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Identifying hybridizing taxa within the *Daphnia longispina* species complex: a comparison of genetic methods and phenotypic approaches

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Abstract *Daphnia galeata* Sars, *D. longispina* O. F. Müller and *D. cucullata* Sars (Crustacea: Cladocera) are closely related species which often produce interspecific hybrids in natural populations. Several marker systems are available for taxon determination in this hybridizing complex, but their performance and reliability has not been systematically assessed. We compared results from identifications by three molecular methods. More than 1,200 individuals from 10 localities in the Czech Republic were identified as parental species or hybrids by allozyme electrophoresis and the analysis of the restriction fragment length polymorphism of the internal transcribed spacer (ITS-RFLP); over 440 of them were additionally analyzed and identified by 12 microsatellite loci. Identification by microsatellite markers corresponded well with allozyme analyses. However, consistent discrepancies between ITS-RFLP and other markers were observed in two out of 10 studied localities. Although some marker discrepancies may have been caused by occasional recent introgression, consistent deviations between ITS-RFLP and other markers suggest a long-term maintenance of introgressed alleles. These results warn against its use as a sole identification method in field studies. Additionally, we quantitatively evaluated the discriminatory power of geometric morphometric (elliptic Fourier) analysis of body shapes based on photos of over 1,300 individuals pre-classified by allozyme markers. Furthermore, a randomly selected subset of 240 individuals was independently determined from photos by several experts. Despite a tendency for morphological divergence among parental *Daphnia* species, some taxa (especially *D. galeata*, *D. longispina*, and their hybrids) substantially overlapped in their body shapes. This was reflected in different

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determination success for particular species and hybrids in discriminant analysis based on shape data as well as from photographs.

Keywords Interspecific hybridization · Introgression · Elliptic Fourier analysis · Internal transcribed spacer · Allozyme electrophoresis · Microsatellites

Introduction

Since its beginning, taxonomy of the cladoceran genus *Daphnia* has been complicated by high morphological variability among individuals within species, causing many discrepancies in the identification of forms and taxonomical grouping. In the literature, one often finds that the status of certain *Daphnia* taxa changed several times within a short period of time (e.g., Flößner & Kraus, 1986; Petrusek et al., 2008a). Because of the occurrence of numerous morphological forms and the high similarity of certain species, the genus *Daphnia* has been considered "… one of the taxonomically most difficult groups of the animal kingdom" (Flößner & Kraus, 1986), though many of the perceived difficulties are likely due to inadequate past taxonomic research. Members of this genus have long been used as model organisms in various fields of basic and applied science, from aquatic ecology and evolutionary biology to ecotoxicology; however, their taxonomy is far from resolved. Many undescribed cryptic species are still being discovered (see, e.g., Adamowicz et al., 2009), and discussions about the status and validity of even very common *Daphnia* species remain controversial (e.g., Nilssen et al., 2007; Petrusek et al., 2008a).

Within the *D. longispina* complex in particular, taxonomical problems are partly caused by a high level of environmentally induced phenotypic plasticity, as natural forms commonly produce various morphs under variable environmental conditions. Genetically identical individuals may thus exhibit different size or different carapace and head shapes (Flößner & Kraus, 1986). Environmental conditions may also cause the development of specialized chitinous forms. For instance, the development of head helmets in *Daphnia cucullata* can be induced by increased turbulence of water (Brooks, 1947; Hrbáček, 1959; Laforsch & Tollrian, 2004) or as a response to the presence of fish (Brooks, 1965; Jacobs, 1965) and invertebrate predators (Tollrian & Laforsch, 2006).

However, environmentally induced phenotypic plasticity is only one of the sources of intraspecific morphological variability. Variation among phenotypes is often genetically based (Gießler, 1997, 2001; Petrusek et al., 2008a), and substantial morphological changes may be attributed to interspecific hybridization, possibly followed by introgression. Based on morphology, this has long been suspected in the *D. longispina* species complex (Lieder, 1956, 1983; Einsle, 1966), in which morphologically intermediate forms are particularly common. Genetic studies confirmed the presence of interspecific hybrids among some taxa of this complex (e.g., Wolf & Mort, 1986; Schwenk & Spaak, 1997; Hobæk et al., 2004) and showed that parthenogenetically reproducing hybrids often occur in syntopy with parental species. The most common hybridizing species in Europe are *D. galeata*, *D. cucullata*, and *D. longispina* (the taxon including both *D. hyalina* and *D. rosea* according to Petrusek et al., 2008a; see "Materials and methods" section for details on taxonomy). Sexual reproduction of hybrids and backcrossing followed by nuclear introgression also occurs, but seems to be relatively rare (Spaak, 1996; Jankowski & Straile, 2004; Keller et al., 2007). However, it may have long-lasting consequences for the evolution of this group (Schwenk et al., 2000). Gene flow among parental species may cause reticulate patterns of evolution which potentially lead to increased levels of genetic and morphological variability in local populations (Schwenk & Spaak, 1997). Although some studies suggest reticulate evolution plays an important role for the *D. longispina* complex (Schwenk et al., 1995; Gießler et al., 1999; Gießler & Englbrecht, 2009), gene pools of parental species seem to remain distinct (Keller et al., 2007), and might be well distinguished by various molecular methods (e.g., Thielsch et al., 2009).

In order to improve taxon determination in the *D. longispina* group, in particular to separate parental species and interspecific hybrids, and in order to obtain reproducible results, several molecular marker systems have been used. Early studies used electrophoresis of one or two species-specific allozyme markers (Wolf & Mort, 1986; Gießler, 1997). Despite a small number of fixed diagnostic loci and sampling limitations imposed by the need to deep-freeze

samples, this method has become widespread in studies on *Daphnia* hybridization (e.g., Spaak, 1996; Spaak, 1997; Gießler, 2001; Winder et al., 2001; Keller & Spaak, 2004). It still remains in use and continues to provide valuable results (Seda et al., 2007; Keller et al., 2008; Petrusek et al., 2008b). However, alternative methods for taxon identification have been recently developed, for example ITS-RFLP, the restriction fragment length polymorphism (RFLP) of the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (Billiones et al., 2004; Skage et al., 2007). This approach, inspired by successful use in other hybrid systems (e.g., King et al., 2001; Pfenninger et al., 2002), and allowing analysis of ethanol-preserved material, has been considered promising, although some limitations of the original protocol have quickly been identified (Skage et al., 2007).

Furthermore, methods enabling indirect determination of *Daphnia* taxa are also available, especially the recently developed set of 32 microsatellite markers for the *D. longispina* complex (Brede et al., 2006). Hybridizing species of the complex can be well distinguished when several microsatellite loci are analyzed (Brede et al., 2009; Thielsch et al., 2009), confirming the suitability of these markers for the identification of hybrid genotypes. Additionally, DNA microsatellite analysis provides detailed data on clonal composition and genotypic richness and is therefore particularly suitable for evolutionary studies (Brede et al., 2009).

The choice of an appropriate method ensuring correct and reproducible results is an important step in every study employing molecular markers. In studies on hybridizing *Daphnia*, it is even more crucial, as some mismatch of the markers can be expected (Schwenk & Spaak, 1995). In our previous studies of the *D. longispina* complex (Skage et al., 2007; Petrusek et al., 2008b), we showed that unexpected intraspecific variation may substantially influence the interpretation of restriction patterns from ITS-RFLP (Billiones et al., 2004). As no detailed study has directly compared results of various molecular approaches for taxon identification in this species complex so far, the real extent of these discrepancies remains unknown.

Here, we present a comparison of three molecular methods for genetic identification of common species and hybrids of the *D. longispina* complex (in particular, *D. galeata*, *D. longispina* and *D. cucullata*): allozyme electrophoresis (according to Wolf & Mort, 1986; Gießler, 1997), ITS-RFLP (according to Skage et al., 2007), and the analysis of 12 microsatellite markers (from Brede et al., 2006). In particular, we define the extent to which the results of DNA-based methods deviate from those of allozyme electrophoresis, as this method was employed in most studies on interspecific hybridization within the *D. longispina* complex. Given the fact that the general phenotype of individuals is often used for identification in routine screening of samples in ecological research, we also compared two phenotype-based approaches testing how body shape reflects taxon identity: a subjective evaluation by several experts and geometric morphometric analysis of *Daphnia* body outlines. In the discussion, we assess the limitations of each technique, hypothesize on the causes of the inconsistencies observed, and evaluate the applicability of all methods with regard to specific research questions.

For this purpose, we used samples collected from 10 canyon-shaped reservoirs in the Czech Republic. Ranges of studied *Daphnia* species overlap in this area and interspecific hybrids are thus common in various habitats. Nevertheless, localities harboring all three parental species and at least some of their interspecific hybrids are relatively rare. The canyon-like morphology of studied reservoirs results in longitudinal environmental gradients offering diverse microhabitats for zooplankton within individual water bodies. Such conditions promote the co-occurrence of parental *Daphnia* as well as hybrids (Seda et al., 2007; Petrusek et al., 2008b) and therefore make the reservoirs excellent localities for studies on these hybridizing taxa.

Materials and methods

Taxonomy and nomenclature

In this study, we compared various methods to identify three species of the *Daphnia longispina* complex and their interspecific hybrids. While the taxonomy and nomenclature of *D. galeata* and *D. cucullata* has been stable in recent decades, the taxonomy of *D. longispina* has recently undergone a revision (Petrusek et al., 2008a). In most papers on

hybridization, the name *D. hyalina* has been used for this taxon. However, recent studies (Petrusek et al., 2008a; Thielsch et al., 2009) suggest that the lake-inhabiting form *D. hyalina*, the mostly pond-inhabiting *D. rosea*, as well as various intermediate morphotypes just represent morphs of the single phenotypically variable biological species *D. longispina.* This conclusion is supported by the lack of genetic divergence in various molecular markers, such as mitochondrial DNA (Petrusek et al., 2008a), allozymes (Gießler et al., 1999), 13 unlinked microsatellite loci (Thielsch et al., 2009), and nuclear ITS sequences (Gießler & Englbrecht, 2009). Individuals identified as *D. longispina* from localities sampled in this study included typical *D. hyalina*-like morphotypes as well as transitional forms closer to *D. rosea* morphology.

Sampling and preservation of samples

Daphnia individuals were collected by a plankton net (mesh size 170 μm) from 10 reservoirs (Table 1) situated in the Czech Republic (Central Europe). Localities included in this study varied in a number of environmental parameters, such as size, depth and trophic level. In order to collect all potential *Daphnia* species and interspecific hybrids, we sampled at three locations along the longitudinal axis of each reservoir (except of Sedlice with one sampling site only), thus covering the main environmental gradients. Zooplankton samples were frozen in liquid nitrogen shortly after collection and stored in a deep-freezer (sampling details are given in Seda et al., 2007). From each reservoir (except of Sedlice with 25 analyzed individuals), 160–210 adult females from the *Daphnia longispina* species complex, randomly selected from the collected samples, were analyzed. First, they were photographed from the lateral side, and then used for allozyme electrophoresis and DNA preparation (for subsequent PCR-based methods). The photographs were analyzed by geometric morphometrics, and a subset was then used for determination using the general phenotype. Out of the total number of 1,276 individuals analyzed by more than one molecular method, 444 were simultaneously identified by all three, which enabled direct comparisons of the markers.

Each individual was determined as one of the parental species (*D. galeata*, *D. longispina*, and *D. cucullata*) or as a hybrid (recombinant) genotype. For the comparability of different molecular methods, further differentiation among hybrid classes (e.g., F_1, F_2, or backcrosses) would be neither practical nor sufficiently reliable. However, more detailed information about taxon composition obtained by the analysis of 12 microsatellite markers was used for interpretation of some results.

Allozyme electrophoresis

All individuals were primarily identified by allozyme electrophoresis on cellulose acetate gels (Hebert & Beaton, 1993). Four allozyme loci were analyzed: sAAT (amino aspartate transferase, EC 2.6.1.1), AO (aldehyde oxidase, EC 1.2.3.1), GPI (glucose-6-phosphtase isomerase, EC 5.3.1.9), and PGM (phosphoglucomutase, EC 2.7.5.1). AO and sAAT loci are fixed for at least some species and can therefore be used for direct taxon identification (for details see Seda et al., 2007). Individuals homozygous for both alleles of the AO and sAAT loci were scored as pure species, heterozygotes were considered to be hybrids. A small proportion of the animals with patterns suggesting backcrosses or later-generation hybrids were pooled with other hybrids for the reasons described above.

ITS-RFLP

In order to obtain a direct comparison of allozymes and DNA-based methods, we took aliquots of *Daphnia* whole-body homogenates prepared for the allozyme electrophoresis as substrates for DNA preparation. We transferred 2.5 μl of the homogenate to 30 μl of a solution containing H3 buffer and proteinase K (Schwenk et al., 1998). Samples were incubated at 55°C for 6–10 h followed by inactivation of proteinase K at 95°C for 10 min. DNA isolates were used for both ITS-RFLP and microsatellite analyses.

Amplification and restriction of the nuclear ribosomal ITS mostly followed the protocol by Skage et al. (2007). However, in order to clearly differentiate between *D. galeata* and possibly uncut PCR products, we used an alternative forward primer ITS-NEW (see Appendix in Skage et al., 2007) to produce a ~190 bp band instead of a ~75 bp band as the smallest fragment. Amplicons were digested by

Table 1 Sampling sites, their geographical location, presence of species and hybrids of the *Daphnia longispina* complex and list of methods applied to individuals from each particular locality: allozyme electrophoresis (allo), DNA microsatellite analysis (μsats), ITS-RFLP, phenotype-based determination (morph), and geometric morphometric analysis of body shape (EFA, elliptic Fourier analysis)

Locality	Latitude	Longitude	Taxa present	Methods used
Kníničky	49°14′	16°31′	g, l, c, gxl, gxc	allo, ITS-RFLP, morph, EFA
Římov	48°50′	14°30′	g, c, gxc	allo, ITS-RFLP, morph
Seč	49°50′	15°39′	g, c, gxc	allo, ITS-RFLP, morph, EFA
Sedlice	49°31′	15°12′	g, c, gxc	allo, ITS-RFLP, morph, EFA
Stanovice	50°11′	12°53′	g	allo, ITS-RFLP, morph, EFA, μsats
Šance	49°31′	18°25′	g, gxl	allo, ITS-RFLP, morph
Trnávka	49°31′	15°13′	g, c, gxl, gxc	allo, ITS-RFLP, morph, EFA
Vír	49°34′	16°19′	g, c, l, gxl	allo, ITS-RFLP, morph, EFA, μsats
Vranov	48°54′	15°49′	g, c, l, gxl, gxc	allo, ITS-RFLP, morph, EFA, μsats
Želivka	49°43′	15°06′	g, l, gxl	allo, ITS-RFLP, morph, EFA

The list of taxa present at the localities is based on the results of allozyme analysis using information from two allozyme loci (sAAT and AO). Names of the species (*D. galeata*, *D. longispina*, *D. cucullata*) are abbreviated by the first letter of their species names. More details about studied localities, apart from Sedlice (length 3 km, area 0.4 km^2, max. depth 14 m) are available in Seda et al. (2007)

overnight incubation with the restriction enzymes *Mbi*I and *Eco*52I (Fermentas, Burlington, Canada) and electrophoresed on an agarose gel. The banding patterns were interpreted according to Skage et al. (2007) and Petrusek et al. (2008b). Individuals exhibiting clear additive patterns were scored as hybrids even if the bands varied in intensity. However, very weak and poorly visible bands were not considered. We also occasionally used the original ITS-RFLP method designed by Billiones et al. (2004) in order to confirm results obtained by the new protocol, especially in populations where results of ITS-RFLP strongly differed from those obtained by other markers (see "Results" section).

Microsatellite analysis

Samples from the Stanovice reservoir apparently containing a single species, and samples from the Vranov and the Vír Reservoirs containing all three parental species plus interspecific hybrids (as determined by allozyme electrophoresis), were analyzed in detail with a set of 12 microsatellite markers: DaB10/14, Dp281NB, SwiD14, DaB17/17, Dp196NB, Dp519, SwiD6, SwiD12, SwiD18, Dgm105, Dgm109, and Dgm112. Amplification and length assessment of the fragments mostly followed the protocol by Brede et al. (2006) and Thielsch et al. (2009). After amplicons had been obtained, they were appropriately diluted and electrophoresed on a CEQ 2000 capillary sequencer (Beckman Coulter, Fullerton, CA, USA) with self-designed size standards based on Lambda virus DNA (Symonds & Lloyd, 2004).

As none of the microsatellite loci were fixed for species-specific alleles, we used the NewHybrids software (Anderson & Thompson, 2002) to compute the posterior probabilities of individuals belonging to parental species or hybrid genotypes. This software is designed to work with two parental species only; we therefore analyzed only subsets of individuals belonging to one pair of species or their respective recombinant genotypes (see Fig. 1). To facilitate selection of individuals for such analyses, we performed a factorial correspondence analysis (FCA) in the software Genetix 4.01 (Belkhir et al., 1996–2004). Before each analysis in NewHybrids, we excluded all individuals possibly sharing characters specific for the third parental species; this included individuals carrying the respective species-specific allozyme allele(s) and individuals, the position of which in the FCA plot (Fig. 1) suggested likely introgression from the third species.

Taxon origin was estimated in nine separate NewHybrids analyses combining all potential parental pairs (*D. galeata* and *D. longispina*, *D. galeata* and *D. cucullata*, *D. cucullata* and *D. longispina*; see

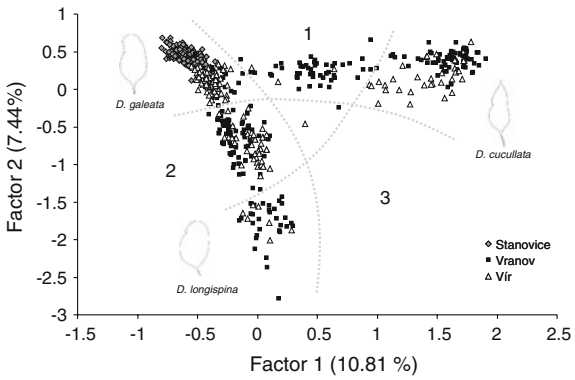

Fig. 1 The first two axes of the factorial correspondence analysis based on twelve microsatellite markers, showing 651 individuals of the *Daphnia longispina* complex from three reservoirs (symbols mark the origin of each individual). Typical body shapes from the three marginal clusters (i.e., parental taxa) are illustrated by outlines. *Dotted gray lines* and numbers approximately indicate groups of individuals involved in separate runs of NewHybrids analyses (see "Materials and methods" section)

Fig. 1) and differing by the sampling site (individuals from all localities pooled, Vranov Reservoir only, Vír Reservoir only). Two random numbers defined the starting position and at least 10^4 iterations were carried out after a burn-in period of 10^4 iterations. In each run, an individual was considered to be identified if its posterior probability of belonging to a certain taxon (parental species or recombinant genotype) was equal to or higher than 0.8. If no posterior probability exceeded 0.8, the taxonomic status of such an individual was considered undetermined in that particular run. Finally, results of different NewHybrids analyses were compared to each other. Each individual was considered unambiguously identified if the results from different runs did not contradict each other.

Photo-based identification and geometric morphometric analysis of body shapes

To compare identification by molecular markers with identification based on general phenotype, which is often used for the routine identification of zooplankton samples in ecological studies, we asked six experts with experience in identification of crustacean zooplankton (including *Daphnia*) to determine individuals from photographs that were taken before genetic analyses. Altogether, we used a subset of 240 lateral-view photographs of genetically identified *Daphnia*. Origin and taxon of the animals was kept secret and the choice of six taxa was given: *D. galeata*, *D. longispina*, *D. cucullata* or any of their hybrids. Results were consequently added to the data set and compared with the results of genetic determination. We are aware that determination based on a photograph is not directly comparable to determination of an individual under a microscope, as some morphological features cannot be examined in detail from an image. However, parameters reflected in body shapes, such as head-to-body ratio, outline of the head and shape of the rostrum, have been considered useful (though not necessarily primary) characters for identification of females in the *Daphnia longispina* complex, and are provided in important identification keys (e.g., Margaritora, 1985; Glagolev, 1995; Alonso, 1996; Flößner, 2000; Benzie, 2005). As hybridization results in intermediate phenotypes (Gießler, 2001; Schwenk et al., 2001), perceived differences in body shapes are sometimes taken into account by experienced persons when screening zooplankton samples for *Daphnia* taxon composition (i.e., presence of parental species and hybrids).

Additionally, we compared this subjective taxon discrimination by human eyes with a geometric morphometric analysis of body shape variation, based on outlines extracted from photographs of 1,328 animals determined by allozyme analysis. Body outlines in lateral view (excluding tail spines) were characterized by 50–60 equally spaced points, and subjected to an elliptic Fourier transformation (Kuhl & Giardina, 1982; Ferson et al., 1985) in the program EFAWin (Isaev, 1995). Size (i.e., scale), location of the outline, rotation, and start position were selected as invariant factors. Normalized coefficients of five harmonic functions (altogether 17 variable parameters) were obtained; none of these alone has a biological interpretation, but together they sufficiently characterize the whole outline of the studied object. The results were analyzed by principal component analysis (PCA) and discriminant analysis (DA). Both methods used the 17 normalized coefficients from elliptic Fourier analysis as input data. Taxon identification by allozymes was used as a grouping variable in DA, and a priori classification probabilities were set equal for all taxa to make results more directly comparable with expert assessment of photographs.

Multivariate analyses were calculated in the software package Statistica 6.1 (StatSoft, Tulsa, USA).

Data comparison

Results of allozyme electrophoresis, ITS-RFLP, and microsatellite analysis were compared with each other. The agreement between each pair of methods was always expressed as the percentage of matching identifications. All *Daphnia* individuals for which results from at least two different methods were available were included in the comparisons. A set of 444 individuals was identified by all three molecular methods. Agreement among molecular markers was calculated for the whole data set, but also for each sampling site and for each taxon separately. If calculated for separate taxa, taxon assignment was based on allozyme data for two reasons. First, these results were available for the whole data set; second, a vast majority of publications on hybridization in the *D. longispina* complex have relied on allozyme markers for taxon identification. We therefore tested to what extent the use of other markers deviates from this most widely used method (though choosing identification based on microsatellite markers as the baseline would not change the patterns substantially—see "Results" section). Determination based on phenotypic traits was compared to results from allozyme electrophoresis; the agreement between the methods was also expressed in percentages.

Results

Molecular markers

Altogether, we analyzed 1,276 individuals from 10 reservoirs by two or more molecular markers: allozymes and ITS-RFLP patterns were compared for 1,275 individuals, allozymes, and microsatellites for 636 individuals, and microsatellites and ITS-RFLP for 445 individuals. A total of 444 individuals were simultaneously determined by all three molecular methods, providing an opportunity to identify a deviating marker in cases where two methods did not correspond to each other. Allozymes, microsatellites, and ITS-RFLP analyses split the data set into five taxa: *D. galeata*, *D. longispina*, *D. cucullata*, *D. galeata* × *longispina* hybrids, and *D. galeata* × *cucullata* hybrids. No individual representing a *D. longispina* × *cucullata* hybrid was present in the data set (see also Petrusek et al., 2008b).

In general, the molecular markers corresponded well to each other (Table 2). The highest fit (97.0%) was observed between results of the allozyme electrophoresis and microsatellite analysis. Allozymes and ITS-RFLP corresponded to each other in 86.8% of cases, and microsatellites and ITS-RFLP in 82.7%. In most examined localities, however, ITS-RFLP matched relatively well to other methods (Table 2, Fig. 2), and results based on this marker would not substantially influence the interpretations.

The majority of deviations among the molecular markers were nonrandom, only affected certain taxa, or were only pronounced in some localities (Table 2). Results of allozyme electrophoresis and microsatellite analysis completely agreed in the Stanovice Reservoir, which was inhabited only by *D. galeata*. The agreement was also high in two other reservoirs—the Vír Reservoir (98.8%) and the Vranov Reservoir (93.8%), both occupied by all three parental species and interspecific hybrids. On the contrary, comparisons of identification based on allozymes and ITS-RFLP showed occasionally marked disagreement. Interestingly, in reservoirs where hybridization frequently occurred and all three *Daphnia* parental species were present, the mismatch of molecular methods did not distinctly exceed that in reservoirs with less frequent hybridization (Table 2, Fig. 2). Actually, the trend, if any, was opposite. The largest deviations occurred in the Trnávka Reservoir (agreement 54.8%), where the proportion of hybrids identified by allozyme markers was below 3% but where ITS-RFLP suggested it to be over 40%, followed by the Stanovice Reservoir (agreement 81.0%), in which no hybrids were detected by other markers but 19% would be detected by ITS-RFLP. In the latter reservoir, the pattern was very similar (agreement 81.8%) when the results from ITS-RFLP were compared to those from the microsatellite analysis. In the Vranov and Vír Reservoirs, the agreement between microsatellite analysis and ITS-RFLP exceeded 80% (81.2% and 86.0%, respectively).

In order to clarify why the fit among markers varied among different reservoirs, we also evaluated the success of all three molecular methods in identifying each parental species and their hybrids. The taxon for which the determination by allozymes

Table 2 Agreement between allozyme electrophoresis (allo), DNA microsatellite analysis (μsats), and ITS-RFLP in determination of individuals belonging to the *Daphnia longispina* complex

Marker	Identification by molecular markers (number of determined individuals/agreement of markers)		
	allo × μsats	allo × ITS-RFLP	μsats × ITS-RFLP
Total	636/97%	1275/86.8%	445/82.7%
Locality			
Knínički	N/A	134/90.3%	N/A
Římov	N/A	134/97.8%	N/A
Seč	N/A	124/92.7%	N/A
Sedlice	N/A	25/96.0%	N/A
Stanovice	190/100%	163/81.0%*	159/81.8%
Šance	N/A	131/90.1%	N/A
Trnávka	N/A	124/54.0%*	N/A
Vír	171/98.8%	139/88.5%	121/86.0%
Vranov	275/93.8%	172/95.9%	165/81.2%
Želivka	N/A	129/85.3%	N/A
Taxon			
D. galeata	330/99.7%	928/88.7%	253/88.1%
D. cucullata	119/84.9%*	154/87.7%	90/77.8%
D. longispina	41/100%	30/83.3%	21/71.4%
D. galeata × *longispina*	37/100%	32/84.4%	54/63.0%
D. galeata × *cucullata*	109/100%	131/74.1%	26/84. 6%

The whole data set contained 1,276 individuals; 444 individuals were determined using all three molecular methods. Relative agreement for each pair of compared methods is shown as the percentage of individuals determined identically by both methods. Results were calculated for the whole data set, for each reservoir separately, and for each taxon separately. Results highlighted by asterisks indicate disagreement of the methods caused by consistent trends for misclassification of a certain taxon (see "Results" section)

and microsatellites deviated most strongly was *D. cucullata* (Table 2). Only 84.7% individuals identified as *D. cucullata* by allozyme markers were identified as such by microsatellite analyses, whereas the agreement of identification between these two marker systems was almost perfect in the other taxa (99.7% for *D. galeata* and 100% for the rest). All of the "atypical" *D. cucullata* individuals originated from the Vranov Reservoir and were determined as pure species by allozymes and ITS-RFLP, but as hybrid genotypes by microsatellites. In a detailed analysis of hybrid classes, computed by NewHybrids from microsatellite data, all these individuals were assigned as backcrosses or F_2 hybrids. When looking in more detail, an unexpected pattern was also observed for individuals identified by allozymes as *D. galeata* × *cucullata* hybrids: all of them were classified as backcrosses or F_2 hybrids based on microsatellites, and no individual was identified as an F_1 *D. galeata* × *cucullata* hybrid out of 300 individuals from the two reservoirs in which all three *Daphnia* species co-occurred.

Agreement of ITS-RFLP with the other two markers, if calculated for each taxon separately, ranged between 74.1% and 88.7% for ITS-RFLP/allozymes, and between 63.0% and 88.1% for ITS-RFLP/microsatellites. In general, hybrids were the most problematic group, where discrepancies between identification by ITS-RFLP and other markers were common (Table 2).

Among the taxa, identification of *D. galeata* was most successful overall; however, this pattern changed when the agreement among markers was calculated separately for each taxon within each reservoir. Two reservoirs, Stanovice and Trnávka, exhibited a large proportion of inconsistent identifications of *D. galeata* in particular, but not of other taxa, which also resulted in overall low agreement of determinations from these localities (see above). The only *Daphnia* species found in the Stanovice Reservoir in

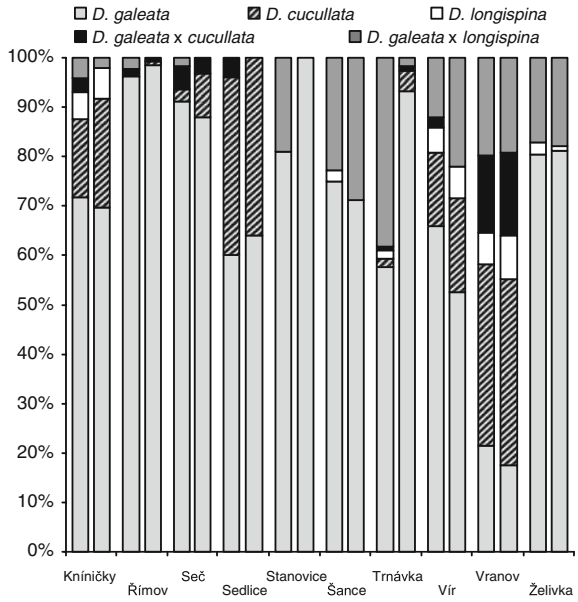

Fig. 2 Relative abundances of *Daphnia* taxa in ten reservoirs as identified by either ITS-RFLP (left columns) or allozyme electrophoresis (right columns). Numbers of analyzed individuals from each sampling site are given in Table 2. Note the marked disagreement of the two methods for individuals from Stanovice and Trnávka

summer 2004 was *D. galeata.* This was independently confirmed by both allozyme and microsatellite analyses (and also agreed with morphological traits). ITS-RFLP analysis of the same individuals nevertheless showed that over 19% of the animals also carried the ITS allele considered typical for *D. longispina*, and the resulting restriction pattern was thus the same as in *D. galeata* × *longispina* hybrids. An analogous situation was found also in the Trnávka Reservoir, where over 40% of *D. galeata* individuals (based on allozyme determination) exhibited the same restriction pattern as observed in Stanovice. Although allozyme-based determination was not simultaneously confirmed by another molecular marker in this case, the morphology of those individuals corresponded more to *D. galeata* than to interspecific hybrids.

Variation in body shape

Photograph-based determination provided by six experts was usually substantially less successful than molecular methods (Table 3). On average, it agreed with allozyme electrophoresis in only 63.5% of cases. The success differed widely among experts and ranged between 40.8% and 82.5%. The most successful determination was very similar to results of discriminant analysis (DA) based on geometric morphometrics (83.4%; see below), and had success comparable to the agreement between allozymes and ITS-RFLP (86.8%). Determination of hybrids was less successful than that of the parental species: two experts did not attempt to differentiate them at all, and results of the other four persons varied substantially. *D. galeata* × *cucullata* hybrids were easier to recognize (33–100% success) than *D. galeata* × *longispina* hybrids (18–49%). *D. galeata* × *longispina* hybrids were apparently most difficult to identify by their phenotype.

Body shape variation among *Daphnia* individuals, as summarized by PCA of parameters from elliptic Fourier transformation of body outlines, was high. Dots representing single individuals formed a relatively compact cluster in the PCA plots (Fig. 3), from which only individuals of *D. cucullata* were clearly separated. Clusters representing other species and hybrids (labeled according to the identification by allozyme markers) at least partly overlapped. However, hybrid clusters were situated between those of the parental species, confirming that hybrids are often morphologically intermediate. *D. galeata* and *D. longispina* were mostly separated from each other, but heavily overlapped with the cluster of *D. galeata* × *longispina* hybrids, the most poorly separable taxon. These patterns were confirmed by results of the DA (Table 3, last column), in which most misclassifications were observed among the two above-mentioned species and their hybrids. The pattern in the plot of the first two canonical axes of DA (results not shown) was similar to that showing the first and the third component of PCA (Fig. 3, top).

Discussion

Processes following interspecific hybridization, such as backcrossing and occasional gene flow, may significantly influence the accuracy of genetic and morphological determination in hybridizing species (Billiones et al., 2004; Mallet, 2005; Skage et al., 2007). At the phenotypic level, the occurrence of intermediate morphological forms may complicate the taxonomy in such groups (Mallet, 2005; Arnold,

Table 3 Success of taxon determination from photographs provided by six experts experienced in cladoceran taxonomy or ecology, based on 240 randomly selected lateral views of the *Daphnia* body, and results of the DA based on the elliptic Fourier analysis of body outlines

Taxon	Identification from body shape (photographs or geometric morphometrics) (number of determined individuals/agreement with allozyme markers)						
	Expert 1	Expert 2	Expert 3	Expert 4	Expert 5	Expert 6	Computer (DA)
All taxa	240/82.5%	223/62.3%	240/64.6%	238/72.3%	133/58.6%	196/40.8%	1328/83.4%
D. galeata	138/96.4%	123/76.4%	138/71.7%	136/97.1%	66/60.6%	106/31.1%	817/84.5%
D. cucullata	22/81.8%	22/95.5%	22/95.5%	22/81.8%	15/100%	21/76.2%	137/90.5%
D. longispina	24/75.0%	24/100%	24/75.0%	24/91.7%	20/50.0%	22/100%	183/82.0%
D. galeata × *longispina*	51/49.0%	49/0%	51/23.5%	51/0%	27/29.6%	44/18.2%	172/72.7%
D. galeata × *cucullata*	5/80.0%	5/0%	5/100%	5/0%	5/100%	3/33.3%	19/100%

Relative success of the determination is shown as the percentage of individuals determined identically by both phenotype and molecular methods from the total number of determined individuals of that particular taxon. In cases where the total N is <240, the person did not determine all individuals from the data set. Species and interspecific hybrids were primarily identified by allozyme electrophoresis using two species-specific loci (sAAT, AO)

2006; Schwenk et al., 2008). At the genotypic level, backcrossing and gene flow may lead to mismatches among different molecular methods (Harrison, 1990; Arnold, 1992), especially if only a limited number of loci are analyzed. For instance, in methods using restriction length polymorphism, recombinant genotypes may be determined as interspecific hybrids or pure species (Bert et al., 1996; Boecklen & Howard, 1997). As later-generation hybrids exhibit a mosaic of parental alleles, an insufficient number of loci may give incomplete information. In the *D. longispina* species complex, such an underestimation of recombinant classes has been observed in studies applying commonly used molecular methods—ITS-RFLP (Billiones et al., 2004) as well as the analysis of two species-specific allozyme loci (e.g., Seda et al., 2007). The observed inconsistencies among markers have therefore been mostly attributed to their insufficient discriminatory power, unable to reveal backcrossing and introgression (Billiones et al., 2004). In our study, detailed analysis of microsatellites nevertheless showed that the proportion of later-generation hybrids and introgressed individuals was low in the studied localities. Thus, other possible causes of observed discrepancies need to be taken into account.

ITS-RFLP

The possibility that mismatches among ITS-RFLP and the other two molecular markers in our samples was caused by a high frequency of hybridization, by backcrossing or by recent gene flow, can be rejected by a comparison of results from various sampling sites. If that was true, localities with more frequent hybridization should exhibit more discrepancies among different markers. However, the observed trend was not consistent with this assumption—the biggest inconsistencies between ITS-RFLP and allozymes were actually observed in reservoirs with no or a very low proportion of hybrids in the active population (Stanovice and Trnávka, respectively). The majority of those inconsistencies were caused by a large proportion of *D. galeata* individuals exhibiting ITS-restriction patterns supposedly characteristic for *D. galeata* × *longispina* hybrids. It is unlikely that this pattern could have been caused by a chance convergence of *D. galeata* ITS alleles, as the hybrid-like patterns were obtained by two proposed ITS-RFLP protocols (Billiones et al., 2004; Skage et al., 2007), which differ in the position of several restriction sites.

Past introgression of ITS alleles between species of the complex is more likely responsible for the patterns described above. Evidence of such processes can also be found in other populations of the *D. longispina* complex. Gießler & Englbrecht (2009) recently reported similar results: they analyzed five individuals of *D. cucullata* from Germany and the Netherlands, all of which carried ITS alleles indistinguishable from those of *D. galeata*. Kraus (2007) also observed ITS-RFLP patterns supposedly typical for *D. galeata* or *D. galeata* × *cucullata* hybrids (according to the

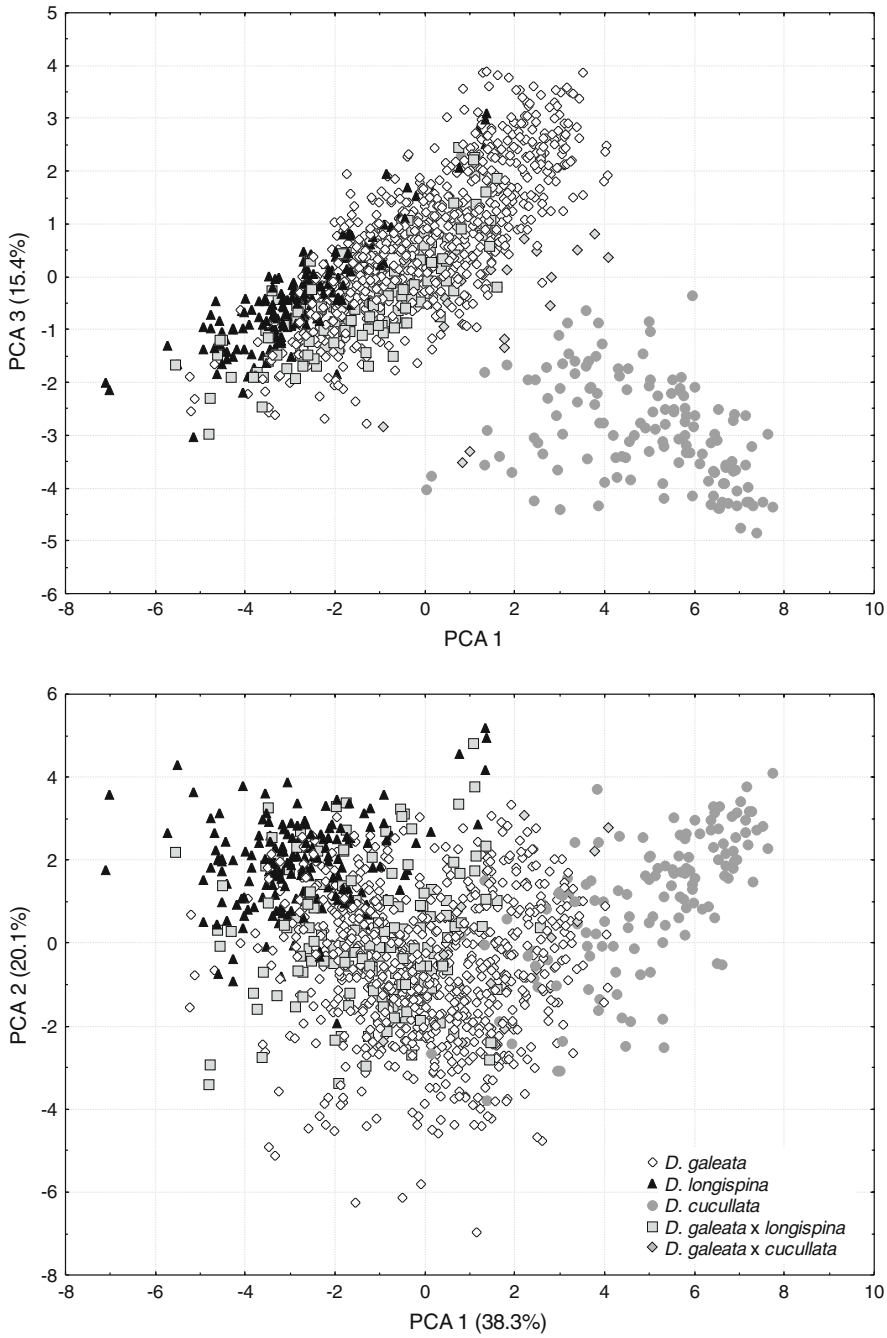

Fig. 3 The first three components of the PCA showing the body shape variability of 1,328 individuals of the *Daphnia longispina* species complex from eight canyon-shaped reservoirs. Normalized coefficients of five harmonic functions from the elliptic Fourier analysis of individual body outlines (altogether 17 variables) were used as input for the PCA. The taxon of each individual (as identified by two allozyme markers) is indicated by different symbols and shading. The first three components explain 73.8% of the variation

protocol of Skage et al., 2007) in several European populations of *D. cucullata* (as determined by microsatellites). However, not all individuals of *D. cucullata* carry a "*D. galeata*-like" ITS, as identification by ITS-RFLP was mostly in accordance with alternative molecular markers or morphology in other populations (including those we studied; see Fig. 2).

Gene flow between species in the distant past may explain inconsistent ITS-RFLP patterns. The question nevertheless remains how an "alien" allele is maintained in the genome of the recipient species instead of being lost due to genetic drift. ITS regions are segments of ribosomal DNA occurring in many copies within a genome. On the one hand, the

multicopy character of the marker facilitates amplification, but on the other hand, such genomic regions may be prone to deviations from expected patterns due to processes such as concerted evolution (Arnheim, 1983; Dover et al., 1993; Murti et al., 1994) and gene conversion. In the latter process, which is thought to have evolved as part of DNA repair mechanisms, sequence heterogeneity between two strands of different chromosomes is removed by recombination, and sequence information of one of the chromosomes is re-written according to the template of the other (Holliday, 1964). This may lead to non-Mendelian inheritance and to an increasing number of template copies in a population (Stacey, 1994). Concerted evolution and gene conversion have been recorded in variety of taxa, from bacteria to mammals (Liao, 1999). Specifically for the ITS region, this process has been studied for example in *Drosophila* (e.g., Polanco et al., 1998); however, we are not aware of data available for animal hybrid genomes. Non-Mendelian inheritance may also be caused by other mechanisms favouring selfish genetic elements (Hurst & Werren, 2001). It has been shown, for instance, that meiotic drive causes segregation distortions in mice (Morita et al., 1992; Futuyma, 2005) or in the dipteran *Cyrtodiopsis* sp. (Wright et al., 2004). Some of these mechanisms may have been also responsible for the conservation of introgressed ITS rDNA alleles in *Daphnia*.

Microsatellites and allozymes

The fit between taxon determination from allozyme and microsatellite data was in general very high, suggesting that both methods are reliable if used for basic determinations of species and hybrids in the *D. longispina* species complex. Inconsistencies between allozymes and microsatellites nevertheless occurred, significantly affecting individuals related to *D. cucullata*. Some apparently pure *D. cucullata* individuals (as determined by two allozyme loci), were suggested to be hybrids with *D. galeata* by Bayesian inference calculated in NewHybrids, and all apparent *D. galeata* × *cucullata* hybrids as backcrosses or F_2 hybrids. A similar pattern persisted even if the same analysis was performed with four allozyme loci (Š. Dlouhá et al., unpublished results), suggesting that these inconsistencies were not caused by the marker system, but were genetically based. Difficulties with determination of hybrid classes among *D. galeata* × *cucullata* recombinant genotypes, inferred by the Bayesian approach from allozyme markers, is also apparent from results presented by Keller et al. (2008).

The observed patterns of disagreement between allozymes and microsatellites within the Vranov Reservoir suggest some horizontal gene flow between species of the *D. longispina* species complex via backcrossing. In such cases, gene pools of the parental species might be partly fused and inconsistencies among the species-specific markers may occur (Harrison, 1990; Arnold, 1992). The importance of this process has been emphasized in some studies (Schwenk et al., 1995; Gießler et al., 1999; Gießler & Englbrecht, 2009) which consider the *D. longispina* species complex as a group of taxa with incomplete reproductive isolation. Thus, species phylogeny could exhibit a reticulate rather than hierarchical pattern of evolution. On the contrary, Keller et al. (2007) recently suggested that levels of effective gene flow within the complex are very low, and that parental species remain reproductively isolated despite hybridization.

Our observations support previous results that backcrosses and later-generation hybrids occasionally sexually produce viable offspring (e.g., Spaak, 1996; Schwenk & Spaak, 1997; Jankowski & Straile, 2004). Substantial deviations of some ITS-RFLP patterns suggest long-term maintenance of introgressed alleles in the genomes of parental species by non-Mendelian inheritance rather than recent gene flow. However, inconsistencies of allozyme and microsatellite markers observable in *D. cucullata* and their hybrids with *D. galeata* apparently result from ongoing processes. A pattern requiring further attention is the absence of individuals identified as F_1 hybrids. We speculate that this result is a consequence of either assortative mating or, more likely, a reproductive incompatibility between certain *D. cucullata* and *D. galeata* genotypes, leading to biased combinations of parental markers in viable hybrids.

Shape variation and overlap among taxa

Comparisons of body shapes of parental species and hybrids clearly show that despite some phenotypic divergence, variation within all groups was high and caused partial overlaps of all clusters except for

parental *D. cucullata*. Apparently, individuals of several taxa within the complex may exhibit nearly identical body shapes, including those belonging to different parental species (*D. galeata* and *D. longispina*; see Fig. 3).

This phenotypic similarity between individuals from different taxa was reflected in the relatively low level of success in species determinations based on body shape (both by expert assessment of photographs and by DA) in comparison to molecular methods. Consistent with the geometric morphometrics, most incorrectly determined individuals were among *D. galeata*, *D. longispina*, and their hybrids. These results nevertheless illustrate that differences in body shape, although hardly describable, can be—and are—used in routine discrimination among taxa even when limited numbers of species-specific characters are visible. The addition of more characters, such as details of the rostrum and antennules, or the pigmentation of swimming setae, would certainly further increase the identification success. However, identification of hybrids currently remains highly subjective and dependent on experience, despite attempts to provide formalized identification keys based on morphological characters (Flößner, 1993, 2000). Further research in this field is therefore warranted.

Conclusions and recommendations

All tested molecular markers, as well as determination based on phenotype, are applicable under certain conditions for taxon determination of hybridizing members of the European *D. longispina* complex. However, as we show in this study, the discriminatory power of different methods has limitations, and the selection of an appropriate method thus depends on specific research questions.

Allozyme markers, the oldest molecular method in use, proved to be very robust for basic determination of species and hybrids in the *D. longispina* complex across various populations. Despite certain inconveniences, in particular the requirement for live or deep-frozen samples, the speed, relatively low cost and good reproducibility of results make this method appropriate for community-level studies.

In contrast, ITS-RFLP, originally suggested as a simple and low-cost DNA-based alternative to allozyme electrophoresis, showed substantial deviations from other molecular methods in some populations, in which its application would give spurious results. We agree with the conclusions of Gießler & Englbrecht (2009), who warn against using ITS-RFLP for taxon identification. Nevertheless, if properly validated for specific study questions, it may remain a convenient method for genotyping large-sized samples, especially from ethanol-preserved material. For example, it can be very useful for analyses of individuals originating from controlled experiments, in which the stocked animals had been genotyped in advance. The use of ITS-RFLP as the sole method of taxon identification in field samples should be discouraged, unless it is accompanied by verification of the results by other methods, at least on a subset of analyzed individuals from each studied locality.

Microsatellite analysis, being the most sophisticated of the tested methods, also provided appropriate results at the level of crude taxon determination. Despite this, it is not necessarily the most convenient method for routine screening, due to the still relatively high costs, and the absence of species-specific markers preventing a simple and straightforward interpretation of observed patterns. On the other hand, microsatellite data provide a wealth of additional information on population structure and clonal composition. It will therefore certainly become the method of choice for evolutionary studies focusing on detailed patterns and consequences of interspecific hybridization and introgression.

Our results of photo-identification suggest that even superficial screening of body shapes may provide useful insights into taxon composition and the potential presence of hybrid genotypes in *Daphnia* populations. However, the accuracy of such an approach strongly depends not only on the species and hybrids present, but also on experience of the person providing determinations. At present, we are not aware of any unambiguous morphological characters allowing reliable identification of at least first-generation hybrids of *D. galeata* × *longispina*. Detailed studies focusing on changes in daphnid morphology resulting from hybridization would therefore be particularly helpful. With the present lack of knowledge on reliable determination characters, molecular methods should be applied if information on taxon composition is crucial, and especially where proportions of parental taxa and hybrids should be known more precisely.

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