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Cryptic species within the *Chydorus sphaericus* species complex (Crustacea: Cladocera) revealed by molecular markers and sexual stage morphology

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ABSTRACT

The cosmopolitanism paradigm in the biogeography of freshwater invertebrates is currently being replaced by non-cosmopolitanism or continental endemism. Benthic water fleas (Cladocera) from the family Chydoridae were the first group of freshwater invertebrates for which non-cosmopolitanism and cryptic diversity was substantiated by morphological studies. Yet, little is known about genetic differentiation and evolutionary history of chydorid species complexes. Here we present the first analysis of the genetic versus morphological differentiation in a benthic cladoceran species complex—*Chydorus sphaericus* s. str. using sequence variation in a nuclear (ribosomal internal transcribed spacer 2, ITS-2) and a mitochondrial (cytochrome *c* oxidase subunit I, COI) genes in 50 Holarctic localities. We tested for continental endemism and cryptic diversity predicted by previous morphological studies. We found evidence for the presence of at least seven putative regional species in the Holarctic, at least three of them being distributed beyond a single continent. While the molecular and sexual stage characters showed general concordance on species lineages, parthenogenetic female characters lacked resolution or were unassociated with molecular lineages. We conclude that cryptic regional lineages of benthic cladocerans are apparent and that the sexual stages represent the most informative morphological source of species characters for this environmental indicator group.

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1. Introduction

Darwin famously concluded that priority effects and local selection led to the frequent evolution of independent lineages among terrestrial islands. His Galapagos bird example illustrates that lineage formation can occur despite the possession of strong dispersal abilities and short distances among islands. Yet, Darwin reasoned a different evolutionary scenario for the inhabitants of freshwater islands or lakes. Here, he proposed that migration would serve to unify aquatic species separated by vast geographic distances because the rates of modification are inherently much slower among freshwater species (mostly invertebrates) compared to terrestrial species. But, lineage formation in the "lower forms" could merely be difficult to detect by humans. Indeed, genetic studies have revealed considerable cryptic diversity in freshwater invertebrates (e.g. Gómez et al., 2002; Jackson and Resh, 1998; Lefébure et al., 2006; Taylor et al., 1998). The failure to distinguish sibling species has partly accounted for the long-standing concept of cosmopolitanism in freshwater invertebrates (Bohonak and Jenkins, 2003). Later, as evidence of strong geographic structuring accumulated, the opposite concept of non-cosmopolitanism or continental endemism became generally accepted (Bohonak and Jenkins, 2003). Likewise, models of lineage formation in freshwater invertebrates now more closely resemble Darwin's model for strongly dispersing vertebrates on oceanic islands (e.g. De Meester et al., 2002; Taylor et al. 1996).

Still, our understanding of the mechanisms and evolutionary consequences of dispersal is limited. Some phylogeographic studies indicated that there might be a high variation in rates, magnitude and success of dispersal among aquatic insects employing similar dispersal strategies (Hughes et al., 1999; Wishart and Hughes, 2003). Pelagic zooplankton groups, such as rotifers and cladocerans, contain well-documented cases of both endemism and cosmopolitanism (Colbourne et al., 1998; Gómez et al., 2002; Schwenk et al., 2000; Taylor et al., 1996). Benthic passively dispersing groups remain poorly studied. Benthic Cladocera mostly show lower growth rates and rely on different food resources than pelagic zooplankton (Smirnov, 1971). According to the monopolization hypothesis cyclic parthenogens that require more time to exhaust the available resources should exhibit less pronounced geographic structure (De Meester et al., 2002). On the other hand, intimate associations of benthic Cladocera with substrata may restrict the choice of habitats suitable for colonization and thus enhance structuring. Therefore, generalizations about freshwater invertebrate biogeography remain elusive.

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1.1. State of the art in the taxonomy and evolution of chydorid Cladocera

Benthic Cladocera of the family Chydoridae are species-rich and abundant in freshwater habitats (Smirnov, 1971, 1996). They have been widely used as indicator and test species in ecological, ecotoxicological and paleolimnological studies (De Eyto et al., 2003; Dekker et al., 2006; Frey, 1960; Hofmann, 1987; Walseng et al., 2003). However, uncertainties in chydorid taxonomy have often hampered the applied research on this group, for instance, cryptic diversity may bias results of ecological studies if sibling species possess different ecological characteristics. The establishment of the non-cosmopolitanism concept (Frey, 1986), substantiated by morphological studies, encouraged further detailed investigations on conspecifics with inter-continental distributions (Frey, 1995). As a result, substantial cryptic diversity was revealed, greatly advancing the taxonomic knowledge of this group. Yet, the state of the chydorid taxonomy is immature (Korovchinsky, 1996, 2000). The morphological approach appears to underestimate the diversity in the most species-rich chydorid taxa, as the latter may often display a combination of morphological stasis and high intra-specific polymorphism (Frey, 1980). Ecological and genetic investigations are likely to provide more taxonomic resolution within closely related species complexes (e.g. Gómez et al., 2002; Taylor et al., 1996).

Frey (1995) noticed, that "demonstrating that cosmopolitanism... is less common than formerly believed...is only the first (and possibly the easiest) part of the problem to tackle. The major question now is: How did these species groups come into being, over what time periods, and why do we have so many of them?". The existing hypotheses on the evolution in benthic cladocerans are based on the assumption of the ancient age of all present taxa. Frey (1995) proposed that most diversifications were promoted by continental drift of both Pangea and Gondwanaland. He noticed that dispersal into yet-uninhabited areas might be "the initiator of many of the troubling species groups", but such events must have been rare and occurred many millions of years ago. Sacherová and Hebert (2003) regarded continental drift as an important factor promoting diversification at the subfamily level. Chydorid genera and species are thought to evolve sympatrically via niche differentiation within the same water bodies. The most recent hypothesis explaining biogeography and evolution of chydorids is the 'theory of ejected relicts' (Korovchinsky, 2006). It considers continental drift an important factor, but emphasizes the role of mass existinctions and range contractions in shaping chydorid biogeography. Korovchinsky's hypothesis predicts that species diversity was lost during the climatic changes in the Tertiary. None of these hypotheses explains how closely related complexes with several co-existing sibling taxa on a continent might have evolved. However, such species complexes are common within species-rich chydorid genera such as *Alona*, *Chydorus*, *Pleuroxus* and others (Smirnov, 1996).

1.2. Diversity of the C. sphaericus species complex

Chydorus sphaericus s. l. is a widely distributed species complex in the Holarctic. A few additional records exist from Africa, Australia and South America (Smirnov, 1996). Regional studies have suggested that these small chydorids (200–500 μm) occur under a widest range of environmental conditions and inhabit almost every investigated lake or pond in the Holarctic (e.g. Alonso, 1996; Chengalath, 1987; Duigan, 1992; Flößner, 2000; Walseng et al., 2003). Like many other zooplankton taxa, *C. sphaericus* was previously regarded as a single cosmopolitan species, but detailed morphological studies revealed cryptic diversity and continental endemism. Even a new genus (*Ephemeroporus*) and a few *Chydorus* species were described from what was previously regarded as *C. sphaericus* (Frey, 1995). At least three potentially valid species are presently recognized within the complex: *C. sphaericus* s.str. (Palearctic distribution), *Chydorus biovatus* (northern Nearctic distribution), and *Chydorus brevilabris* (southern Nearctic distribution) (Frey, 1980, 1985). Frey also distinguished *sphaericus* and *brevilabris* species groups within the complex. He argued that *C. sphaericus* s.str. and *C. biovatus* belong to the former and *C. brevilabris*—to the latter, and each group consists of many undescribed cryptic species (Frey, 1980, 1985). The taxonomic status of three recently described or redescribed taxa *Chydorus latus*, *Chydorus arcticus* and *Chydorus patagonicus* is unclear. They display the same general morphology of females and males that is characteristic for the complex, but their present descriptions are insufficient for the reliable discrimination of these taxa from *C. sphaericus* s. str. (Korovchinsky, 1996; Smirnov, 1996). Some morphotypes, which were previously regarded as separate species,— *Chydorus caelatus*, *Chydorus herrmanni*, *Chydorus mutilus* and *Chydorus rylovi*—are now synonymized with the *C. sphaericus* s.str. (Brancelj, 1996; Frey, 1980; Smirnov, 1996).

The *C. sphaericus* complex is a challenging group for taxonomists. Differences in morphology of parthenogenetic females between the species are subtle, while considerable within-species variation has been described (Belyaeva, 2003; Duigan and Murray, 1987; Flößner, 2000; Frey, 1980; Hann, 1975; Smirnov, 1971, 1996). Consequently, the majority of morphological characters are of unknown taxonomic utility, as there is a possibility that they are characteristic just for the described populations, but not necessarily for the entire species. Alternatively, convergence may have occurred between closely related species (Frey, 1980). The distinct morphology of the ephippial females and males suggests independent taxonomic status of the three species named above. However, it is not unlikely that there might be unrevealed sibling taxa displaying similar morphology of gamogenetic individuals. Overall, a large amount of morphological variation remains unexplained and could be either due to the presence of sibling species, phenotypic plasticity or simple morphotypes. Based on the analysis of morphological variation in Nearctic populations, Frey predicted that many more species still masquerade under the present species names. Yet, recent investigations have failed to find morphological evidence for further separation of taxa within the *C. sphaericus* complex (e.g. Belyaeva, 2003; Duigan and Murray, 1987). It seems that applicability of the morphological approach is limited in this particular case. On the contrary, the genetic approach seems a promising tool for taxonomy, as many chydorid species are believed to be ancient (Frey, 1995; Sacherová and Hebert, 2003) and, consequently, they should be well-differentiated genetically.

In the present study we analysed sequence variation in one mitochondrial—cytochrome *c* oxidase I (COI) and one nuclear—rDNA internal transcribed spacer 2 (ITS-2) gene in the *C. sphaericus* complex in the Holarctic. We also examined the morphological characters, which were previously proposed to be useful for species identifications. The objectives of this study were to (1) provide a phylogenetic framework for the diversity of the *C. sphaericus* complex; (2) test the predictions based on morphological investigations of continental endemism and cryptic diversity; and (3) obtain insights into the morphological and molecular evolution in a benthic cladoceran species complex.

2. Materials and methods

2.1. Sampling and scoring of morphological characters

Specimens were collected from 50 Holarctic localities (Table 1; Fig. 1) and either preserved in 95% ethanol, acetone

or liquid nitrogen. Prior to DNA extractions, all specimens were placed into a drop of water on a slide, examined under a light microscope to assign to either the *brevilabris* or the *sphaericus* morphotype *sensu* Frey (1980) (Fig. 2) and photographed (Leica equipment and software). The available gamogenetic populations were scored for the following morphological characters: number of ephippial eggs, shape of male postabdomen, presence of post-abdominal claw in males and shape of male rostrum. These are the major characters on which species diagnoses are based in the currently known species within the *C. sphaericus* complex (Frey 1980, 1985). We also examined some morphological traits of parthenogenetic females, suggested by Frey (1980, 1985) as being of potential taxonomic utility, such as shell sculpture (polygons along the ventral margin, dimples, lines, connecting major head pores) and shape of the tip of the rostrum (sharply emarginated versus weakly emarginated).

Table 1
List of the sampling localities and morphotype of collected *Chydorus* populations.

Code	Locality, state/region	Latitude	Longitude	Morphotype	COI clade
Germany					
Ger1	Senftenberger See, Brandenburg	51°29′03″N	14°01′14″E	"*sphaericus*"	A1
Ger2	Großer Barsch See, Brandenburg	53°07′01″N	13°00′04″E	"*sphaericus*"	A1
Ger3	Tiefer See, Brandenburg	52°09′12″N	13°59′42″E	"*sphaericus*"	A1
Ger4	Oder flood plain, Brandenburg	53°02′46″N	14°16′45″E	"*sphaericus*"	A1
Ger5	Großer Fuchskuhle, Brandenburg	53°06′23″N	12°59′07″E	"*sphaericus*"	A1
Ger6	Scharmützelsee, Brandenburg	52°17′28″N	14°03′05″E	"*sphaericus*"	A1
Ger7	Felixsee, Brandenburg	51°36′48″N	14°32′48″E	"*sphaericus*"	A1
Ger8	Wolziger See, Brandenburg	52°15′07″N	13°48′07″E	"*sphaericus*"	A1
Ger9	Langer See, Brandenburg	52°14′28″N	13°47′10″E	"*sphaericus*"	A1
Ger10	Melangsee, Brandenburg	52°09′36″N	13°59′18″E	"*sphaericus*"	A1
Ger11	Kleiner Milasee, Brandenburg	52°09′14″N	13°57′26″E	"*sphaericus*"	A1
Norway					
Nor12	Nameless pond, Finnmark	70°06′N	28°37′E	"*sphaericus*"	A2
Nor13	Nameless pond, western Swalbard	78°55′N	11°56′E	"*sphaericus*"	A2
Nor14	Lake, Bear Island	74°25′N	19°02′E	"*sphaericus*"	A2
Iceland					
Ice15	Nameless pond, central Iceland	64°44′N	19°26′W	"*sphaericus*"	A2
Ice16	Nameless pond, northern Iceland	66°58′N	20°22′W	"*sphaericus*"	A1, A2
Ice17	Lake Fljótsbötn, southern Iceland	63°39′59″N	18°17′0″W	"*sphaericus*"	A1
Finland					
Fin18	Lake Kejtele	63°02′41″N	25°49′16″E	"*sphaericus*"	A1
Fin19	Lake Orajärvi	66°54′19″N	24°05′48″E	"*sphaericus*"	A1, A2
Greenland					
Gree20	Nameless pond, Disko Island	69°16′N	53°50′W	"*sphaericus*"	A1
Gree21	Nameless pond, Disko Island	69°15′N	53°38′W	"*sphaericus*"	A1
Gree22	Nameless pond, Uummannaq	70°41′24″N	52°10′12″W	"*sphaericus*"	A1, A2
Russia					
Rus23	Nameless pond, Tomsk	56°29′N	84°58′E	"*sphaericus*"	A2
Rus24	Nameless pond, Belyj Island	73°06′N	70°06′E	"*sphaericus*"	A2
Rus25	River Eruslan, Saratov Area	50°43′N	46°46′E	"*sphaericus*"	A1
Rus26	Lake Krasnogo Plastika, Arkhangelsk Area	68°35′N	52°18′E	"*sphaericus*"	A2
Rus27	Nameless lake, Khanty-Mansi Area	61°14′N	73°26′E	"*sphaericus*"	A2
Rus28	Lake Primorskoe, Region of Primorsky	42°49′N	132°53′E	"*sphaericus*"	A3
Japan					
Jap29	Lake Midori-ga-ike, Toyama	36°33′N	137°39′E	"*sphaericus*"	A3
Jap30	Lake Misuma-Ike, Yamagata	38°22′05″N	139°49′16″E	"*sphaericus*"	A3
Canada					
Yuk31	Lake Kookatsoon, Yukon Territory	60°33′28″N	134°52′32″W	"*sphaericus*"	A3
Yuk32	Nameless pond, Canol Road, Yukon	61°44′53″N	133°04′21″W	"*sphaericus*"	B6
Yuk33	Nameless pond, Yukon Territory	63°03′29″N	136°25′42″W	"*sphaericus*"	B5
Yuk34	Fox Lake, Yukon Territory	61°10′38″N	135°23′29″W	"*sphaericus*"	A3, B6
Yuk35	Lapie Lake, Yukon Territory	61°39′34″N	133°03′37″W	"*sphaericus*"	A3, A4
Newf36	Octagon Pond, St. John's, Newfoundland	47°31′00″N	52°52′60″W	"*brevilabris*"	C7
Newf37	Nameless pond, Newfoundland	49°07′14″N	55°05′19″W	"*brevilabris*"	C7
ON38	Silver Lake, Ontario	43°05′23″N	80°31′48″W	"*brevilabris*"	C7
USA					
Ala39	Nameless pond, Teller, Alaska	65°14′30″N	166°19′46″W	"*sphaericus*"	A3
Ala40	Council pond, Alaska	64°57′27″N	163°41′41″W	"*sphaericus*"	A3
Ala41	Nameless pond, Teller, Alaska	65°01′13″N	166°08′31″W	"*sphaericus*"	A3
Ala42	Nameless pond, Nome, Alaska	64°33′37″N	165°29′14″W	"*sphaericus*"	A3
Ari43	Nameless pond, Coconino, Arizona	34°47′30″N	111°29′19″W	"*brevilabris*"	C7
Ari44	Nameless pond, Yavapai, Arizona	34°34′22″N	111°51′22″W	"*brevilabris*"	C7
NY45	Nameless pond, Buffalo, NY	43°01′40″N	78°42′39″W	"*brevilabris*"	C7
NY46	Nameless pond, Buffalo, NY	43°01′42″N	78°45′16″W	"*brevilabris*"	C7
NY47	Irondequoit Bay, NY	43°11′01″N	77°31′58″W	"*brevilabris*"	C7
NY48	Deep pond, Mendon, NY	43°01′29″N	77°34′18″W	"*brevilabris*"	C7
OK49	Swan Lake, Oklahoma	36°08′06″N	95°58′06″W	"*brevilabris*"	C7
NH50	Sunapee Lake, New Hampshire	43°22′32″N	72°04′12″W	"*brevilabris*"	C7

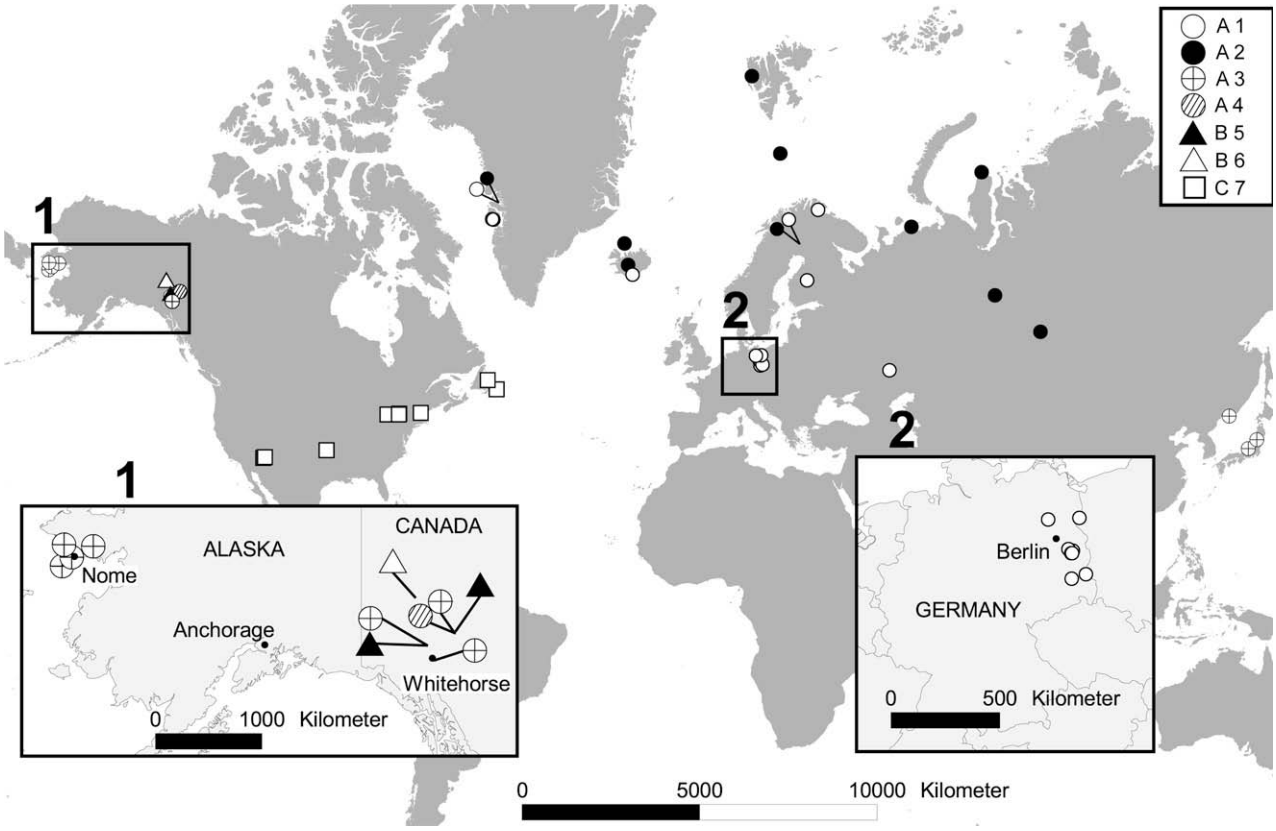

Fig. 1. Collection sites for the studied Holarctic populations of the *C. sphaericus* species complex. (A1–C7)—putative species lineages identified from mtDNA and ncDNA phylogenies and the morphology of sexual stages.

2.2. DNA sequencing

For DNA extraction specimens were transferred into reaction tubes and dried. For each extraction 25 μl of Quickextract (Epicentre Technologies) was added, the specimens were homogenized, and the tubes were placed for 2 h at 65 °C, then for 10 min at 95 °C.

DNA extracts from the same individuals were used to obtain PCR products for both genes. A total of 158 individuals were sequenced for the mitochondrial cytochrome oxidase subunit I (COI) gene. A subsample of 60 individuals were sequenced for the nuclear fragment, which included the complete sequence of internal transcribed spacer 2 (ITS-2) as well as small partial sequences of 5.8S and 28S ribosomal genes. In the text below we refer to the nuclear fragment as ITS-2 for convenience. The following taxa were sequenced for the outgroups: *Chydorus pubescens*, *Paralona pigra* and *Pleuroxus procurvus*—COI (GenBank Accession Nos. EU719119, EU719117 and EU719118, respectively), and *C. pubescens* and *P. pigra*—ITS-2. Other available chydorid COI sequences, obtained from the GenBank or kindly provided by Veronika Sacherová from her published (Sacherová and Hebert, 2003) and unpublished data, were also tested as outgroups. As COI amplification had a low success with universal arthropod primers (Folmer et al., 1994), internal primers—Chy-f 5′-TTG GGG ATG ATC AAA TTT ATA ATG T-3′ and Chy-r 5′-AGA GGT ATT CAG ATT TCG ATC TGT CA-3′—were designed from the conserved regions in the sequences of *Eurycercus lamellatus* (Seiji Ishida, unpublished data) and *C. sphaericus* from Germany. These primers were used for amplification of all ingroups and outgroups, except for some specimens from the Yukon belonging to clade B6. Those failed to amplify with our specific primers due to mutations in the primer regions. Yet, we obtained PCR products with the universal COI primers from Folmer et al. (1994). The primers 5.8SF (Taylor et al., 2002) and D2r (Omilian and Taylor, 2001) were used to amplify the ITS-2 fragment. Each 50 μl PCR reaction consisted of 35 μl dd H2O, 5 μl PCR buffer, 1.5 μl each primer, 1 μl dNTPs, 1 μl Taq DNA polymerase and 5 μl DNA extract. The PCR conditions for the COI amplification were 40 cycles of 30 s at 94 °C (denaturation), 30 s at 50 °C (annealing) and 90 s at 72 °C (extension) followed by 1 cycle of 7 min at 72 °C. The PCR conditions for the amplification of ITS-2 were the same, but the annealing temperature was 60 °C. PCR products were sequenced on an ABI 3700 sequencer. DNA sequences were submitted to the GenBank database (Accession Nos. EU719117–EU719163, EU822324–EU822330 for COI and EU719164–EU719188 for ITS-2 sequences).

2.3. Phylogenetic analyses

The authenticity of the sequences was verified by BLAST comparisons. Sequences were edited and assembled in Sequencher 4.1 (Gene Codes Corporation), Bioedit (Hall, 1999) and MEGA 4.0. (Tamura et al., 2007). COI sequences were aligned manually in MEGA 4.0. ITS-2 sequences were aligned in CLUSTAL X (Thompson et al., 1997) using default options and after that manually adjusted in MEGA 4.0. The best-fit models of nucleotide substitution were selected in jModelTest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) based on likelihood scores for 88 different models and the AIC criterion. Within- and among-clade distances were calculated and neighbor-joining (NJ) phylogenetic analyses were carried out in MEGA 4.0. using Kimura 2-parameter (K2P) model and gamma rates distribution with the shape parameter estimated by jModelTest and with pairwise deletion of gaps. All phylogenetic analyses were performed separately for each gene. Maximum parsimony (MP) analyses were performed in PAUP* 4.0b10 (Swofford, 2003). Heuristic MP searches were done using equal weighting, 10

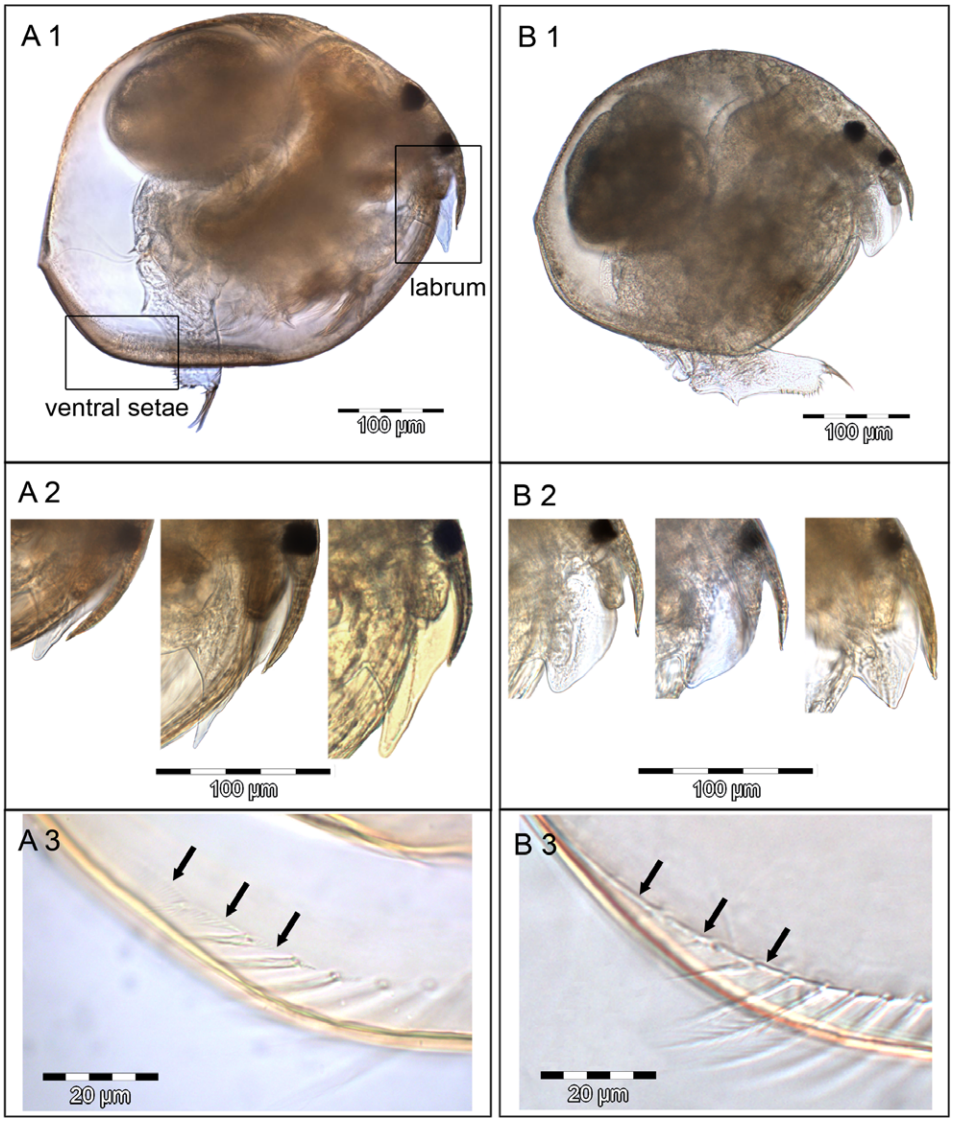

Fig. 2. Diagnostic morphological characters of the two *Chydorus* morphotypes indicating the major groups of species according to Frey (1980). (A1–A3)—"*sphaericus*", (B1–B3)—"*brevilabris*". (A1 and B1)—adult parthenogenetic female, lateral view; (A2 and B2)—labrum (note the difference between the two species groups despite the within-group variability); (A3 and B3)—ventral setation on the inner shell surface: (A3)—ventral setae not connected by a line (inter-setal ridges), (B3) "connected" ventral setae. The position of the line is indicated by arrows.

random sequence addition replicates and TBR branch swapping. Non-parametric bootstrapping was performed to assess the nodal support using 1000 pseudoreplicates for NJ and 100 for MP. Bayesian analyses (BI) were performed in MrBayes v.3.0b3 (Huelsenbeck and Ronquist, 2001). The number of substitution types (nst) was set to 6 and rates were set to gamma with a proportion of invariable sites, all priors were left default to allow estimation of the parameters from the data. Four independent Markov chain Monte Carlo (MCMC) analyses were run simultaneously for 5 million generations and sampled every 100 generations. The first 20% of the generations were discarded as the burn-in and a 50% majority rule consensus tree was calculated from the remaining trees. Additionally BI analysis of a mixed ITS-2 data set was performed, where indels were coded as binary characters and added to the nucleotide data set. In the binary partition the model was corrected for the ascertainment bias (lset coding = variable), the settings for the nucleotide partition were as before.

Both data sets were tested for recombination events using GENECONV (Sawyer, 1989) and GARD (Kosakovsky Pond et al., 2006a,b) software. In GENECONV default settings were used (10,000 permutations, no mismatches allowed) and with mismatches allowed and penalties set to 1–4. Prior to the analysis the first 295 bases were deleted from the ITS-2 alignment as they contained only very few polymorphisms and caused apparently false detection of gene conversion. GENECONV has a reasonable rate of false positives (ca. 5%) at a sequence divergence range of 5–20% (Posada and Crandall, 2001). As sequence divergences ranged from 5% to 11% between the main clades in our data, the program should reliably detect recombination between the sequences belonging to different clades. However, within-group recombination might remain undetected, for the method has a low power at divergences <5%. Besides, rare recombination events may also remain undetected (Posada and Crandall, 2001). The GARD algorithm detects putative recombination break points based on phylogenetic incongruence among partitions within the data set (Kosakovsky Pond et al., 2006a). The method was shown to be more sensitive than most other recombination tests, however, it sometimes may detect fragments that are underlined by significantly different phylogenies due to substitution rate variation rather than recombination (Kosakovsky Pond et al., 2006b). To check for this we applied a

conservative SH test implemented in HyPhy software (Kosakovsky Pond et al., 2005). The model selection tool available on the GARD server was used to obtain the input nucleotide substitution model. The other settings were: general discrete model for rate variation with four rate classes.

3. Results

3.1. Sequence variation and alignments

Of 158 ingroup specimens from 50 locations sequenced for COI, 51 unique haplotypes were detected. A summary of the diversity is presented in Table 2, but only unique sequences were included in the phylogenetic analyses. The COI alignment for all ingroups and 3 outgroups was 461 bp long, unambiguous and contained no indels. There were 178 variable and 141 parsimony informative sites. The average base composition for the 51 ingroup sequences was as follows: T = 37.4%; C = 21.0%; A = 22.8%; G = 18.7%. The observed A–T content around 60% is common for COI of chydorids (Sacherová and Hebert, 2003). The translated amino-acid alignment had 153 characters, of which 12 were variable and 5 parsimony informative.

Of 60 individuals, for which ITS-2 was amplified, 27 ingroups and 3 outgroups were homozygous in ITS-2. These gave good-quality sequences with no or few ambiquities. The rest of the ITS-2 sequences were unreadable due to within-individual variation in the sequence and its length, resulting in ca. 60% of double electropherogram peaks. Such ITS-2 heterozygotes were not associated with a particular clade, but rather they were present in each of the COI-defined clades. The final ITS-2 alignment included only non-identical sequences from homozygous individuals, and all putative species lineages revealed by the COI phylogeny were represented. Outgroups were excluded as well, because their inclusion substantially decreased the quality of the alignment. The length of the sequences varied from 900 to 1016 bp. The ITS-2 alignment was 1057 characters long, with 197 variable and 126 parsimony informative sites and contained numerous indels 1–46 bp long. There were both very variable regions, which were difficult to align, and very conserved ones which displayed no variation. The average ITS-2 base composition was: T = 23.2%; C = 29.4%; A = 22.6%; G = 24.7%.

The K2P distances among COI ingroup taxa varied between 0% and 22.8%. Between ingroups and outgroups the following sequence divergences were observed: *C. pubescens*—21.1–25.4%, *P. procurvus*—21.9–29.2%, *P. pigra*—25.8–29.9%. The ITS-2 sequence divergences varried between 0% and 26.2% among ingroup taxa.

3.2. Mitochondrial gene tree

The best-fit model selected by jModeltest for the COI data set was TIM3 + I + G with a relative AIC weight of 0.3343, the next best model was GTR + I + G with a weight of 0.2338. Based on the cumulative relative weight, five more models were within the 95% confidence interval, all of them assuming the presence of invariable sites and gamma substitution rates.

All phylogenetic methods resulted in trees that did not differ in their main topology, i.e. all specimens were assigned to the same main clades and the relationships between these clades held. In the MP analysis 7791 best trees each of 473 steps were found, with a consistency index (CI) of 0.567 and retention index (RI) of 0.858. Three major clades within the *C. sphaericus* complex, named A, B and C were well-supported in all phylogenetic analyses of the COI data set (Fig. 3). These clades were very distinct from each other, the mean between-group K2P distances being 18.4–20.0%. The phylogenetic relationships between the major clades remained unresolved. In the phylogeny based on the COI amino-acid composition (not shown) clades A and C were unresolved in a single clade and clade B was divergent.

Clade A contained four well-supported lineages with the mean K2P-distances of 4.9–15.6% between and 0–4.1% within them (Table 3; Fig. 3): (A1)—all specimens from Germany and some from Finland and Greenland; (A2) Finland, Norway, Iceland, Greenland, Russian Arctic and Siberia; (A3) Alaska, Yukon, Japan and eastern Siberia; (A4) one specimen from the Yukon. Clades A1 and A2 were sister groups with high support for this node. The relationships between clades A1 + A2, A3 and A4 were unresolved (Fig. 3). Clade B contained two lineages from Yukon: (B5) was represented by twelve identical sequences sampled in one location and (B6)—by five identical sequences from two locations. Despite the high nucleotide distance of 6.4% between clades B5 and B6, they had an identical amino-acid composition, which was, however, distinct from all other clades. A highly supported Clade C included only Nearctic specimens and constituted just one putative species lineage, showing moderate sequence variation comparable with that observed in other putative species lineages (Table 2). Also, no phylogeographic subdivision was apparent within the *brevilabris* clade C7.

The age of the *C. sphaericus* species complex and the time scale for the diversification can be only approximately estimated, as no fossil calibration exists for Cladocera. For Arthropoda the rates of 1.4–2.6% per MY have been reported (Knowlton and Weigt, 1998; Schubart et al., 1998). Then a crude estimation of diversification time of the major clades A, B and C, given that the Kimura-2-parameter model is appropriate for the data, is

Table 2
Number of the sampled *Chydorus* populations, specimens, and detected COI haplotypes, and sequence divergence within the putative species lineages in the *C. sphaericus* complex. For the definition of the clades A1-C7 see Fig. 3 and the text.

COI clade	Putative species lineages	No. populations	No. specimens	No. haplotypes	Max within-group COI sequence divergence, %
A1	*C. sphaericus* s.str.	18	75	19	4.1
A2	Undescribed species	12	22	13	2.5
A3	Underscribed species	10	27	6	1.8
A4	Undescribed species	1	1	1	–
B5	Undescribed species	1	12	1	–
B6	Undescribed species	2	5	1	–
C7	*C. brevilabris*	11	16	10	2.9
	Total	55	158	51	

Table 3
Between-group Kimura 2-parameter distances (in percent) among the putative species lineages within the *C. sphaericus* complex for COI/ITS-2 genes. Distances were calculated with assuming gamma distributed rates and the gamma parameter estimated in jModelTest. ITS-2 distances were calculated with pairwise deletion of gaps. For the definition of the clades A1–C7 see Fig. 3 and the text.

Clade	A1	A2	A3	A4	B5	B6
A1						
A2	4.9/0.7					
A3	10.5/3.4	10.3/3.4				
A4	15.6/3.3	14.3/3.2	15.2/2.3			
B5	20.0/24.8	19.1/24.6	19.8/22.0	25.2/26.0		
B6	20.3/15.1	19.9/15.4	19.2/13.9	24.8/15.9	6.4/14.9	
C7	20.8/15.6	20.2/16.1	17.1/16.1	20.9/16.0	18.8/19.2	18.0/6.7

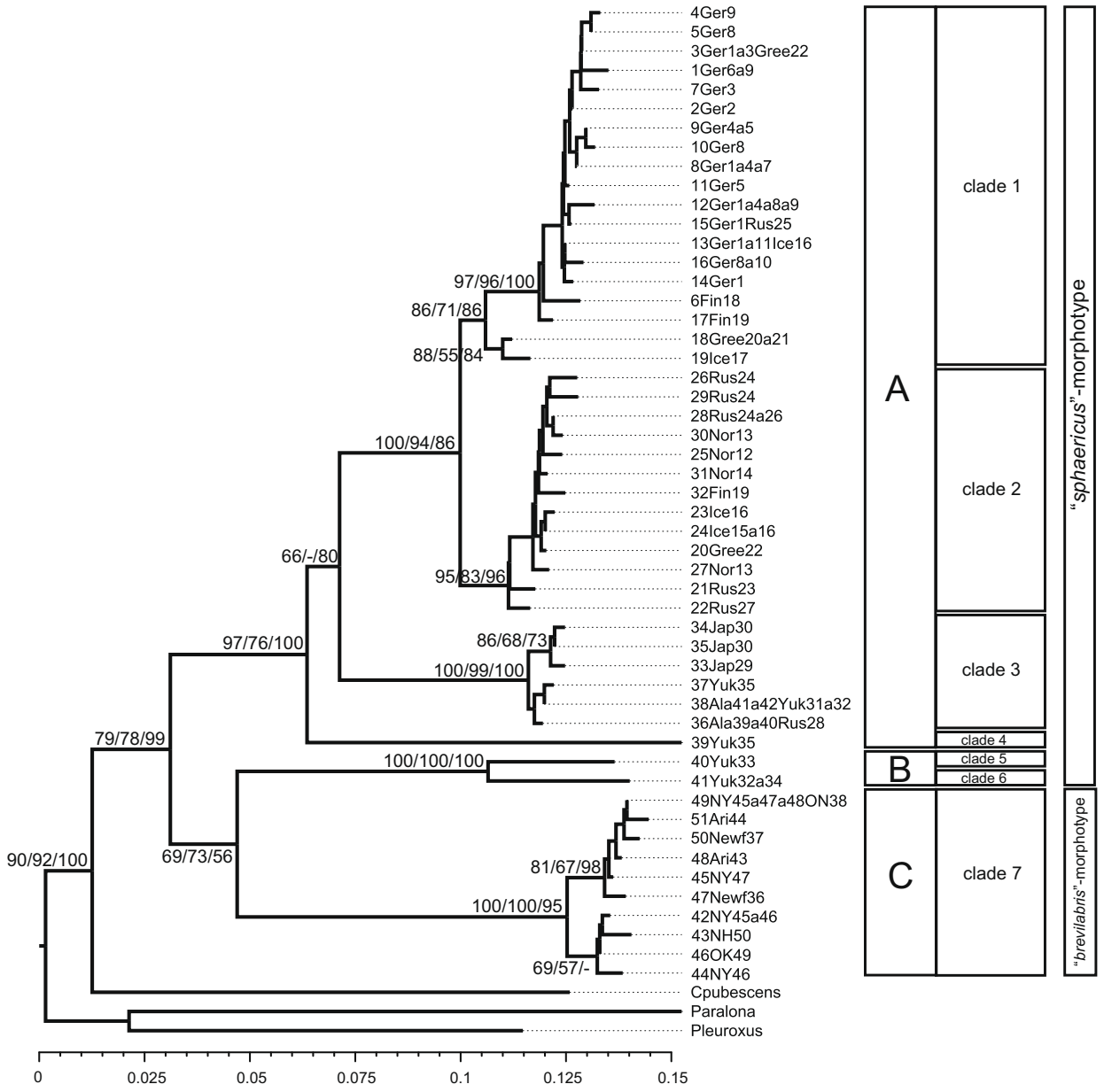

Fig. 3. COI phylogeny: NJ tree based on Kimura 2-parameter distances as indicated by the scale axis. Gamma rates were assumed with a gamma parameter of 1.838 (estimated in jModelTest). The tree is rooted using *Pleuroxus* and *Paralona* sequences as outgroups. The node support: bootstrap NJ/MP/Bayesian posterior probabilities. (A–C)—main branches in the *C. sphaericus* complex; 1–7—putative species lineages (see Table 1 for the location codes and the text for further explanations).

ca. 5–15 MYA and subsequent speciation within clades A and B—ca. 2–10 MYA. Thus, the youngest putative species detected by our study are *C. sphaericus* s.str. (A1) and the undescribed species from the European Arctic, Greenland and Siberia (A2), which possibly diverged from a common ancestor in late Pliocene–early Pleistocene.

GENECONV recombination test applied to the COI data set detected no significant global fragments under either of the settings used. Some significant pairwise fragments were found, involving the outgroup *Pleuroxus* and C7; A1 and A4; A2 and A4; sequences within A1; sequences within A3. Nevertheless, as the more conservative global test detected no recombination, and also because pairwise fragments did not involve species found to co-exist, we consider the test results as providing no reliable evidence for recombination. GARD detected no significant recombination breakpoints by either criteria used.

3.3. ITS-2 gene tree and concordance among the mitochondrial and the nuclear phylogenies

The best-fit model selected by jModeltest for the ITS-2 data set was TPM2uf + G with a relative AIC weight of 0.3178, the next best model was TIM2 + G with a weight of 0.2181. Six more models were within the 95% confidence interval, all of them assuming gamma substitution rates and some of them also assuming a proportion of invariable sites.

MP analysis of the ITS-2 data set resulted in 40 best trees with a length of 270 steps, CI of 0.848, and RI of 0.924. All phylogenetic analyses of the ITS-2 data set revealed four major clades that corresponded to A, B5, B6 and C7 mitochondrial clades (Fig. 4). Thus, A and C clades were recovered and well-supported in both gene trees, whereas clades B5 and B6 were much more divergent from each other in the nuclear than in the mitochondrial tree (14.9% ver-

sus 6.4%, respectively). The monophyly of clade B received no support in the ITS-2 phylogeny. Similar to the mitochondrial phylogeny, the relationships among the major ITS-2 clades remained unresolved, i.e. neither grouping yielded any significant support. BI analysis of a mixed ITS-2 data set that included binary coded gaps resulted in higher posterior probabilities for the groupings A1 + A2 (100%) and (A1 + A2)A3 (99%) and (B5, B6) (95%). Overall, the nuclear phylogeny provided even less resolution than the mitochondrial one. For example, most recent, but well-separated in the COI tree A1 and A2 clades merged in one well-supported clade. A3 and A4 clades were also, respectively, divergent in ITS-2, but no relationships among putative species lineages were resolved except the 99% support for the (A1 + A2)A3 in BI analysis of the mixed data set that received no support at all in other analyses.

Age estimations based on ITS-2 would probably be even more unreliable than those based on mitochondrial DNA divergences. A great variation in substitutional rates for this gene has been observed in insects (Schlötterer et al., 1994). Although a rate of 2.4% per MY is reported for *Drosophila* and it has been used for age estimations in other arthropods (e.g. Schwenk et al., 2000), it was based on a sophisticated algorithm aiming to exclude invariable sites assumed to be "conserved" by chance (Schlötterer et al., 1994). Thus, a straightforward application of the reported rate would give (negatively) biased results. Also our ITS-2 data suggest the presence of a considerable rate heterogeneity, as indicated by the highly divergent sequences representing B5 clade (see above). Even though we give only very rude age estimations for the putative species clades, nevertheless, we decided to limit them to the mitochondrial gene phylogeny, as it seems much more reliable.

GENECONV recombination test detected no significant global inner fragments (*P*-values <0.05) and one significant outer fragment involving a sequence from clade B6. Pairwise significant fragments were detected between sequences belonging to clade A and between clades B5, C and B6. Thus, no reliable evidence for recombination between the clades have been found. GARD test detected two significant breakpoints. Of these only one fragment was found to be significant by SH test. Furthermore, close examination of the significant breakpoint revealed that it corresponded to the boundary between the ITS-2 and 28S genes, the latter being very conservative with almost no variation within this alignment fragment.

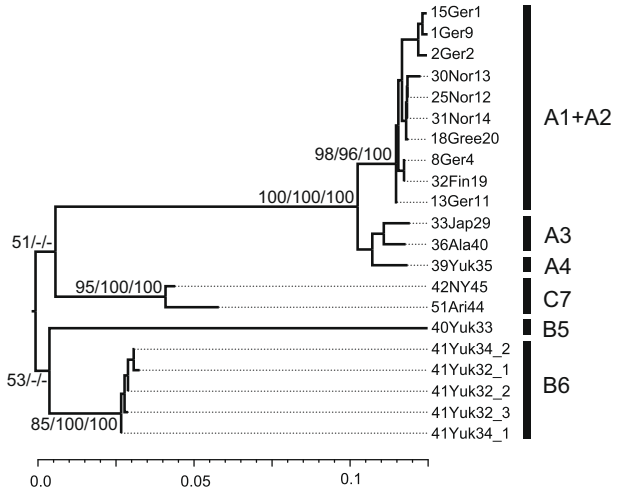

Fig. 4. ITS-2 phylogeny: NJ tree based on Kimura 2-parameter distances as indicated by the scale axis, calculated with pairwise deletion of gaps and assuming gamma rates with a gamma parameter of 0.129 (estimated in jModelTest). The tree is rooted on the midpoint. The node support: bootstrap NJ/MP/Bayesian posterior probabilities for the nucleotide data set.

Thus, the significance of the test most likely results from the rate variation rather than recombination events. We conclude that no unequivocal evidence for recombination was found by either of the tests applied.

3.4. Biogeographical patterns

We found a greater diversity of the *C. sphaericus* s.l. in the Nearctic than in the Palearctic, both regarding the presence of the major evolutionary clades (three in the Nearctic versus one in the Palearctic) and the number of the putative species lineages (7 in the Nearctic versus 3 in the Palearctic). The *C. sphaericus* complex comprises both rather widely distributed species (A1, A2, A3 and C7) and those that are likely to be more restricted geographically (A4, B5 and B6). Lineages A4, B5 and B6 were found only in Yukon and might represent Beringian glacial relict species. We detected clade A3 from both the western Nearctic and the eastern Palearctic, yet, it was restricted to the Beringian region. Among the widely distributed lineages *C. brevilabris* (C7) was restricted to the North American continent, whereas *C. sphaericus* s. str. (A1) and clade A2 were encountered both in Nearctic and Palearctic locations. *C. sphaericus* s. str. was restricted to sites in western Eurasia and Greenland.

Each of the widely distributed species showed considerable intraspecific variation in mitochondrial DNA (Table 2; Fig. 3). The lack of observed variation within the geographically restricted lineages is probably the result of the low number of the sampled locations and analysed specimens (Table 2), the real variation present in nature remaining unknown. Some phylogeographic structuring was observed in the *C. sphaericus* s. str., as northern populations possessed COI haplotypes that were distinct from those in the Central- and East-European populations. Yet, no phylogroups were apparent within other species lineages, perhaps due to the low number of sampled populations (Table 2).

3.5. Morphological variation within the C. sphaericus species complex and differentiation of the newly discovered lineages

Examination of the specimens prior to DNA extractions assigned clades A and B to the *sphaericus*-morphotype *sensu* Frey (1980) and clade C to the *brevilabris*-morphotype (Table 1; Figs. 2 and 3). The *sphaericus*-morphotype was generally larger (230–480 μm versus 220–420 μm in the *brevilabris*-morphotype according to Frey (1980)), possessed a longer labrum plate of variable shape and the ventral setae were not connected by a line (inter-setal ridges), whereas the *brevilabris*-morphotype could be readily distinguished by its somewhat smaller size, short, broadly rounded labrum and "connected" ventral setae (Fig. 2).

Where possible, the morphology of the newly found genetically divergent lineages was studied. Only those characters that are helpful for the delimitation of these lineages are described here, all of them being traits of gamogenetic individuals—ephippial females and males. The latter occurred in the sampled populations of the two new species found in this study—lineages A2 and A3, thus enabling their reliable delimitation from the *C. sphaericus* s. str. (clade A1). The formal taxonomic descriptions of these two new species as well as a detailed assessment of the intra-species morphological variation within *C. sphaericus* s. str. (A1) will be published elsewhere. The other newly discovered putative species—clades A4, B5 and B6 were represented each by just one or a few parthenogenetic individuals, therefore, no morphological investigations were conducted.

Gamogenetic individuals belonging to clade A2 could be readily distinguished from the occasionally co-existing sister species *C. sphaericus* s. str. based on the characters described below. The ephippial females carried two eggs and the males lacked the prea-

nal angle of postabdomen (Fig. 5). Although these particular characters are diagnostic of *C. biovatus*, the males from A2 populations also showed some substantial morphological differences from this species. The shape of rostrum was similar to that of *C. sphaericus* s.str. and not to *C. biovatus* (Fig. 5). The postabdomen of males had a well-developed basal spine on the claw, which was approximately of the same size as that of females. All five adult males studied, all had a basal spine, suggesting normal development. Male postabdomens with basal spines are unknown in the well-described species of the *C. sphaericus* complex. However, this feature was mentioned in Lilljeborg's description of *C. latus* (Lilljeborg, 1900, cited in Flößner, 2000), which is a poorly investigated species and is not regarded as valid according to the modern taxonomic standards (Smirnov, 1996). However, the specimen drawn by Lilljeborg had a well-developed preanal angle and none of our specimens had it. Therefore, we conclude, that clade A2—is a new yet-undescribed species belonging to the *sphaericus*-branch of the *C. sphaericus* species complex.

Beringian clade A3 was very similar to *C. sphaericus* s. str., yet, differing in the shape of the rostrum tip of adult males that was narrowly rounded as in *C. biovatus* and *C. brevilabris*, rather than broadly truncate as in A1 and A2 clades. Five adult males were examined, all possessing narrowly rounded rostrum. Juvenile males from the studied A3 clade population also displayed rostrum morphology similar to that described for *C. biovatus* (Frey, 1985, Figs. 16 and 17) and not to *C. sphaericus* s. str. (Frey, 1985, Figs. 19 and 20). However, other characters, such as the shape of male postabdomen and one egg in the ephippium clearly delineate the new species from *C. biovatus* (Fig. 5).

4. Discussion

4.1. Phylogenetic framework for the taxonomy of the C. sphaericus species complex: is there an agreement between the morphological and genetic data?

The *C. sphaericus* species complex, as it is defined by morphological criteria, also proved to be monophyletic in our phylogenetic analyses. However, it should be noted that rigorous testing for the monophyly of the species complex would require inclusion of all species from the genus *Chydorus*. The nuclear gene tree contained only ingroups and thus monophyly could not be established. Nevertheless, a rather high similarity among the ingroup sequences might be an indication of their monophyletic origin.

Both nuclear and mitochondrial phylogenies were concordant in revealing three major lineages within the *C. sphaericus* complex (Figs. 3 and 4): (A) *sphaericus*-lineage with a Holarctic distribution, (B) a previously undescribed Yukonian lineage and (C) a *brevilabris*-lineage—widely distributed in North America, but possibly absent from the northernmost part of the continent, where it is replaced by the two other major lineages. The major clades A, B and C probably reflect an ancient radiation within the *C. sphaericus* complex and correspond to a higher than specific taxonomic level, as at least the *sphaericus*-branch (A) shows clear morphological, geographical, and phylogenetic evidence for further speciation. Two of the three major lineages revealed by our study—A and C—correspond to the traditional separation of the complex into two species groups (Frey, 1980)—*sphaericus* and *brevilabris*, respectively. Thus, there is some agreement between morphological and genetic approaches in recognition of supra-specific lineages. However, our study showed that some deeper divergences in both nuclear and mitochondrial genomes might have arisen without any recognizable morphological changes in parthenogenetic individuals. Specimens belonging to the Yukonian clade B, which was genetically the most divergent one, did not display any substantial morphological differences from those belonging to the *sphaericus*-branch (A). However, taking into account the evolutionary patterns within the complex (see below) it can be expected that gamogenetic individuals would be morphologically distinct also in clade B. Overall, an important taxonomic implication is that the morphology of parthenogenetic females, even if one studies fine morphological structures, may sometimes be missleading or of no taxonomic use, even in case of relatively ancient diversifications. It seems possible that more major lineages belonging to the complex can be still found in the regions, which were not sampled in this study. In particular, there are some records of *C. sphaericus* from Africa, Asia, South America and Australia, and those might prove to comprise yet undetected highly divergent lineages.

Several independent lines of evidence support the species status of seven lineages belonging to the three major clades within the *C. sphaericus* complex. These are concordance between the phylogenies of two independently evolving genes, high between-group divergences versus lower within-group divergences and high support for the proposed specific lineages in the COI phylogeny. Further evidence of the independent status of the Beringian lineages (A3, A4, B5 and B6) as well as two Palearctic lineages (A1 and A2) comes from their occurrence in the same sampling sites. All these arguments are commonly used as a prove of an independent evolutionary history and specific status of lineages (Avise and Ball, 1990). Furthermore, the youngest phylogenetic lineages—A1 and A2—proved to display substantial differences in the morphology of gamogenetic individuals. These can possibly lead to reproductive isolation between closely related species (Van Damme and Dumont, 2006). Unfortunately, one of the three of the currently described valid species in the *C. sphaericus* species complex—*C. biovatus*—was lacking from our samples. According to the morphological description (Frey, 1980, 1985) it is likely to belong to clade A in our phylogenetic framework (Fig. 5). Further studies are required to clarify the taxonomic status (a single species or a group of cryptic species) and the position of this taxon within the complex.

Recombination tests failed to detect any reliable evidence for ongoing recombination between the putative species lineages, thus, inter-specific hybridization should be absent or rare despite the frequent sympatry. However, it should be noted that recombination rates are likely to be underestimated in our ITS-2 data set, which included only homozygous individuals (see Section 2). In spite of the observed morphological differences, which should hamper hybridization between the most closely related lineages A1 and A2, an intermediate position of some haplotypes from Greenland and Iceland (Fig. 3: 18Gree20a21 and 19Ice17) might indicate a mitochondrial introgression or, more likely, a recent speciation event. It is also unknown, whether allopatric lineages belonging to the *sphaericus*-branch (A) would be capable of hybridization once they are brought into a secondary contact, for example, via human-mediated introduction. Although inter-specific hybridization is poorly studied in chydorids, there is experimental evidence for it in a chydorid genus *Pleuroxus* (Shan and Frey, 1983) and in the well-studied *Daphnia* it is a wide-spread phenomenon (Schwenk and Spaak, 1995). Thus, we cannot completely exclude the possibility of hybridization in the studied species complex. Nevertheless, our study clearly shows that even if inter-specific hybridization does occur within the *C. sphaericus* complex, hybrids are rare in nature, and putative species lineages are capable of maintaining genetic differentiation in sympatry.

4.2. Cryptic diversity and non-cosmopolitanism within the C. sphaericus complex

Prior morphological investigations on populations of the *C. sphaericus* s. l. from a wide range of locations in North America indicated that each of the two major lineages—*sphaericus* and

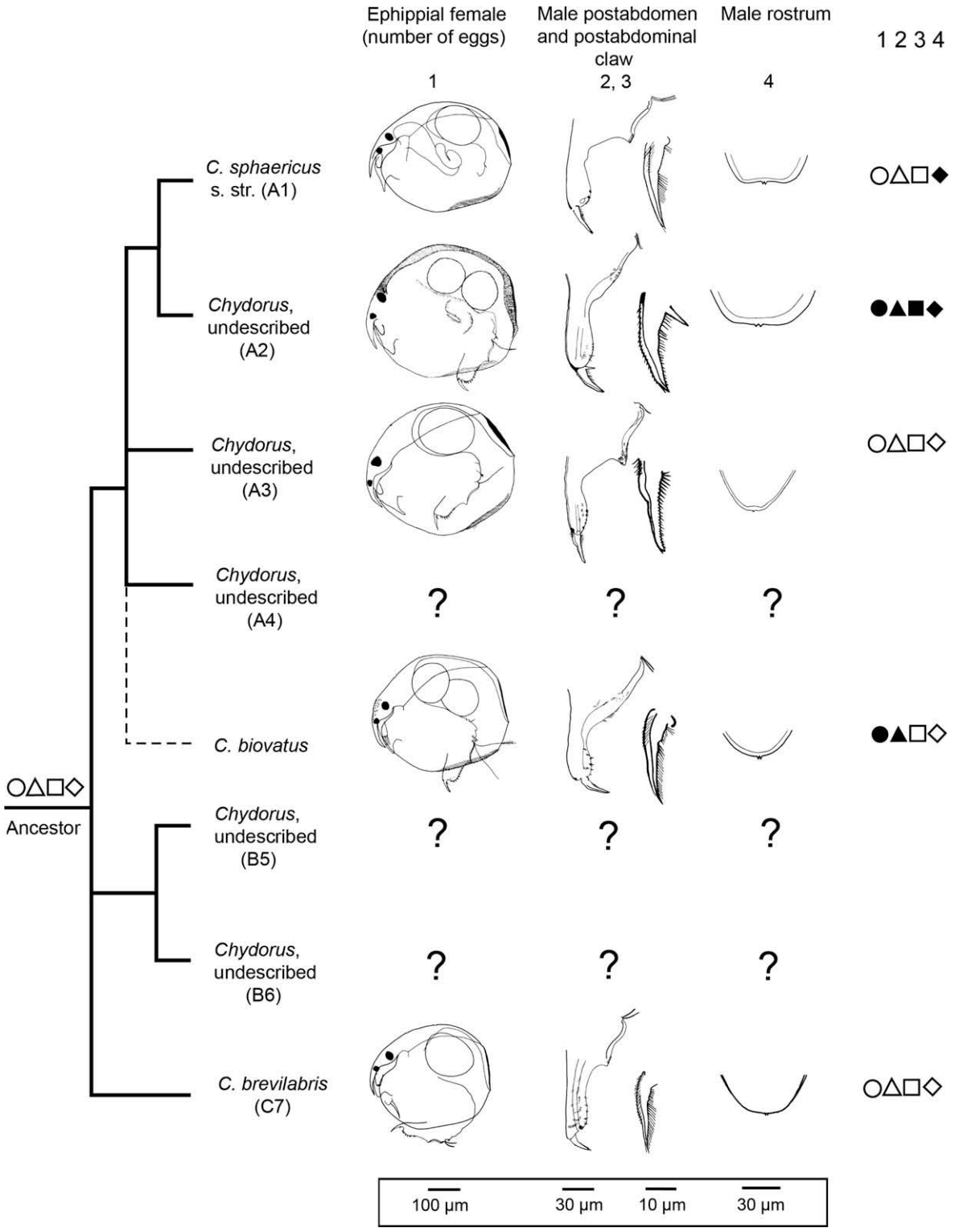

Fig. 5. Morphological versus molecular evolution within the *C. sphaericus* complex. The summary tree shows the phylogenetic relationships between the putative species lineages (A1–C7) estimated from the mitochondrial (COI) and nuclear (ITS-2) sequence variation. Branches with support <70% in the COI phylogeny are collapsed. Genetic data on *C. biovatus* were lacking, hence, its position on the tree is unknown (shown by a dotted line), possibly it belongs to the major clade A. Morphological characters: 1—number of ephippial eggs; 2—shape of preanal angle of male postabdomen (position indicated by an arrow); 3—presence of a spine on postabdominal claw (position indicated by an arrow); 4—shape of male rostrum. On the right: empty symbols are ancestral character states, filled symbols—derived characters. All drawings of *C. sphaericus* s. str., *C. biovatus* and *C. brevilabris* except for male rostrums of *C. sphaericus* s. str. and *C. biovatus* are modified from Frey (1980), with kind permission from Springer Science and Bisiness Media. Male rostrums are redrawn from SEM photos in Frey (1985), with kind permission from Wiley-VCH Verlag GmbH & Co. KGaA. All drawings for Clades A2 and A3 are original.

brevilabris—may consist of numerous sibling species (Frey, 1980, 1985). We have tested this prediction with molecular data and found that the *sphaericus*-clade (A) is comprised of several independent lineages, though not so numerous as anticipated. How-

ever, the *brevilabris*-clade (C), despite its ancient origin, included just one species, widely distributed in North America. This was an unexpected result, given the high phenotypical polymorphism and wide distribution of this taxon (Fig. 1; Frey, 1980, 1985). This finding is also in agreement with other studies, which detected only one *brevilabris*-like species with a low level of COI diversity in different climatic zones in Mexico (Elías-Gutiérrez et al., 2006; Elías-Gutiérrez, pers. comm.). Although our analysis did reveal two subclades within *C. brevilabris*, they were shallow, not well-supported and did not correspond to any geographic structuring (Fig. 3).

Frey (1986, 1995) argued that cladoceran species, particularly chydorids, display continental endemism, restricted distributions within continents and narrow ranges of environmental preferences. Indeed, continental endemism and regionalism were found to be a common pattern for cladocerans with sexual propagules (Adamowicz et al., 2004; Cox and Hebert, 2001; Haney and Taylor, 2003; Taylor et al., 1996, 2002). In our first assessment of this pattern in a benthic cladoceran we find that three species from the *C. sphaericus* clade A are present in both the Nearctic and the Palearctic, and thus they are not continental endemics in the strict sense. Yet, these species lack cosmopolitan status as they appear to be restricted to Greenland and Eurasia (clades A1 and A2) or eastern and western parts of the former Beringia (clade A3). The remaining species (A4, B5, B6 and C7) appear to be restricted to the Nearctic. Also, contrary to Frey's predictions, at least two species in the complex—*C. sphaericus* s.str. and *C. brevilabris* are widely distributed within each continent and capable of living under a broad range of conditions. Hence, our results provide evidence for isolation among the European and North American faunas and for some regionalism, on the other hand, they also show that chydorid Cladocera are capable of dispersing over large distances within continents and to isolated islands.

Whereas studies on pelagic cladocerans detected several cases of recent inter-continental introductions (Haney and Taylor, 2003; Taylor et al., 1996), we did not find any evidence for these in the *C. sphaericus* complex, nor local faunistic studies ever reported of any indications of such introductions. A number of thorough recent revisions of the local faunas in the northern, western and southern Europe (e.g. Alonso, 1996; Duigan, 1992; Flößner, 2000) failed to detect the presence of *C. brevilabris* or *C. biovatus* in Europe. On the other hand, there are numerous records of *C. sphaericus* from North America, but it seems probable that all of them are in fact misidentifications of *C. biovatus* or even *C. brevilabris*. There is still a possibility that some recent human-mediated introductions are undetected, but the presence of *C. sphaericus* s.str. in North America so far lacks any reliable evidence, so we suggest that the name *Chydorus sphaericus* s. str. should not be applied to North American populations.

4.3. Taxonomic and phylogenetic utility of morphological characters

The phylogenetic framework developed in this study allows us to assess the taxonomic and phylogenetic utility of the previously proposed morphological characters (Frey, 1980, 1985). A number of characters in the morphology of parthenogenetic females, which have been traditionally used in chydorid taxonomy (such as armament of the first and second antennae, trunk limbs and postabdomen), appear to have remained unchanged in all investigated species from the *C. sphaericus* complex (Frey, 1980). Conversely, other characters (shell sculpture, shape of rostrum, postabdomen armament) show high intra-specific variability, at the same time, similar morphotypes occurred across several distantly related lineages (Belyaeva, unpublished data), indicating convergence. Hence, only very few of presumably selectively neutral characters in the morphology of parthenogenetic females seem invariable within the species and are diagnostic of the major monophyletic lineages (e.g. the short labrum and "connected" ventral setae in *C. brevilabris*). On the contrary, the morphology of gamogenetic individuals proved very useful for distinguishing species, although, due to frequent convergences, it probably has less if any value for phylogenetic reconstructions. Convergences might have occurred in such characters as the number of eggs in the ephippium and presence/absence of the preanal angle in the postabdomen of males. Furthermore, both gains and losses of characters have occurred in the evolution of chydorids (Adamovicz and Sacherova, 2006), that may complicate phylogenetic reconstructions. The second egg in the ephippium and the spine at the base of male postabdomen are examples of such gains in the *C. sphaericus* complex. Taking into account the above considerations, a joint genetic and morphological approach would be advisable for determining the affinities of newly discovered species in the *C. sphaericus* complex.

4.4. Evolution in the Holarctic C. sphaericus complex

Our results suggest that the major branches within the *C. sphaericus* complex evolved from a common ancestor in the Tertiary. Furthermore, the resistance to cold temperatures displayed by all studied taxa including the most southern species *C. brevilabris* possibly developed, when in Tertiary sharp climatic gradients established between high and low latitudes and promoted adaptation to cold climate in a number of freshwater invertebrate taxa (Korovchinsky, 2006). There are also some indications for mass extinctions and range contractions during Tertiary suggested by Korovchinsky (2006) for Cladocera. Namely, the major branch B has a very restricted distribution in Beringia and was possibly more widely distributed in the past. Besides, the observed lack of genetic variation in the ancient lineage of *C. brevilabris* (C) as compared to the other two major lineages can be explained by elimination of the previous diversity by severe climate changes in Tertiary and survival of just one species. It is possible that more species from the two other major lineages survived, because they were better adapted to cold climate, as suggested by their more northern distributions at present.

We detected not only ancient, but also some putatively recent speciation events in the *C. sphaericus* species complex. The youngest species A1 and A2 probably diverged ca. 2 MYA. These results show that not all existing chydorid species might deserve a relict status, as it was proposed by Korovchinsky (2006). It is not unlikely that a substantial proportion of chydorid taxa will prove to be recently evolved, and there is still ongoing speciation in some benthic Cladocera. With the moderately evolving DNA markers used in this study we could not reliably determine whether the variation that had accumulated within the complex during the Pleistocene lead to further speciation.

Analysis of the genetic versus phenotypical differentiation within the *C. sphaericus* complex reveals some evolutionary patterns and trends characteristic of this group of chydorids. The striking morphological similarity of parthenogenetic females from genetically divergent lineages is most likely plesiomorphic, as a near- identical morphology is shared by some *Chydorus* species, which probably do not belong to the complex, such as *C. ovalis*. It seems, that there is a strong selective constraint, which preserves the bauplan of parthenogenetic females, even given the great variety of abiotic and biotic conditions, under which these animals live in Holarctic water bodies. This constraint possibly includes two components. Conservatism in body shape and size is characteristic of the family Chydoridae, which do not display such environmentally induced phenotypical changes, common for pelagic bosminids and daphniids. The specific conservatism in the *C. sphaericus* complex probably includes the constraint on the antennae and trunk limb morphology and ventral valve setation—the features, which

are invariable in the *C. sphaericus* complex, but commonly vary among other closely related chydorids (Frey, 1980; Smirnov, 1971, 1996). Thus, the conservatism in the morphology of parthenogenetic females common for chydorids (Frey, 1986; Smirnov, 1971) seems to have reached its maximum in the presumably advanced *C. sphaericus* complex. More investigations on the functional morphology of this species complex are needed to answer, why this particular bauplan is so strongly preserved and how it has contributed to its apparent ecological success.

Whereas parthenogenetic females have remained almost unchanged for millions of years, the morphology of gamogenetic individuals has undergone considerable evolutionary changes. Ephippial females in at least two species within the *sphaericus*-branch have gained a second egg and males have undergone changes in the shape and armament of the postabdomen, the armament of the postabdominal claw, the shape and structures of the copulatory hook, the shape of the rostrum and possibly some other yet undetected changes. Unfortunately, the lack of sequence data for *C. biovatus* does not allow a more detailed inference of evolution of these morphological traits. Nevertheless, it seems likely that a two-egged ephippium has evolved in parallel in two species in the *C. sphaericus* species complex, as it is also the case in several other chydorid species (Fryer and Frey, 1981), whereas one-egged ephippium is clearly the ancestral state. A two-egged ephippium may have an advantage in the cold climate, where the growing season is short and the importance of sexual reproduction increases in chydorids (Sarmaja-Korjonen, 2004). An increased number of eggs in the ephippium in some chydorids may ensure a faster re-establishment of the population in spring and a better chance for colonizing new habitats. Interestingly, another *Chydorus* species with two ephippial eggs is *C. ovalis*, which also has a northern Holarctic distribution (Smirnov, 1996). However, a two-egged ephippium, even if it is advantageous in the cold climate, is apparently not a necessity, as one-egged *C. sphaericus* s.str. co-occurs with the two-egged species in northern Palearctic water bodies (this study). Considerable evolution of the male morphology was probably mainly associated with advances in the mating process and may ensure reproductive isolation between closely related species (Van Damme and Dumont, 2006). Indeed, the latter study showed that males of *C. ovalis* were even not able to attach to females of *C. sphaericus*, though they reacted to the chemical signal of heterospecific females. A narrow postabdomen of males lacking denticles is probably a major synapomorphy of the *C. sphaericus* complex. This type of postabdomen appears to be the most derived one in the genus *Chydorus*, whereas a postabdomen similar to that of females is the most primitive one (Van Damme and Dumont, 2006). Some other species of *Chydorus* also show the tendency for narrowing of the postabdomen and decreasing its armament, e.g. *C. pizzari*, *C. faviformis, C. baicalensis*, but this is most pronounced in the *C. sphaericus* complex. Within the latter the armament of the postabdomen is reduced to few fine setules within the *sphaericus*-branch, while further reduction of the preanal angle of the postabdomen leads to the narrowing of its proximal part. The functional meaning of these morphological changes is still unknown, but it is likely related to a more specialized mating behaviour in this species (Van Damme and Dumont, 2006).

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