m but the authors did not comment on this finding. Knowing the long-term history of the complicated *Daphnia* hybridisation processes in Lake Constance (Jankowski & Straile, 2003, 2004) we cannot exclude that these *D. galeata* in the deep layers at the end of the 1980s in fact represented backcrosses or later-generation hybrids.

The concept of *D. galeata* as a typical epilimnetic species has also influenced the choice of *Daphnia* species in experimental studies regarding depth distributions and diurnal migration patterns within the *D. longispina* complex. In papers where the specific aspects of depth distribution or vertical migration were addressed, the taxa of choice for the experiments were *D. hyalina* or *D. galeata* × *hyalina* hybrids (Dawidowicz & Loose, 1992; Dodson et al., 1997; De Meester & Weider, 1999; Lass et al., 2000; Lampert et al., 2003; Kessler, 2004; Kessler & Lampert, 2004).

Bast et al. (1993) reported significant differences between day and night vertical distributions of *D. galeata* in the eutrophic lake Meerfelder Maar (south-western Germany). They found *D. galeata* in low numbers in relatively deep strata at the beginning of the vegetative season (May) when there was still enough oxygen. However, these deep strata animals disappeared with further seasonal development (July, October). Unfortunately, the authors' findings on *Daphnia* vertical distribution were poorly documented by data on temperature and oxygen stratification.

Müller & Seitz (1993) found that electrophoretically identified clonal groups of *D. galeata* were not homogeneously distributed and showed distinct differences in diel vertical migratory behaviour in the eutrophic lake Neuhofener Altrhein (south-western Germany). The vertical distribution pattern of *D. galeata* was characterised by their preference for the mid- or deep metalimnion during the day and subsequent upward migration to the upper metalimnion, or deep epilimnion, during the night. No *D. galeata* were reported to live in the hypolimnion in the lake. Surprisingly, however, the deepest occurring *Daphnia* in this particular study was a hybrid clone of *D. galeata* × *cucullata*, although *D. cucullata* is always regarded as an obligatory epilimnetic species.

Additional indications that *D. galeata* cannot be regarded as an exclusively non-migrating epilimnetic species were recently shown by Winder et al. (2004), investigating the vertical behaviour of *D. galeata* in the lake Oberer Arosasee (Switzerland). They found that *D. galeata* inhabited deep water strata with a maximum chlorophyll concentration during the day. At night, these *Daphnia* migrated upwards, out of the food-rich environment, showing a trade-off between food and temperature.

The present study documents another, not very well known aspect of the vertical distribution of *D. galeata*. This species occurs in the deep eutrophic Římov Reservoir (Czech Republic) throughout the year (Sed'a & Kubečka, 1997) with a great majority of the population living in the epilimnion in summer. However, detailed and intensive sampling of deep-water strata has revealed that during most of the period of summer stratification a certain, though minor, part of the *D. galeata* population was more or less permanently segregated to the deep hypolimnion. The aim of our study was to analyse the clonal structure of this segregated part of the population, its dynamics within a season, and compare it with the majority of the *D. galeata* population in the epilimnion; to test the hypothesis that the deep hypolimnetic *D. galeata* are genetically distinct from other *D. galeata* in the reservoir.

Methods

Římov Reservoir is located in south-west Czechia (48°48–50′ N, 14°29′ E), at an elevation of ca. 470 m

a.s.l. The reservoir is approximately 9 km long, with a surface area of 2.1 km^2, a volume of 33.1×10^6 m^3, and a maximum depth of 45 m. It was formed after damming of the river Malše, which is the main and only significant inflow. Its long-term annual average water flow varies from 2.8 to 7.0 m^3 s^{-1}. The reservoir retention time usually ranges between 50 and 165 days (on average approx. 100 days), depending on hydrological conditions. More hydrological data on Římov, and its comparison with other Czech reservoirs, are available in Brandl et al. (1989) and Hejzlar & Straškraba (1989).

Samples of *Daphnia* for analysis of their vertical distribution and population genetic structure were collected during the vegetation period (May–September) in two consecutive years, 2003 and 2004. The samples were taken four times a year at the deepest point of the reservoir near the dam (ca. 42 m—depending on the reservoir water level). Temperature and oxygen profiles were measured before each sampling, which enabled us to distinguish four discrete depth strata: epilimnion (usually 0–4 m), metalimnion (4–8 m), hypolimnion (12–17 m) and deep hypolimnion (25 or 30 m to bottom). Unfortunately, data on oxygen concentration from July 26, 2004 are not available because of an oxygen probe failure.

Zooplankton samples were collected with closing nets (mesh size 170 μm) from four defined depth strata during the day, and preserved in 4% formaldehyde. As the abundance of *Daphnia* sometimes differed by nearly two orders of magnitude between the epilimnion and deep strata, two different closing nets were used for zooplankton sampling. A net with a mouth diameter of 24 cm was used for the epilimnion, and one with a diameter of 40 cm for the deep strata. Animals for genetic analyses were collected only from the epilimnion and the deep hypolimnion. Immediately after sampling, *Daphnia* were frozen in liquid nitrogen. From each sample, we used about 50–60 randomly selected adult females for genetic analysis.

The sampling always started from the surface layer to avoid contamination among the sampled strata. For the same reason, each sampled layer was separated from the next one by an unsampled region. Samples from adjacent lower layers (metalimnion, upper hypolimnion and deep hypolimnion) were always separated by several meters; those from epilimnetic and metalimnetic layers were closer to each other, especially in cases of a strong thermal gradient in the metalimnion at the beginning of the year. Potential diurnal vertical migration behaviour was checked by three additional mid-night samplings in 2003 (June, July and August), which allowed us to compare the day and night distribution.

The genetic structure of the *Daphnia* population was assessed by allozyme electrophoresis on cellulose acetate gels (Hebert & Beaton, 1989). Three allozyme loci were scored: sAAT—amino aspartate transferase, EC 2.6.1.1; GPI—glucose-6–phosphate isomerase, EC 5.3.1.9; and PGM—phosphoglucomutase, EC 5.4.2.2. The sAAT locus was used for taxon identification; only individuals homozygous for FF allele (considered specific for *D. galeata*; Wolf & Mort, 1986) were used in further analyses.

Bi-locus genotypes (BLG) were assembled for each individual from the allele composition at two polymorphic loci (GPI and PGM). The TFPGA software package (Miller, 1997) was used for most data analyses (computation of allelic frequencies, exact test of the differences in allele frequencies, calculation of F_{ST} as the measure of genetic differentiation between populations, hierarchical analysis of F_{ST} with depth nested in sampling date nested in year to see what level contributes most to the genetic differences, and the test for Hardy–Weinberg equilibrium). Significance of differentiation between the epilimnetic and subhypolimnetic subpopulations was tested by randomisation of individual BLGs between samples (i.e., without expectations of Hardy–Weinberg equilibrium) in the program FSTAT version 2.9.3 (Goudet, 2001). F_{IS} values per locus and each epilimnetic and hypolimnetic subpopulation (sample) were calculated from the formula $F_{IS} = (H_e - H_o)/H_e$ (H_e—heterozygosity expected, H_o—heterozygosity observed, obtained from TFPGA). Positive values of F_{IS} indicate heterozygote deficiency, negative values indicate heterozygote excess.

The significance of differences in BLG frequencies was evaluated by RxC tests in the software BIOMstat v. 3.1 (Rohlf & Slice, 1996). The cluster analysis of the analysed samples based on their BLG composition was calculated in STATISTICA 5.5, using the pairwise Euclidian distances between samples clustered by Ward's method. The BLG diversity within samples was calculated as a complement of the maximum likelihood estimator of Simpson's index ($1 - D$).

Changes in the BLG structure in the same layer between adjacent sampling dates (turnover rate) were expressed by the complement of Morisita–Horn index (1 − MH) to take into account not only presence or absence of individual BLGs but also changes in BLG frequencies. Both diversity indices (Magurran, 2004), including their standard error estimates, were calculated in the program SPADE (Chao & Shen, 2003–2005).

Results

Daphnia vertical distribution

The seasonal variation of daytime abundance of *D. galeata* in the vertical profiles, together with the temperature and oxygen characteristics of their environment, is shown in Fig. 1A. An overwhelming majority of the daphnids inhabited the epilimnion. However, some specimens of *D. galeata* were also found in deep strata. The abundance of this deep hypolimnetic subpopulation, at 25–35 m, can be even higher than the abundance of *Daphnia* in the upper hypolimnetic layer, i.e., 10–25 m, as demonstrated by 2003 samples. The seasonal development of abundance of this hypolimnetic population shows a slight gradual increase from May to September. Temporal changes of hypolimnetic *Daphnia* abundance are clearly different from the development in the epilimnion where *Daphnia* peaked in June (Fig. 1A).

The data in Fig. 1A illustrate the *D. galeata* distribution during the day, we also determined the night time abundances of *D. galeata* in the vertical profile on three sampling dates in 2003 (Fig. 1B). There were no significant differences between vertical distributions of *D. galeata* during the day and night when tested with a RxC test.

Genotype variability and *Daphnia* clonal structure

The analysis of *Daphnia* polymorphism at the sAAT locus was used as a diagnostic marker to discriminate

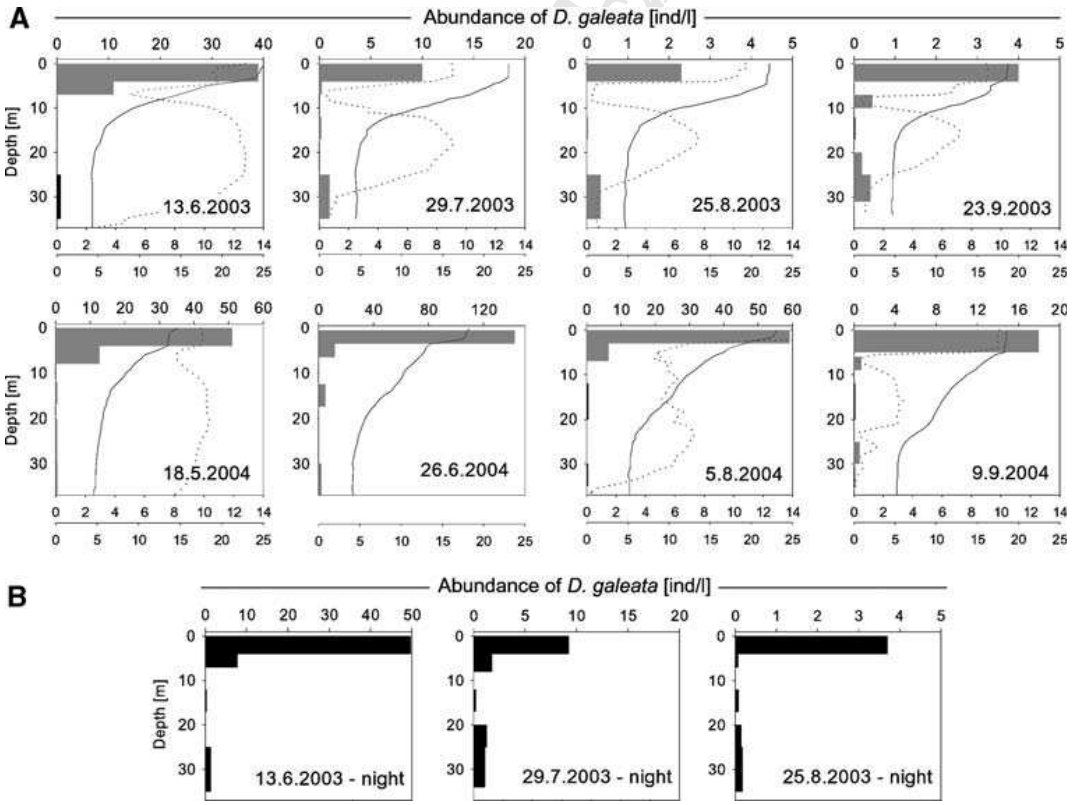

Fig. 1 Vertical profiles of daytime (**A**) and mid-night (**B**) abundance of *D. galeata* in Římov Reservoir (*horizontal bars*). Identification of *D. galeata* was based on morphological characteristics. Vertical profiles of temperature (*solid lines*) and oxygen concentration (*dotted lines*) are shown. *X*-axis: the upper one is oxygen (0 till 14) and the lower one is temperature (0 till 25). Oxygen data are unavailable for the June 2004 sampling

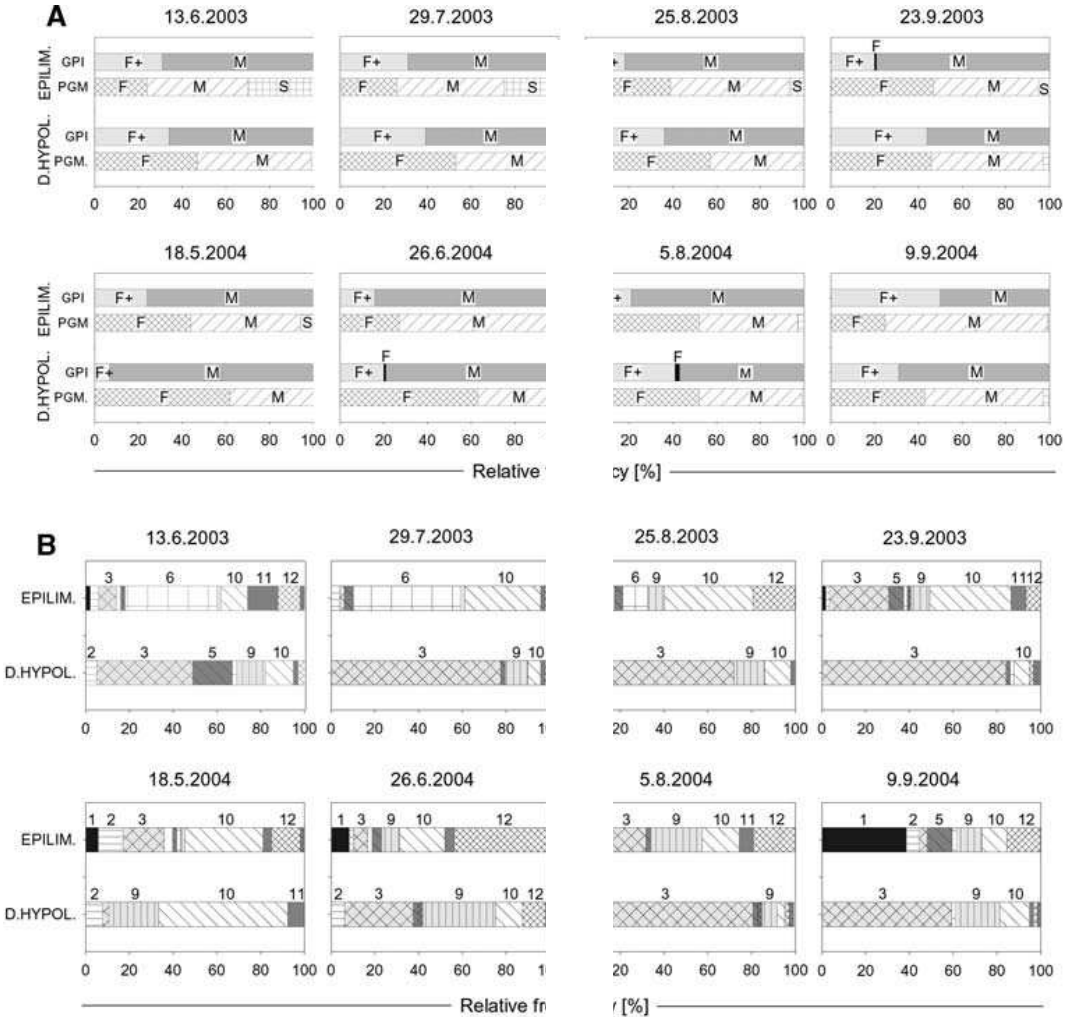

Fig. 2 Differences in genetic structure of *D. galeata* in the epilimnion (*upper bars*) and deep hypolimnion (*lower bars*) at all sampling dates. (**A**) Allele frequencies at the studied polymorphic loci (GPI, PGM). (**B**) Proportions of bi-locus genotypes (GPI + PGM). Only BLGs above 5% are indicated

umbers: 1 = F + F + MM, 2 = F + MFF, 3 = F + MFM, F + MFS, 5 = F + MMM, 6 = F + MMS, 7 = F + MSS, FMFF, 9 = MMFF, 10 = MMFM, 11 = MMFS, MMMM, 13 = MMMS, 14 = MMSS

between *D. galeata* and its potential interspecific hybrids with *D. longispina/hyalina* and *D. cucullata*. The sAAT analyses revealed that *D. galeata* was overwhelmingly the dominant taxon. Out of 930 specimens of *Daphnia* analysed, only three individuals were identified as *D. galeata* × *cucullata* hybrids, and no other members of the *D. longispina* complex were found. The hybrid genotypes were found in the sample from August 2004, and we excluded them from further analyses to eliminate the effect of multi-species comparisons on the intrapopulation variability of *Daphnia.*

The polymorphism of the dimeric enzyme GPI was expressed by the presence of three alleles (F^+, F and M). The F allele was extremely rare and was

ıd in only four specimens in the whole data set limnion–September 2003; hypolimnion–June and ;ust 2004). PGM, a monomeric enzyme, was ent in three alleles (F, M and S). Although there no specific allele occurring exclusively in imnetic or subhypolimnetic populations, the allele uencies in the two layers differed (Fig. 2A). se differences were significant on all eight pling dates, as revealed by exact tests (Table 1). Ve calculated F_{ST} values to quantify the genetic erentiation occurring between the epilimnetic and hypolimnetic populations (Table 1). Except for sampling date, in August 2004, all F_{ST} values e above 0.05, which indicates a certain degree of etic differentiation. The randomisation tests have

Table 1 The probability values of exact tests for differences in allele frequencies between epilimnetic and hypolimnetic subpopulations of *D. galeata* and F_{ST} values quantifying the genetic differentiation of epilimnetic and deep hypolimnetic subpopulations

2003	June-13	July-29	Aug-25	Sept-23
Exact test	<0.001	<0.001	<0.001	<0.001
F_{ST}	0.058	0.063	0.063	0.051
2004	May-18	June-26	Aug-5	Sept-9
Exact test	<0.001	<0.001	0.004	<0.001
F_{ST}	0.069	0.138	0.047	0.133

F_{ST} values were significant ($p < 0.001$) on all sampling dates

shown that this differentiation is highly significant ($p < 0.001$ after sequential Bonferroni correction for all sampling dates). For the whole data set we have done a hierarchical analysis of F_{ST} with depth nested in sampling date nested in year, to evaluate which of these levels contributed most to the genetic differences. This yielded the F_{ST} values of 0.0005, 0.015 and 0.063 for year, sampling date and depth, respectively. Apparently, depth was the most important component partitioning the *Daphnia* genetic variation in our data set.

Bi-locus genotypes (BLGs) constructed from allelic composition at two enzymes (GPI and PGM) were used to identify clonal groups within the *D. galeata* population. In total, we distinguished 14 BLGs; 10 of them were shared between the epilimnion and deep hypolimnion (Fig. 2B). Two BLGs were found exclusively in the epilimnion, and two more only in the hypolimnion. The relative frequencies of these "layer-specific" BLGs were always below 5%, except for the "epilimnetic" BLG 1 – F^+F^+MM (Fig. 2B). We used the RxC test to evaluate the significance of differences in BLG frequencies between epilimnetic and deep hypolimnetic subpopulations of *D. galeata*. These were highly significant ($p < 0.001$) in all sample pairs in the comparison.

The BLG diversity (Table 2) was almost always lower in the deep hypolimnion than in the epilimnion, with the exception of one sampling date (June 26, 2004). Similarly, the number of detected BLGs (BLG richness) was lower in the deep layer in all but one case (Table 2). In both years, the dominant BLGs in the deep hypolimnetic samples were nos. 3, 9 and 10 (Fig. 2B). Although their relative abundance fluctuated, these three BLGs always formed more than 70%

Table 2 Number of analysed individuals (N), BLG richness (BLG), BLG diversity and turnover rate of *D. galeata* in the epilimnion and deep hypolimnion

	Epilimnion				Deep hypolimnion			
Date	*N*	BLG	BLG diversity	Turnover rate	*N*	BLG	BLG diversity	Turnover rate
13.6.2003	50	11	0.770 (0.061)		55	8	0.739 (0.065)	
				0.138 (0.151)				0.170 (0.092)
29.7.2003	49	8	0.635 (0.157)		49	6	0.383 (0.332)	
				0.305 (0.156)				0.010 (0.132)
25.8.2003	52	8	0.766 (0.042)		50	5	0.475 (0.223)	
				0.139 (0.139)				0.036 (0.329)
23.9.2003	59	10	0.765 (0.060)		57	7	0.284 (0.490)	
				0.073 (0.119)				0.881 (0.311)
18.5.2004	53	11	0.798 (0.052)		66	5	0.587 (0.104)	
				0.320 (0.122)				0.499 (0.185)
26.6.2004	48	9	0.743 (0.058)		48	8	0.760 (0.067)	
				0.242 (0.071)				0.375 (0.164)
5.8.2004	47	8	0.835 (0.023)		58	8	0.336 (0.407)	
				0.400 (0.151)				0.071 (0.086)
9.9.2004	52	8	0.783 (0.037)		59	7	0.587 (0.192)	

BLG diversity at different sampling dates is expressed as a complement of the maximum likelihood estimator of Simpson's index ($1 - D$), turnover rate between the sampling dates is expressed by the complement of Morisita–Horn index ($1 - MH$). Standard error estimates are given in parentheses

of deep hypolimnetic *Daphnia*. The pattern in the epilimnion was more variable; however, the calculated turnover rates of BLGs seem to be comparable for both layers (Table 2). The strongest change in BLG composition (indicated by the highest value of the turnover rate) was found between deep hypolimnetic samples from autumn 2003 and spring 2004, separated by two mixing events (autumn, spring) and the winter season.

The seasonal development of the genetic structure within epilimnetic and deep hypolimnetic subpopulations was also apparent in the changing pattern of deviations from the Hardy–Weinberg equilibrium expressed as significant heterozygote excess or deficiency (Table 3). In general, the deviations were much less pronounced in the epilimnion, which usually did not show significant divergence from equilibrium, although both a heterozygote excess (in July 2003) and a strong heterozygote deficiency (at the end of the 2004 season) were observed as well. On the other hand, the deep hypolimnetic subpopulation was characterised by significant heterozygote excess in the second half of both years, apparently due to the dominance of BLG 3, heterozygous on both PGI and PGM loci.

Table 3 Wright's F_{IS} statistics for measuring the relative deviations of heterozygote frequencies from Hardy–Weinberg equilibrium (negative values: heterozygote excess, positive values: heterozygote deficiency)

2003	June-13	July-29	Aug-25	Sept-23
Epilimnion				
GPI	−0.36	−0.42	−0.20	−0.17
PGM	−0.29	−0.71*	−0.24	−0.32
Deep hypolimnion				
GPI	−0.53*	−0.66*	−0.56*	−0.78*
PGM	−0.16	−0.72*	−0.72*	−0.90*
2004	May-18	June-26	Aug-5	Sept-9
Epilimnion				
GPI	−0.02	+0.45	+0.14	+0.54*
PGM	−0.17	+0.36	+0.19	+0.55*
Deep hypolimnion				
GPI	−0.06	−0.26	−0.75*	−0.44*
PGM	−0.31	+0.06	−0.72*	−0.53*

Significant ($p < 0.01$) deviations from HWE after sequential Bonferroni correction are marked by asterisks

We analysed the similarity among the BLG composition of all samples using cluster analysis, by calculating the pairwise Euclidian distances between samples (Fig. 3). Two major clusters can be recognised, generally separating samples from the deep hypolimnion and epilimnion. This pattern has some exceptions, as two samples from the deep hypolimnion (May 18, 2004 and June 26, 2004) are nested among epilimnetic samples. On the whole, however, the separation between the upper and lower strata is very pronounced.

Discussion

The present study unambiguously shows that *D. galeata* does not live exclusively in the epilimnion, but that a small part of the population also inhabits the deep hypolimnetic layers in Římov Reservoir. We have also shown that these two subpopulations were genetically differentiated in the studied years. Two hypotheses may be formulated to explain both the spatial segregation and genetic differentiation on a vertical gradient: (1) there may be segregation (spatial and/or reproductive) between epilimnetic and deep hypolimnetic subpopulations resulting in completely or partially separated gene pools; (2) there could be frequent mixing and strong gene flow between both subpopulations, with yearly recurrent selection causing differences in the spatial genetic structure of *D. galeata*. The latter hypothesis, however, would predict that after independent events of clonal erosion, different clones would survive in the hypolimnion. This is in contrast with the observed genetic structure of the hypolimnetic subpopulation: the prevalence of similar BLGs in the deep hypolimnion (Figs. 2B, 3), together with significant heterozygote excess at the end of both seasons (Table 3), suggests that the clonal structure of this subpopulation was at least partially preserved over the consecutive years. Alternatively, a non-random BLG composition in the deep hypolimnion could result also from selection processes, if the studied allozyme markers were not selectively neutral. PGI and PGM allele frequencies may be significantly correlated with some environmental factors, as demonstrated by Weider et al. (1997) on the relationship between *Daphnia* genetic structure in Lake Constance and P concentration. In either case, however, we may

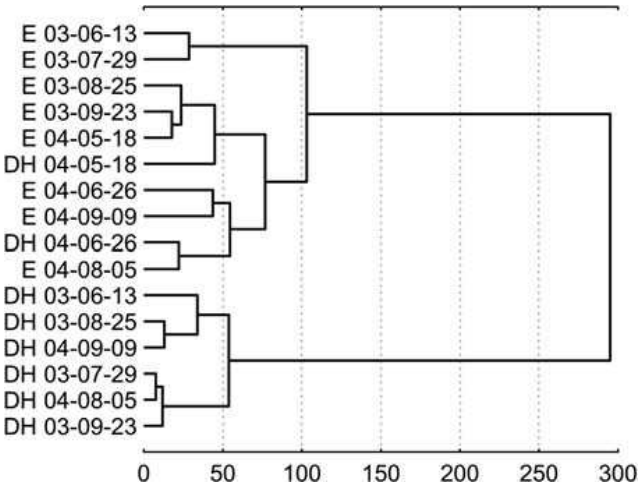

Fig. 3 The cluster analysis of the BLG composition of all samples (Ward's method based on the pairwise Euclidian distances between samples of BLGs). The horizontal scale shows the dissimilarity (Euclidian distance) between samples. E—epilimnion, DH—deep hypolimnion

conclude that at least some clones that make up a significant part of the deep hypolimnetic subpopulation are very likely specialists adapted to the deep-water environment.

The deep, cold, low-food hypolimnion is usually considered to be a hostile environment for *Daphnia*, and their occurrence there is interpreted as a trade-off between high risk of predation in the otherwise favourable epilimnion and survival under suboptimal temperature and food conditions (e.g., Zaret & Suffern, 1976; Stich & Lampert, 1981; Lampert, 1989; Guisande et al., 1991). On the other hand, living in a cold hypolimnion can be regarded as the colonisation of a broader spectrum of habitats available within a lake or reservoir, and thus as a mechanism facilitating the co-occurrence and increased diversity of *Daphnia* clones and species (Tessier and Leibold, 1997). There are three indications that the presence of *D. galeata* in the deep hypolimnion of Římov Reservoir fits the ecological specialisation for broader habitat use rather than the trade-off strategy. First, the deep hypolimnetic subpopulation does not migrate. Second, the animals occupy much deeper strata (25–35 m) than would be necessary for a simple trade-off strategy (10–15 m). Third, the species diversity of *Daphnia* in the deep hypolimnion of Římov Reservoir is higher than in the epilimnion. *D. galeata* in the deep hypolimnion is frequently accompanied by other non-migrating species, *D. pulicaria* and *D. ambigua*, which almost did not occur in the upper strata.

In general, there is a positive correlation between the vertical distribution of *Daphnia* and fish in Římov Reservoir (Čech & Kubečka, 2002). The overwhelming majority of fish live in the warm, *Daphnia*-rich, epilimnion. Only a very few isolated fish are detectable by echosounding below the thermocline down to 20 m depth, and we do not know whether these fish are actively feeding. The strata below 25 m are completely fishless, irrespective of oxygen concentration (Čech, 2006). Life in the deep hypolimnion therefore means a complete absence of fish predators for daphnids, regardless of the proximate mechanism for the depth selection.

Several mechanisms, which are not mutually exclusive, may contribute to the founding and continued presence of the deep subpopulation of *D. galeata*, which is further maintained by parthenogenetic reproduction. Hypolimnetic specialists may live permanently in the deep layers or some specimens may regularly migrate (or simply sink) down from upper layers, not returning back. Additionally, we cannot rule out some contribution of hatchlings from the sediment egg bank to the hypolimnetic subpopulation.

The seasonal dynamics of the vertical distribution of *Daphnia* suggest that no *D. galeata* in Římov Reservoir remain exclusively in the hypolimnion all year round. The summer pattern of *Daphnia* abundance during summer is shown in Fig. 1. In winter, the picture is reversed: almost all *Daphnia* are in the deep strata and hardly any are present in the upper

5 m. After the ice break (end of March), however, the whole population moves quickly to the epilimnion and no *Daphnia* can be found in the deep strata. The first detectable specimens of *D. galeata* appear in the hypolimnion only at the beginning of May (J. Seda, unpublished data).

The seasonal pattern of *Daphnia* abundance therefore suggests that the deep hypolimnetic subpopulation is regularly re-established from "colonists" migrating from the epilimnion in spring. This is supported by the similarity of epilimnetic and hypolimnetic samples from the beginning of the 2004 season (Fig. 3). The hypothesis of "colonisation from the epilimnion" is also backed by a strong BLG turnover in the deep hypolimnetic subpopulation between autumn and spring of the two seasons (Fig. 2B, Table 2). On the other hand, this does not rule out the possibility that specialists adapted to deepwater conditions, which had been only temporarily present in upper layers, formed a significant part of the subpopulation founders.

Although the BLG composition of the deep hypolimnetic subpopulation at the beginning of summer was different between the years (Fig. 2B), the general pattern of seasonal development of the clonal groups in the deep hypolimnion was similar in both seasons. Three BLGs (3, 9 and 10) always formed more than 70% of deep hypolimnetic *Daphnia*. BLG 3 (F + MFM) seemed to be the best performing one, especially in 2003. We observed a tendency of increased dominance of this BLG towards the end of the season accompanied by a reduction of the BLG diversity (Table 2) and low F_{IS} (Table 3), indicating strong clonal selection. This suggests that some elimination of the co-occurring real clonal lines may have taken place, although this was not reflected by a reduction of the number of detected BLGs (Table 2). We therefore presume that the genetic differentiation at the beginning of the season reflects different composition of individuals founding the deep hypolimnetic subpopulation (either migrating from above or hatching from ephippia), possibly affected by different depth preferences of various clones; while the subsequent changes in the genetic structure are results of differential selection in the upper and lower strata.

Low temperature is the most likely explanation for the slow selection process in the deep hypolimnion. The four and a half month period of segregation at a temperature of 5°C is probably not long enough for complete elimination of all "outsiders" (i.e., clones with suboptimal adaptation to the local conditions) from the deep hypolimnion.

The BLGs described in this study were distinguished on the basis of two loci, GPI and PGM. It is almost certain that every lineage represents a family of clones rather than the progeny of a single female. The method used is therefore very limited in the detection of clonal selection, which likely happens within these families of clones. Macháček & Seda (unpublished) found significant differences in life history traits (maturation time, fecundity, size at first reproduction) of two laboratory clones isolated from the deep hypolimnion at different times, in May and in September 2004, both belonging to the BLG 3 (F + MFM). The differences of life history traits under laboratory conditions indicate that these two lines, although identical in studied allozyme markers, most likely represented two unique, ecologically divergent clones.

Given significant differences in the genetic composition of *D. galeata*, as well as in environmental conditions between the epilimnion and deep hypolimnion, the possibility that the subpopulations in these layers may be at least partially reproductively isolated is intriguing. Although there are no physical barriers between the layers, differences in spatial segregation of sexual individuals could reduce gene flow between the two subpopulations. The spatial distribution of males and ephippial females in spring differs in Římov Reservoir, with males being produced in the epilimnion and migrating downwards, and ephippial females being present predominantly in the deeper layers (J. Macháček, unpublished data). Differences in the distribution of males and sexual females have also been observed in other *Daphnia* species (Brewer, 1998) or populations (Spaak & Boersma, 2001). However, such a pattern does not seem to provide for the reproductive isolation between epilimnetic and hypolimnetic clones. It is therefore unclear whether the spatial segregation of *D. galeata* is maintained only by the long-term survival of specialised deep hypolimnetic clones, or whether some degree of genetic isolation between epilimnetic and hypolimnetic subpopulations exists.

The occurrence of *D. galeata* in the hypolimnion is not just a case unique to Římov Reservoir. We have surveyed 11 dammed valley reservoirs in Czechia,

including Římov, to find taxon-specific preferences in *Daphnia* spatial distribution across environmental gradients (Seda et al., in prep.). The presence of *D. galeata* in hypolimnetic samples was relatively common, they were found in seven out of eleven studied reservoirs. The absence of *D. galeata* in the hypolimnion was always coincident with an overwhelming dominance of *D. longispina* in that particular reservoir, or with more eutrophic conditions resulting in a hypolimnetic oxygen depletion. This indicates that vertical structuring of *D. galeata* populations, and the intraspecific specialisation of part of the planktonic population to seemingly unfavourable conditions, might be a more common phenomenon than we had originally expected.

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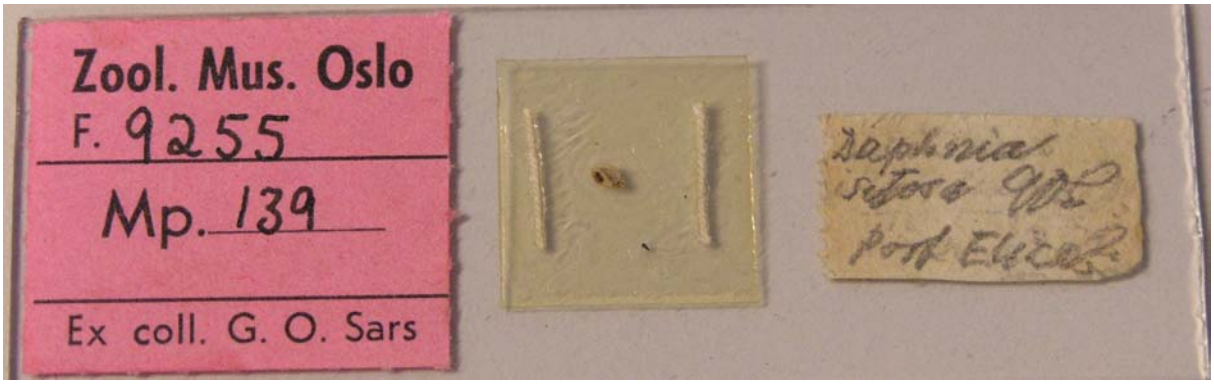
CHAPTER 8

Forró L., Korovchinsky N. M., Kotov A. A., Petrusek A.:

Global diversity of cladocerans (Cladocera; Crustacea) in freshwater

Hydrobiologia, doi: 10.1007/s10750-007-9013-5

(part of the special issue “A global assessment of animal diversity in freshwater”)



This slide made by G. O. Sars contains an enigma: a South African *Daphnia* with an unusually-shaped ephippium. Labelled as *Daphnia setosa*, it is likely one of many undescribed African endemic species.

Note: final version of the paper should contain drawings of all four cladoceran orders

Hydrobiologia
DOI 10.1007/s10750-007-9013-5

FRESHWATER ANIMAL DIVERSITY ASSESSMENT

Global diversity of cladocerans (Cladocera; Crustacea) in freshwater

L. Forró · N. M. Korovchinsky · A. A. Kotov · A. Petrusek

Abstract Cladocera is a primarily-freshwater monophyletic group, an important component of the microcrustacean zooplankton. They inhabit most types of continental fresh and saline water habitats, occurring more abundantly in both temporary and permanent stagnant waters. Cladocera is an ancient group of Palaeozoic origin. About 620 species are currently known, but we estimate that the real number of species is 2–4 times higher. A number of currently-recognised widespread species can be expected to harbour extensive cryptic diversity.

Keywords Cladocera · Species richness · Global assessment · Biogeography · Endemicity

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Freshwater Animal Diversity Assessment

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Introduction

Cladocerans ("water fleas") are primarily-freshwater small-sized (0.2–6 mm, and up to 18 mm in single case of *Leptodora kindtii*) branchiopod crustaceans, inhabiting pelagic, littoral, and benthic zones. Four cladoceran orders are recognised (Fryer, 1987a, b): Anomopoda, Ctenopoda, Onychopoda, and the monotypic Haplopoda (see Figs. 1, 2, 3 and 4 for representatives of each order). Most species occur in continental fresh or saline waters, although two ctenopods and several onychopods from the family Podonidae are truly marine, and a few more ctenopod and anomopod species occur in brackish waters. Seven known species may be regarded as true inhabitants of subterranean environment, and a few others (of the family Chydoridae) live in semi-terrestrial conditions.

The trunk and appendages of most cladocerans (Anomopoda and Ctenopoda) are enclosed in a bivalved carapace. Tagmosis of the body is obscure (except in *Leptodora kindtii*, the single representative of Haplopoda), and a single eye and ocellus are usually present. Antennules are uniramous, while antennae are biramous (except in females of *Holopedium*), natatory, with 2–4 segments per branch. Four to six pairs of trunk limbs are either mostly similar in shape (Ctenopoda, Onychopoda, Haplopoda) or modified individually for various functions (Anomopoda).

Water fleas are important components of the fauna of fresh waters; they are particularly significant in the

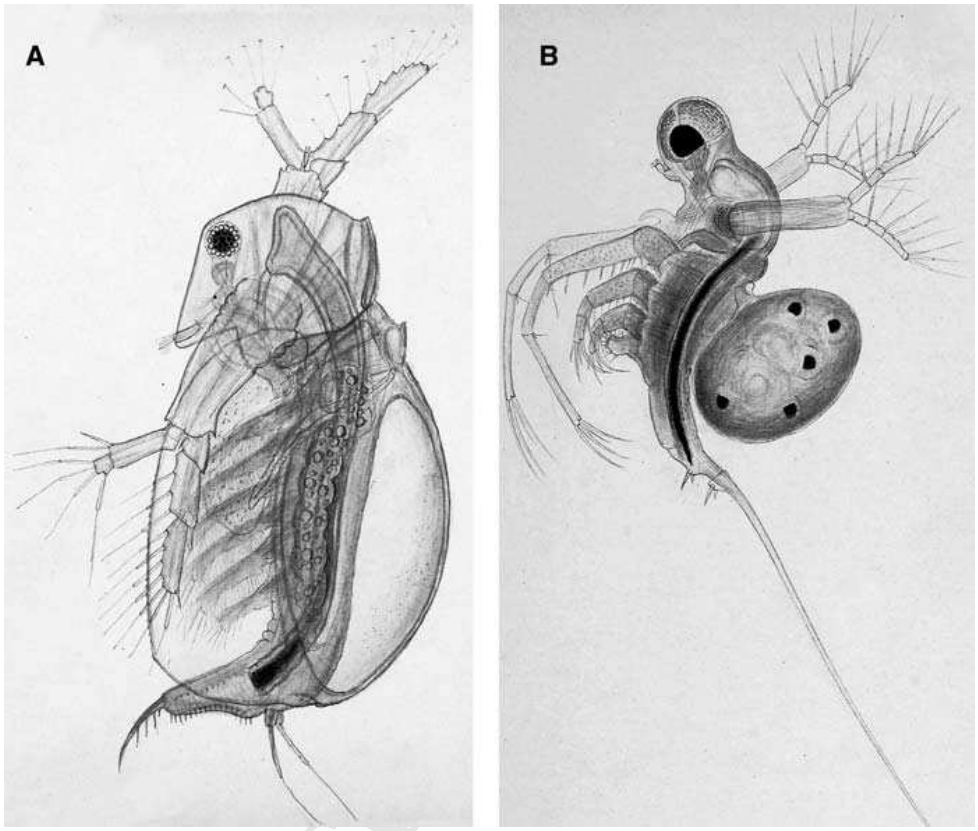

Fig. 1 A—*Sida crystallina.* B—*Bythotrephes longimanus* (original drawings by by G O Sars)

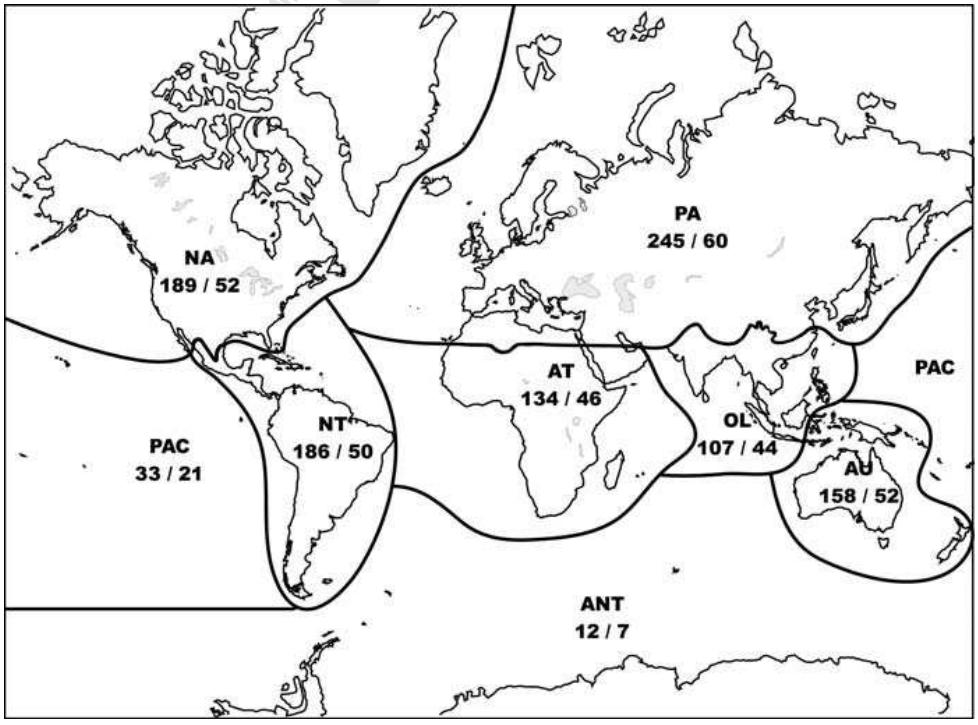

Fig. 2 Global Distribution of species and genus diversity by zoogeographic region (Species Number/ Genus Number). PA—Palaearctic; NA—Nearctic; NT—Neotropical; AT—Afrotropical ; OL—Oriental; AU—Australasian; PAC—Pacific Oceanic Islands; ANT—Antarctic

food web of stagnant waters. Most species are filter-feeders; onychopods and haplopods are predatory. They usually reproduce by cyclical parthenogenesis (but asexual lineages are known as well), and populations are mostly dominated by females. Sexual dimorphism is normally rather distinct. Sexually

produced diapausing eggs are resistant to desiccation and other unfavourable conditions, and may even survive passage through the digestive track of birds (Figuerola & Green, 2002); thus, they are important propagules for passive dispersal.

The first information on Cladocera date from the 17th century; the history of research has been divided into three to seven major phases (for a detailed discussion see Korovchinsky, 1997; Dumont & Negrea, 2002). An important change of paradigm, characterised by the rejection of the prevailing assumption of cosmopolitanism of cladoceran species, occurred around the 1950–1980s with a new approach to the taxonomy and phylogeny of Chydoridae (Frey, 1959, 1982, 1987a, b). Subsequently, the concept of non-cosmopolitanism has been supported by numerous morphological, as well as molecular, studies. The increasing use of molecular tools in recent years has and will continue to have a strong impact on our understanding of cladoceran diversity, phylogeny and biogeography (e.g., Adamowicz et al., 2004; Cox & Hebert, 2001; Schwenk et al., 2000; Taylor et al., 2002).

Species diversity

The currently accepted number of cladoceran species based on existing descriptions is around 620. The tables summarize the currently known number of species and genera within orders and families of the group (Tables 1, 2), based on recent major publications (Smirnov, 1992a, 1996; Korovchinsky, 1996, 2004; Orlova-Bienkowskaja, 2001; Dumont & Negrea, 2002; Benzie, 2005; Kotov & Stifter, 2006) and additional published or as yet unpublished sources. The described taxonomic diversity of Cladocera, however, underestimates the reality, and even higher-

Table 1 Number of Cladocera species currently known in the main biogeographic areas. PA: Palaearctic; NA: Nearctic; NT: Neotropical; AT: Afrotropical ; OL: Oriental; AU: Australasian; PAC: Pacific Oceanic Islands; ANT: Antarctic. (numbers in parentheses indicate endemic species)

	PA	NA	NT	AT	OL	AU	PAC	ANT	World
Order Anomopoda	195 (83)	169 (66)	170 (89)	125 (24)	89 (20)	149 (78)	29 (0)	12 (6)	537
Family Daphniidae*	58 (21)	58 (25)	32 (13)	25 (1)	17 (1)	26 (13)	6 (0)	3 (2)	121
Family Moinidae*	13 (6)	7 (2)	10 (5)	10 (1)	3 (0)	7 (3)	4 (0)	0	29
Family Dumontiidae	0	1 (1)	0	0	0	0	0	0	1
Family Ilyocryptidae	11 (3)	10 (2)	9 (4)	8 (3)	5 (3)	5 (3)	1 (0)	1 (0)	28
Family Bosminidae	4 (0)	8 (3)	7 (3)	3 (0)	4 (1)	3 (0)	1 (0)	0	14
Family Acantholeberidae	1 (0)	1 (0)	0	0	0	0	0	0	1
Family Ophryoxidae	3 (1)	3 (1)	0	0	0	0	0	0	3
Family Macrothricidae	16 (10)	10 (5)	21 (12)	12 (2)	12 (4)	20 (9)	2 (0)	3 (1)	60
Family Neothricidae	0	0	0	0	0	3 (3)	0	0	3
Family Eurycercidae	4 (2)	5 (3)	2 (1)	1 (0)	1 (0)	0	0	0	8
Family Chydoridae	85 (40)	66 (24)	89 (51)	66 (17)	48 (11)	85 (47)	15 (0)	5 (3)	269
Order Ctenopoda	17 (5)	18 (7)	16 (9)	9 (0)	15 (4)	9 (5)	4 (1)	0	50
Family Holopediidae	1 (0)	2 (1)	1 (1)	0	0	0	0	0	3
Family Sididae	16 (5)	16 (6)	15 (8)	9 (0)	15 (4)	9 (5)	4 (1)	0	47
Order Haplopoda	1 (0)	1 (0)	0	0	1 (0)	0	0	0	1
Family Leptodoridae	1 (0)	1 (0)	0	0	0	0	0	0	1
Order Onychopoda	32 (31)	1 (0)	0	0	1 (0)	0	0	0	32
Family Polyphemidae	2 (1)	1 (0)	0	0	1 (0)	0	0	0	2
Family Podonidae	17 (17)	0	0	0	0	0	0	0	17
Family Cercopagidae*	13 (13)	0	0	0	0	0	0	0	13
Total	245 (119)	189 (73)	186 (98)	134 (24)	107 (24)	158 (83)	33 (1)	12 (6)	620

* Invasive species not considered

Table 2 Number of Cladocera genera currently known in the main biogeographic areas. PA: Palaearctic; NA: Nearctic; NT: Neotropical; AT: Afrotropical ; OL: Oriental; AU: Australasian; PAC: Pacific Oceanic Islands; ANT: Antarctic. (numbers in parentheses indicate endemic genera)

	PA	NA	NT	AT	OL	AU	PAC	ANT	World
Order Anomopoda	44 (4)	43 (3)	44 (3)	42 (1)	36 (1)	48 (11)	19 (0)	7 (0)	76
Family Daphniidae	5 (0)	5 (0)	4 (0)	5 (0)	4 (0)	4 (0)	4 (0)	2 (0)	5
Family Moinidae	1 (0)	2 (0)	2 (0)	2 (0)	2 (0)	2 (0)	1 (0)	0	2
Family Dumontiidae	0	1 (1)	0	0	0	0	0	0	1
Family Ilyocryptidae	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1
Family Bosminidae	2 (0)	2 (0)	2 (0)	2 (0)	2 (0)	2 (0)	1 (0)	0	2
Family Acantholeberidae	1 (0)	1 (0)	0	0	0	0	0	0	1
Family Ophryoxidae	1 (0)	2 (1)	0	0	0	0	0	0	2
Family Macrothricidae	6 (0)	6 (0)	7 (2)	5 (0)	4 (0)	5 (0)	1 (0)	1 (0)	11
Family Neothricidae	0	0	0	0	0	1 (1)	0	0	1
Family Eurycercidae	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	0	0	0	1
Family Chydoridae	26 (4)	22 (1)	27 (1)	26 (1)	22 (1)	33 (10)	11 (0)	3 (0)	49
Order Ctenopoda	7 (1)	7 (0)	6 (0)	4 (0)	6 (0)	4 (0)	2 (0)	0	8
Family Holopediidae	1 (0)	1 (0)	1 (0)	0	0	0	0	0	1
Family Sididae	6 (1)	6 (0)	5 (0)	4 (0)	6 (0)	4 (0)	2 (0)	0	7
Order Haplopoda	1 (0)	1 (0)	0	0	1 (0)	0	0	0	1
Family Leptodoridae	1 (0)	1 (0)	0	0	0	0	0	0	1
Order Onychopoda	8 (5)	1 (0)	0	0	1 (0)	0	0	0	10
Family Polyphemidae	1 (0)	1 (0)	0	0	1 (0)	0	0	0	1
Family Podonidae	5 (3)	0	0	0	0	0	0	0	**7**
Family Cercopagidae	2 (2)	0	0	0	0	0	0	0	2
Total	60 (10)	52 (3)	50 (3)	46 (1)	44 (1)	52 (11)	21 (0)	7 (0)	95

ranked taxa are still being discovered, e.g., a new family, Dumontiidae (Santos-Flores & Dodson, 2003).

Only about 45–50% of the species may be considered to be more or less well described and valid, while the status of other species is vague, and many of them likely represent cryptic complexes (Korovchinsky, 1996). The families Chydoridae, Daphniidae, Ilyocryptidae, and Sididae have been studied comparatively better. The largest number of valid species is known from Europe, North America, Australia, and South America, and the smallest number from Africa and Southern Asia. This, however, at least partly reflects the intensity of research rather than real patterns of diversity.

Adamowicz & Purvis (2005) estimated three correction factors to extrapolate global branchiopod diversity from the diversity of described species, and predicted that there are about 2.1 times more branchiopod species in nature than currently known. The overall cladoceran species richness is probably up to 4 times higher than currently known. This is supported by the results of molecular studies. Detailed studies, combining morphological analyses and molecular tools, are especially promising for delineating species boundaries in groups with relatively uniform morphology, fewer qualitative characters, and widespread phenotypic plasticity. Although most molecular analyses have so far focused on a single model genus, *Daphnia*, within a relatively short time this led to the discovery of an unprecedented number of cryptic lineages. According to Hebert & Taylor (1997), the global total for the genus *Daphnia* (including *Daphniopsis*) is likely closer to 200 species instead of 75 included in the last monograph on the genus (Benzie, 2005). Similar patterns of widespread cryptic diversity and high numbers of undescribed lineages can be seen in other groups, e.g., in *Moina* (Petrusek et al., 2004 and unpublished data),

Ilyocryptus (Kotov & Štifter, 2006) and several genera of the Chydoridae.

Phylogeny and historical processes

Cladocerans probably derived from large bodied branchiopod ancestors. Recent molecular analyzes have suggested two alternative phylogenetic relationships among cladoceran orders. The monophyly of Gymnomera (Haplopoda and Onychopoda) is supported in both cases but one hypothesis suggests a sister relationship between Anomopoda and the remaining three orders (Swain & Taylor, 2003), while the other clusters Anomopoda and Ctenopoda together (De Waard et al., 2006). Other authors (see review in Negrea et al., 1999) have recently proposed alternative hypotheses on ordinal-level relationships for the Cladocera; these were, however, based only on cladistic analyses of morphological traits.

Cladocera is an ancient group of Palaeozoic origin (Dumont & Negrea, 2002), but their unambiguous fossil remains are known only from the Mesozoic (Smirnov, 1971, 1992b; Kotov & Korovchinsky, 2006). Recently, Anderson et al. (2004) described crustaceans similar to the Cladocera from the Early Devonian. Molecular phylogenetic data have revealed that the subfamilies of Chydoridae (Anomopoda) were separated in the Middle Palaeozoic (about 400 Myr ago; Sacherová & Hebert, 2003) and representatives of the genus *Daphnia* differentiated at least 200 Myr ago (Colbourne & Hebert, 1996). Any Mesozoic scenarios, such as 'Gondwana-Laurasia' (e.g., Benzie, 2005), are only moderately applicable to cladoceran groups, especially at a generic and subgeneric level. In spite of the general antiquity of Cladocera, radiation within some groups is only recent or even contemporary, e.g., in some Holarctic *Daphnia* and *Bosmina* (Colbourne & Hebert, 1996; Taylor et al., 2002).

Present distribution and main areas of endemicity

The distribution and patterns of endemicity of higher-level taxonomic groups are relatively well-known. The known species diversity, as well as the number of endemic taxa, is nevertheless bound to increase with further faunistic research, especially from non-northern temperate regions, and with the application of detailed morphological and molecular tools to resolve cryptic species complexes. Some endemic species have narrow distributions, and it is therefore likely that many remain overlooked.

The Holarctic cladoceran inland fauna is rich and composed of all four orders. Two orders (Haplopoda and Onychopoda), three families, 13 genera (including those of the Caspian Sea and Lake Baikal), and about 250 known species are endemic for the region. Many taxa are presumably old and phylogenetically divergent, monotypic, or composed of a few species. The Palaearctic taxa are more diverse than those of the Nearctic due to, first of all, the presence of numerous Caspian and Baikalian endemics. Among other zoogeographical regions, Australasia is rich in endemics, represented by one family, one subfamily, one tribe, 11 genera, and 83+ species, while known endemics in Oriental and Neotropical regions are of a lower rank or fewer (one tribe, one genus and 21+ species, and three genera and 98+ species, respectively). The Afrotropical region, though poorly studied, seems to be especially deprived of known higher-level endemic cladoceran taxa, being represented by a single endemic genus and 24+ endemic species.

Cladoceran species richness does not change evenly with latitude but concentrates in the warm temperate to subtropical zone of both hemispheres (~25–50°, including mountain areas within the true tropics) (Korovchinsky, 2006). In the belt from the Mediterranean through Central Asia including the Pontocaspian region, northern India to East Asia (Amur region and China), five genera and over 100 known endemic species occur, while those in the North Palaearctic do not exceed 55–60. In North America, the area embracing the United States, Mexican plateau, and southern Canada, is inhabited by many endemics, including one family (Dumontiidae), two genera, and over 70 species. Southern Australia, Tasmania, and New Zealand are rich in endemics of high taxonomic rank: one subfamily (Sayciinae), one tribe (Australospilini), 8 genera, and about 80 currently known species compose altogether most of the known Australasian endemics. Only five of them are shared between Australia and New Zealand, which itself has seven endemic species/subspecies. As the African cladoceran fauna

has been relatively poorly studied, there are only a few described endemics (one genus and about 10 species), but e.g., endemic *Daphnia* fauna of the Ethiopian biogeographic region is certainly significantly more numerous (Mergeay et al., unpublished). Cladocerans of subtropical and temperate South America include two endemic genera and 17+ endemic species. In total, the species richness of the southern temperate—subtropical zone amounts to more than 100 endemic species. The intermediate tropical zone, from which altogether 163 species are known, is characterized by fewer endemic taxa of comparatively lower taxonomic rank: one tribe (Indialonini) and nine genera (Korovchinsky, 2006).

A bipolar (antitropical) disjunct distribution of faunal complexes and taxa (*Daphnia, Pleuroxus, Tretocephala* etc.), the wide ranges of some species (though some of these likely form species complexes) and the narrow restriction of others, the presence of isolated populations, and concentration of endemics in the warm temperate—subtropical zone of both hemispheres, are typical traits of cladoceran zoogeography.

Such patterns stimulated the analysis of cladoceran faunal formation by the modern version of the concept of 'ejected relicts' instead of vicariance. This hypothesis consider the extant Cladocera as a relict group (Korovchinsky, 2006), whose taxa were widely distributed in the past. Tertiary climatic changes, primarily within the present tropical and boreal latitudes, resulted in mass extinction of their biotas, while the warm temperate—subtropical regions remained comparatively unchanged. Additional factors (e.g., the radiation of freshwater planktivorous fish) could have operated in conjunction with climate changes as well.

While this scenario might be likely for a number of cladocerans, molecular data suggest that vicariance processes and allopatric speciation at both the intercontinental level and in regional refugia within continents plays a significant role in shaping species diversity in at least some genera (e.g., *Daphnia*). Sweepstake intercontinental dispersals, followed by a local radiation, seems to have been important factors in augmenting the diversity in the different biogeographic regions. Founder effects coupled with habitat shifts, such as pond-lake transitions (Lynch, 1985) or, possibly, shifts among substrates in littoral groups, are also regarded as potentially important drivers of speciation. Finally, interspecific hybridization and hybrid speciation plays an important role in dynamic young species complexes in *Daphnia*, though reports of other hybridizing cladocerans are scarce (Schwenk & Spaak, 1995).

Human related issues

Cladocerans (especially *Daphnia*) are important model organisms in both basic and applied research, due to their easy culturing, short generation time, and clonal reproduction. Species of *Daphnia* have been widely used in ecological and evolutionary studies (e.g., on trophic interactions, diel vertical migration, interspecific hybridisation, polyploidy and asexuality, host-parasite interactions etc.), and the soon to be available sequence of the whole *Daphnia pulex* s.l. genome will open further research possibilities in genomics and other fields. Cladocerans have also gained certain economic importance as they are also widely used in aquaculture, and large filter-feeding planktonic species have an indirect economic impact as important fish food or phytoplankton-controlling group. These animals as intermediate hosts of some parasites may potentially pose a threat to human health.

A high diversity of cladocerans can be found in the littoral zone of stagnant waters, as well as in temporary water bodies. These habitats are often negatively influenced by human activities, and especially the loss of temporary waters may lead to a decrease of diversity or even local extinction of some species.

Some cladocerans have recently invaded successfully other continents through human-mediated dispersal, and it is likely that this trend will increase. For example, non-indigenous species of *Daphnia* are widespread in Europe, North America or Africa (e.g., Havel et al., 1995; Mergeay et al., 2005), though mostly without a strong ecological impact. The invasion of predatory onychopods (especially *Bythotrephes*) from the Palaearctic into the Laurentian Great Lakes and those of the Canadian Shield, however, have influenced the native fauna significantly (Yan et al., 2002).

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