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Genetic markers, genealogies and biogeographic patterns in the cladocera

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Abstract

Cladoceran crustaceans are an important component of zooplankton in a wide range of freshwater habitats. Although the ecological characteristics of several cladoceran species have been well studied, biogeographical studies have been hampered by problematic taxonomic affiliations. However, recently developed molecular techniques, provide a powerful tool to subject aquatic taxa to comparative analyses. Here we highlight recent molecular approaches in aquatic ecology by presenting a simple method of DNA preparation and PCR amplification of the mitochondrial DNA (16S rDNA) in species from nine different families within the cladocera. On a broad taxonomic scale, sequence analysis of this mtDNA fragment has been used to produce the first molecular based phylogeny of the cladocera. This analysis clustered the cladoceran families in a fashion similar to that suggested by previous systematic classifications. In a more detailed analysis of the family Daphniidae, nuclear randomly amplified polymorphic DNA (RAPD), mitochondrial restriction fragment length polymorphism (RFLP) and morphological analyses were combined to identify species and interspecific hybrids within the *Daphnia galeata* species complex across 50 lakes in 13 European countries and one lake in Africa. The study revealed interspecific hybridization and backcrossing between some taxa (*D. cucullata* and *D. galeata*) to be widespread, and species and hybrids to frequently occur in sympatry. Genetic, as well as morphological information, suggests the occurrence of *D. hyalina* outside the Holarctic.

Introduction

Cladocerans, comprising the orders Anomopoda, Ctenopoda, Haplopoda and Onychopoda are of great importance in aquatic food chains. They mostly feed on algae, detritus or both and are in turn consumed by planktivorous fish and invertebrate predators. Some cladoceran genera (e.g. *Daphnia*) have been utilized as model organisms in ecology, ecotoxicology and ecological genetics (e.g. Mort, 1991), yet several aspects of their biology, such as population ecology, gene flow and population genetics, have been studied in only a few species (Mort, 1991). Also, the evolutionary history and phylogenetic relationships of several groups are unresolved (e.g. chydorids: Korovchinsky, 1996) or in a state of flux (e.g. *Daphnia*: Hrbáček, 1987; Colbourne & Hebert, 1996). These uncertainties are caused by the high phenotypic plasticity of species, the occurrence of local races and natural interspecific hybridization (e.g. Hebert, 1985; Frey, 1986; Hann, 1987; DeMelo & Hebert, 1994). Many *Daphnia* species, such as *D. galeata*, *D. cucullata*, *D. hyalina*, *D. rosea* and *D. longispina*, belonging to the *D. galeata* species complex, are known to form interspecific hybrids (Wolf & Mort, 1986; Hebert et al., 1989), which can be considered guilds since hybrids represent ecological units that differ from their parental species in traits such as susceptibility to predation, competition and behavior (e.g. Weider, 1993;

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Spaak & Hoekstra, 1995). However, identification of species and their hybrids appears to be difficult using morphological traits alone, since syntopic populations are characterized by gradual transitions of supposedly species-specific traits (e.g. Flößner, 1993).

During the last decade, several polymerase chain reaction (PCR) based techniques have found widespread application in population genetics and ecology (e.g. Avise, 1994). These techniques include amplification of mitochondrial DNA and subsequent sequencing or detection of restriction fragment length polymorphism (RFLP) and amplification of nuclear DNA (random amplified polymorphic DNA, microsatellites, and anonymous loci; Arnheim et al., 1990; Avise, 1994). Research programms which require high genetic resolution (e.g. niche differentiation of clonal organisms) or cases where only small amount of tissue is available (e.g. microcrustaceans) have made PCR based techniques the preferred method for population genetic analyses (e.g. Schierwater et al., 1994). Among the cladocerans, species of the genus *Daphnia* have been subjected to a variety of molecular DNA analyses (e.g. Crease et al., 1989; Taylor & Hebert, 1993a; Schwenk, 1993; Weider & Hobaek, 1994; Ender et al., 1996) but other genera have been neglected.

This paper highlights recent molecular approaches used in aquatic ecology and evolution by describing first a simplified DNA preparation method and second a genetic analysis of 16 cladoceran species at two different levels (i.e. species and populations). In a broad approach, using 'universal' mtDNA primers on species from four cladoceran orders comprising nine families, we used DNA sequence information to determine both divergence among species and their phylogenetic relationships. In the second part, data on the magnitude of interspecific hybridization among members of the genus *Daphnia* and the distribution of several species and their hybrids from 64 European lakes are presented. For each individual used in this analysis, three independent data sets on morphological, mitochondrial and nuclear DNA were obtained to reveal the discriminatory power of each character set and compare genetic and phenotypic information.

Materials and methods

Cladoceran collections

Individuals of 23 different cladoceran species, belonging to all four orders (Anomopoda, Ctenopoda, Haplopoda and Onychopoda), were subjected to a simple DNA preparation method. Specimens came from a variety of geographic regions and originated either from recent field sampling or from zooplankton collections (animals preserved in 70–98% ethanol: Table 1) that were sent to our laboratory. Ethanol samples which were only a few weeks old constituted the majority of the samples. However, some samples had been stored for periods exceeding two decades at room temperature.

Daphnia *collections and morphological analysis*

Individual *Daphnia* from the *D. galeata* species complex were selected from ethanol preserved field samples and characterized for 10 morphological traits, which are considered to be characteristic for the species and interspecific hybrids (Flößner, 1972, 1993; Flößner & Kraus, 1986). These traits included the number of anal spines, the shape of the antennulae mound, insertion and length of aesthetasks, presence of ocellus, the crest in frontal view, rostrum shape and length, head shape near the eye and the ventral margin of the head. Two hundred and nine individuals were analyzed for all morphological characters, and a sub-sample of ninety-four individuals was subjected to DNA analysis. A large geographic range of samples (i.e. Portugal to northern Finland, Scotland to Greece; Table 2) was used in order to examine the range of within and between species variation.

DNA preparation, amplification and RFLP

For all the studies presented here, individual animals were transferred to 1.5 ml reaction tubes, containing 30 to 400 μl H3-buffer (10 mm Tris-HCl, pH 8.3 at 25 °C, 0.05 M potassium chloride, 0.005% Tween-20 and 0.005% NP-40; Replitherm Reaction Buffer, Biozym) and 15 μg proteinase K. The volume of the H3-buffer is dependent on the amount of tissue per sample, i.e. the size of the animals (size range: 0.1 to 10 mm). Optimal volumes are given in Table 1. Specimens were homogenized with a Perspex pestle that fits precisely into the reaction tube. After brief grinding, samples were incubated overnight in a 50 °C waterbath with mild shaking. Finally, the proteinase

Table 1. Species used in DNA preparation method and in DNA sequencing analysis (SA), including systematic grouping, origin of samples and optimal volume of homogenization buffer (H3). Starting material for PCR reactions were either DNA preparations from fresh material (fresh), alcohol preserved specimens (alcohol) or DNA preparations which had been stored at 4 °C (homogenate)

Order	Family	Species	Origin	Starting material	H3 (μL)	SA
Anomopoda	Chydoridae	*Chydorus sphaericus*	Loosdrecht, NL	Fresh	50	–
		Alona quadrangularis	IJsselmeer, NL	Fresh	100	Yes
	Daphniidae	*Daphnia galeata*	Tjeukemeer, NL	Fresh	100	Yes
		Daphnia cucullata	Tjeukemeer, NL	Fresh	100	Yes
		D. cucullata x galeata (one resting egg)	Tjeukemeer, NL	Fresh	30	–
		Daphnia hyalina (Africa)	Lake Tana, Ethiopia	Alcohol	100	Yes
		Daphnia hyalina	Hartsee, Germany	Alcohol	100	Yes
		Daphnia rosea	Pond Ismaning, Germany	Alcohol	100	Yes
		Daphnia magna	Sneekermeer, NL	Homogenate	300	Yes
		Daphnia similis	Mbrillo Observatorio, Spain	Alcohol	100	Yes
		Daphnia pulex	Institute pond, NL	Homogenate	300	–
		Ceriodaphnia pulchella	IJsselmeer, NL	Fresh	100	–
		Scapholeberis mucronata	Bladel, NL	Fresh	100	Yes
	Moinidae	*Moina micrura*	Tissawewa, Sri Lanka	Alcohol	100	Yes
	Bosminidae	*Bosmina coregoni*	IJsselmeer, NL	fresh	100	Yes
	Macrothricidae	*Acantholeberis curvirostris* Bladel, NL	fresh	100	–	
		Ilyocryptus sordidus	Bladel, NL	fresh	100	–
Ctenopoda	Holopedidae	*Holopedium gibberum*	Western Siberia, Russia	Alcohol	100	Yes
	Sididae	*Sida crystallina*	Maarsseveen, NL	Fresh	100	Yes
		Diaphanosoma brachyurum	IJsselmeer, NL	Alcohol	100	–
Haplopoda	Leptodoridae	*Leptodora kindti*	Biesbos, NL	Fresh	100	Yes
Onychopoda	Ceropagidae	*Bythotrephes longimanus*	Biesbos, NL	Fresh	200	Yes
	Polyphemidae	*Polyphemus pediculus*	Maarsseveen, NL	Fresh	200	Yes

K was irreversibly denatured via a 10 min incubation at 95 °C. The homogenate was stored at 4 °C before being used in a PCR reaction. Animals which were preserved in alcohol were subjected to a minimum of 4 h incubation in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) prior to the DNA preparation. In some cases, we extracted DNA from the resting eggs of *Daphnia cucullata* × *galeata* hybrids. These eggs were dissected from ephippia and transferred individually into reaction tubes containing 30 μl H3-buffer (Table 1).

Individual homogenates were used in two kinds of PCR reactions: (1) amplification of mitochondrial DNA (16S rDNA gene) and (2) random amplified polymorphic DNA analysis (RAPD). Mitochondrial DNA was amplified using the conserved primers (S. Pääbo T.D. Kocher, pers. comm.) S1 (5′-CGG CCG CCT GTT TAT CAA AAA CAT-3′) and S2 (5′-GGA GCT CCG GTT TGA ACT CAG ATC-3′). Amplification of mtDNA was performed in 35 or 45 μl reaction volumes containing 5 or 8 μl homogenate, respectively, 1× reaction buffer (Boehringer Mannheim, BM), 0.6 U Taq polymerase (BM), 3 mM $MgCl_2$, 0.25 mM of each dNTP, and 0.5 μM of each primer. A thermocycler (OmniGene, Hybaid) was run at 93 °C for 2 min 30 s, 55 °C for 1 min and 72 °C for 2 min (1 cycle), followed by 93 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min (40 cycles). PCR products were separated on 2% agarose 1× TBE gels (LE agarose, Biozym). For RFLP-analysis, amplified products (10 μl) were cut using the restriction enzymes *Rsa*I, *Dde*I and *Mnl*I (New England Biolabs, NEB).

Amplification of mitochondrial DNA (using primers S1 and S2) of various cladoceran species resulted in segments of approximately 540 to 580 bp. Although we initially varied the volume of H3 buffer (three volumes between 25 and 400 μl, depending on the size of the animals) most amplifications were successful.

Mitochondrial DNA sequencing analysis of Cladocerans

PCR products from the cladocerans (Table 1) were directly sequenced by fluorescence sequencing following standard protocol procedures for a VISTRA DNA Sequencer 725 (Amersham International plc 1995). An internal primer (S3; 5′-GTA CCG CCT GCT CAA TGA -3′) was applied to the S1-S2 products and a segment of ±450 bp was sequenced. DNA sequences were first automatically aligned and then manually adjusted using Sequencher (Gene Codes Cooperation, Ann Harbor, Michigan, U.S.A.). Estimates of sequence divergence between all pairs of sequences and construction of neighbor-joining phylograms (Saitou & Nei, 1987) were carried out using MEGA 1.02 (Kumar et al., 1994). DNA sequences are available from the authors.

RAPD analysis of nuclear markers of the European D. galeata *complex*

RAPD reactions were performed in 0.5 ml reaction tubes with a total reaction volume of 12.5 μl containing 1× reaction buffer (BM), 0.4 U Taq polymerase (BM), 2 mM $MgCl_2$, 0.25 mM of each dNTP, and 0.2 μM of one of the three 10 bp primer (B-03: 5′-CAT CCC CCT G-3′, C-04: 5′-CCG CAT CTA C-3′ and B-14 5′-TCC GCT CTG G-3′; Operon Technologies Inc.) known to be informative in the *D. galeata* complex (see below). Two amplifications per individual with different homogenate concentrations (3 μl homogenate per reaction of a $\frac{1}{3}$ and a $\frac{1}{10}$ dilution) were routinely used. A PCR was performed using a Hybaid thermocycler (OmniGene) with tube-controlled program with (1) one cycle of 85 °C, 2 min 30 s, and (2) 40 cycles of 92 °C, 20 s; 38 °C, 15 s; ramp 3 s/ °C; and 72 °C, 1 min.

The initial selection of nuclear species specific-markers for *D. galeata* and *D. cucullata* was developed using allopatric populations and screening of more than 20 different primers (Ender, 1993; Schwenk et al., 1995; Schwenk, 1997). In addition, species-specific RAPD markers and fixed allozyme loci for *D. galeata* and *D. cucullata* were highly correlated (Schwenk, 1997). Identification of informative markers with the three primers used in this study (Table 3) enabled discrimination between species and hybrid classes (F_1-hybrids and backcrosses). In order to evaluate the reproducibility and consistency of RAPD bands, we repeated amplifications and scored only those bands that appeared in all four replicates. Furthermore, species-specific bands were found independent of DNA preparation method (either standard phenol-chloroform methods, commercial kits or the simple method presented), DNA polymerase, PCR thermocycler or laboratory (three laboratories, six different investigators; K. Schwenk, unpubl.). However, only species-specific markers of high intensity were selected and weak bands, which are more sensitive to variation in reaction conditions (e.g. DNA concentration), were omitted. We grouped individuals according to the presence of species-specific markers into four taxa: (G) *D. galeata*: all three *D. galeata* markers, but no *D. cucullata* marker, (C) *D. cucullata*: three *D. cucullata* but no *D. galeata* marker, (CG) *D. cucullata* × *galeata*: three *D. cucullata* plus three *D. galeata* markers, and (GX) *D. galeata*-like individuals: all three *D. galeata* markers, but one *D. cucullata* marker (Table 3). The remaining individuals were grouped either as: *D. hyalina* (one species-specific marker), *D. hyalina*-like (one *D. hyalina* and one ore more of the other species-specific markers) or as unknown daphnids (exhibiting none of the species specific markers; Table 3).

Principal co-ordinate analysis (Gower, 1966) of the nuclear DNA data was performed using the NTSYS-PC program (Rohlf, 1993). Similarity coefficients (s) among individuals were calculated using the formula

$$s = 2a/(2a + b + c),$$

where a = number of shared bands between two individuals $a = 1/1$; b and c = number of 'unmatches' $b = 0/1$, $c = 1/0$.

Results

Quantity and quality of DNA preparation

Regardless of sample origin, either field samples (homogenization and amplification within a week), homogenate (stored for several months), or alcohol samples (animals preserved in 70–98% ethanol), sufficient PCR product was obtained for analysis. Starting with only a single *Daphnia* or even a single resting egg on average twenty PCR (at minimum ten) reactions of nuclear or mitochondrial gene regions are feasible. Apparently no PCR inhibiting factors remain in the homogenate and DNA preparations stay intact for several months, i.e. in 1993, *D. magna* and *D. pulex* samples were collected, DNA prepared and stored at 4 °C until successful DNA amplification in November

Table 2. Geographic locations of *Daphnia* samples and morphological (morph), RAPD (ncDNA) and mitochondrial RFLP (mtDNA) classifications of selected individuals. G: *D. galeata*, C: *D. cucullata*, CG: *D. cucullata x galeata*, GX: *D. galeata*-like individuals, H: *D. hyalina*, HX: *D. hyalina*-like individuals and X: unknown species (probably belonging to *D. rosea* or *D. longispina*). Not all samples could be subjected to genetic analyses because of insufficient preservation methods (e.g. low quality ethanol). Abbreviations of mtDNA composite haplotypes are given in Table 3

Country	Location	N	Morph	ncDNA	mtDNA
Czech Republic	Cerna Spring Pond (Ceské Budějovice)	2	X, GX	G, X	g1, h1
	Knínicky Reservoir (Brno)	2	G	G	g1
	Rimov Reservoir (Ceské Budějovice)	4	G	GX	g1
	Sykovec Pond	3	C, G	C, G	c1, g1
	Uhlistsky Pond (Ceské Budějovice)	3	X, GX	HX, X	h1, g1
	Xerr Pond (Ceské Budějovice)	1	G	G	g1
Germany	Dobben (Oldenburg)	1	GX	G	g1
	Dümmer (Saxony)	2	G	G	g1
	Fischteich Am Ende (Oldenburg)	2	GX	X	x5
	Großer Bornhorster See (Oldenburg)	4	G	G	g1
	Hartsee (Bavaria)	1	H	HX	h1
	Höftsee (Holstein)	1	H	H	h1
	Meedengroden (Varel)	2	HX, X		x1
	Mühlenteich (Varel)	2	G		g1
	Obinger See (Bavaria)	2	CG, G	CG	c1
	Schöhsee (Plön)	1	H	H	h1
	Wahnbecker Teich	2	G	G	g1
	Winkelsheide (Varel)	1	H	G	g1
	Zwischenahner Meer (Saxony)	2	CG	CG	g1, c2
Spain	Amadadorio (Northwest)	6	HX, X, G		
	Guadalest (Northwest)	4	CG, G, CX		
	Loriguilla (Northwest)	3	GH, G		
France	Cormier (Roissy en Brie)	2	X		
	Etang de Bellebouche	15	G, CG, C, GX	G, C, CG	g1, c1
	Gour de Tazenat	2	G	GX	g1
	Lake Aydat (Massif Central)	1	GX	GX	c1
	Pereuse (Normandie)	7	G, X		
	Torcy (Normandie)	3	X		
Finland	clone from Mekkojarvi (South)	2	X	X	x6
	Lake Mekkojarvi (South)	2	X	X	h1
	Lammi Pond (South)	2	X	H	h1
Greece	Lake Ioannina (West)	3	CG, GX		g1
	Lake Mikri Prespa (North West)	3	C		
	Lake Tavropos (Central)	3	CG		
	River Aliakmonas (North)	5	CX, G, GX	GX	c2, g1
Hungary	Kis Balaton reservoir	7	G, X	G	g1
	Lake Balaton	3	C, CG		
Norway	Grimevatnet	2	G	G	g2
	Havardsvatnet	12	X		
	Myrdalsvatnet	3	X	HX	h2
	Skranevatnet	3	G, X	G	c1
	Stendavatnet	3	G, X	G	g2
	Finse Alpine area (pond)	6	X		x2, x3
	Ringebu	2	X	HX	h1
	Jotunheimen	2	X	X	x4

Table 2. Continued

Country	Location	N	Morph	ncDNA	mtDNA
The Netherlands	Biesbos	1	G	G	g1
	Maarsseveen	1	GH	G	g1
	Tjeukemeer	4	CG, C	CG, C, GX	c1, g1
	Vechten	1	C		
	Zwarte Meer	2	H, HX	H, HX	h1
Portugal	C. Bode, clone A (Central)	1	X	GX	g1
	Divor (Central)	5	G, X	GX, G	g1
	Fratel (Central)	3	G, X	GX	g1
	Meimoa (Central)	3	G, X	G, GX	g3
Poland	Sulejów Dam Reservoir (Central)	15	G, CG	G, CG	c1, g1
Sweden	Lake Erken (Uppsala)	5	GX, G	GX, G	g1
	Lake Norrviken (Uppsala)	1	GX	GX	c1
United Kingdom	HGB Exclosure	8	G, GX		
	Llangorse Lake (South Wales)	1	G	g1	
	Llyn Llagi (North Wales)	2	X	X	h1
	Loch Leven (Scotland)	3	H, HX	H, X	h1
	Pound End (South East)	2	X		
	Rollesby (South East)	4	G, X		
	Upton Broad (South East)	3	G	CG, HX	g1
Sum of individuals		209			
Sum of locations			64	46	50

1994 (Table 1). However, for animals stored in alcohol for more than five years, amplification of larger segments (>1 kb) was difficult.

Phenetic analysis of cladoceran mitochondrial DNA sequences

Comparison of mtDNA sequences revealed the characteristic molecular structure of the mitochondrial large rDNA subunit (e.g. Parker & Kornfield, 1996), several highly conserved segments (among nine cladoceran families), as well as short segments which exhibited interspecific and intraspecific variation. A phylogram based on similarity of 16S sequences revealed a cluster pattern similar to systematic classifications of cladoceran species based on morphology (e.g. Martin, 1992; Fryer, 1995; Figure 1). Bootstrap analysis of genetic distances (Figure 1), as well as parsimony and maximum likelihood analyses (data not presented) support the monophyletic groupings of the Ctenodaphnia (*D. magna* and *D. similis*) with *Hyalodaphnia*, *Alona* with *Bosmina*, *Polyphemus* with *Bythotrephes*, and *Holopedium* with *Sida*. *Leptodora kindti* (Haplopoda) formed a sister taxon to the Onychopoda, however, this branching order was not well supported by bootstrap analysis. Sequence divergence among species of the *D. galeata* complex (*D. rosea*, *D. hyalina*, *D. galeata* and *D. cucullata*) averaged 5.9% (±3.2), whereas among all daphnids, including species of the subgenus *Ctenodaphnia*, sequence divergence was around (12.1%, ±6.5). Among the different genera of the Anomopoda divergence was 19.4% (±3.3) on average (Figure 2).

Morphological differentiation of the European D. galeata *complex*

The discriminatory power of morphological characters for species and interspecific hybrids was tested based on the genotypic and haplotypic classification of individuals (see Tables 2 and 3). Although it was not possible to verify discrete diagnostic morphological traits for all species within the *D. galeata* complex, a few traits proved to be diagnostic for the *D. cucullata*/*galeata* complex and these showed gradual transitions from one parental species, via various hybrid classes, to the second parental species (Figure 3). The number of anal spines (AS), rostrum shape (RS)

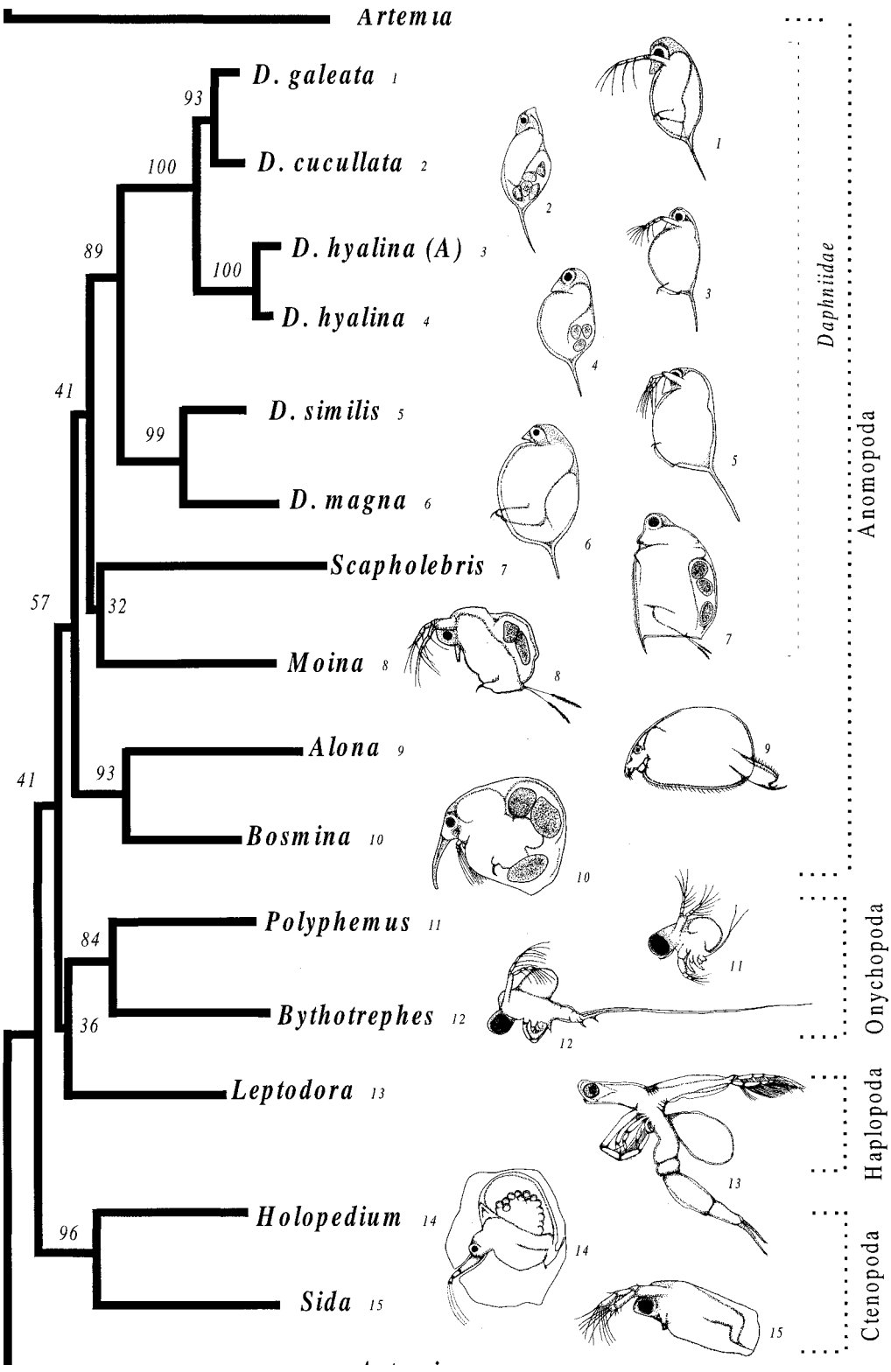

Figure 1. A neighbour-joining tree of 16S rDNA sequences of 16 cladoceran species. *D. hyalina* (A) represents the sequence of a specimen collected in Africa, Ethiopia (Lake Tana). Phylogenetic tree is based on Kimura's 2-parameter distances, the numbers above branches represent the bootstrap confident limit (1000 replicates with distances based on pairwise deletion of gaps and missing sites. Illustrations were produced by A. Sand from photographs of the actual specimens sequenced.

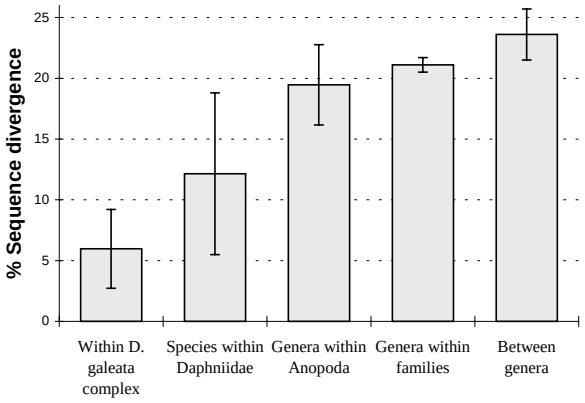

Figure 2. Percent sequence divergence among different taxonomic groups. Error bars represent standard deviation from arithmetic means. Pairwise sequence divergence was estimated based on both transitions and transversions (450 nucleotides), gap sites and missing data were pairwise deleted.

and ventral margin of the head (VM) showed a gradual transition between species. The number of anal spines increases from 6 to 8 in *D. cucullata* and 7 to 13 in *D. cucullata* × *galeata* to 8 to 14 in *D. galeata*. A similar pattern was found in rostrum shape and the ventral margin of the head (Figure 3). In contrast, traits such as rostrum length (RL) or headshape near the eye (HE) are not informative with respect to taxon affiliations. Most morphological traits investigated appeared to be polymorphic, even at higher taxonomic levels. For example, the presence of a crest (in frontal view), which is supposed to distinguish the group of *D. galeata, D. hyalina*, and *D. cucullata* from *D. longispina* and *D. rosea* (e.g. Flößner, 1972) showed a gradual transition of presence to absence, except in *D. cucullata* (only presence). This phenomenon might also be the consequence of interspecific hybridization and introgression, since it is known that *D. longispina* and *D. galeata* form interspecific hybrids (Hebert et al., 1989).

Nuclear and mitochondrial DNA analysis of the European D. galeata *complex*

Selection of restriction enzymes for mitochondrial DNA analysis was based on 16S mtDNA sequences. After cutting the approximately 560 bp segment of the mitochondrial DNA with the restriction enzymes *Rsa*I, *Dde*I and *Mnl*I (New England Biolabs), variation both between species and within species, was revealed (Table 3). Population genetic studies and DNA sequence comparisons have established that composite haplotypes of *D. cucullata, D. galeata* and *D. hyalina* differ significantly in their DNA sequences (Schwenk, 1993; Taylor et al., 1996). In total thirteen composite haplotypes were identified. Reference haplotypes (*c1, g1* and *h1*) and identification of previously unknown haplotypes (*c2*, *g2*, *g3* and *h2*) were achieved by DNA sequence comparisons (Schwenk et al., in prep.). The remaining haplotypes (*x1*, *x2*, *x3*, *x4*, *x5* and *x6*) could not be associated with any known DNA sequence. They belong most probably to other species within the *D. longispina* complex (e.g. *D. longispina*, *D. rosea*). For *D. galeata* individuals four mitochondrial types were found: *g1* (the reference), *g2*, *g3* and *c1* (which appears to be a *D. cucullata* marker; Table 3).

The analysis of the 94 daphnids revealed 20 unique cytonuclear genotypes (= unique combinations of nuclear and mitochondrial markers: Table 4). Genotypic characterization of individuals belonging to the *D. galeata*/*cucullata* complex revealed 12 cytonuclear genotypes, one belonging to *D. cucullata*, three to *D. cucullata* × *galeata* hybrids, four to *D. galeata* and three to *D. galeata*-like individuals (Table 4). Individuals of the GX-group share not only the three nuclear species-specific *D. galeata* markers, but also one of the *D. cucullata* markers. They could have been either F_2-hybrids, backcrossed individuals (between *D. cucullata x galeata* hybrids and *D. galeata*), or the additional *D. cucullata* marker might not have been species-specific.

Principal co-ordinate analysis revealed *D. galeata* genotypes to form a cluster, with *D. cucullata* × *galeata* hybrids showing an intermediate position between the parental species (Figure 4). *D. cucullata* × *galeata* hybrids and *D. galeata*-like genotypes exhibited either mtDNA from *D. cucullata* or *D. galeata*, indicating bidirectional hybridization. Since only one species-specific *D. hyalina* marker was available, classification of *D. hyalina* and *D. hyalina* hybrids was less efficient. However, *D. hyalina*-like genotypes, which either exhibited one *D. galeata* or *D. cucullata* nuclear marker or mtDNA haplotype, probably represent interspecific hybrids. These genotypes were found either between the reference genotype of *D. hyalina* and *D. galeata* or between *D. hyalina* and *D. cucullata* (Figure 4).

Interspecific hybrids of the *D. galeata* species complex were found in most countries investigated (Figure 5). *D. galeata*-like genotypes were found in several countries; some cytonuclear genotypes seem far distributed (e.g. *G-g1*, eight countries), whereas others seem restricted to certain areas (e.g. *G-g3*, Portugal). *Daphnia* specimens originating from Lake

Table 3. Species specific nuclear DNA markers and composite haplotypes (mtDNA) for *Daphnia* taxa. Restriction fragments for three restriction endonucleases (*Rsa* I, *Dde* I, and *Mnl* I) and seven RAPD markers (sizes in base pairs). RFLP-fragments smaller than 50 bp have not been considered. B03, C04 and B14 are RAPD primers used for amplification of species-specific markers. * = reference genotypes/haplotypes

Nuclear DNA				Mitochondrial DNA				
Taxa	*B03*	*C04*	*B14*	Taxa	Composite haplotype	*Rsa* I	*Dde* I	*Mnl* I
*D. cucullata**	480	850	1000	*D. cucullata*	c1*	560	290–180–100	250–230–100
*D. galeata**	980	1150	1050		c2	560	290–180–100	230–180–100–90
*D. hyalina**		1400		*D. galeata*	g1*	510–50	290–180–100	250–210–100
					g2	510–50	290–180–100	230–180–100–90
					g3	560	390–100–80	250–230–100
				D. hyalina/rosea	h1*	560	390–100–80	230–180–100–90
					h2	510–50	450–100	230–180–100–90
				D. ssp.	x1	560	390–100–80	210–120–90
					x2	510–50	290–180–100	250–230–100
					x3	510–50	450–100	250–230–100
					x4	560	290–180–100	250–120–100
					x5	560	300–160–90	250–120–100
					x6	510–50	300–160–90	250–120–100

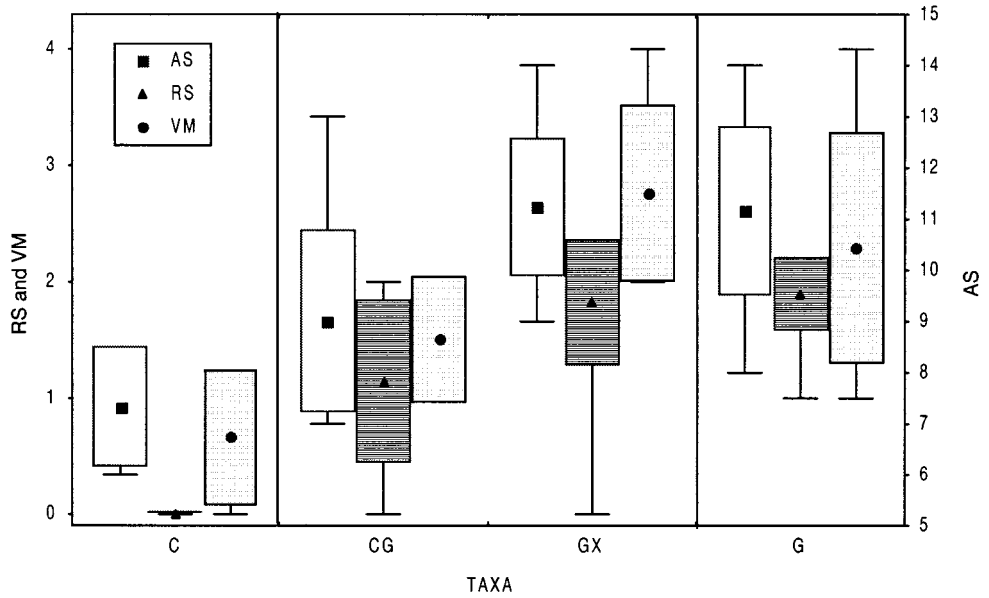

Figure 3. Comparison of morphological variation among *D. cucullata* (C), *D. galeata* (G), *D. cucullata* × *galeata* (CG) and *D. galeata*-like (GX) genotypes. Grouping of taxa is based on nuclear DNA markers (RAPD). Points are arithmetic means, boxes are standard deviations and whiskers are minimum and maximum values, RS = rostrum shape (0 = blunt, 1 = short and obtuse, 2 = sharp), VM = ventral margin of the head (0 = convex − straight, 1 = straight/concave, 2 = concave, 3 = concave with slight angle, 4 = concave with distinct angle) and AS = number of anal spines.

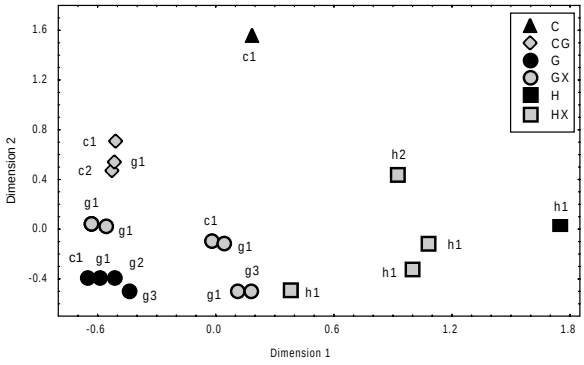

Figure 4. Principal co-ordinate analysis of species diagnostic RAPD markers. Species are represented by symbols C: *D. cucullata*, G: *D. galeata*, GX: *D. galeata*-like, CG: *D. cucullata* × *galeata*, H: *D. hyalina* and HX: *D. hyalina*-like. Characters adjacent to symbols represent mitochondrial composite haplotypes (c: *D. cucullata*, g: *D. galeata*, h: *D. hyalina*). In order to display identical nuclear but different mtDNA haplotypes, some symbols were moved slightly.

Tana, Ethiopia, exhibited DNA sequences and morphological traits characteristic for *D. hyalina*.

Discussion

DNA preparation for freshwater microcrustaceans

Sequence analysis of mtDNA showed that our simple DNA preparation method and subsequent amplification with the universal primers (S1 and S2) is sufficient to obtain DNA sequences of various cladoceran species. Since no substances in cladoceran homogenates appear to inhibit polymerase chain reactions, DNA preparation is simple, fast and enables population based studies using sequence information to differentially cut amplified products. In addition, alcohol-preserved material, species of extremely small size and resting eggs can be examined using this approach. In particular, the simple way of storing field samples over long periods of time (even at room temperature), the possibility to work with individuals (instead of clonal cultures) and the quick and easy method of DNA preparation, should greatly facilitate studies on evolutionary processes, biogeography and ecological genetics of freshwater microcrustaceans.

Cladoceran phylogeny

Clustering of 16S DNA sequences of sixteen cladoceran species and sequence divergence between species and genera are consistent with traditional classifications of cladoceran taxa (e.g. Martin, 1992; Fryer, 1995). Preliminary results show that classifications based on morphological criteria seem consistent with genetic information, and that the major branching among the Daphniidae is in agreement with previous genetic studies based on either cytochrome *b* (Schwenk, 1993), 12S mtDNA (Colbourne & Hebert, 1996) or PCR-RFLP analysis of 12S and 16S mitochondrial DNA (Schwenk, 1997).

However, at higher taxonomic levels, such as families and orders, some previous phylogenetic relationships based on morphology differ from those based on mtDNA sequence information. At the family level within the Anomopoda, the molecular phylogeny is in agreement with recent morphological classifications (e.g. Fryer, 1995) which suggest that the Moinidae belong to the family of the Daphniidae and that the Chydoridae and the Bosminidae form a sister group of the Daphniidae. The orders Onychopoda and Haplopoda are usually grouped into the so-called Gymnomera, and the Ctenopoda and the Anomopoda were grouped into the Calyptomera (e.g. Fryer, 1987). Although the molecular phylogeny clusters the Onychopoda and Haplopoda into one monophyletic group as well, the Ctenopoda, however, form a ancestral group to the Gymnomera and the Anomopoda. It is also remarkable that all predatory cladoceran species (*Leptodora*, *Polyphemus* and *Bythotrephes*) form a monophyletic group. Since the transversion/transition ratio of the 16S genes reach saturation for comparisons of higher taxa, the branching is only weakly supported (e.g. low bootstrap samples, polytomies in parsimony analyses), information from slower evolving genes is required to unambiguously resolve the phylogenetic relationships among orders.

The mtDNA sequence information from different *Daphnia* species was successfully used to select restriction enzymes to differentially cut amplified products. The application of selected restriction enzymes allows identification of species-specific haplotypes for ecological studies, or to access intraspecific variation for biogeographic studies. This approach allows for population studies without the need for DNA sequencing. As for *Daphnia*, sequences from any other cladoceran species can be used to investigate species affiliations of problematic groups (e.g. the chydorids) and to test hypotheses on biogeography and ecological differentiation.

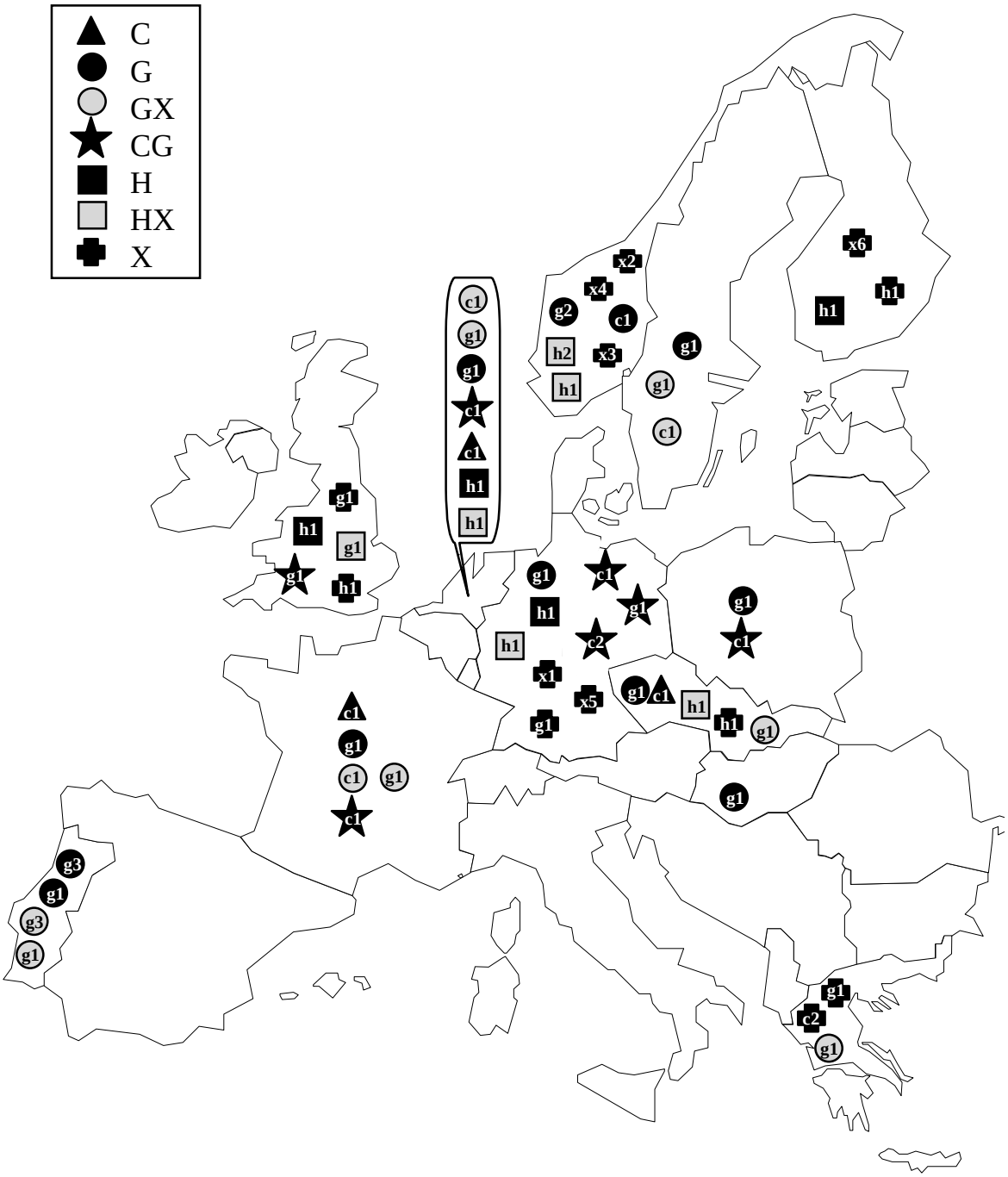

Figure 5. Biogeographic pattern of species and hybrid distribution across Europe. Taxa defined by RAPD markers are represented by symbols C: *D. cucullata*, G: *D. galeata*, GX: *D. galeata*-like, CG: *D. cucullata* × *galeata*, H: *D. hyalina*, HX: *D. hyalina*-like and X: unknown (probably *D. longispina*/*D. rosea*). Characters within symbols represent mitochondrial composite haplotypes (*c*: *D. cucullata*, *g*: *D. galeata* and *h*: *D. hyalina*; see Table 2).

Table 4. Association of mitochondrial DNA composite haplotypes (rows) and nuclear genotypes (columns). Nuclear genotypes are C: *D. cucullata*, CG: *D. cucullata x galeata*, GX: *D. galeata*-like individuals, G: *D. galeata*, H: *D. hyalina*, HX: *D. hyalina*-like individuals, X: unknown taxa (probably belonging to *D. rosea* or *D. longispina*). Composite haplotypes (mtDNA) are *c*: *D. cucullata*, *g*: *D. galeata*, *h*: *D. hyalina* and *x*: haplotypes of unknown species (see Table 2)

Haplotypes	C	CG	GX	G	H	HX	X	n
c1	3	5	2	2				12
c2		1						1
g1		2	13	30		1		46
g2				5				5
g3			2	1				3
h1					6	6	6	18
h2						3		3
x3							2	2
x4							2	2
x6						1	1	2
n	3	8	17	38	6	11	11	94

The D. galeata *species complex*

In order to obtain a sufficiently high discriminatory power for species and hybrids, we combined standard morphological classification together with nuclear and mitochondrial markers. Although combining characters from multiple data sets (total-evidence approach) enhances the discriminatory power for species and hybrids (e.g. Bert et al., 1996), we analyzed the data separately (taxonomic-congruence approach). The latter approach enables detection of introgression, directionality of hybridization and the opportunity to access the process responsible for discordance among data sets (Bert et al., 1996). Comparing nuclear and mitochondrial DNA data, we found mitochondrial DNA haplotypes of *D. cucullata* among *D. galeata* nuclear genotypes (directional introgression; Figure 4), and by comparing nuclear DNA with morphological data we could characterize the consequences of interspecific hybridization on morphological variation among taxa (Figure 3). However, when a taxonomic-congruence approach is applied to the data set, the nuclear and mitochondrial markers are found to be strongly associated, and discrimination of species and hybrids is usually possible (Table 4).

The distributional patterns of parental species and hybrids indicates that interspecific hybridization between *D. galeata* and *D. cucullata* is not restricted to a certain geographical zone of hybridization, but is widespread in Europe. These data are consistent with allozyme studies on several lake districts across Europe (for review see Schwenk & Spaak, 1995). In addition to various incidences of interspecific hybridization, backcrossed (*GX-g1*; *GX-c1*; *GX-g3*) and introgressed genotypes (*G-c1*) are also found at several locations (Figure 1). In addition to molecular data, morphological data of daphnids from Greece, Spain and Sweden (Table 2), as well as other studies (e.g. Flößner & Kraus, 1986; Lieder, 1987; Flößner, 1993), indicate that interspecific hybrids between *D. cucullata* and *D. galeata* have a broad European distribution. Similar patterns of distribution are found for the North American *D. galeata mendotae* species complex. Interspecific hybrids and parental species are found across large areas and parental species differ in habitat associations (Taylor & Hebert, 1993b; Hebert, 1995).

Because our sampling did not cover all major European habitats and different sampling techniques were applied, the results concerning distributional pattern could be biased. In addition, we focused on *D. galeata* and *D. cucullata*, since no *D. longispina* and *D. rosea* markers are available so far. However, *D. galeata* appears to be more abundant and more widely spread than *D. cucullata* and *D. hyalina*. Cytonuclear genotypes of *D. galeata* show an interesting geographic pattern: some genotypes seem to be widely distributed, but others show a restricted distribution. The genotype *G-g3* was found only in Portugal and *G-g2* only in Norway, whereas *G-g1* was found in eight countries (Figure 5). Further studies will determine whether these distributional patterns are based on differential postglacial expansion of haplotypes (vicariance events) or on current ecological processes, such as niche differentiation.

Although species and hybrid discrimination among *D. hyalina*/*D. rosea*/*D. longispina* was limited (a few species-specific markers), distinct cytonuclear genotypes were found indicating the presence of these three species in our sample (Table 4). In particular, *D. longispina* possesses an accumulation of different morphotypes (e.g. melanic forms) and genotypes (e.g. presumably mtDNA *x2* and *x3*) which are characterized by a patchy or endemic distribution compared with the more continuous distribution of other taxa (e.g. *D. galeata*). These findings seem to contradict the general picture of the widely distributed taxon *D. longispina*, since it has been described from Scandinavia (Korpelainen, 1986; Hobaek & Wolf, 1991), France (Lair et al., 1992), Spain (King & Miracle,

1995) Eastern Europe (Hebert et al., 1989) even Mali, West Africa (Dumont et al., 1981). However, more information on biogeographic pattern is required to reveal whether *D. longispina* was not detected because of the different classification methods used, or whether the species range has been overestimated due to misidentifications.

Finally, the presence of sequence and morphological characteristics for *D. hyalina* in specimens from Lake Tana, Ethiopia, provided the first evidence that the taxon is not restricted to the Holarctic. These findings suggest that besides the systematic uncertainties, distributional patterns have to be reconsidered. Such findings pose questions regarding whether the occurrence of *D. hyalina* in Africa has been undetected because of the taxonomic problems mentioned or whether recent intercontinental dispersal events of *Daphnia*, as shown for *D. galeata* between Europe and North America (Taylor & Hebert, 1993c), occurred between Europe and Africa.

From phenotypes to genotypes and back

In concordance with more detailed studies on phenotypic variation among hybridizing *Daphnia* taxa (e.g. Gießler, 1997), we found no single universal diagnostic morphological character. Instead, a combination of morphological traits is required to successfully discriminate among taxa. Since hybridization involves not only the production of intermediate phenotypes, but also introgressed and backcross genotypes, the total array of phenotypes resembles a gradual transition rather than distinct groups. For instance, cytonuclear genotypes of *D. galeata* and *D. cucullata* showed a gradual transition from *D. cucullata*, *via D. cucullata* × *galeata* hybrids and *D. galeata*-like hybrids to *D. galeata* in three traits (rostrum shape, ventral margin of the head and number of anal spines: Figure 3).

The application of molecular markers in combination with morphological data facilitates not only the evaluation of discriminative phenotypic traits but also enables the study of the morphological consequences of interspecific hybridization. *Daphnia* species are capable of altering their phenotype in response to the presence of visual predators (e.g. Brooks & Dodson, 1965) by producing neck teeth, spines or altering their life history characteristics. Spaak & Hoekstra (1995) have shown that, under high fish predation, interspecific hybrids exhibit a combination of life-history traits and body size which results in higher relative fitness compared to parental species. The fact that interspecific hybrids of several *Daphnia* species seem to successfully coexist with their parental species (even in syntopy) in various habitats raises the possibility that other combinations of genetically based characters, such as body shape (visibility for predators) and anti-predator behavior (dial vertical migration), are responsible for the maintenance of interspecific hybridization in *Daphnia*.

Conclusion

One general cause of the taxonomic uncertainties among several cladoceran species is the unknown extent of phenotypic variation within species and the potential overlap in phenotypic variation of closely related species. This problem is accentuated by the restricted geographical basis of most studies, which have often examined only one aquatic habitat, such as a lake district (e.g. Wolf & Mort, 1986; Hann, 1987). Consequently, there exists considerable variation among studies. Thus, various 'local' taxonomic keys that have been developed (e.g. Christie, 1983; Flößner & Kraus, 1986; Glagolev, 1986) are often of limited use on a broader geographic scale and may be contradictory due to the variation in analyzed traits. In addition, although interspecific hybridization is known to occur frequently among several cladoceran taxa (Schwenk & Spaak, 1997), only a few taxonomic keys have incorporated this information (e.g. Flößner & Kraus, 1986; Hebert, 1995).

Interspecific hybrids are found in many locations across Europe, and frequently in syntopy with parental species. However, our data on the diversity and distribution of taxa are certainly underestimates, not only because of the limited number of samples, but more importantly due to the formation of diapausing eggs. By producing resting eggs, *Daphnia* species, but also other Anomopoda, are able to temporarily escape detection from population genetic surveys. Populations are often characterized by large temporal fluctuations, succession and extinction of clonal lineages, species and interspecific hybrids (e.g. Spaak, 1996). This phenomenon complicates studies on genetic variation both within populations and among populations. To overcome this methodological problem, analysis of genetic variation of 'dormant populations', as well as contemporary populations are required.

The application of molecular markers, such as allozymes, mtDNA or RAPD analysis, enables a higher

degree of discrimination than morphological traits alone and have been used to establish comparable data sets for the classification of taxa (e.g. Avise, 1994). However, the application of molecular markers offers more than just an increase in taxonomic resolution. In particular, the combination of morphological, ecological and genetic data sets offers a powerful method to test hypotheses related to ecological differentiation, character evolution and biogeography.

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