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Three new cryptic species of the freshwater zooplankton genus *Holopedium* (Crustacea: Branchiopoda: Ctenopoda), revealed by genetic methods

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Abstract

Molecular approaches have greatly advanced our understanding of species diversity and biogeography in the cladoceran crustaceans. Here, we provide the first large-scale examination of taxonomic diversity in the genus *Holopedium* Zaddach, 1855, by characterizing patterns of allozyme, mtDNA, and morphological variation from a total of 193 sites from three continents, including collections from near the type localities for the two generally recognized species, *Holopedium gibberum* Zaddach, 1855, and *Holopedium amazonicum* Stingelin, 1904. Allozyme data were only available for North American samples but revealed the presence of four species. Divergence patterns in the mitochondrial cytochrome *c* oxidase subunit I (COI) gene supported those species, as well as a fifth taxon endemic to South America. The five putative species are separated by substantial sequence (8.7–24.5%) and allozyme (0.36–1.54 Nei's distance) divergences, while intraspecific genetic diversity was generally limited in comparison. Although two of these species exhibited little morphological differentiation from their closest relatives, and diagnostic traits were not found among the characters considered, a population-level approach revealed significant morphological differences among all pairs of taxa. We therefore

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present both an allozyme key and a morphological/geographic key to all species, as well as new or augmented descriptions for all five species. *H. gibberum* s.s. is distributed in Europe and across arctic North America, while its cryptic sister species, *H. glacialis* **n. sp.**, is widely distributed across temperate North America. *H. amazonicum* s.s. is apparently restricted to the Amazon basin, *H. atlanticum* **n. sp.** occurs in lakes along the eastern margin of North America, while *H. acidophilum* **n. sp.** occurs sporadically across North America along a narrow band of middle latitudes. Due to high morphological variability within species, as well as the detection of cryptic diversity, we suggest that genetic analyses should be performed on populations from other geographic regions and should always accompany the recognition of new species of *Holopedium*.

Key words: allozymes, Cladocera, COI, Crustacea, cryptic species, freshwaters, mitochondrial DNA, morphology, species description, taxonomy

Introduction

The past twenty years have seen a paradigm shift in taxonomic perceptions for the cladoceran crustaceans and many other freshwater invertebrate groups. The traditional view of low species diversity and cosmopolitan distributions was derived from the observations of early workers who noted that many freshwater invertebrates exhibited little morphological variation over vast geographic distances (Lyell 1832; Darwin 1859), as well as great dispersal ability (Darwin 1882). Mayr (1963) described this biogeographic pattern as arising from the homogenizing effects of gene flow, and, indeed, the resting eggs of these organisms do possess several characteristics that would appear to make them ideal passive agents of dispersal (Fryer 1996). Capable of being transported by wind and surviving passage through avian digestive systems (reviewed in De Meester *et al.* 2002), resting eggs also often possess sticky spines or protuberances that facilitate attachment to waterfowl, and they are produced in the greatest numbers when waterfowl migration is at its peak (Fryer 1996). Propensity for dispersal is also supported by the rapid colonization of northern habitats following deglaciation for many cladocerans.

However, despite this capacity for dispersal, detailed morphological and genetic investigations have revealed high levels of taxonomic diversity and endemicity (e.g. Frey 1982, 1985, 1987; DeMelo & Hebert 1994; Taylor *et al.* 1996, 1998; Colbourne *et al.* 1998; Petrusek *et al.* 2004). Moreover, genetic information has challenged the view that gene flow is sufficient to maintain genetic cohesion among cladoceran populations on a continental — let alone a global — scale (Boileau *et al.* 1992; Hebert & Taylor 1997). Founder effects, combined with rapid population increase and local adaptation, may be important factors that restrict gene flow in the face of dispersal of propagules (Boileau *et al.* 1992; De Meester *et al.* 2002). Thus, local genetic differentiation and continental or regional endemism, as opposed to cosmopolitanism, have become established features of our understanding of cladoceran diversity.

Despite the high biotic and abiotic variability among aquatic habitats, such genetic divergence is often not associated with morphological change in zooplankton species. Detailed phylogenetic frameworks have allowed researchers to address key questions regarding how molecular and morphological evolution proceeds in these lineages (e.g. King & Hanner 1998). For example, is morphological similarity among species due to convergence, cosmopolitanism, introgression, or shared ancestry (Taylor *et al.* 1996)? Habitat-linked convergence and introgression have played important roles in morphological evolution in the cladocerans (Colbourne *et al.* 1997; Schwenk *et al.* 2000) and these processes have tended to be associated with cases of more rapid morphological evolution. However, the overwhelming answer in most cases has been that shared ancestry, combined with a slow pace of morphological evolution, is the culprit for past cases of diversity underestimation. Thus, combined genetic and morphological approaches have proven both necessary and fruitful for assessing species diversity and for investigating the evolutionary history of the Cladocera.

The genus *Holopedium* Zaddach, 1855 is an example of a cladoceran taxon still regarded as being both broadly distributed and species poor. Its representatives are widely distributed in softwater lakes throughout

the northern hemisphere, and also in the Amazon River basin (reviewed in Rowe 2000). Until recently, only two species were generally recognized, *Holopedium gibberum* Zaddach, 1855 and *Holopedium amazonicum* Stingelin, 1904. Described from a pond in the region of Kaliningrad, Russia, *H. gibberum* is thought to have a broad distribution in Eurasia and North America, from the high arctic to temperate latitudes, while *H. amazonicum* is thought to inhabit lakes in the Amazon River basin and along the Atlantic coast of North America. Although Rao *et al.* (1998) described a new species (*Holopedium ramasarmii*) from India, its status is *incertae sedis* (corroborated by Korovchinsky 2004), due to an incomplete description based upon an unspecified "body shape" and "head structure." Korovchinsky (2005) recently described another species from Greenland, *Holopedium groenlandicum*. Genetic analysis will be required to ascertain if these are in fact distinct species or if they are synonymous with described taxa.

The taxonomy of the genus *Holopedium* has long been in flux. Populations exhibiting morphological anomalies and intermediate features were reported following descriptions of the original species (Scheffelt 1909; Carpenter 1931; Coker 1938; Bunting 1970; Hegyi 1973). Such inconsistencies have prompted some workers to call for the recognition of just a single phenotypically plastic taxon (Hegyi 1973; Havel in Dodson & Frey 1991). Early studies on *Holopedium* were restricted to small geographical areas and provided little opportunity for a critical examination of taxonomic diversity. Upon examining morphological and ecological diversity in *Holopedium* from several continents, Hegyi (1973) concluded that there was insufficient variation to warrant the recognition of two species. A decade later, however, he and others described an additional characteristic, microsculpturing on the jelly coat, which permitted reliable differentiation of the two then-described species of *Holopedium* (Montvilo *et al.* 1987). Tessier (1986) suspected the presence of different species of *Holopedium* in his samples, but noted that some individuals had morphological affinities to two different species. More recently, Korovchinsky (1992 and 2004) provided summaries of *Holopedium* taxonomy but no new diagnostic characters were presented. He did note a few specimens from eastern North America with abnormal morphologies. Thier (1994) was the first to carry out population genetic analyses on *Holopedium*, examining assemblages from New England. Populations showed marked heterozygote deficits, which he attributed to low levels of gene flow and sporadic sexual recruitment within a single species. However, such extreme deficits are unlikely within single habitats in cyclic parthenogens like *Holopedium*, which reproduce sexually approximately yearly. Hebert & Finston (1997) later showed that the Hardy-Weinberg disturbances reflected the occurrence of at least two species of *Holopedium* in eastern North America.

Because of the reported existence of intermediate forms, and the uncertain taxonomy due to the limited geographical scope of past studies, this study seeks to resolve species boundaries in the genus *Holopedium* by analyzing material from across North America and from a few sites in Europe and in the Amazon basin. Following allozyme and mitochondrial DNA analyses aimed at diagnosing putative species boundaries, morphological analysis of select characters is carried out to assess whether there are significant differences among species and to search for diagnostic traits. We next present new and revised descriptions for five species, along with identification keys. We conclude by returning to the issue of patterns of cladoceran diversification and by suggesting avenues for further research.

Methods

Sample collection

Samples of *Holopedium* were collected from 193 localities mainly in the summers (May-September) of 1990–1998, including sites in all eleven North American freshwater biogeographic provinces (Burr & Mayden 1992) where this genus has been reported (Fig. 1; and see Rowe 2000). An attempt was made to sample from the type localities of the two initially described species, but logistical difficulties made this impossible. Samples from the other continents were therefore obtained from sites as close to the type localities as possible. The three European sites range from approximately 600 to 1,050 km from the type locality of *H. gibberum*

(which is near Kaliningrad). Amazonian samples were obtained from about 1,300 km from the type locality of *H. amazonicum*. Nevertheless, it is assumed here that these habitats have a higher probability than the North American sites of containing the species that were originally described.

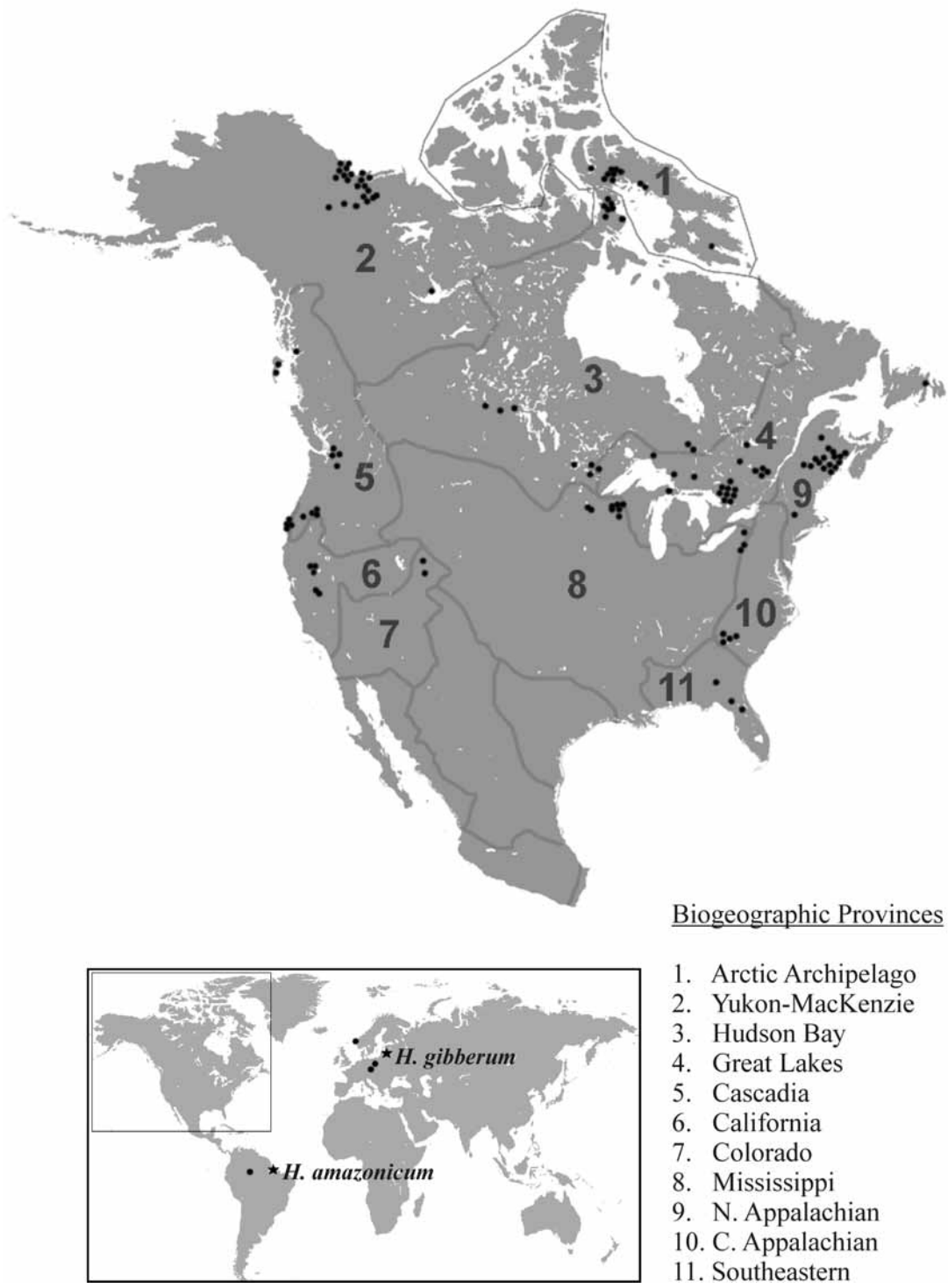


FIGURE 1. Maps of *Holopedium* populations sampled for genetic analyses. Exact geographic coordinates and habitat names are provided in Appendix A. Populations sampled from outside North America are shown on the inset global map. Type localities for the two initially described species of *Holopedium* are indicated with stars on the inset map and were not included in this study, although (relatively) nearby collections were available. North American freshwater biogeographic provinces (after Burr & Mayden 1992) are shown, and the names of those from which samples were collected are listed in the legend.

Populations are identified by their official name or by habitat number in a particular sampling region. Each name is followed by a two-letter provincial or state abbreviation if in Canada or the United States; otherwise it is followed by a three-letter international country code (e.g., Inuvik 5 NT refers to a particular waterbody in the proximity of Inuvik, Northwest Territories, Canada). Most localities were sampled only once, except for Paint ON, Prospect ON, Lac La Ronge SK, and Rest WI, which were sampled in both 1993 and 1997. Samples from these sites are identified with two additional digits indicating the year in which the collection occurred. Abbreviation codes, geographical coordinates, and sampling dates for all habitats are listed in Appendix A.

All specimens were identified according to Pennak (1989) and Korovchinsky (1992), and individuals were removed and stored in liquid nitrogen for genetic analyses or in 70% ethanol for morphological analyses. The three European populations and one Brazilian population were preserved in ethanol only, allowing mtDNA analysis but precluding allozyme analyses. Thirteen additional populations from Brazil were stored in formalin, thus allowing only for morphological characterization.

Allozyme analysis

Electrophoresis. One hundred and sixty-four collections were available for allozyme analyses (see Appendix A), including the four habitats that were sampled twice in different years. Individuals were thawed and their jelly coat removed before being separately homogenized in 4–8 μL of sterile distilled water. A mean of 24 individuals (ranging from 1–133 depending upon availability) was analyzed from each site. Allozyme variation was examined using cellulose acetate electrophoresis with a Tris-glycine buffer (pH 8.5), using standard methods (Hebert & Beaton 1993) and an electrophoretic run time of 15 min.

Preliminary screening of *Holopedium* populations resulted in the identification of seven allozyme loci that both stained reliably and exhibited polymorphism. Thus, variation was examined at the following loci, with the abbreviations and Enzyme Commission (EC) numbers provided in parentheses: amino aspartate transferase, supernatant form (*sAat*, EC 2.6.1.1); arginine phosphate kinase (*Apk*, EC 2.7.3.3); glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9); malate dehydrogenase, supernatant form (*sMdh*, EC 1.1.1.37) and mitochondrial form (*mMdh*, EC 1.1.1.37); mannose-6-phosphate isomerase (*Mpi*, EC 5.3.1.8); and phosphoglucomutase (*Pgm*, EC 5.4.2.2). Four individuals from a reference population, Prospect ON, were included in each assay as mobility standards. Alleles were identified by their mobilities (R_f) relative to those of the standard population, averaged over several electrophoretic runs. Of the 164 collections, 93 are new to this study, while 71 have been previously published (Hebert & Finston 1997) by members of the same research group, who were assisted by the first author of the present study. These data were included here to establish the species identity of all populations, and to examine patterns of genetic diversity and structure using as many populations as possible. Alleles within 2% mobility could not be reliably distinguished for members of the *H. gibberum* group. Therefore, three alleles detected in Hebert & Finston (1997) have been combined with alleles detected here (i.e. their *Pgm 90* was combined with *Pgm 92* here; their *Gpi 112* has been combined with *Gpi 110*; and their *sAat 91* has been combined with *sAat 93* here).

Genotypic characterization of populations. Allozyme data were entered in the NEXUS computer file format (Maddison *et al.* 1997) for use with Genetic Data Analysis (GDA) software (Lewis & Zaykin 1999). Genotypic frequencies in each population were tested against Hardy-Weinberg (H-W) expectations using Fisher's exact test (Fisher 1935), excepting populations with sample sizes of less than five. Although cladocerans are cyclically parthenogenetic, one of the assumptions of H-W is sexual reproduction. Nevertheless, these tests are expected to be informative in studying *Holopedium* species boundaries. Regular (generally yearly) sex serves to restore heterozygosity, and H-W equilibrium is often observed in cyclic parthenogens. Severe departures from expectations are usually indicative of multi-species assemblages, hybridization, and breeding system shifts. Fixation indices were also calculated from the expected and observed heterozygosities. The fixation index varies from -1 for populations that consist solely of heterozygotes to 1 for populations that show polymorphism but lack heterozygotes.

All collections that were either invariant or in H-W equilibrium were assumed to represent single-species collections. For these populations, a phenogram was contructed based upon Nei's (1972) genetic distances, using the unweighted pair group method with arithmetic averaging (UPGMA), performed in GDA. Levels of intraspecific and interspecific genetic divergence were examined. Allelic profiles of species were initially summarized prior to investigation of those assemblages showing departures from H-W expectations.

Species composition of Hardy-Weinberg deviant assemblages. The results obtained from the allozyme analysis of single-species populations provided a means to probe the nature of those assemblages showing H-W deviations. Upon finding four major allozyme lineages showing diagnostic allelic differences (and noting that these lineages corresponded to major mitochondrial DNA clusters), this made it possible to inspect deviant populations to ascertain whether they contained one or more of the taxa detected in single-species populations. Any genotype (or mtDNA haplotype) from a deviant population that was genetically identical or similar to one from a recognized species was assigned to that taxon. Similarity matrices employing Nei's (1978) genetic identity were constructed for individuals within each population with H-W deviations to ensure that species assignments were concordant with the ranges of intraspecific variability observed for the single-species assemblages.

Genetic differentiation among populations. Wright's (1978) F-statistics were computed using Cockerham & Weir's (1993) methods to examine levels of genetic differentiation among conspecific populations at three hierarchical levels: 1) the population, 2) the biogeographic province, and 3) all North American populations. Biogeographic provinces are defined in Figure 1. Each hierarchical level above the population must contain at least two populations for the F-statistic calculation. In two cases, a biogeographic province (BP) was represented by a single population, and so these populations were grouped with neighbouring biogeographic provinces: Great Slave Lake NT in Yukon-MacKenzie was included in Hudson Bay, and Soldier Pond MI in the Great Lakes was included in N. Appalachian. 95% confidence intervals (C.I.) for all F-statistics were obtained in GDA by bootstrapping with 1,000 pseudoreplicates. When a resulting 95% C.I. did not include the value zero, its F-statistic was considered to indicate significant genetic structure at that hierarchical level.

Mitochondrial DNA analysis

Sequencing. A single individual from several populations of the four lineages identified through allozyme analysis were sequenced. In addition, individuals were sequenced from fifteen populations lacking allozyme data because of their preservation in ethanol. Three of these populations were from western Europe, and a fourth was from the Amazon. As allozyme analysis and DNA extraction were often performed at different times, only a few individuals have corresponding allozymic and haplotypic data.

For frozen specimens, total genomic DNA was extracted from single individuals by aliquoting 1–2 µL of whole-body homogenate (which totaled 4–8 µL) into 30 µL of 6% Chelex-100 (BioRad Inc.). This solution was incubated at 55° C for 12 hrs., next boiled for 10 min. at 100° C, then centrifuged for 1 min. at 14,000 rpm, and finally incubated at 4° C overnight. The supernatant was then used directly in the polymerase chain reaction (PCR) (Saiki *et al.* 1988). The remainder of the whole-body homogenate (2–7 µL) was immediately used in electrophoresis for those individuals with accompanying allozyme data. DNA from samples preserved in ethanol was extracted using the methods of Shiozawa *et al.* (1992). Preserved animals were soaked individually in a Tris buffer for 12 hrs., and then homogenized and incubated at 55° C for 18 hrs. in a buffer containing 75 µL 20% SDS and 5 µL of 20 mg/mL proteinase K solution. DNA was then extracted with two phenol:chloroform and one chloroform wash. DNA was precipitated overnight at -20° C in 70% ethanol, and resuspended in 50 µL of Tris-EDTA pH 8.0. Total DNA was also extracted from a specimen of *Sida crystallina* from Lake Rosseau ON (45.25° N, 79.24° W) for use as an outgroup in subsequent phylogenetic analyses.

A 658 base pair (bp) fragment (excluding primer sites) of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was PCR amplified using the primer pair (LCO1490: 5'- GTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'- TAAACTTCAGGGTGACCAAAAAATCA-3') described by Folmer *et al.* (1994). Each 50 µL PCR reaction contained 3–5 µL of DNA template, 4.5 µL 10x PCR buffer (Hillis *et al.* 1996), 0.2 µM of each primer, 2.2 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 unit of *Taq* DNA polymerase. Amplifications, which were carried out on a Perkin Elmer Cetus thermal cycler, consisted of an initial denaturation step of 94° C for 1 min., followed by five cycles of denaturation at 94° C for 1 min., annealing at 45° C for 1.5 min., and extension at 72° C for 1.5 min. The annealing temperature was then raised to 50° C for another 35 cycles, followed by a final extension at 72° C for 5 min. Two replicate PCR amplifications were performed on each individual to increase yield; PCR products were separately electrophoresed and excised from a 2% agarose gel, and then combined for purification using the Qiaex II reagents (Qiagen Corp.). 100–200 ng of purified DNA were subsequently sequenced with the LCO1490 primer on an ABI Prism 377 automated sequencer using the *Taq* FS dye rhodamine kit.

Characterization of mtDNA divergence. Sequences were verified as cladoceran COI fragments using the NCBI BLAST search (Altschul *et al.* 1990) and were aligned using Sequence Navigator (Perkin Elmer, version 1.01), resulting in a final alignment of 616 (bp). A pairwise distance matrix for all sequences was constructed, based upon Kimura's (1980) two-parameter model (K2P) of sequence divergence (with pairwise deletion of missing sites), in the program MEGA version 1.02 (Kumar *et al.* 1993). This commonly used distance metric was selected to facilitate comparisons of genetic patterns across studies and taxa and because it has previously been found useful at low taxonomic levels. Firstly, the mean pairwise divergence among major lineages (which we will later argue correspond to species) was calculated, and standard errors for these means were obtained using 1,000 bootstrap replicates. Secondly, the mean and maximum divergences *within* each of those lineages were calculated, such that patterns of within-lineage versus between-lineage genetic variability could be compared. All sequences were included in these calculations (rather than just unique haplotypes), in order to represent the typical divergence values in the frequencies encountered.

Four sequences from each of five major mtDNA lineages (when available) were selected for inclusion in a phenogram, constructed using the neighbour-joining (NJ) method (Saitou & Nei 1987) performed in MEGA. These haplotypes were selected to maximize the extent of intraspecific variation presented. Complete deletion of missing sites was employed, and bootstrap support for nodes was calculated using 10,000 replicates. Further details regarding intraspecific mitochondrial patterning, as well as geographic distributions of mtDNA lineages based upon all available sequences, will be presented elsewhere.

Morphological analyses

Morphology was examined to assess whether the five divergent lineages of *Holopedium* recognized through genetic analysis can be discriminated using morphological characters. The potential taxonomic value of the array of morphological characters used in previous studies is reviewed in Rowe (2000), where further justification is also provided regarding our choice of characters for inclusion.

Eight to 29 individuals from 16 populations representing the five species were selected for morphological analyses. Poor quality of preserved animals prevented many collections, specifically those from the European sites, from being included in the morphological study. Each animal was sexed and examined for eight structural or morphometric characters that were found to be variable in prior taxonomic studies: carapace length (mm), carapace height (mm), carapace (H/L) ratio, number of anal spines on the post-abdomen (counting those on the left and right sides as separate characters), ventral carapace margin spinules, basal spines on the post-abdominal claw, and denticles on the post-abdominal claw. Measurements and meristic counts were performed under a 40x or 100x oil immersion objective lens, depending on the sizes of the structures being examined. Colour images of each individual were captured and length measurements were made using the Image-Pro Plus 3.0 image analysis software package (Media Cybernetics).

Summary statistics (mean and variance) for each character were calculated for all populations. Variance components analysis was performed to determine at what hierarchical level (individuals within populations, populations within species, or among species) most morphological variability occurs for each character. Species-level character means were tested for heterogeneity using a one-way analysis of variance, and Tukey's honestly-significant differences test was performed to determine which pairs of means differed significantly (at a family-wise $\alpha = 0.05$) using the program R version 2.2.1 (R Development Core Team 2005). Discriminant function analysis was performed to determine the characters that best separate individuals into the predefined species groups. The percentage of individuals accurately assigned to the genetically identified species based upon post-hoc morphological classification was also recorded. Due to many missing values, two characters (number of anal spines on postabdomen side #2 and the number of denticles on the post-abdominal claw) were omitted from the discriminant function analysis.

Results

Overview and species identities

Large allozyme and mtDNA divergences separated populations belonging to the two initially described species of *Holopedium*, *H. gibberum* and *H. amazonicum*. However, further deep and concordant allozyme and mtDNA splits were apparent within each of these groups, indicating the presence of cryptic lineage diversity (see further Results below). We will propose that the five main genetic clusters detected here correspond to species (see Discussion). For clearer presentation and discussion of results, we assign names to these lineages here and provide justification later.

In order to assign names to these species in accordance with the existing taxonomy, phenetic mtDNA placements were examined for those individuals collected from regions near the type localities of the two initially described taxa. The inclusion of three haplotypes from Europe from the region of the type locality of *H. gibberum* suggests that one of the species from the *H. gibberum* group represents *H. gibberum* s.s., while the other cluster corresponds to an undescribed species, *H. glacialis* **n.sp.** Similarly, the sole haplotype collected from the Amazon basin was presumed to correspond to *H. amazonicum* s.s., while the two other (North American) clusters within the *H. amazonicum* group are proposed to represent new species (*H. atlanticum* **n.sp.** and *H. acidophilum* **n.sp.**). Rationale for the etymology of all species and criteria permitting their discrimination are presented with the species descriptions.

Allozyme analyses

Hardy-Weinberg tests on assemblages

Allozyme phenotypes were concordant with those expected based on the known quaternary structure of the enzymes (Ward *et al.* 1992). The 164 *Holopedium* collections available for allozyme analysis showed extensive genetic variation, with up to nine alleles detected at one locus (*Gpi*). Eighty-six populations exhibited polymorphism at one or more loci. Five of these populations had sample sizes of fewer than five individuals and were excluded from H-W tests (Herschel Island 7 NT, First NB, Bee ON, Ellery CA, and Blue Ridge GA). Genotypic frequencies at 121 sites were either in H-W equilibrium (44 populations) or were invariant (77 populations), indicating that each contained a single species. Thirty-eight other populations exhibited significant ($p < 0.05$) H-W deviations (Table 1) and were classed as deviant. The populations out of H-W and those with sample sizes of fewer than five individuals were temporarily removed from the data set, but were revisited after examination of populations in H-W equilibrium.

TABLE 1. Fisher's exact tests for deviations in allozyme genotype frequencies from Hardy-Weinberg expectations. Of 164 *Holopedium* collections assayed for allozyme variation, 38 were found to exhibit significant ($p < 0.05$) departures. Five additional collections had a sample size of less than five, precluding statistical tests, but showed both polymorphism and a lack of heterozygotes. The "diagnosis" column indicates the nature of the deviation (heterozygote excess, heterozygote deficit, or a complete lack of heterozygotes). The next column indicates the presumed reason for these departures: "2 spp." designates the presence of mixed-species assemblages (based upon the presence of fixed allozyme differences among species in the analysis of single-species assemblages); "br. sys." indicates that a breeding system shift (to selfing or a similar system) is the most likely explanation for the complete lack of heterozygotes in arctic *H. gibberum* (see Hebert *et al.* 2007); and "?" indicates a within-species deviation of unknown cause (possibly a long bout of asexual reproduction within a habitat). Abbreviations for the species at each site are: A- *H. atlanticum*; C- *H. glacialis*; G- *H. gibberum* s.s.; L- *H. acidophilum*.

Site No.	Locality	n	Locus	P-value	Diagnosis	Reason	Species
2	Mayer, BC	42	*sAat*	0.000 *	no hets	?	G
5	Reed, MB	20	*Gpi*	0.000 *	no hets	?	C
12	Lomond, NB	6	*Pgm*	0.025	no hets	2 spp.	A,C
			Gpi	0.027	no hets		
			Mpi	0.025	no hets		
			Apk	0.027	no hets		
			sMdh	0.025	no hets		
9	First, NB	2	*Pgm*	--	no hets	2 spp.	A,C
			Gpi	--	no hets		
			Mpi	--	no hets		
			Apk	--	no hets		
			sMdh	--	no hets		
18	Utopia, NB	8	*Pgm*	0.012	no hets	2 spp.	A,C
			Gpi	0.012	no hets		
			Mpi	0.018	no hets		
			Apk	0.018	no hets		
			sMdh	0.020	no hets		
46	Great Slave, NT	40	*Gpi*	0.000 *	no hets	?	C
			Mpi	0.016	no hets		
47	Inuvik 1, NT	20	*sMdh*	0.026	no hets	br. sys.	G
			sAat	0.000 *	no hets		
49	MacKenzie Delta 13, NT	20	*sMdh*	0.000 *	no hets	br. sys.	G
53	Parry Bay 2, NU	44	*Gpi*	0.000 *	no hets	2 spp.	C,G
54	Quartzite Lk 7, NU	22	*sMdh*	0.000 *	no hets	br. sys.	G
55	Sarcpa Lk 8, NU	22	*Gpi*	0.000 *	no hets	2 spp.	C,G
			sMdh	0.000 *	no hets	(and br. sys. in G)	
56	Sarcpa Lk 13, NU	22	*Gpi*	0.000 *	no hets	2 spp.	C,G
63	Bee, ON	2	*Pgm*	--	no hets	?	C
79	Lac des Milles Lacs, ON	41	*Mpi*	0.016	no hets	?	C
			sAat	0.000 *	no hets		
80	Lk of the Woods, ON	40	*Pgm*	0.036	het deficit	?	C
93	White, ON	40	*Pgm*	0.001	het excess	?	C
			Gpi	0.016	no hets		

96	Archambault, QC	19	*Gpi*	0.007	het excess	?	C
97	Beland, QC	21	*sAat*	0.000 *	no hets	?	C
98	Chibougamau, QC	20	*Pgm*	0.002	het deficit	?	C
102	Amisk, SK	10	*Gpi*	0.001	no hets	?	C
104	Lac La Ronge 97, SK	39	*Gpi*	0.000 *	het deficit	?	C
105	Herschel Island 7	3	*sAat*	--	no hets	br. sys.	G
106	Herschel Island 8, YK	41	*sAat*	0.001	no hets	br. sys.	G
109	Shingle Pt 4, YK	21	*sMdh*	0.000 *	no hets	br. sys.	G
			sAat	0.021	no hets		
110	Shingle Pt 7, YK	40	*sMdh*	0.000 *	no hets	br. sys.	G
			sAat	0.003	no hets		
111	Shingle Pt 8, YK	22	*sMdh*	0.025	no hets	br. sys.	G
113	Shingle Pt 12, YK	22	*sAat*	0.023	no hets	br. sys.	G
114	Shingle Pt 13, YK	22	*sMdh*	0.000 *	no hets	br. sys.	G
115	Shingle Pt 16, YK	42	*sMdh*	0.011	no hets	br. sys.	G
			sAat	0.000 *	no hets		
116	Shingle Pt 17, YK	40	*sMdh*	0.000 *	no hets	br. sys.	G
			sAat	0.000 *	no hets		
117	Shingle Pt 18, YK	42	*sMdh*	0.000 *	no hets	br. sys.	G
			sAat	0.000 *	no hets		
121	Ellery, CA	4	*Mpi*	--	no hets	?	C
122	George, CA	18	*Pgm*	0.004	no hets	?	C
			Gpi	0.027	no hets		
124	Mary, CA	19	*Gpi*	0.000 *	no hets	?	C
130	Blue Ridge, GA	4	*sAat*	--	no hets	?	A
134	Flying Pond, ME	19	*sAat*	0.030	no hets	?	A
147	James, NC	25	*Gpi*	0.000 *	het excess	?	A
148	Santeetlah, NC	21	*Gpi*	0.000 *	het excess	?	A
153	Link, OR	41	*Gpi*	0.000 *	no hets	?	C
157	Saunders Pond, OR	40	*Apk*	0.001	no hets	?	L
			sAat	0.013	no hets		
167	Big Muskellunge, WI	40	*sAat*	0.000 *	no hets	?	C
171	Merrill, WI	43	*Pgm*	0.000 *	het deficit	?	C
175	North Turtle, WI	44	*Pgm*	0.046	het deficit	?	C

* Significant following sequential Bonferroni correction (Rice 1989), based upon the total sample size of polymorphic loci across sites.

Allozyme divergence among species

The UPGMA phenogram based upon Nei's (1972) genetic distances among populations that either exhibited H-W equilibrium or were invariant showed two major groups (Fig. 2). As the geographic distributions of populations comprising these groups were consistent with the presumed distribution of the two initially described species of *Holopedium* (Korovchinsky 1992), these groups were assigned the names *H. gibberum* and *H. amazonicum*. However, the presence of population clusters showing marked genetic divergence within each of these groups suggested that both harbour cryptic diversity. Dividing each of these groups into two

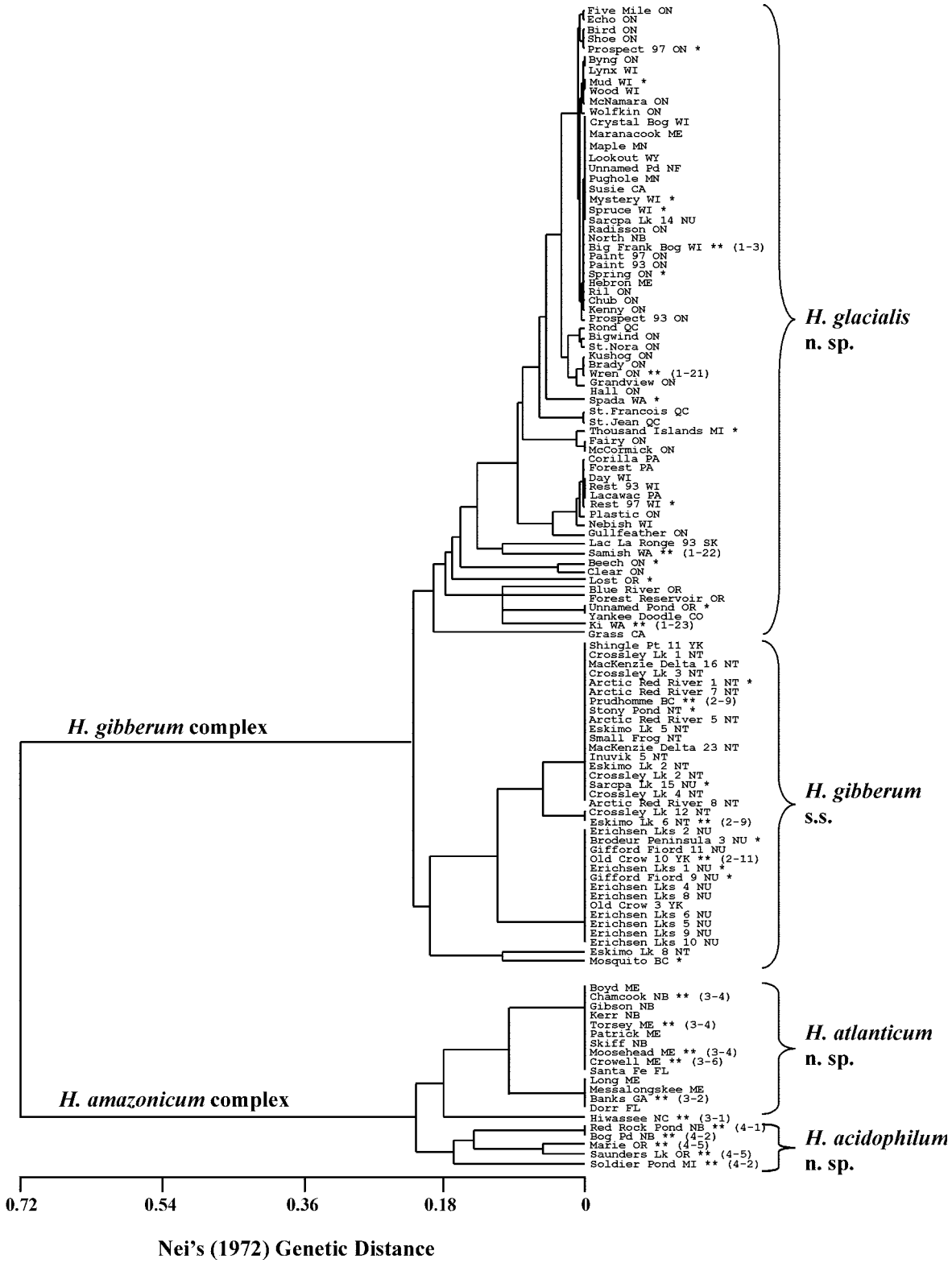


FIGURE 2. UPGMA phenogram showing genetic distances, based upon allozyme data, among 121 *Holopedium* populations. Only those collections known to contain a single species, due to being either invariant or in H-W equilibrium, are included here. Nei's (1972) genetic distance is indicated on the scale bar. The geographic distributions of the two major groups are consistent with those of the two initially described species of *Holopedium*, and these groups are therefore named after these species, *H. gibberum* and *H. amazonicum*. New species names are assigned to two clusters on the basis of allozyme, mtDNA, distributional, and morphological information (see text for further information and justification). Asterisks designate those populations for which mtDNA results are available; allozyme and mtDNA clusters are concordant. Double asterisks and haplotype numbers are given for those populations having sequence data presented in Fig. 4.

putative species resulted in four clusters of populations, each exhibiting both substantial allozyme divergence from the others (Table 2) and at least one fixed allozyme difference from all others (Table 3). Moreover, these groupings were concordant with deeply divergent clusters based upon mtDNA (see below), suggesting that they correspond to different species that are reproductively isolated and have separate evolutionary trajectories. Average Nei's distances among species ranged from 0.36–1.54, while average distances among populations within species were far more limited, ranging from 0.05–0.19 (Table 2). Maximum intraspecific divergences were also generally lower than for interspecific comparisons, with the exception of *H. glacialis*, which exhibited some larger pairwise distances of up to 0.57 (Table 2).

Patterns of allele frequency variation

Average allele frequencies for each species are shown in Table 3, while allele frequencies within every population are available in Rowe (2000) or may be obtained from CLR. Members of the *H. gibberum* and *H. amazonicum* species complexes could be distinguished by examining allozyme variation at *sMdh*. All alleles found in the *H. gibberum* complex were slower than those in the *H. amazonicum* complex. The two species within the *H. gibberum* complex could themselves be distinguished at *Gpi*, as *H. gibberum* was monomorphic for *Gpi 114*, while *H. glacialis* never possessed this allele. All but one allele at *Gpi* (*Gpi 119*) in *H. glacialis* was slower than the sole *H. gibberum* allele, *Gpi 114*. The two North American members of the *H. amazonicum* complex could be distinguished at *Pgm*. *H. acidophilum* was fixed for *Pgm 90*, while *H. atlanticum* was fixed for *Pgm 85*.

Allozyme statistics across populations, organized by species and biogeographic province, are summarized in Table 4. Average heterozygosities (both observed and expected) were low across all species, indicating both low genetic variability and a tendency towards heterozygote deficit. No heterozygotes were found at all within populations of *H. gibberum* s.s. from the Canadian arctic; these populations are discussed in more detail in Hebert *et al.* (2007).

Diagnosis of assemblages showing Hardy-Weinberg deviations

The results obtained from analysis of the single-species populations provided a means to investigate the genetic composition of 43 additional collections, 38 that were out of H-W and 5 that had sample sizes of less than five (Table 1). Among these, 24 sites contained at least one locus exhibiting significant H-W deviation even following sequential Bonferroni correction. Thus, a large proportion of the 86 sites exhibiting polymorphism deviated from expectations. Based upon the allelic profiles from single-species populations, no F1 hybrids were detected in any of the deviant assemblages screened.

Six of the 43 deviant collections contained two divergent groups of genotypes which were individually similar to genotypes detected in single-species analysis. Verification of this fact was obtained when mtDNA sequencing showed that the same co-occurring individuals possessed haplotypes characteristic of these species (although not all isolates were sequenced) (Appendix A). Therefore, three sites were concluded to consist of co-habiting *H. atlanticum* and *H. glacialis*, while three sites contained both *H. glacialis* and *H. gibberum* s.s. (Table 1).

Seventeen of the 43 populations appeared to consist solely of *H. glacialis*, but were in H-W disequilibrium. Eleven of these had no heterozygotes at certain loci, but subsequent mtDNA sequencing verified their assignment as *H. glacialis*. These populations were spread across the range of this species. Seven populations had a heterozygote deficit or excess at either *Pgm* or *Gpi*.

Fourteen other populations contained allelic arrays typical of *H. gibberum* but possessed two or more genotypes homozygous for alternate alleles at *sMdh, sAat,* or both. These populations were restricted to the arctic, and heterozygotes were never detected within them. Four genotypes were identified, with varying proportions of each genotype among populations. The significance of these particular results is discussed in detail in Hebert *et al.* (2007); in brief, the combined mtDNA and allozyme evidence indicates a shift in breeding system

TABLE 2. Genetic distances within and among *Holopedium* species. Nei's (1972) genetic distances are used for allozymes (with variances given in parentheses), while Kimura's (1980) 2-parameter (K2P) model was used for COI sequence divergences (with standard errors for mean distances provided in parentheses). *H. amazonicum* specimens were not available for allozyme analysis. The highlighted areas compare maximum within-species distances with the range of average interspecific divergences observed.

	Within species		**Between species average distance**			
	Average distance	Maximum distance	*H. glacialis*	*H. gibberum*	*H. atlanticum*	*H. acidophilum*
Allozymes (Nei)				Summary:	Within species max.:	0.57
H. glacialis	0.11 (0.01)	0.570	--		Between species ave.:	0.36-1.54
H. gibberum	0.14 (0.01)	0.336	0.36 (0.03)	--		
H. atlanticum	0.05 (0.01)	0.154	1.39 (0.08)	1.53 (0.11)	--	
H. acidophilum	0.19 (0.01)	0.328	1.19 (0.07)	1.54 (0.03)	0.74 (0.01)	--
mtDNA – Nucleotides (K2P in %)				Summary:	Within species max.:	6.0 %
H. glacialis	0.90 (0.15)	4.28	--		Between species ave.:	8.7-24.5 %
H. gibberum	0.40 (0.12)	1.92	13.08 (1.58)	--		
H. atlanticum	1.66 (0.32)	4.79	23.04 (2.27)	22.94 (2.10)	--	
H. acidophilum	3.20 (0.56)	5.97	24.54 (2.32)	21.43 (2.25)	10.54 (1.19)	--
H. amazonicum	*	*	24.07 (2.44)	22.64 (2.30)	12.32 (1.54)	8.75 (1.28)

* Only a single sequence of *H. amazonicum* was available.

TABLE 3. Allele frequencies at seven loci for the four species of *Holopedium* surveyed for allozyme variation. Relative mobility values (R_f values) for all alleles are given in relation to a reference population (see Methods). Allele frequencies are based upon average frequency across all populations of each species (rather than based upon the total number of individuals, which would be more sensitive to differential sample sizes among sites). Asterisks indicate alleles unique to each species. The number of populations, followed by the total number of individuals, analyzed is indicated in parentheses following each species name. The dominant allele for each locus for each species is highlighted in bold. Allele frequencies for each population are available in Rowe (2000) or directly from CLR.

Locus and R_f value of each allele	*H. glacialis* (N = 90; 2686)	*H. gibberum* (N = 52; 1184)	*H. atlanticum* (N = 22; 271)	*H. acidophilum* (N = 6; 142)
Pgm				
85	0.001	--	**1.00**	--
90	--	--	--	**1.00***
92 [1]	0.203*	--	--	--
96	0.069*	--	--	--
100	**0.715**	**1.00**	--	--
103	0.0003*	--	--	--
116	0.011*	--	--	--
Gpi				
83	0.0044*	--	--	--
89	0.0003*	--	--	--
97	0.011	--	--	0.333
100	**0.878**	--	0.049	0.132
103	0.001*	--	--	--
110 [2]	0.0751*	--	--	--
112	--	--	**0.951***	--
114	--	**1.00**	--	**0.534**
119	0.030*	--	--	--
Mpi				
91	0.0005*	--	--	--
96	0.012	--	**0.994**	--
100	**0.864**	**1.00**	0.006	**1.00**
106	0.123*	--	--	--
Apk				
78	0.00016*	--	--	--
92	--	0.0002*	--	--
100	**0.9998**	**0.9998**	--	0.008
105	--	--	**1.00**	**0.992**
sMdh				
75	--	0.019*	--	--
85	0.00014	0.373	--	--
100	**0.9999**	**0.607**	--	--
113	--	--	**1.00**	**1.00**

mMdh				
75	--	0.019*	--	--
100	**1.00**	**0.981**	**1.00**	**1.00**
sAat				
91	--	--	0.267	**0.994**
93 [3]	0.013*	--	--	--
100	**0.983**	**0.825**	**0.733**	0.006
107	--	0.175*	--	--
109	0.0037*	--	--	--

* alleles unique to each species.

[1-3] Alleles within 2% mobility could not be reliably distinguished within members of the *H. gibberum* group (i.e. *H. gibberum* s.s. and *H. glacialis*). Therefore, three alleles detected in Hebert & Finston (1997) have been equated with those superscripted 1-3: (1) *Pgm 90* from their paper has been combined with *Pgm 92* here; (2) *Gpi 112* has been combined with *Gpi 110* here; and (3) their *sAat 91* has been combined with our *sAat 93*.

either to selfing or automictic parthenogenesis. The corresponding haplotypes of each genotype were very similar or identical, and all were within the *H. gibberum* clade (although not all isolates were sequenced). A single temperate-zone population of *H. gibberum*, Mayer BC, was also in H-W disequilibrium due to a single individual homozygous for an alternate allele at *sAat*. Only one population of *H. acidophilum* (Table 1) was in H-W disequilibrium, involving a lack of heterozygotes at *Apk* and *sAat*. Three populations of *H. atlanticum* were in H-W disequilibrium. Of these, James NC and Santeetlah NC had only heterozygotes at *Gpi*. The H-W deviation in the Flying Pond ME population resulted from a single individual homozygous for an alternate allele at *sAat*.

Geographic genetic structure

The extent of variance in gene frequencies for each species was calculated for all populations, using a three-level hierarchy, increasing from the population level, to the biogeographic province, to all of North America. Two-species assemblages were divided into their component species for this analysis. There was substantial variation in the incidence of H-W disturbances among individuals within local populations, with F_{IP} (=F_{IS}) values ranging from 0.21– 0.99 (Table 5). The North American populations of *H. gibberum* were distinguished by a very high F_{IP} (0.99), suggesting that populations of this species consist of inbred lines (see Hebert *et al.* 2007). Two other species (*H. glacialis* and *H. acidiphilum*) displayed lesser, but still significant, evidence of inbreeding.

More overall genetic sub-structuring was due to allozymic differences among populations within biogeographic provinces (F_{PB} values 0.69–0.89) than among provinces within all of North America (F_{BT} values 0.22–0.59), signifying marked local but limited regional genetic differentiation in all taxa. However, these values were only occasionally significantly different from zero (see Table 5).

Intraspecific allelic patterning

Pgm, *Gpi*, and *Mpi* were monomorphic in all populations of *H. gibberum*. The four arctic inbred lines can be differentiated by fixed differences at the *sMdh* and *sAat* loci (see Hebert *et al.* 2007). The *sMdh 85* allele was dominant in the eastern arctic, but was also present in a few western arctic populations. The *sAat 107* allele was restricted to the western arctic.

H. glacialis showed higher allelic diversity than any other species, but it was also the species most represented in the present study. The same allele was dominant at each locus across its North American range, excepting a few populations in Oregon and Washington, which were fixed for novel alleles at *Pgm* and *Gpi*.

Due to the method of preservation, allozyme analysis was not performed on any *H. amazonicum* populations.

TABLE 4. Summary of allozyme statistics for four *Holopedium* species, arranged by biogeographic province. All populations, including those found to be in H-W disequilibrium and those with sample sizes of fewer than five individuals are included. Two-species assemblages are separated into their component species. Abbreviations are: Pops- the number of populations sampled; n- the average number of individuals for which data were obtained across all loci; P- the average proportion of polymorphic loci; A- the mean number of alleles per locus; Ap- the mean number of alleles per polymorphic locus; He- the overall expected heterozygosity; Ho- the overall observed heterozygosity; and f- the overall inbreeding coefficient or fixation index.

Species, Biogeogr. Prov.	Pops	n	P	A	Ap	He	Ho	f
H. glacialis								
Hudson Bay	15	25.05	0.10	1.19	2.61	0.04	0.02	0.33
Great Lakes	33	28.44	0.21	1.30	2.07	0.08	0.07	0.07
Cascadia	8	30.26	0.01	1.05	--	0.00	0.00	0.00
California	5	12.17	0.11	1.11	2.00	0.04	0.00	0.60
Colorado	2	39.14	0.00	1.00	--	0.00	0.00	0.00
Mississippi	20	36.98	0.09	1.13	2.00	0.03	0.02	0.10
N. Appalachian	7	10.86	0.02	1.02	2.00	0.00	0.00	0.00
H. gibberum								
Arctic Archipelago	11	25.61	0.00	1.00	--	0.00	0.00	0.00
Yukon-MacKenzie	33	19.82	0.07	1.08	2.00	0.03	0.00	0.36
Hudson Bay	5	21.54	0.06	1.06	2.00	0.02	0.00	0.40
Cascadia	3	20.33	0.05	1.10	2.00	0.01	0.00	0.29
H. atlanticum								
N. Appalachian	15	10.86	0.01	1.01	2.00	0.00	0.00	0.07
C. Appalachian	4	14.25	0.21	1.21	2.00	0.09	0.12	-0.36
Southeastern	3	15.24	0.00	1.00	--	0.00	0.00	0.00
H. acidophilum								
Cascadia	3	23.75	0.07	1.10	2.00	0.02	0.01	0.33
N. Appalachian	3	19.00	0.00	1.00	--	0.00	0.00	0.00

Populations of *H. acidophilum* showed considerable variation in allozyme allele frequencies and surprisingly high divergence in mtDNA haplotypes, reflecting the fragmented range of this species. The *Gpi* locus was, for example, dominated by different alleles on the east and west coasts. The eastern populations were genetically invariant, while the central population had a novel allele at *Gpi*. One population of *H. acidophilum* was in H-W disequilibrium. A lack of heterozygotes at *Apk* and *sAat* resulted in disequilibrium in Saunders Pond OR, but COI haplotypes of these homozygous genotypes showed only 0.1% divergence.

Populations of *H. atlanticum* showed little variation at most loci, except for *sAat*, which showed a marked north-south shift from *sAat 100* to *sAat 91*, a pattern first recognized by Hebert & Finston (1997). Three populations of this species exhibited H-W disequilibrium. Flying Pond ME had no heterozygotes at *sAat*, while two North Carolinian populations (James and Santeetlah) had only heterozygotes at *Gpi*. These populations are likely not obligately parthenogenetic due to the presence homozygotes at other loci.

Mitochondrial DNA analysis

COI sequences were obtained for 118 *Holopedium* specimens from 77 sites. A single individual was analyzed from 58 of these sites, but multiple individuals were sequenced from 19 other habitats to determine the extent of intra-population haplotypic diversity. Sequence alignments and amino acid translations were unambiguous, as there were no gaps or nonsense codons. The length of the alignment was 614 bp, of which 209 sites were variable within the ingroup. Fifty unique haplotypes were detected, with the localities for each provided in Appendix A.

Patterns of COI divergence

The NJ phenogram (Fig. 3), which was based upon a sample of the most divergent haplotypes within each major lineage and employed complete deletion of missing sites (resulting in a 544 bp alignment), showed patterns similar to the allozyme results, indicating that all haplotypes belonged to two major groups. The geographic distribution of populations within the two major groups also corresponded to the presumed distributions of the two initially described species of *Holopedium*. However, there was evidence that each group itself included genetically divergent lineages, supported by high bootstrap values. The *H. gibberum* group included two main clusters, while the *H. amazonicum* group was split into three clusters. The additional cluster identified by mtDNA analysis did not have corresponding allozyme data (frozen South American specimens were not available). We suggest that these five clusters correspond to species, whose genetically confirmed localities, as well as hypothesized overall distributions, are shown in Fig. 4. Selecting different sequences from each cluster for the NJ analysis produced the same result, and the pruned datasets also agreed with the phenogram based on all available sequences (not shown here as intraspecific phylogeography will be addressed elsewhere).

Analyzing K2P genetic distances among all haplotypes indicated that levels of genetic divergence within populations were low (< 2 %). Divergences within the major clusters were also modest, with the average distances among sequences ranging from 0.9–3.2% (Table 2). Maximum value within the five main clusters ranged from a low of 1.9% in *H. gibberum* s.s. up to a high of 6.0% in *H. acidophilum* n.sp. (Table 2). By contrast, average divergences among these clusters were higher and did not overlap with the within-cluster results, ranging from 8.7 to 24.5% s (Table 2).

Diversity and distributions of haplotypes within species

Twelve COI haplotypes were detected in *H. gibberum*. Haplotype 2-1 was restricted to populations in the eastern arctic, and the only other haplotype (2–4) from this area differed from it by two transversions. Haplotype 2–2 was the dominant haplotype in the western arctic, but several others were present in this region (2–3, 2–4, and 2–7 to 2–11). There was no association between mitochondrial haplotypes and allozyme genotypes for those arctic individuals having joint determination of genetic data (Hebert *et al.* 2007). European populations consisted of closely-related COI haplotypes in Norway and the Czech Republic (2–5 and 2–6, respectively), while a slightly-divergent haplotype (2–12) was detected in Poland.

Twenty-five COI haplotypes were found in *H. glacialis*. Sixteen of these (1–1 to 1–6, 1–10 to 1–17, 1–20, 1–21) were widespread, ranging from Ontario to the Northwest Territories, and south to California. However three haplotypes (1–7 to 1–9) were only found in west-central regions of Canada, and two others (1–18, 1–19) were restricted to the arctic. Another group of haplotypes (1–22 to 1–25) was restricted to the Pacific Northwest.

Eighteen individuals from nine populations of *H. glacialis* having corresponding genotype and haplotype data were examined for association between these markers. However, the small sample size precluded statistical tests, as only a single sequence was available for 8 of the 11 multilocus allozyme genotypes detected. Nevertheless, there did not appear to be any clear relationship between nuclear genotype and mitochondrial

haplotype. For example, individuals with 6 different allozyme genotypes possessed haplotype 1–2, while a different genotype with a larger sample size of individuals (n = 5) exhibited four different COI haplotypes.

No COI haplotype variation was detected in *H. amazonicum*, but sampling was limited. Three individuals from a ria lake on the Amazon River possessed the same haplotype (5–1).

The six haplotypes of *H. acidophilum* clustered into two groups, one including the eastern and central populations (4–1 and 4–2), and the other consisting of western populations (4–3 to 4–6).

The six COI haplotypes of *H. atlanticum* fell into two groups, a southeastern (3–1 to 3–3) and a northeastern (3–4 to 3–6).

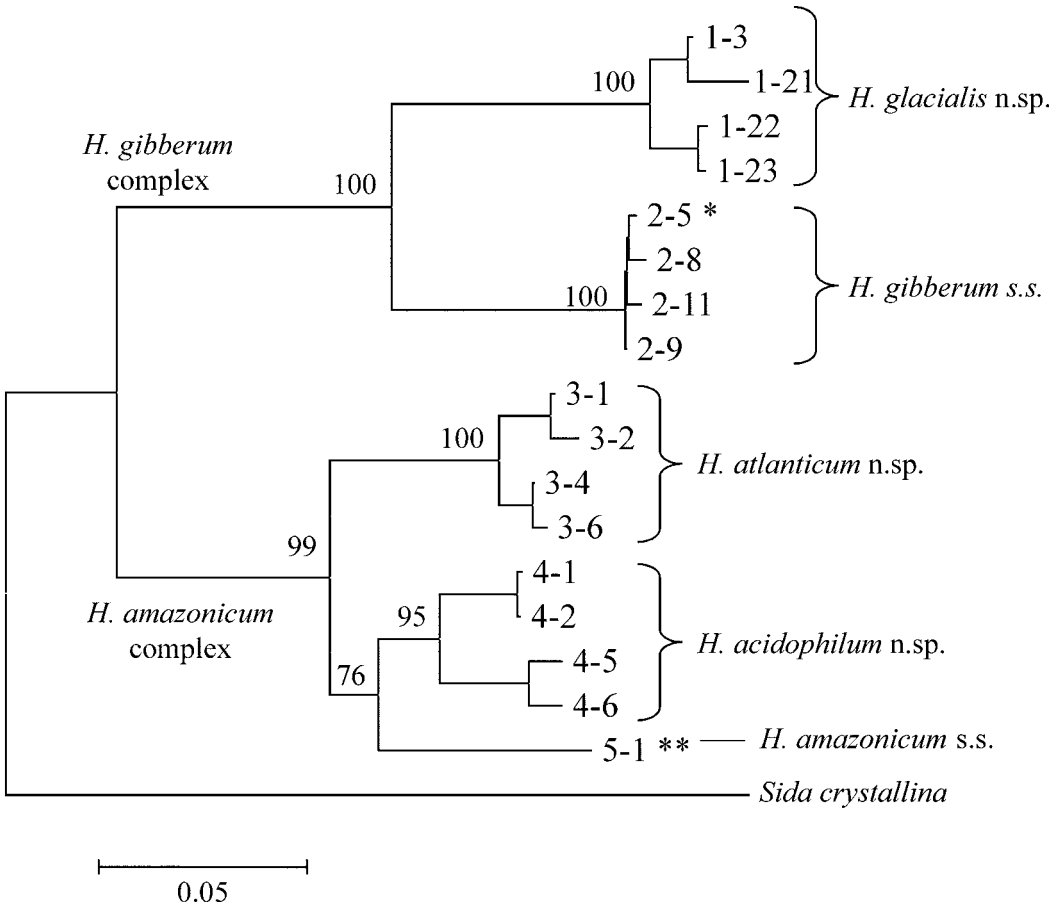


FIGURE 3. NJ phenogram of *Holopedium* based upon 17 COI haplotypes, rooted using *Sida crystallina*. Boostrap support values (based upon 10,000 replicates) are shown for major clusters and deeper nodes, and the scale bar shows K2P genetic distance. Terminal branch labels indicate the clade number, followed by the haplotype number. Haplotypes of reference populations collected from relatively near the type localities of *H. gibberum* and *H. amazonicum* are indicated with one and two asterisks, respectively. The two clusters containing these haplotypes are considered to represent these species *sensu strictu*, while the remaining clusters are described here as new species.

Morphology

Morphometrics

Table 6 summarizes the morphological data for 15 populations of *Holopedium*. There was substantial variation in the 8 morphological traits examined. For four of the characters, the majority of variation occurred between species, rather than among populations within species or among individuals within populations (Table 7). These traits were the numbers of anal spines on both sides of the postabdomen, the number of denticles on the post-abdominal claw, and the number of basal spines on the post-abdominal claw. The variation in the last of these traits was overwhelmingly at the species level (97.4%). Thus, these traits may be especially informative for differentiating among genetically identified species.

TABLE 5. Hierarchical F-statistics with variances given in parentheses for the four species of *Holopedium* included in allozyme analyses. Each species was separated out for those assemblages found to consist of two species. F-statistics describe the reduction in heterozygosity of one hierarchical level relative to another. F_{IP} (=F_{IS}) describes inbreeding in individuals within sub-populations; F_{IT} characterizes inbreeding among individuals relative to the total population; F_{PB} concerns inbreeding among sub-populations within a biogeographic province; and F_{BT}(=F_{ST}) concerns inbreeding among sub-populations within a biogeographic province, with the total being all North American conspecific populations. Dashes indicate conspecific populations which were fixed at a particular locus. Biogeographic provinces are defined in Fig. 1. F-statistics that differ significantly from zero are marked with an asterisk (as indicated by a 95% confidence interval that did not include zero).

Species, n	F_{IP}	F_{IT}	F_{PB}	F_{BT}
H. glacialis, n = 90 pops				
Pgm	0.16 (0.14)	0.74 (0.03)	0.69 (0.23)	0.27 (0.14)
Gpi	0.44 (0.03)	0.87 (0.03)	0.78 (0.14)	0.24 (0.06)
Mpi	0.08 (0.09)	0.63 (0.01)	0.60 (0.12)	0.11 (0.03)
Apk	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
sMdh	0.01 (0)	0.00 (0)	-0.01 (0)	0.00 (0)
mMdh	--	--	--	--
sAat	0.56 (0.01)	0.78 (0.01)	0.49 (0.02)	-0.05 (0)
All	**0.21 (0.27) ***	**0.75 (0.07) ***	**0.69 (0.50)**	**0.22 (0.23) ***
H. gibberum, n = 52 pops				
Pgm	--	--	--	--
Gpi	--	--	--	--
Mpi	--	--	--	--
Apk	0.01 (0)	0.01 (0)	-0.01 (0)	0.01 (0)
sMdh	1.00 (0)	1.00 (0.07)	0.87 (0.20)	0.56 (0.37)
mMdh	--	--	--	--
sAat	1.00 (0)	1.00 (0.07)	0.80 (0.19)	0.24 (0.08)
All	**0.99 (0) ***	**1.00 (0.16) ***	**0.84 (0.39) ***	**0.45 (0.45)**
H. atlanticum, n = 22 pops				
Pgm	--	--	--	--
Gpi	-0.10 (0.17)	0.15 (0.09)	0.57 (0.02)	0.49 (0.10)
Mpi	-0.12 (0.01)	0.00 (0)	0.11 (0)	-0.01 (0)
Apk	--	--	--	--
sMdh	--	--	--	--
mMdh	--	--	--	--
sAat	0.22 (0.02)	0.96 (0.01)	0.95 (0.36)	0.32 (0.19)
All	**-0.63 (0.21)**	**0.74 (0.08)**	**0.84 (0.38)**	**0.36 (0.28) ***
H. acidophilum, n = 6 pops				
Pgm	--	--	--	--
Gpi	0.32 (0.04)	0.96 (0.02)	0.94 (0.28)	0.63 (0.56)
Mpi	--	--	--	--
Apk	1.00 (0)	1.00 (0.03)	0.01 (0)	0.01 (0)
sMdh	--	--	--	--
mMdh	--	--	--	--
sAat	1.00 (0)	1.00 (0.02)	-0.03 (0)	0.01 (0)
All	**0.66 (0.04) ***	**0.96 (0.07) ***	**0.89 (0.28)**	**0.59 (0.56)**

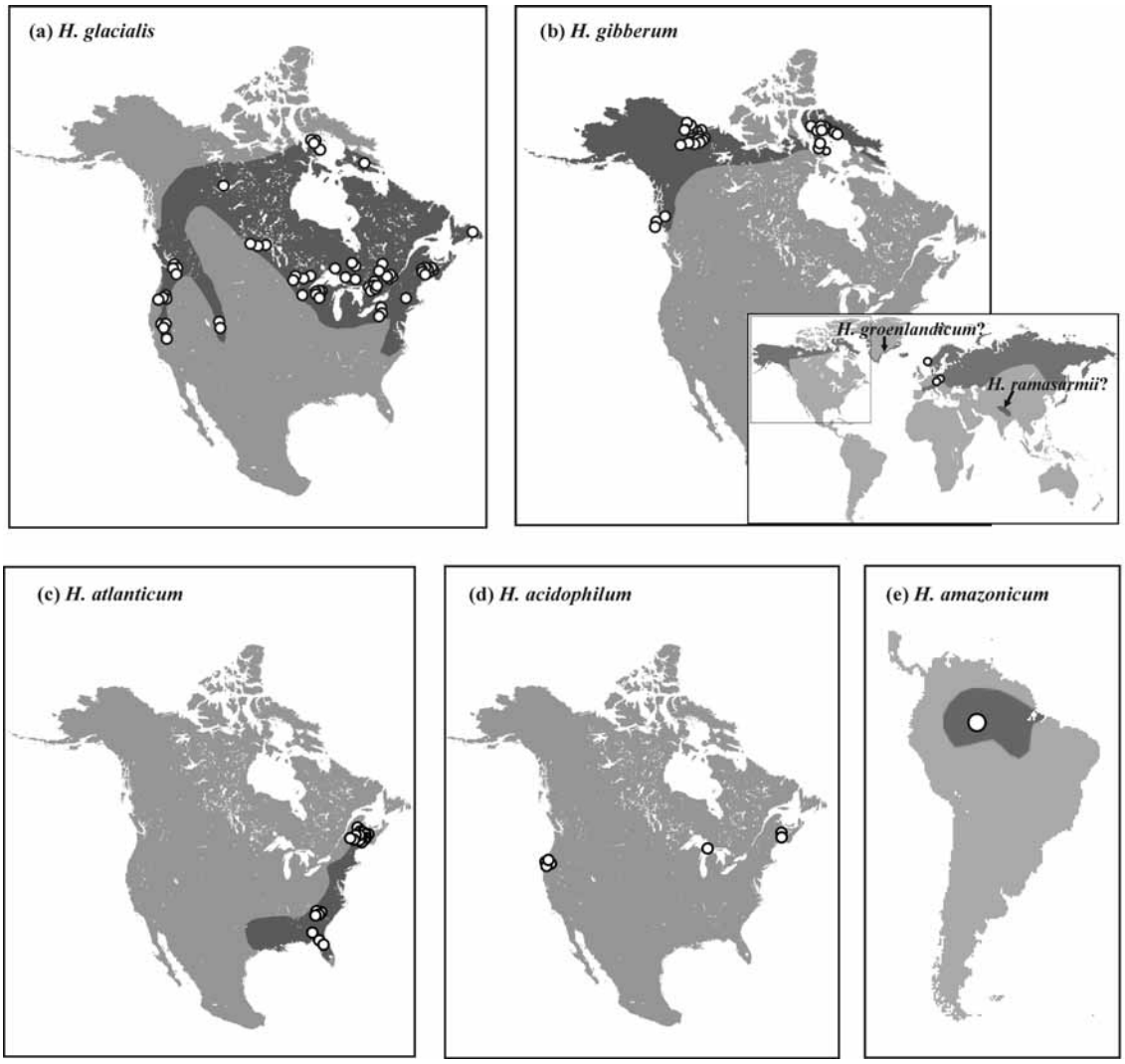


FIGURE 4. Putative geographic distribution of *Holopedium* species. Dots indicate populations where species assignments were confirmed by genetic analyses. Dark shaded areas represent the hypothesized range of each species based on results from this study and from distribution data from 1,827 localities inhabited by *Holopedium*, obtained from literature reports or by sampling (see Rowe 2000). Since several species are morphologically cryptic while the species complexes can be readily distinguished, the areas between or adjacent to genetic localities are tentatively marked as that same species, provided that there are *Holopedium* records there belonging to the same complex. Pending further evidence, all South American localities are here shaded as *H. amazonicum* and most Eurasian localities as *H. gibberum* s.s., but further cryptic species may be detected in the future. Definitive species assignments for populations in Greenland and India, which are currently described as separate species, require genetic evidence (see text), but morphological traits indicate that they do belong to the *H. gibberum* complex. Their distributions are shown along with *H. gibberum* s.s. (inset map in part b). a) *H. glacialis* **n.sp.**, b) *H. gibberum* s.s., c) *H. atlanticum* **n.sp.**, d) *H. acidophilum* **n.sp.**, and e) *H. amazonicum* s.s.

There were significant morphological differences among species for all 8 of the characters examined (Table 8). Although most variability in carapace length occurred among populations (52.6%), there were significant differences at the species level. In general, individuals from northern taxa (*H. glacialis*, *H. gibberum*, and *H. acidophilum*) were significantly larger in size than the taxa with more southerly distributions (*H. amazonicum* and *H. atlanticum*) (Table 8). Two species in the *H. amazonicum* complex, *H. acidophilum* and *H. atlanticum*, were significantly different in size, despite their neighbouring sympatry in New Brunswick. Carapace height followed the same pattern as carapace length. The height/length ratio was significantly greater in *H. glacialis* than in all other species (Table 8).

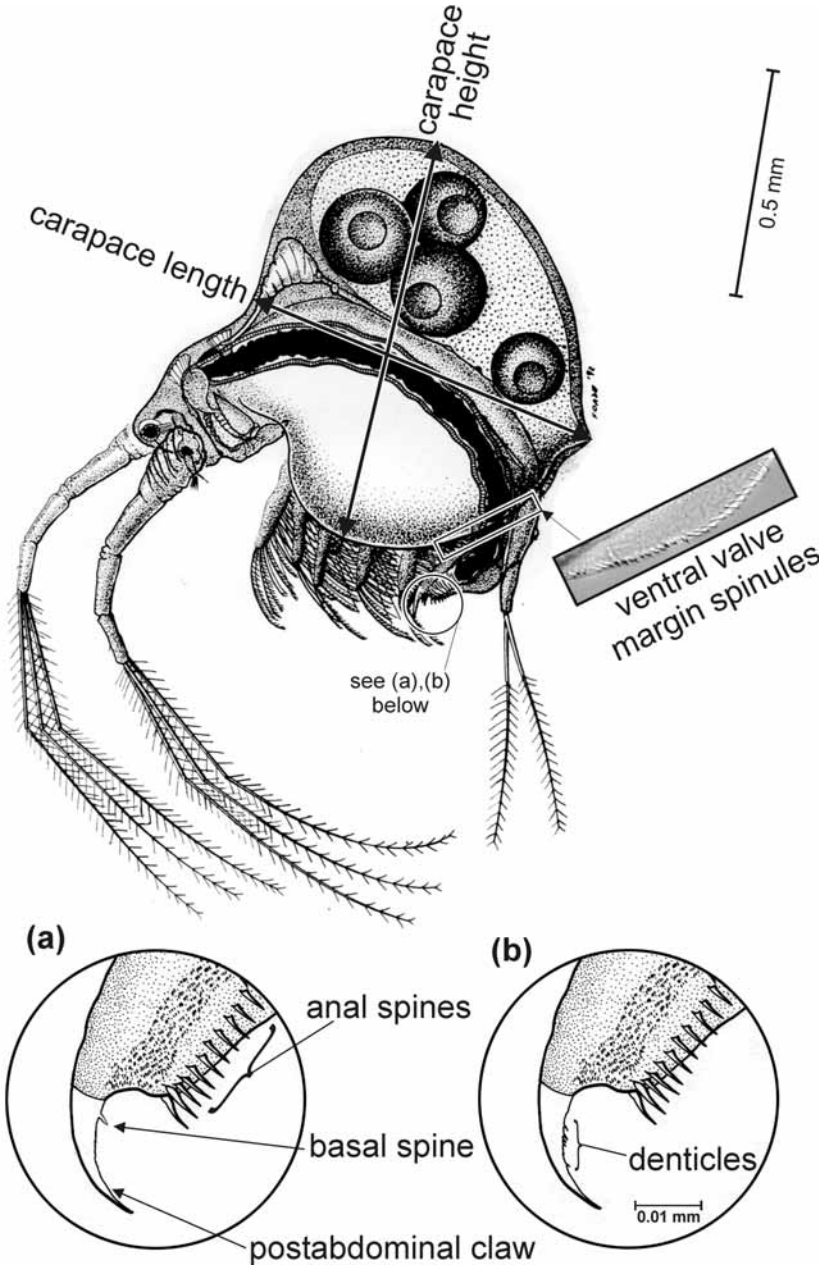


FIGURE 5. Morphological characters used in the discrimination of *Holopedium*. Postabdominal claws (a) with and (b) without a basal spine are illustrated. The jelly coat is not illustrated.

Structural-count characters also revealed differences among species (Tables 6, 8). There was often within-individual bilateral asymmetry in the numbers of anal spines on the sides of the postabdomen, but the count never differed by more than three. Although there was some overlap among species, there were significant species-level differences in the anal spine counts. For the postabdomen side #1, within the *H. gibberum* complex, *H. gibberum* s.s. had significantly more spines than *H. glacialis*, but this difference was due to the higher number of anal spines in the sole European population of *H. gibberum* (Table 6). In the *H. amazonicum* complex, *H. acidophilum* possessed more anal spines than either *H. amazonicum* or *H. atlanticum*. For the postabdomen side #2, there were only data for members of the *H. amazonicum* complex (due to preservation problems for some samples of the *H. gibberum* group); again, *H. acidophilum* possessed significantly more spines than either of the other species within this group.

TABLE 6. Summary of morphological data collected for female specimens from five species of *Holopedium*. Column values are means of trait values except for ventral carapace margin spinules and denticles on postabdominal claw, which are given as a proportion of individuals possessing the character (presence/ absence characters). The variance is shown in parentheses, and missing data are indicated with dashes.

Species and Locality (n)	Carapace length (mm)	Carapace height (mm)	Carapace (H/L) ratio	No. anal spines (post-abdomen side #1)	No. anal spines (post-abdomen side #2)	Ventral carapace margin spinules	Basal spines on post-abdominal claw	Denticles on post-abdominal claw
H. glacialis								
Great Slave, NT (8)	0.97 (0.02)	1.18 (0.06)	1.25 (0.08)	17.67 (4.33)	- (-)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
Wren, ON (10)	0.97 (0.02)	1.14 (0.04)	1.18 (0.02)	15.00 (3.56)	- (-)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
Rest, WI (10)	1.00 (0.03)	1.43 (0.04)	1.45 (0.06)	14.22 (3.19)	- (-)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
All (28)	**0.98 (0.02)**	**1.25 (0.06)**	**1.30 (0.06)**	**15.05 (4.43)**	**- (-)**	**1.00 (0.00)**	**1.00 (0.00)**	**1.00 (0.00)**
H. gibberum								
Mayer, BC (10)	0.78 (0.01)	0.79 (0.02)	1.01 (0.01)	14.25 (3.07)	- (-)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
Inuvik 1, NT (6)	0.96 (0.02)	1.16 (0.07)	1.21 (0.02)	17.00 (6.50)	- (-)	0.80 (0.20)	1.00 (0.00)	1.00 (0.00)
Bergen, Norway (10)	1.60 (0.09)	1.80 (0.03)	1.17 (0.07)	29.00 (39.78)	- (-)	1.00 (0.00)	1.10 (0.10)	1.00 (0.00)
All (26)	**1.14 (0.18)**	**1.27 (0.24)**	**1.12 (0.04)**	**21.26 (67.66)**	**- (-)**	**0.96 (0.04)**	**1.04 (0.04)**	**1.00 (0.00)**
H. acidophilum								
Red Rock Pond, NB (12)	1.17 (0.04)	1.20 (0.09)	1.01 (0.03)	15.17 (3.97)	- (-)	1.00 (0.00)	0.00 (0.00)	1.00 (0.00)
Soldier Pond, MI (10)	1.19 (0.03)	1.27 (0.02)	1.08 (0.02)	12.00 (1.20)	13.50 (0.33)	1.00 (0.00)	0.00 (0.00)	1.00 (0.00)
Saunders Lake, OR (10)	0.92 (0.05)	0.75 (0.06)	0.81 (0.04)	14.00 (17.78)	- (-)	0.80 (0.18)	0.00 (0.00)	1.00 (0.00)
All (32)	**1.10 (0.05)**	**1.08 (0.11)**	**0.97 (0.04)**	**14.07 (9.25)**	**13.50 (0.33)**	**0.93 (0.10)**	**0.00 (0.00)**	**1.00 (0.00)**
H. amazonicum								
Coari, Brazil (10)	0.91 (0.05)	0.85 (0.03)	0.96 (0.02)	8.40 (5.38)	7.17 (0.57)	1.00 (0.00)	0.00 (0.00)	0.33 (0.25)
Rio Negro, Brazil (10)	0.58 (0.03)	0.57 (0.04)	1.01 (0.12)	8.33 (1.50)	7.86 (0.48)	1.00 (0.00)	0.00 (0.00)	0.00 (0.00)
All (20)	**0.74 (0.07)**	**0.71 (0.05)**	**0.99 (0.07)**	**8.37 (3.36)**	**7.54 (0.60)**	**1.00 (0.00)**	**0.00 (0.00)**	**0.17 (0.15)**
H. atlanticum								
Dorr, FL (10)	0.68 (0.02)	0.67 (0.03)	0.99 (0.03)	7.10 (1.88)	7.83 (1.37)	0.30 (0.23)	0.00 (0.00)	0.10 (0.10)
Moosehead, ME (10)	0.84 (0.02)	0.86 (0.03)	1.01 (0.01)	9.50 (2.72)	9.17 (2.17)	0.56 (0.28)	0.00 (0.00)	0.40 (0.27)
James, NC (1)	0.96 (0.00)	0.98 (0.00)	1.02 (0.00)	10.00 (0.00)	10.00 (0.00)	1.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Hiwassee, NC (8)	0.64 (0.03)	0.66 (0.05)	1.01 (0.04)	8.25 (1.36)	8.75 (0.92)	0.86 (0.14)	0.00 (0.00)	0.50 (0.30)
All (29)	**0.73 (0.03)**	**0.74 (0.04)**	**1.00 (0.02)**	**8.25 (2.95)**	**8.65 (1.74)**	**0.56 (0.26)**	**0.00 (0.00)**	**0.30 (0.22)**

At least some individuals in every population possessed spinules on their ventral carapace margins, but when present, these spinules were always restricted to the posterior half of the carapace. *H. atlanticum* possessed significantly fewer spinules than all other species (Table 8). However, there was overlap in spinule counts among populations of different species (Table 6). The presence (*H. gibberum* complex) or absence (*H. amazonicum* complex) of one, or rarely two, basal spines on the postabdominal claw reliably differentiated the two species complexes (Tables 6,8; see Fig. 5). All individuals of *H. glacialis*, *H. gibberum*, and *H. acidophilum* had denticulated claws, while some individuals of *H. amazonicum* and *H. atlanticum* lacked denticulation. Thus, *H. amazonicum* and *H. atlanticum* possessed significantly fewer denticulated individuals (Table 8), but species diagnosis could not be made on an individual-level basis.

Discriminant function analysis

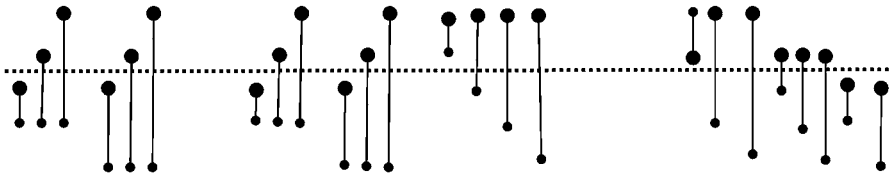
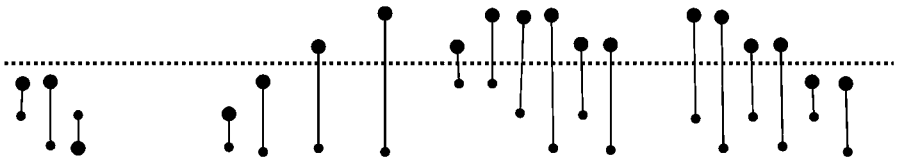
Discriminant function (DF) analysis, followed by post-hoc classification, indicated that a majority of individuals (76%) could be assigned to the correct species on the basis of six morphological characters (the number of anal spines on postabdomen side #2 and denticles on the claw were omitted due to many missing values) (Table 9). The proportion of individuals correctly classified ranged from 59% (for *H. atlanticum*) to 95% (for *H. gibberum* s.s.). Thus, while there were significant morphological differences among all species, there were few diagnostic differences allowing reliable species discrimination among individuals.

The number of basal spines on the post-abdominal claw was the most important character, being most strongly associated with the first DF (Table 9b) and allowing the separation of members of the *H. gibberum* and *H. amazonicum* species groups (Table 6). The presence of at least one basal spine on the claw was an invariable characteristic of the *H. gibberum* complex, while members of the *H. amazonicum* complex never possessed a basal spine. Carapace length was most closely associated with the second DF and assisted in the differentiation of species. The number of anal spines on postabdomen side #1 was another important character (Table 9b), helping to differentiate *H. acidophilum* from *H. amazonicum* and *H. atlanticum* (see also Table 8). There was no overlap for this trait in population means between *H. acidophilum* and these other two species (Table 6).

TABLE 7. Variance components analysis for 8 morphological traits of five species of *Holopedium*. The percentage of total variation occurring among species, among populations within species, and among individuals within populations is given. The dominant level at which variation occurs is highlighted in bold for each character.

Trait	Total sample size	Species (d.f.)	Populations (d.f.)	Individuals (d.f.)
Carapace length	135	15.62 % (4)	**52.63 % (10)**	31.75 % (120)
Carapace height	135	22.08 % (4)	**53.03 % (10)**	24.89 % (120)
Carapace (H/L) ratio	135	24.82 % (4)	10.50 % (10)	**64.69 % (120)**
No. anal spines (post-abdomen side #1)	121	**50.25 % (4)**	32.95 % (10)	16.80 % (106)
No. anal spines (post-abdomen side #2)	34	**80.33 % (2)**	4.22 % (4)	15.46 % (27)
Ventral carapace margin spinules (pres/abs)	124	19.52 % (4)	13.22 % (10)	**67.26 % (109)**
No. basal spines (post-abominal claw)	127	**97.44 % (4)**	0.00 % (10)	2.56 % (112)
Denticles on post-abdominal claw	89	**63.40 % (4)**	4.35 % (8)	32.24 % (76)

TABLE 8. Tests for significant differences in morphological traits among five species of *Holopedium*. Statistics are first presented for ANOVA tests to identify whether there are any significant differences in traits among species. The vertical lines represent the results of Tukey's honestly significant differences tests to identify which pairs of species differ significantly from one another (at a family-wise $\alpha = 0.05$). The sizes of dots on the ends of the vertical lines designate which species has the larger trait value. The dotted horizontal lines separate the two groups of *Holopedium* species (*H. gibberum* group and *H. amazonicum* group).

Species and statistics	**Traits**			
	Carapace length	**Carapace height**	**Ratio (height/ length)**	**No. anal spines (postabdomen side #1)**
ANOVA				
F-statistic (and d.f.)	14.37 $_{4, 130}$	18.78 $_{4, 130}$	11.62 $_{4, 130}$	39.88 $_{4,116}$
R^2	0.3066	0.3662	0.2633	0.579
P-value	< 0.001	< 0.001	< 0.001	< 0.001
Tukey test				
1- *H. glacialis*				
2- *H. gibberum*				
3- *H. acidophilum*				
4- *H. amazonicum*				
5- *H. atlanticum*				
	No. anal spines (post-abdomen side #2)	**Ventral carapace margin spinules (pres/abs)**	**No. basal spines (postabdominal claw)**	**Denticles on post-abdominal claw**
ANOVA				
F-statistic (and d.f.)	46.97 $_{2,31}$	8.777 $_{4,119}$	983.4 $_{4,122}$	33.28 $_{4,84}$
R^2	0.7519	0.2278	0.9699	0.6131
P-value	< 0.001	< 0.001	< 0.001	< 0.001
Tukey test				
1- *H. glacialis*				
2- *H. gibberum*				
3- *H. acidophilum*				
4- *H. amazonicum*				
5- *H. atlanticum*				

Taxon descriptions

Descriptions of the family Holopediidae Sars, 1863 and genus *Holopedium* Zaddach, 1855 are found elsewhere. For the most recent summaries, see Korovchinsky (1992 and 2004). A discussion of the five species included in this study follows, focusing on traits of relevance for species discrimination, and concludes with a note concerning two additional *Holopedium* species.

TABLE 9. Discriminant function analysis using morphological data to distinguish the genetically defined species of *Holopedium*, based upon 113 individuals with complete data for the 6 characters used (see text). A) Post-hoc classification analysis, showing the percentage of individuals assigned to each species. Cases of correct assignment are shown in bold along the diagonal. B) Standardized canonical coefficients for the first two discriminant functions, with the most important characters highlighted in bold.

A)		Predicted species				
Actual species	Sample size	1	2	3	4	5
1- *H. glacialis*	21	**20 (95%)**	1 (5%)	0 (0%)	0 (0%)	0 (0%)
2- *H. gibberum* s.s.	20	7 (35%)	**13 (65%)**	0 (0%)	0 (0%)	0 (0%)
3- *H. acidophilum*	27	0 (0%)	0 (0%)	**22 (81%)**	2 (7%)	3 (11%)
4- *H. amazonicum* s.s.	18	0 (0%)	0 (0%)	1 (6%)	**15 (83%)**	2 (11%)
5- *H. atlanticum*	27	0 (0%)	0 (0%)	1 (4%)	10 (37%)	**16 (59%)**

B)Variable	DF I	DF II
Carapace length	-0.199	**0.902**
Carapace height	0.264	-0.673
Carapace H/L ratio	-0.024	-0.008
Number of anal spines (post-abdomen side #1)	-0.022	0.704
Ventral carapace margin spinules	0.016	0.271
Number of basal spines on postabdominal claw	**0.992**	-0.115

Holopedium gibberum Zaddach, 1855

Historical literature descriptions.

Animals described as *H. gibberum* from the temperate zone of North America are properly assigned to *H. glacialis* **n. sp.** (see below), but the following records represent *H. gibberum* s.s.:

Zaddach (1855): 159–187, Table VIII, Figs. 1–7, Table IX, Figs. 8–19
Eurén (1861): 118, Table III, Fig. 3
Sars (1865): 57–67, Table IV, Figs. 1–19
Müller (1868): 103
Hellich (1877): 19
Beck (1883): 778, Plate XI
Richard (1895): 384, Fig.2; 383, Plate XVI, Fig. 15
Sars (1890): 31 var. *ornata* (properly *H. gibberum* s.s.)
Lilljeborg (1901): 59–64, Table VI, Figs. 5–10, Table VII, Figs. 1–8
Stingelin (1904*a*): 54–64, Table 1, Figs. 3–4
Stingelin (1904*b*): 577–578, Table 20, Figs. 1–2
Uéno (1926): 274–275, Plates XXI, Fig. 3, Plate XXII, Figs. 3a–e
Pennak (1953): 364–365, Fig. 227c
Pennak (1978): 365–366, Fig. 254c
Pennak (1989): 386–387, Fig. 12c
Korovchinsky (1992): 74–78, Figs. 363–370
Korovchinsky (2004): 345–347, Figs. 139–140

Etymology. *gibberum*—from Greek for undivided rudder, referring to the female's uniramous swimming antennae.

Type locality. A small pond near Königsberg (now Kaliningrad), Russia (approx. 54.72º N 20.62º E). Zaddach (1855) did not specify the exact location of this pond.

Type specimens. Nothing is known about Zaddach's type material despite efforts to find it by contacting present-day researchers in Kaliningrad (Korovchinsky, pers. comm. to CLR). A neotype was not designated in this study due to the inability to collect new material in this vicinity.

Voucher specimens. Five mature females from Inuvik1, NT, Canada (collection date August 19, 1997; 68.127°„ N, 132.430°„ W) deposited in the Canadian Museum of Nature (CMN) under accession number CMNC 2007-0743, as well as twenty-five mature females from Bergen, Norway (collected by A. Hobaek on June 15 1995) deposited under accession number CMNC 2007-0744.

Material examined. Habitats harbouring *H. gibberum* are listed in Appendix A.

Morphological description. FEMALE. Fig. 6 provides representative photomicrographs of *H. gibberum*. The jelly coat is of the Z type, in which the anterior jelly curl is relatively straight and ends in a short curve posteriorly toward the carapace and the lateral lobes are divided (see Montvilo *et al.* 1987).

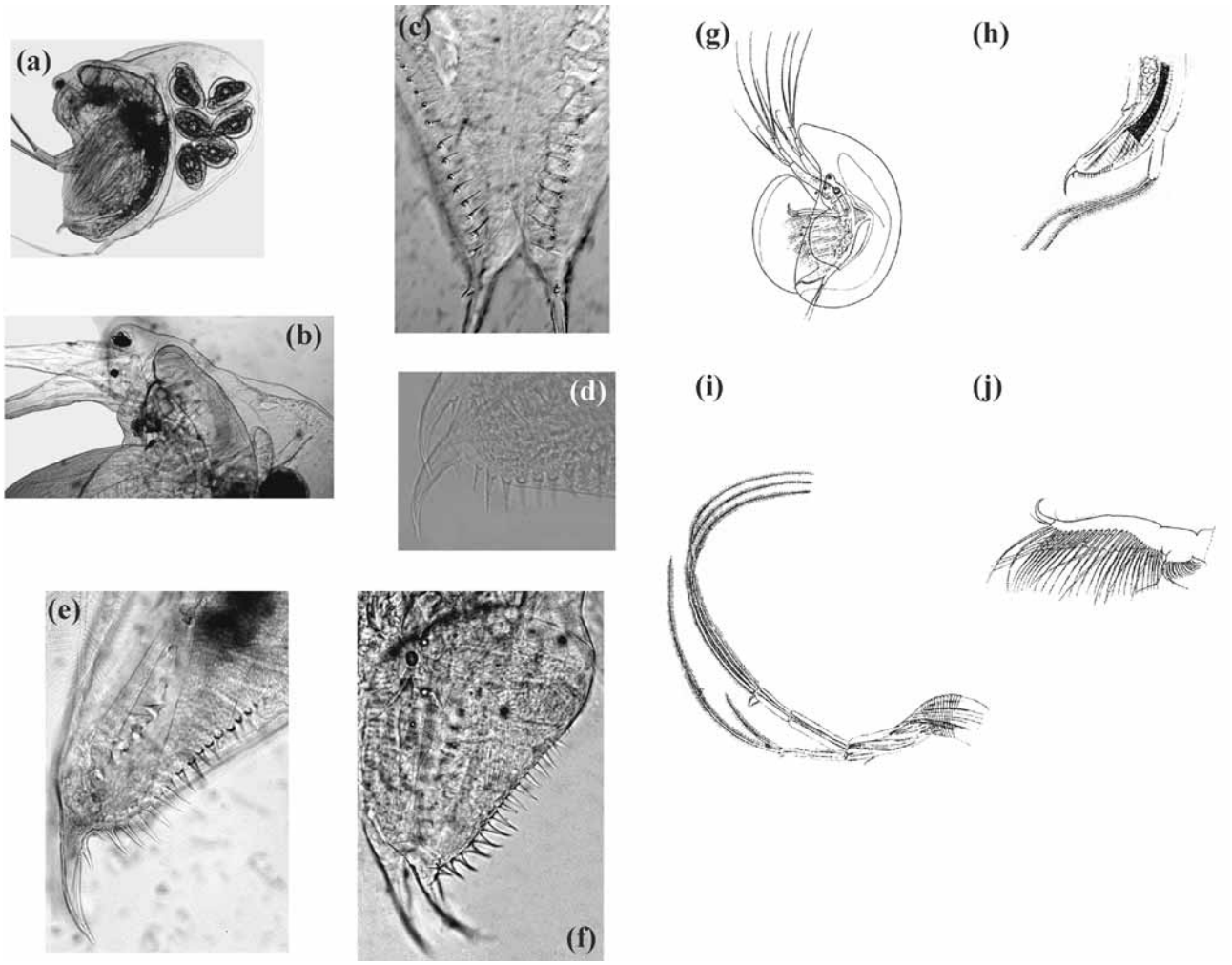


FIGURE 6. Representative photomicrographs and drawings of *Holopedium gibberum*. (a) Lateral view of female with jelly coat removed. Old Crow 10, Yukon, August 15, 1997. (b) Lateral view of female head and anterior jelly curl. Jelly coat stained with fuschian red. (c) Lateral view of female postabdomen. Longstaff Bluff 1, Nunavut, August 16, 1994. (d) Lateral view of female postabdominal claws. Mayer Lake, British Columbia, August 7, 1997. (e) Ventral view of female postabdomen. (f) Lateral view of female postabdomen. (g) Drawing of lateral view of male in jelly coat. (h) Drawing of lateral view of male postabdomen. (i) Drawing of lateral view of biramous antennae of male. (j) Drawing of lateral view of first thoracic limb of a male. (b,e,f) from Steensby 3, Nunavut, August 13, 1994. (g,j) from Lilljeborg 1901. (h,i) from Sars 1865.

Adult female carapace lengths range from 0.53–1.94 mm (mean 1.14 mm), while carapace heights range from 0.50–2.11 mm (mean 1.23 mm). The H/L ratios range from 0.88–1.92 (mean 1.12). The ventral carapace margins have many, closely-spaced spinules posteriorly, but are ordinarily smooth anteriorly. Individuals lacking spinulation along the entire ventral valve margin were encountered.

Anal spine number is variable, ranging from 11–42 (mean 21.3). *Holopedium gibberum* typically possesses at least one basal spine on each postabdominal claw, although there may be up to three, all closely spaced. These spines may be very small, and individual spines may have several points. Each claw has a row of denticles running laterally from the base of the claw to its midpoint.

MALE. Males of *H. gibberum* were described by Sars (1865) and Lilljeborg (1901), and their illustrations are reproduced in Figure 6. Males are common in the spring and autumn in the temperate zone, but are apparently absent from arctic habitats, as evidence suggests these populations produce resting eggs by selfing or automictic parthenogenesis (Hebert *et al.* 2007). The jelly coat is present but microsculpturing has not been determined. Mature males are typically smaller than adult females, ranging from 0.50–1.50 mm in length (Korovchinsky 1992). Body height is much reduced compared to the female as the dorsal carapace is not extended to accommodate a brood cavity.

Differential diagnosis. Although *H. gibberum* appears to be morphologically indistinguishable from *H. glacialis*, the two species have largely allopatric distributions (Fig. 4a,b). Within North America, these species only occur sympatrically in a narrow zone in the arctic. Where they co-occur, there was no evidence of hybridization. *H. gibberum* can be distinguished from all members of the *H. amazonicum* species complex by its possession of at least one basal spine on each postabdominal claw. North American *H. gibberum* can be biochemically distinguished from *H. glacialis* at the *Gpi* locus. *H. glacialis* was never found to possess *Gpi 114*, while *H. gibberum* was monomorphic for this allele. COI mtDNA sequence divergence between *H. gibberum* and *H. glacialis* averages 13.1%. Based on current evidence, individuals showing less than 2% divergence from a representative COI mtDNA sequence (GenBank AF 245354) belong to *H. gibberum*. However, greater divergences may be found in the future, because other *Holopedium* species display higher levels of intraspecific divergence (Table 2).

Distribution. *H. gibberum* appears to be the most widely distributed species in the genus (Fig. 4b). It was found in Poland, the Czech Republic, and Norway, locations that surround the type locality near Kaliningrad, Russia. Zaddach (1855) did not specify the exact location of the type locality. *Holopedium gibberum* also dominates arctic lakes in North America, north of 67° N, but is absent from much of the high arctic archipelago, excepting Baffin Island. However, populations occur as far south as 53° N in the Coastal Mountain Range of British Columbia. This species occurs on the Queen Charlotte Islands, likely reflecting lineages which persisted in a coastal refugium during the Pleistocene.

There are reports of *H. gibberum* from regions of Europe, Asia, and India which were not sampled in this study. Until critical evaluations are undertaken, it is reasonable to continue to assign these lineages to *H. gibberum*. Populations in the Himalayas and Japan merit particular attention due to their geographic isolation from populations which have been studied.

The low mtDNA divergence between North American and European populations of *H. gibberum* suggests a recent (i.e. Pleistocene) exchange of propagules. Such dispersal could perhaps have been influenced by ice floes, as suggested by Weider *et al.* (1999) for *Daphnia*, but greater sampling in Europe would be required to draw conclusions about directionality in *Holopedium*.

Breeding system. Populations of *H. gibberum* from the Canadian arctic possess a breeding system unlike congeneric taxa in the temperate zone. Because all populations from this region consist of one to four allozyme genotypes homozygous for alternate alleles, Hebert *et al.* (2007) concluded hat these lineages have made the transition to either automictic parthenogenesis or self-fertilization, the first record of such a breeding system in the Cladocera.

Temperate-zone populations of *H. gibberum* appear to reproduce via the typical cladoceran mode, cyclic parthenogenesis. Three *H. gibberum* s.s. populations were detected in western British Columbia. Heterozygotes were detected at *Apk* in Mayer BC. However, the population from Mosquito BC was represented by a single individual that was homozygous for a different allele at *sMdh* compared to the arctic populations. Genotypic diversity in Prudhomme BC could not be determined due to poor gel resolution. Nevertheless, the presence of heterozygotes in Mayer BC indicates that populations of *H. gibberum* in the temperate zone likely reproduce by cyclic parthenogenesis, in contrast to automixis employed by their polar counterparts. Males were not detected at these sites (due to the time of year of sampling).

Holopedium glacialis n. sp.

Synonymy. All previous descriptions of *H. gibberum* from temperate North America are properly assigned to *H. glacialis*.

Forbes (1882): 641–642, Plate IX, Figs. 12–15
Herrick (1884): 22–23, Plate N, Fig. 11
Birge (1918): 693, Figs. 1060, 1061a
Brooks (1959): 603, Figs. 27.12a,b
Pennak (1953): 364–365, Figs. 227a,b
Pennak (1978): 365–366, Figs. 254a,b
Pennak (1989): 386–387, Figs. 12a,b
Dodson & Frey (1991): 746–747, Fig. 20.7

Etymology. *glacialis* refers to the near restriction of this species to regions of North America that were glaciated during the Pleistocene.

Type locality. Wren Lake, Ontario (45.183º N, 78.866º W). This lake is situated near Carnarvon, Ontario, approximately 3 km past the Leslie M. Frost Natural Resources Centre on Hwy 35N.

Type specimens. Holotype: an ovigerous female in ethanol deposited in the CMN under accession number CMNC 2007-0745 (collection date Sept. 29th 2007).

Paratypes: Twenty ovigerous females, also from Wren Lake, Ontario, preserved in ethanol, deposited in the CMN under accession number CMNC 2007-0746(collection date Sept. 29th 2007).

Material examined. Other habitats with *H. glacialis* are listed in Appendix A.

Morphological description. FEMALE. Representative photomicrographs are shown in Figure 7. The jelly coat is of the Z type, in which the anterior jelly curl is relatively straight and ends in a short curve posteriorly toward the carapace, and the lateral lobes are divided (see Montvilo *et al.* 1987).

Adult female carapace lengths range from 0.68–1.30 mm (mean 0.98 mm), while carapace heights range from 0.75–1.82 mm (mean 1.26 mm). The H/L ratios range from 0.61–1.95 (mean 1.30). The ventral carapace margins usually have many, tightly-spaced spinules posteriorly, but are often smooth anteriorly.

Anal spine number is less variable than in *H. gibberum*, ranging from 11–20 (mean 15.1). *H. glacialis* typically possesses at least one basal spine on each postabdominal claw. Each claw has a row of denticles running laterally from the base of the claw to its midpoint.

MALE. Males were encountered in autumn collections. The jelly coat is present and resembles that of the female of this species. Mature males are often half the size of adult females, with individuals ranging from 0.40–0.63 mm in length (CLR, pers. obs.). The ventral carapace margin is spinulated posteriorly, but is smooth anteriorly.

The postabdomen is long and terminates posteriorly with two postabdominal claws. A single row of anal spines runs ventro-laterally on each side of the postabdomen (range 13–17). There is one basal spine on each postabdominal claw, and each claw invariably has a row of denticles running laterally from the base of the claw to its midpoint.

Differential diagnosis. *Holopedium glacialis* can be distinguished from all members of the *H. amazonicum* species complex by its possession of at least one basal spine on each postabdominal claw. It is morphologically indistinguishable from *H. gibberum*, although they have largely allopatric distributions (Fig. 4a,b). *Holopedium glacialis* can be biochemically distinguished from North American *H. gibberum* at the *Gpi* locus. *Holopedium glacialis* was never found to possess *Gpi 114*, while *H. gibberum* was monomorphic for this allele. COI mtDNA sequence divergence between *H. glacialis* and *H. gibberum* averages 13.1%. Based on current evidence, individuals showing less than 4.3% divergence from a representative COI mtDNA sequence (GenBank AF 245355) belong to *H. glacialis*.

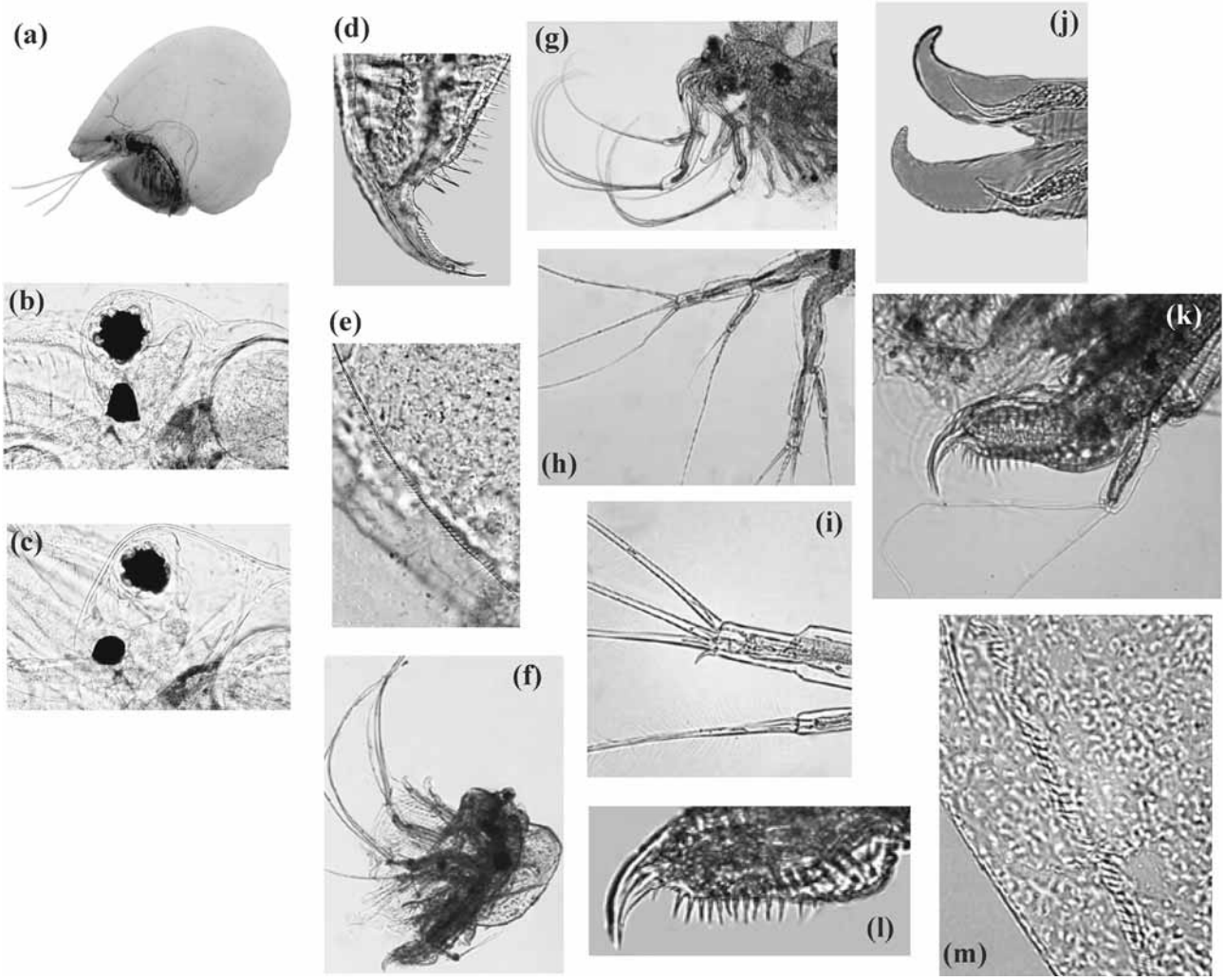


FIGURE 7. Representative photomicrographs of *Holopedium glacialis*. (a) Lateral view of female in jelly coat stained with dilute fuschian red. Wren Lake, Ontario, June 13, 1994. (b,c) Lateral views of female head. (d) Lateral view of female postabdomen. (e) Lateral view of ventral carapace spinules. (f) Lateral view of male with jelly coat removed. (g) Lateral view of male head and antennae. (h) Lateral view of male antennae. (i) Lateral view of hook on male antennae. (j) Lateral view of hooks on first pair of male thoracic limbs. (k) Lateral view of male postabdomen. (l) Lateral view of male postabdominal claw. (m) Lateral view of ventral carapace spines of a male. (b–e) from Como Lake, Ontario, June 28, 1992. (f–m) from Blue Chalk Lake, Ontario, October 17, 1996.

Distribution. *Holopedium glacialis* presumably occurs over most of the formerly glaciated regions of North America (Fig. 4a), from the Coastal and Rocky Mountain ranges in the west through to the Atlantic coast in the east, excepting the central Great Plains, where habitats with suitable water chemistry are absent (see Rowe 2000 for a discussion of the ecological requirements of *Holopedium*). It occurs as far south as the

Sierra Nevada mountains of California and the Rocky Mountains of Colorado, but in the east there are no confirmed populations south of New York State. It occurs sympatrically with *H. atlanticum* in New Brunswick and Maine, with no evidence of hybridization. *H. glacialis* is also present in the Canadian arctic, but not north of 68.5° N latitude. The northernmost habitats were a few lakes on the Melville Peninsula, including three in which it was sympatric with *H. gibberum*, again with no evidence of hybridization.

Breeding system. Many populations were invariant, but 43 of 59 polymorphic populations were in H-W equilibrium, suggesting that at least these populations reproduce by cyclical parthenogenesis. Males were discovered in several populations in the spring and in larger numbers in the autumn. The few single-species populations in H-W disequilibrium were due to occasional heterozygote excesses or deficits, patterns that may result from extended bouts of asexual reproduction.

Holopedium amazonicum Stingelin, 1904

Historical literature descriptions. *Holopedium amazonicum* is restricted to the Amazon River basin. Individuals from North America previously identified as this species are either *H. atlanticum* or *H. acidophilum*.
Stingelin (1904*a*): 54–64, Table 1, Figs. 1–2
Stingelin (1904*b*): 577–578, Table 20, Figs. 1–2
Thomasson (1955): No figures (paper incorrectly identifies specimens as *H. gibberum*)
Korovchinsky (1992): 77–78, Figs. 378–381
Paggi (1995): 912, 930–931. Figs. 14–15
Korovchinsky (2004): 345–348, Fig. 141

Etymology. *amazonicum* refers to the distribution of this species in waterbodies throughout the Amazon River basin.

Type locality. Rio Aramá Grande on Marajó Island at the mouth the Amazon River in Brazil (Stingelin 1904*a*; approx. 1.02° S, 49.24° W).

Type specimens. Stingelin's (1904*a*) type material is housed in the Naturmuseum Olten (Kirchgasse 10, CH–4600 Olten, Switzerland; Dr. D. Vallen, curator). The material consists of two mounts, with some adult females and many juveniles from "Amazonas, 1900". One of the females is designated as the lectotype, and the others as paralectotypes.

Voucher specimens. Twenty ovigerous females from Lago Coari, Amazonas, Brazil (collected by P. Mera, INPA, Manaus, Amazonas, on May 24, 1996; 4.08° S, 63.14° W) were deposited in the CMN under accession number CMNC 2007-0740 and an additional 30 ovigerous females from this collection were deposited at INPA, Manaus, Amazonas.

Material examined. Other habitats with *H. amazonicum* are listed in Appendix A.

Morphological description. FEMALE. Representative photomicrographs are shown in Fig. 8. In this study, due to specimens' preservation in formalin, jelly coat sculpturing was not discernable.

Females are small, with adult carapace lengths ranging from 0.39–1.27 mm (mean 0.74 mm), while carapace heights range from 0.32–1.17 mm (mean 0.71 mm). The H/L ratios range from 0.66–1.64 (mean 0.99). The ventral carapace margin has many, tightly-spaced spinules posteriorly, but is smooth anteriorly.

Anal spine number ranges from 5–12 (mean 8.37). *Holopedium amazonicum* lacks a basal spine on each postabdominal claw. Each claw ordinarily has a row of denticles running laterally from the base of the claw to its midpoint, although individuals were observed lacking claw denticulation.

MALE. Two males were discovered by CLR in a collection from Lago Coari, Amazonas, Brazil (4.08º S, 63.14º W), which constitutes their first report from South America. The jelly coat was present, but due to its preservation in formalin, sculpturing was not discernable. The specimens were small, with a mean length and height of 0.36 mm and 0.21 mm, respectively. The ventral carapace margin has many, tightly-spaced spinules posteriorly, but is smooth anteriorly.

The two males examined possessed 7 and 11 anal spines, respectively, and both lacked a basal spine on each postabdominal claw. Postabdominal claws have a row of denticles running laterally from the base of the claw to its midpoint.

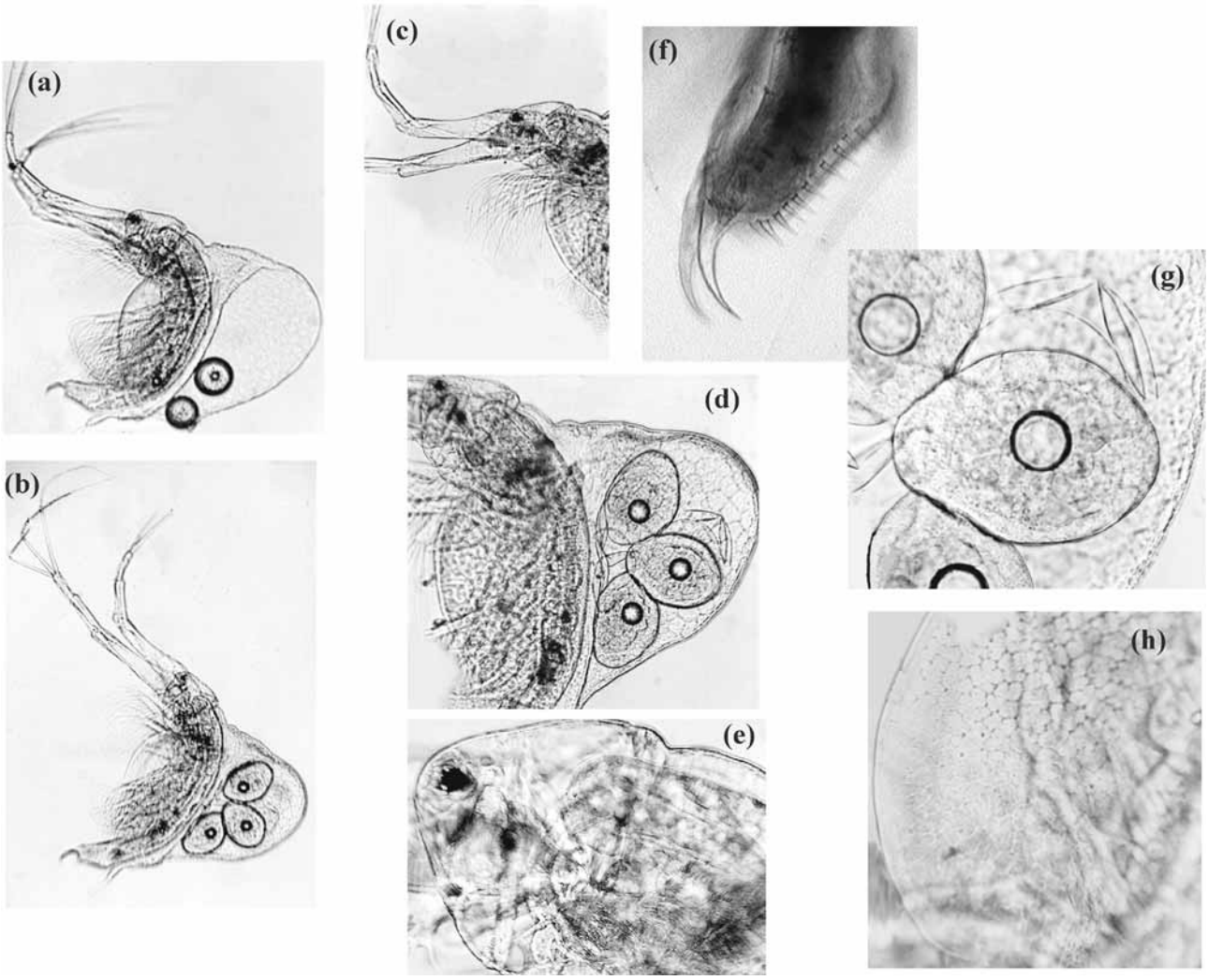


FIGURE 8. Representative photomicrographs of *Holopedium amazonicum*. (a) Lateral view of ovigerous female with jelly coat removed. (b) Lateral view of brooding female with jelly coat removed. (c) Lateral view of female head. (d) Partial lateral view of female. (e) Lateral view of female head. (f) Lateral view of female postabdomen. Lago Coari, Amazonas, May 24, 1996. (g) Lateral view of brood pouch margin and eggs. (h) Lateral view of ventral carapace margin. (a–e, g–h) from Lago Caju, Amazonas, September 24, 1998.

Differential diagnosis. *Holopedium amazonicum* is morphologically indistinguishable from *H. atlanticum*, but these species have allopatric distributions (Fig. 4c,e). *H. amazonicum* is distinguished from *H. acidophilum* by its smaller size and smaller number of anal spines. It differs from members of the *H. gibberum* complex by its absence of a basal spine on either postabdominal claw. COI mtDNA sequence divergence between *H. amazonicum* and *H. atlanticum* averages 12.3%, while the divergence between *H. amazonicum* and *H. acidophilum* averages 8.7%. Levels of genetic variation within this species remain poorly known, but, based on patterns in the other *Holopedium* species, individuals showing less than 4% divergence from a representative COI mtDNA sequence (GenBank AF 245351) are likely to belong to *H. amazonicum*.

Distribution. *H. amazonicum* appears restricted to the Amazon River basin (see also Rowe 2000), from which it was described (Stingelin 1904*a*) (Fig. 4e). It has been reported from several ria lakes throughout the

Amazon River basin, typically associated with highly humic-stained "blackwater". Records indicate that *H. amazonicum* is present throughout the year, with highest densities during the semi-annual rising and lowering of the water level (E.R. Hardy, INPA, Manaus, Amazônia, unpubl. data). It has occasionally been collected in low densities from whitewater ria lakes, but it has likely been washed into these habitats from blackwater habitats during periodic flooding.

Breeding system. Members of this species were not included in allozyme analyses, and so the breeding system cannot be diagnosed in this fashion. However, males of *H. amazonicum* were detected by CLR in a collection made by Pedro Mera (INPA, Manaus, Amazônia) from Lago Coari, Brazil (4.077° S, 63.140° W; May 24, 1996), suggesting that cyclic parthenogenesis may be the mode of reproduction.

Holopedium acidophilum n. sp.

Etymology. *acidophilum* refers to the apparent restriction of populations of this species to acidic bogs, ponds, and lakes.

Type locality. Red Rock Pond, New Brunswick, Canada (45.233º N, 66.733º W), which is located near St. George, NB. It is situated north of Lake Utopia. From Hwy 785, turn onto Red Rock Lake Road. This road bifurcates 3.5 km later. Take the right fork. Proceed 2.9 km to Red Rock Pond which is on the right hand side of the gravel road.

Type specimens. Holotype: an ovigerous female in ethanol deposited in the CMN under accession number CMNC 2007-0738 (collection date June 13, 1992).

Paratypes: 10 ovigerous females, preserved in ethanol, deposited in the CMN under accession number CMNC 2007-0739 (collection date June 13, 1992).

Material examined. Other habitats with *H. acidophilum* are listed in Appendix A.

Morphological description. FEMALE. Representative photomicrographs are shown in Fig. 9. The jelly coat is of the A type, in which the anterior jelly curl arches toward the anterior portion of the jelly coat, and the lateral lobes are undivided (see Montvilo *et al.* 1987).

Adult carapace lengths range from 0.62–1.46 mm (mean 1.10 mm), while carapace heights range from 0.24–1.48 mm (mean 1.08 mm). The H/L ratios range from 0.40–1.29 (mean 0.97). The ventral carapace margin is ordinarily spinulated posteriorly, but smooth anteriorly. Individuals lacking spinulation along the entire ventral valve margin were encountered.

Anal spine number ranges from 8–21 (mean 14.07). *Holopedium acidophilum* lacks a basal spine on each postabdominal claw. Each claw invariably has a row of denticles running laterally from the base of the claw to its midpoint.

MALE. Males were found in the type locality (Red Rock Pond, NB) from collections made in June and September over several years. Body lengths range from 0.40–0.91 mm. The ventral carapace margin is spinulated posteriorly, but smooth anteriorly.

Males possess 9–17 anal spines. *Holopedium acidophilum* lacks a basal spine on each postabdominal claw. Each claw invariably has a row of denticles running laterally from the base of the claw to its midpoint.

Differential diagnosis. *H. acidophilum* can be distinguished from *H. amazonicum and H. atlanticum* by its larger size and greater number of anal spines. It differs from both members of the *H. gibberum* species complex by its lack of a basal spine on the postabdominal claw. *Holopedium acidophilum* can be biochemically distinguished from *H. atlanticum* at the *Pgm* locus. *Holopedium acidophilum* possesses an allele that migrates faster than the allele present in *H. atlanticum*. COI mtDNA sequence divergence between *H. acidophilum* and *H. amazonicum* averages 8.7%, while the divergence between *H. acidophilum* and *H. atlanticum* averages 10.6%. Based on current evidence, individuals showing less than 6% divergence from a representative COI mtDNA sequence (GenBank AF 245352) belong to *H. acidophilum*.

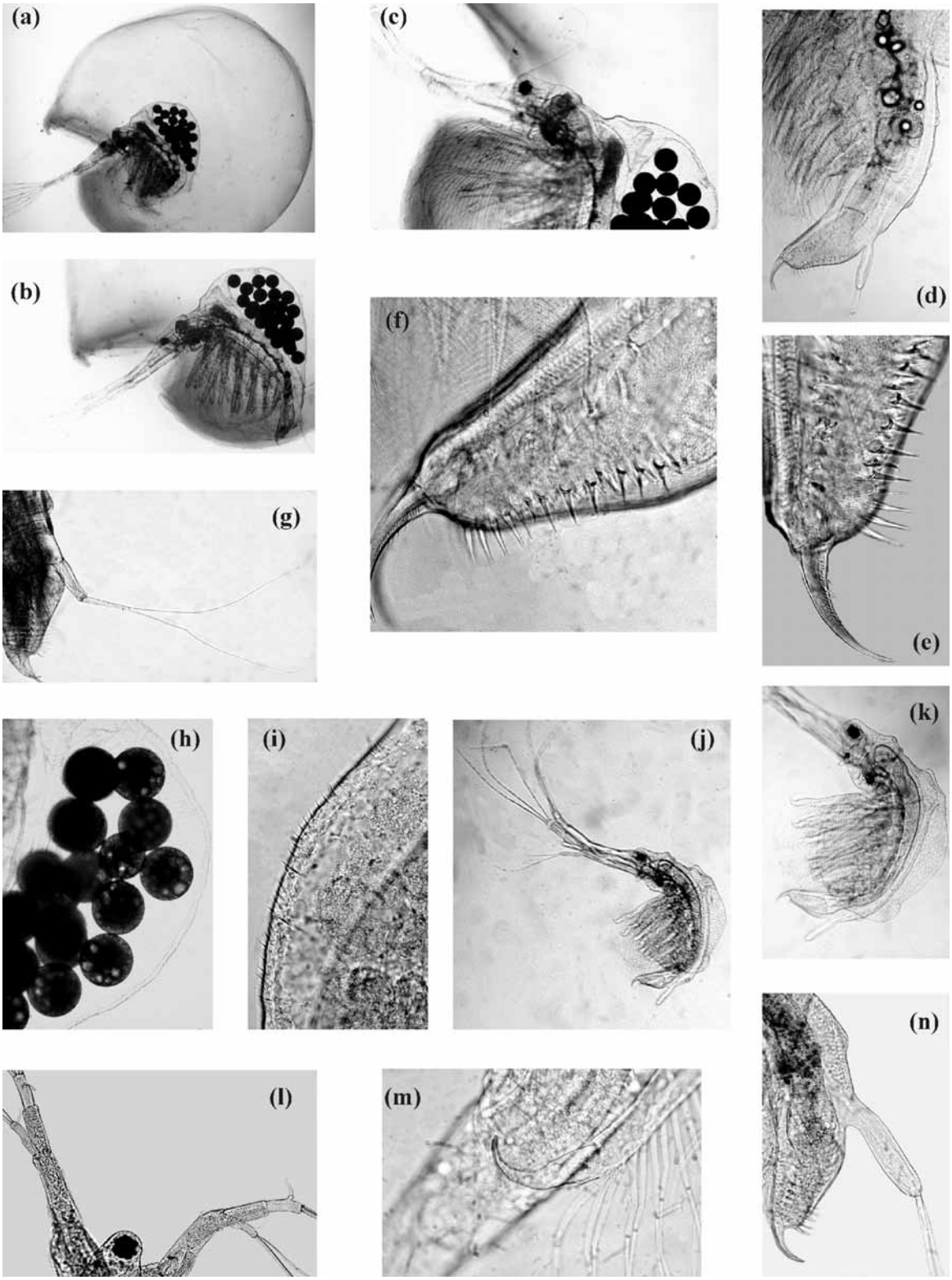


FIGURE 9. Representative photomicrographs of *Holopedium acidophilum*. (a,b) Lateral view of female in jelly coat stained with dilute fuschain red. (c) Lateral view of female head and anterior jelly curl. Jelly coat stained with dilute fuschian red. (d) Lateral view of female abdomen. (e,f) Lateral views of female postabdomen (g) Lateral view of female postabdomen, Red Rock Pond, New Brunswick, June 1, 1992. (h) Lateral view of brood pouch margin and eggs. (i) Lateral view of ventral carapace spinules. (j,k) Lateral views of male with jelly coat removed. (l) Frontal view of male biramous antennae. (m) Lateral view of hook on first thoracic limb of male. (n) Lateral view of male postabdomen. (a–c, h, j–n) from Red Rock Pond, New Brunswick, June 15, 1994. (d–f, i) from Saunders Pond, Oregon, April 16, 1993.

Distribution. *Holopedium acidophilum* appears to be restricted to a narrow latitudinal range (43º to 47º N) spanning North America (Fig. 4d). This species was not found to co-occur with other species of *Holopedium*. Despite concentrated sampling within this range, this species was found rarely. It occurred in three lakes and one pond on the west coast of Oregon, a small pond on the upper Michigan peninsula, and two bogs in south-eastern New Brunswick. The eastern bog habitats were situated within a few kilometers of lake populations of both *H. glacialis* and *H. atlanticum*, but there was no evidence of genetic exchange as indicated by distinctive allozyme and mtDNA profiles. In the west, *H. acidophilum* was found in coastal lakes and ponds in Oregon, while the nearest populations of *H. glacialis* were in lakes in the Coastal Mountains.

Breeding system. Males were detected in the eastern populations in mid June and late September, indicating that members of these populations are cyclical parthenogens. Males were not detected in western and central populations, but this was likely because they were sampled in the summer. Moreover, genotype frequencies were generally concordant with Hardy-Weinberg expectations.

Holopedium atlanticum n. sp.

Synonymy. Individuals from North America previously identified as *H. amazonicum* should properly be identified as *H. atlanticum*.
Birge (1918): 693, Fig. 1061b
Pennak (1953): 364–365, Fig. 227d
Brooks (1959): 603, Fig. 27.13
Pennak (1978): 365–366, Fig. 254d
Pennak (1989): 386–387, Fig. 12d
Korovchinsky (1992): 77–78, Figs. 371–373, 375, 377

Etymology. *atlanticum* refers to the distribution of this species in lakes along the eastern Atlantic seaboard of North America.

Type locality. Moosehead Lake, Maine (45.633º N, 69.683º W). On Hwy ME-6, in close proximity to the town of Moosehead.

Type specimens. Holotype: an ovigerous female in ethanol deposited in the CMN under accession number CMNC 2007-0741 (collection date September 2, 1993).

Paratypes: 10 ovigerous females, preserved in ethanol, deposited in the CMN under accession number CMNC 2007-0742 (collection date September 2, 1993).

Material examined. Other habitats with *H. atlanticum* are listed in Appendix A.

Morphological description. FEMALE. Representative photomicrographs are shown in Fig. 10. The jelly coat is of the A type, in which the anterior jelly curl arches toward the anterior portion of the jelly coat, and the lateral lobes are undivided (see Montvilo *et al.* 1987).

Adult carapace lengths range from 0.44–1.01 mm (mean 0.73 mm), while carapace heights range from 0.30–1.06 mm (mean 0.74 mm). The H/L ratios range from 0.68–1.37 (mean 1.00). The ventral carapace margin is ordinarily spinulated posteriorly, but smooth anteriorly. Individuals lacking spinulation along the entire ventral valve margin were encountered.

Anal spine number ranges from 6–11 (mean 8.35). *Holopedium atlanticum* lacks a basal spine on each postabdominal claw. Each claw ordinarily has a row of denticles running laterally from the base of the claw to its midpoint, although individuals were observed that lacked claw denticulation.

MALE. Males have been found in small numbers in collections from sites in North Carolina in May and June; however, they are typically found in the highest abundance in the autumn (Hegyi 1973). Males of this species were not examined in this study, and thus detailed morphometrics cannot be presented. However, Hegyi (1973) presented a photograph and brief description of a male *Holopedium* which, based on distributional data, is probably *H. atlanticum*.

Differential diagnosis. Although *H. atlanticum* is morphologically indistinguishable from *H. amazonicum*, these two species have allopatric distributions reducing the likelihood of genetic exchange (Fig. 4c,e). *Holopedium atlanticum* is distinguished from *H. acidophilum* by the larger size and greater number of anal spines of the latter species. It differs from members of the *H. gibberum* complex by the absence of a basal spine on either postabdominal claw. *Holopedium atlanticum* can be biochemically distinguished from *H. acidophilum* at the *Pgm* locus, as *H. atlanticum* produces an enzyme which migrates slower than that of the latter species. COI mtDNA sequence divergence between *H. atlanticum* and *H. amazonicum* averages 12.3%, while the divergence between *H. atlanticum* and *H. acidophilum* averages 10.6%. Based on current evidence, individuals showing less than 4.8% divergence from a representative COI mtDNA sequence (GenBank AF 245353) belong to *H. atlanticum*.

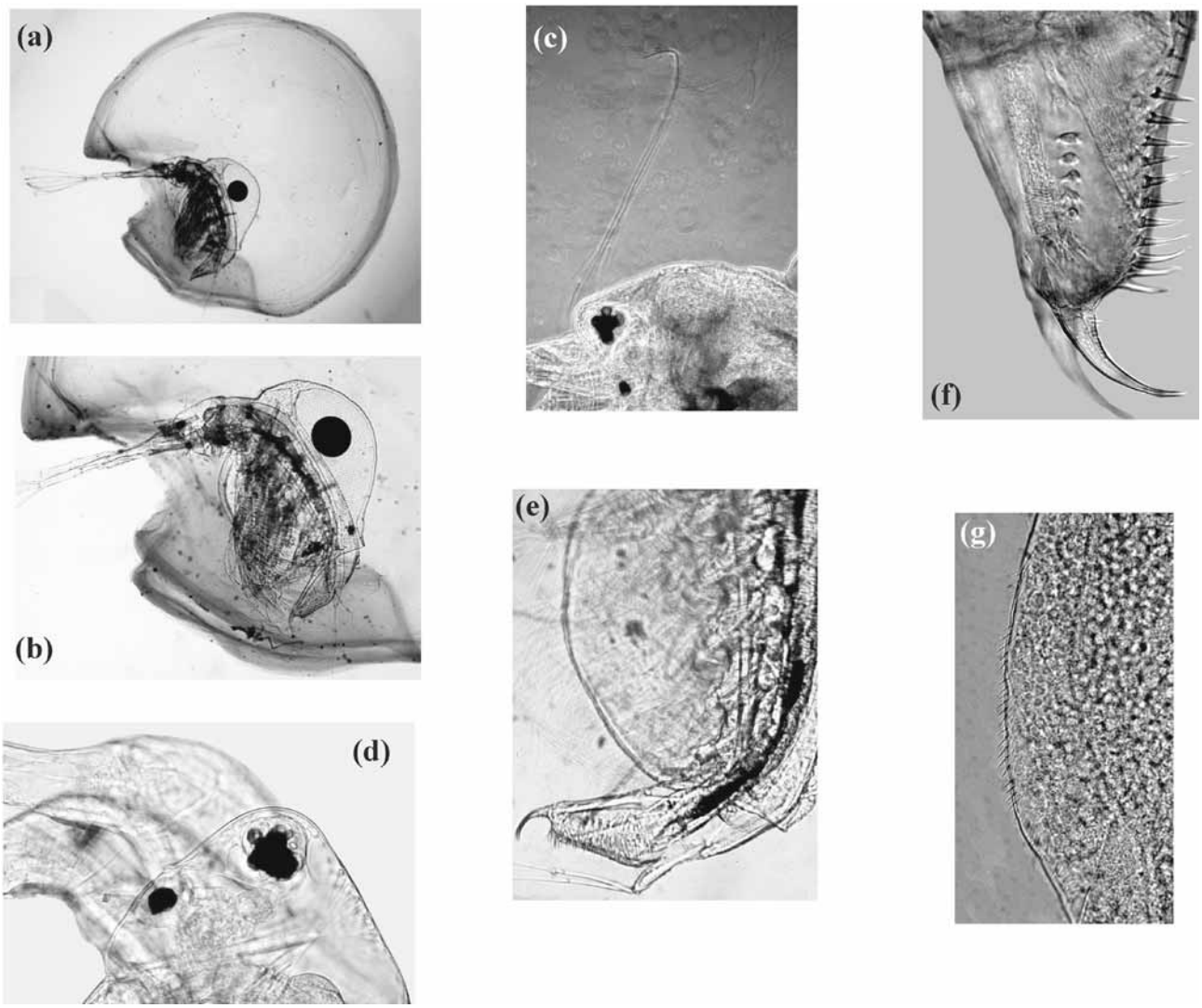


FIGURE 10. Representative photomicrographs of *Holopedium atlanticum*. (a,b) Lateral views of female in jelly coat stained with fuschian red. (c) Lateral view of female head and anterior jelly curl. Santeetlah, North Carolina, October 30, 1993. (d) Lateral view of female head. (e) Lateral view of female abdomen. Lake James, North Carolina, December 13, 1992. (f) Lateral view of female postabdomen. (g) Lateral view of ventral carapace spinules. Lake James, North Carolina, October 31, 1993. (a,b,d,f) from Digdeguash Lake, New Brunswick, June 15, 1994.

Distribution. *H. atlanticum* was found along the Atlantic coast of North America from New Brunswick and Maine south to Florida, (Fig. 4c). Populations of *Holopedium* reported by other workers from the southeastern United States are likely also *H. atlanticum*. Its range overlaps that of *H. glacialis* in the northeastern

USA and southern New Brunswick, where these species occur sympatrically without hybridization. The extent of range overlap with *H. glacialis* is unresolved by this study, but several workers have identified *H. atlanticum* (formerly *H. amazonicum*) as far north as New Brunswick and *H. glacialis* (formerly *H. gibberum*) as far south as Tennessee and possibly South Carolina (Coker 1938, Bunting 1970, Hebert & Finston 1997).

Breeding system. Males were not detected in populations collected throughout the summer in this study. In a life history study spanning two years, males were most abundant in early spring and late autumn (Hegyi 1973). In some southern localities, populations persist throughout the winter. Due to the existence of males, this species likely reproduces by cyclic parthenogenesis, but there is very little allozyme variation, suggesting that either this species engages in sexual reproduction infrequently or that variation has been trimmed due to a population bottleneck.

A note regarding *H. groenlandicum* and *H. ramasarmii*

While individuals from Greenland were not included in the present study, the recently described species *H. groenlandicum* (Korovchinsky 2005) can purportedly be distinguished from *H. gibberum* by its "dorsally low shell and jelly envelope, shorter row of valve marginal spinules which are subdivided in groups, and comparatively longer postabdominal claws." However, shell shape is a highly variable feature, which may be environmentally influenced (Røen 1962) and can depend upon the locality and presence/absence of fish (CLR pers. obs). The body lengths (0.74 to 1.09mm, mean 1.45mm), carapace heights (0.80 to 1.57mm, mean 1.19mm), and H:L ratios (0.641 to 1.000, mean 0.814) found by Korovchinsky (2005) in the Greenland populations fall within the ranges of values found in *H. gibberum* and *H. glacialis* populations in the present study (the preceding ranges and means that were not published in Korovchinsky [2005] were provided to CLR by that author). Jelly coat shape may be influenced by preservation (CLR, pers. obs), and therefore this trait may not be a good feature for diagnosing species. Moreover, the degree of carapace margin spinulation is also a highly variable trait within species (present study), although the discontinuous nature of the spinulation in the Greenland populations is noteworthy. Finally, the length of the postabdominal claws reported by Korovchinsky (2005, his Figure 1) is within the range of claw lengths observed for the *H. gibberum* s.s. populations studied here. Furthermore, the fact that we detected closely related lineages of *H. gibberum* s.s. in both northern Europe and North America suggests that similar lineages may be found in intervening arctic areas.

Individuals from India were also not included in the present study. Consideration of the differences between either of the species in the *H. gibberum* complex and *H. ramasarmii* (Rao *et al.* 1998) is not currently possible due to the poor description of the latter species, lacking in detail. Korovchinsky (2004) labeled this species *incertae sedis*.

We suggest that genetic evidence is required to determine if *H. groenlandicum* and *H. ramasarmii* are distinct species or if they are synonymous with described taxa.

Discussion

Diversity of *Holopedium*

Many past investigations have failed to recognize more than two species of *Holopedium*. In fact, due to their limited geographic scale, most studies only recognized a single species. Early studies that did cover a larger scale focused mainly on morphological criteria, and the lack of such variation did little to advance understanding of the taxonomic diversity in the genus. Initial genetic surveys were also geographically restricted and therefore were unable to critically examine the diversity of the genus on a large scale.

Thier (1994) was the first to examine the patterning of genetic diversity in *Holopedium*. He found marked heterozygote deficiencies in several New England populations, but he did not consider that the two common homozygote classes might be different species. A larger scale allozyme study encompassing lakes from

Thier's study area provided clear evidence for the co-occurrence of two *Holopedium* species in eastern North America (Hebert & Finston 1997). Even this study was limited, however, in that it did not include samples from type localities, so species identification remained uncertain.

In the present study, *Holopedium* was collected from across North America and from a few sites in Europe and South America. In order to validate species identifications, comparisons were made with individuals collected from regions near the type localities of the two species that are generally recognized (since the precise type locality of *H. gibberum* is unknown and that for *H. amazonicum* is subject to seasonal flooding of the Amazon). Patterns of variation in both biochemical and sequence markers provided support for the presence of two major lineages of *Holopedium* that corresponded with the presumed geographic distribution of the two initially-described species, *H. gibberum* and *H. amazonicum*. However, the results also revealed further genetic divergence within each of these lineages.

Allozyme and mtDNA results indicated that the North American populations in the *H. gibberum* complex were divided into two divergent groups. One was distributed throughout temperate North America, while the other was found in arctic North America, and along the west coast of British Columbia. Individuals from European populations, which are much nearer to the type locality for *H. gibberum*, consistently fell within the arctic *Holopedium* group, suggesting that this clade is *H. gibberum* s.s. Its sister taxon, found throughout temperate North America, is apparently an undescribed taxon. This species is described above as *H. glacialis* **n. sp.**, due to its distribution across many formerly glaciated regions.

Allozyme and COI results showed that the *H. amazonicum* complex consisted of two highly distinctive groups in North America. Due to their mtDNA divergence from individuals belonging to a third cluster, collected from the region of the type locality of *H. amazonicum*, these North American taxa apparently represent unknown species. *H. acidophilum* **n. sp.**, described above, occupies bogs, ponds, and lakes across middle latitudes of North America, while *H. atlanticum* **n. sp.** is found along eastern North America. *H. amazonicum* s.s. is apparently restricted to the Amazon basin.

Undescribed species or conspecific populations?

Molecular and biochemical data indicate that there are five distinct evolutionary lineages among our collections of *Holopedium*. Several lines of evidence suggest that these clades merit recognition as distinct species. First, the clades meet the phylogenetic species criterion of diagnosability (Davis & Nixon 1992) as each can be distinguished from all other in-group taxa.

Secondly, the extent of genetic divergence between clades is substantially greater than that within clades, on average by nine-fold for the allozymes and twelve-fold for mtDNA. Moreover, the entire range of mtDNA divergences observed within species is completely non-overlapping with the interspecies range. The allozyme results are similar but with a lone exception. Large allozyme divergences are observed between allopatric groups of *H. acidophilum*, but jointly considered morphological, habitat, and genetic data do not currently justify the splitting of this taxon, although the different groups might be considered incipient species. The patterns of genetic variability within and amongst the five main clades identified here thus substantiate the view that they are distinct phylogenetic species rather than just genetically divergent populations of a single species. Such disjunctions between intra- and interspecific genetic diversity are found in other crustaceans as well. Following large-scale surveys and further methodological development, this pattern is expected to be a highly useful tool for species identification (Costa et al. 2007).

Third, three of the allozyme loci are diagnostic, exhibiting fixed allelic differences. Allelic variation at *Pgm*, *Gpi*, and *sMdh* provide diagnostic markers that allow discrimination of at least the four species which occur in North America. Moreover, several of the clades maintain this genetic divergence despite their neighbouring or actual sympatry. For example, the ranges of *H. glacialis* and *H. gibberum* overlap in the North American arctic, with the two species co-occuring at three sites with no evidence of hybridization. *Holopedium glacialis* and *H. atlanticum* were similarly found to coexist in three lakes in eastern North America with-

out hybridization. Finally, *H. acidophilum* occurs within the range of *H. glacialis* and *H. atlanticum* but never interbreeds with them. Therefore, these clades clearly have unique evolutionary histories and appear to be reproductively isolated. Thus, these concordant pieces of evidence support the conclusion that the five genetic lineages of *Holopedium* represent five non-hybridizing species.

Morphologically intermediate forms revisited

The present study establishes that the *H. gibberum* and *H. amazonicum* species groups can be easily distinguished with morphological criteria. In fact, they are reliably separated by the presence or absence of basal spines on the postabdominal claw, and by the divergent morphologies of their jelly coat (Tessier 1986). Past suggestions that there is only a single *Holopedium* species, due to the presence of morphologically intermediate individuals in eastern North America, were due to flawed taxonomy. The recognition that separate species in the *H. amazonicum* complex have ventral carapace margin spinulation, and vary considerably in the number of anal spines, eliminates the taxonomic puzzle associated with most of these intermediates. In addition, the ranges of *H. glacialis* and *H. atlanticum* overlap extensively in eastern North America, and these species coexist in several habitats. Although they did not recognize it, descriptions given by several authors suggest they may have also encountered these two species in sympatry (e.g., Carpenter 1931; Coker 1938; Bunting 1970; Hegyi 1973; Korovchinsky 1992; Thier 1994).

By contrast, species within each of these groups are largely morphologically cryptic, with the exception of *H. acidophilum* in the *H. amazonicum* group. However, both species in the *H. gibberum* cluster occur in sympatry without evidence of hybridization. Similarly, two species in the *H. amazonicum* group occur in neighbouring sympatry in eastern North America without hybridization. The third species in the *H. amazonicum* cluster (*H. amazonicum* s.s.) has an allopatric geographic range, reducing the probability of genetic exchange, and contributing to the marked mitochondrial divergence from all other species. Therefore, despite the lack of clear, diagnostic morphological differences, we suggest that the five genetic species warrant recognition as species, described above. We present both biochemical and morphological/ distributional keys to these five species below in Appendix B. Future morphological work, perhaps involving scanning electron miscroscopy on the fine structure of limbs, may yet reveal subtle diagnostic traits and will contribute to a greater understanding of the evolutionary divergence of these species.

Summary and future directions

This study presents the first broad, genetics-based assessment of species boundaries in the genus *Holopedium*. Discontinuity in the amounts of within- and between-clade genetic differentiation, as well as the presence of diagnostic molecular characters (despite their adjacent and overlapping ranges), indicate that the five clades found in this study are genetically distinct and represent evolutionarily independent groups. The identification of lineages by both mitochondrial sequence data and multiple allozyme loci indicates that they are not simply a reflection of the history of a single gene, but rather represent separate species.

Such genetic surveys may help to elucidate both the extent and the origins of biological diversity. Previous researchers have proposed that surveys of both highly diverse and species-poor cladoceran groups will be necessary to enhance our understanding of cladoceran diversity and evolutionary history (Hebert & Taylor 1997). This study has indeed revealed that taxonomic diversity was greater than previously supposed for the low-diversity genus *Holopedium*, similar to findings for other branchiopod crustaceans whose diversity has been evaluated from a genetic perspective (reviewed in Adamowicz & Purvis 2005; Korn & Hundsdoerfer 2006). With a ratio of total species: morphologically known species of at least 5:2 among our samples, *Holopedium* is among those branchiopod groups most poorly known from morphology alone (Adamowicz & Purvis 2005). However, interestingly, *Daphnia* had quite similar levels of undescribed species diversity prior to genetic investigation (such as a ratio of 2.33:1 for North America). Thus, levels of (in)completeness of taxonomic knowledge were similar between these genera, both of which are widely distributed but which have

very different levels of global species diversity. Therefore, while these genetic surveys have advanced our understanding of taxonomic diversity, and of the ubiquity of (nearly) cryptic diversity in the branchiopods, they have not yet revealed reasons for differing levels of species diversity among branchiopod groups.

Additional research, taking a phylogenetic perspective, will be required to address diversity patterns among cladocerans. Two avenues that may be fruitful would be to compare the propensity for dispersal and allopatric divergence across groups and to investigate whether there are different potentials for habitat transitions. Habitat shifts between lake and pond environments appear to be an important mechanism of speciation in *Daphnia* (e.g. Taylor *et al.* 1996; Schwenk *et al.* 2000) and possibly in *Bosmina* (DeMelo and Hebert 1994), but do not seem to be operating to the same extent in *Sida* and *Holopedium*. Multidisciplinary surveys including genetic analyses, such as the present one, provide a solid taxonomic foundation for such further studies of distribution and evolutionary patterns that should aid in our understanding of diversification in the freshwater realm.

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Appendix A. List of 193 sites from which *Holopedium* was studied, with genetically-studied populations assigned site numbers 1-180 (as in Rowe 2000) and populations only studied from a morphological perspective assigned letter codes from A-M. North American biogeographic province (BP) numerical codes correspond to those in the legend of Fig. 1. Country codes are: BRA (Brazil), CAN (Canada), CZE (Czech Republic), NOR (Norway), POL (Poland), and USA. Geographic coordinates are based on global quadrants, such that positive values indicate North latitudes and East longitudes. Codes designating the species detected at each site are: A- *H. atlanticum* n.sp., C- *H. glacialis* n.sp., G- *H. gibberum* s.s., L- *H. acidophilum* n.sp., and Z- *H. amazonicum* s.s. Types of data collected were: A (allozymes), D (mitochondrial DNA), and M (morphological). Allozyme data for 71 populations from Hebert and Finston (1997) were included and reanalyzed in this study (marked with an asterisk). For the morphological data, a large X indicates that the population was included in the detailed morphological analysis, while a plus sign (+) indicates that the population was simply identified morphologically/geographically as belonging to one of the five species.

Site code	BP	Country	State/ Prov	Locality	Lat	Long	Date collected	Species	Type of Data		
									A	**D**	**M**
A	--	BRA	AM	Arraja	-2.733	-60.773	22-Sep-98	Z	--	--	+
B	--	BRA	AM	Caju	-3.039	-60.243	23-Sep-98	Z	--	--	+
C	--	BRA	AM	Coari	-4.077	-63.140	24-May-96	Z	--	--	X
D	--	BRA	AM	do Prato	-2.704	-60.752	22-Sep-98	Z	--	--	+
E	--	BRA	AM	Lago Taruma-mirim	-3.005	-60.189	21-Sep-98	Z	--	--	+
1	--	BRA	AM	Muia	-2.754	-60.753	22-Sep-98	Z	--	5-1	+
F	--	BRA	AM	Rio Negro lagoon	-3.100	-60.017	14-Sep-79	Z	--	--	X
G	--	BRA	AM	Taruma-acu	-3.024	-60.111	21-Sep-98	Z	--	--	+
H	--	BRA	AM	Tupe	-3.044	-60.255	21-Sep-98	Z	--	--	+
I	--	BRA	AM	Unnamed Parana	-2.720	-60.714	22-Sep-98	Z	--	--	+
J	--	BRA	AM	Xidaua	-2.722	-60.751	22-Ssep-98	Z	--	--	+
2	5	CAN	BC	Mayer	53.640	-132.056	07-Aug-97	G	X	2-9	X
3	5	CAN	BC	Mosquito	53.070	-132.070	06-Aug-97	G	X	2-7	
4	5	CAN	BC	Prudhomme	54.240	-130.136	08-Aug-97	G	X	2-9	
5	3	CAN	MB	Reed	54.586	-100.496	27-Jul-97	C	X	1-2,1-8(x2)	
6	9	CAN	NB	Bog Pond	45.200	-66.983	12-Jun-93	L	X	4-2	
7	9	CAN	NB	Chamcook	45.150	-67.100	18-Jul-96	A	X*	3-4	
8	9	CAN	NB	Digdeguash	45.217	-66.917	12-Jun-93	A		3-4	
9	9	CAN	NB	First	46.866	-66.600	18-Jul-96	A,C	X*	--	
10	9	CAN	NB	Gibson	45.183	-67.083	12-Jun-93	A	X*	--	
11	9	CAN	NB	Kerr	45.217	-67.033	12-Jun-93	A	X*	--	
12	9	CAN	NB	Lomond	45.333	-65.883	17-Jul-96	A,C	X*	--	
13	9	CAN	NB	Magaguedavic	45.717	-67.200	12-Jun-93	A		3-4	
14	9	CAN	NB	North	46.933	-66.433	12-Jun-93	C	X*	--	
15	9	CAN	NB	Red Rock Pond	45.233	-66.733	12-Jun-93	L	X	4-1	X

16	9	CAN	NB	Second	45.400	-65.817	12-Jun-93	A		3-5	
17	9	CAN	NB	Skiff	45.817	-67.533	12-Jun-93	A	X*	--	
18	9	CAN	NB	Utopia	45.183	-66.783	18-Jul-96	A,C	X*	--	
19	9	CAN	NF	Unnamed	47.683	-53.850	07-Jul-90	C	X*	--	
20	2	CAN	NT	Arctic Red River 1	67.682	-131.879	19-Aug-97	G	X	2-2	
21	2	CAN	NT	Arctic Red River 5	67.746	-132.762	19-Aug-97	G	X	--	
22	2	CAN	NT	Arctic Red River 7	67.742	-132.828	21-Aug-97	G	X	--	
23	2	CAN	NT	Arctic Red River 8	67.840	-132.621	20-Aug-97	G	X	--	
24	1	CAN	NU	Brodeur Peninsula 3	71.367	-84.998	24-Aug-96	G	X	2-1	
25	2	CAN	NT	Crossley Lk 1	68.088	-130.659	12-Aug-93	G	X	--	
26	2	CAN	NT	Crossley Lk 2	68.050	-130.646	12-Aug-93	G	X	--	
27	2	CAN	NT	Crossley Lk 3	68.013	-130.922	12-Aug-93	G	X	--	
28	2	CAN	NT	Crossley Lk 4	68.055	-130.960	12-Aug-93	G	X	--	
29	2	CAN	NT	Crossley Lk 12	67.952	-132.236	19-Aug-97	G	X	--	
30	1	CAN	NU	Dam	63.750	-68.550	28-Aug-96	C		1-18	
31	1	CAN	NU	Eqe Bay 4	69.570	-76.557	11-Aug-94	G		2-1	
32	1	CAN	NU	Erichsen Lks 1	70.451	-82.364	27-Aug-96	G	X	2-1	
33	1	CAN	NU	Erichsen Lks 2	70.447	-81.773	28-Aug-96	G	X	--	
34	1	CAN	NU	Erichsen Lks 4	70.472	-81.388	28-Aug-96	G	X	--	
35	1	CAN	NU	Erichsen Lks 5	70.622	-80.872	28-Aug-96	G	X	--	
36	1	CAN	NU	Erichsen Lks 6	70.666	-81.138	28-Aug-96	G	X	--	
37	1	CAN	NU	Erichsen Lks 8	70.763	-81.629	28-Aug-96	G	X	--	
38	1	CAN	NU	Erichsen Lks 9	70.687	-81.836	28-Aug-96	G	X	--	
39	1	CAN	NU	Erichsen Lks 10	70.561	-81.944	28-Aug-96	G	X	--	
40	2	CAN	NT	Eskimo Lk 2	68.511	-133.623	18-Aug-97	G	X	--	
41	2	CAN	NT	Eskimo Lk 5	68.554	-133.746	18-Aug-97	G	X	--	
42	2	CAN	NT	Eskimo Lk 6	68.679	-133.817	20-Aug-97	G	X	2-8	
43	2	CAN	NT	Eskimo Lk 8	68.759	-134.126	09-Aug-93	G	X	--	
44	1	CAN	NU	Gifford Fiord 9	70.286	-83.401	22-Aug-96	G	X	2-1	
45	1	CAN	NU	Gifford Fiord 11	70.305	-83.432	27-Aug-96	G	X	--	
46	2	CAN	NT	Great Slave[1]	61.383	-115.633	22-Aug-97	C	X	1-2 (x2),1-9	X
47	2	CAN	NT	Inuvik 1	68.127	-132.430	19-Aug-97	G	X	2-2(x2),2-9	X
48	2	CAN	NT	Inuvik 5	68.118	-132.846	19-Aug-97	G	X	--	
K	1	CAN	NU	Longstaff Bluff 1	68.936	-74.354	11-Aug-94	G	--	--	+
49	2	CAN	NT	MacKenzie Delta 13	69.237	-134.704	21-Aug-97	G	X	2-2,2-11	
50	2	CAN	NT	MacKenzie Delta 16	69.438	-133.063	22-Aug-97	G	X	--	
51	2	CAN	NT	MacKenzie Delta 23	69.359	-134.180	22-Aug-97	G	X	--	
52	3	CAN	NU	Nagvaak Lk 1	67.794	-84.498	16-Aug-94	G		2-1	
53	3	CAN	NU	Parry Bay 2	68.322	-82.607	22-Aug-96	C,G	X	(no data for *H. glacialis*);	

									2-1	
54	3	CAN	NU	Quartzite Lk 7	67.582	-81.526	23-Aug-96	G	X	--	
55	3	CAN	NU	Sarcpa Lk 8	68.535	-83.339	23-Aug-96	C,G	X	1-19,2-1	
56	3	CAN	NU	Sarcpa Lk 13	68.341	-83.329	23-Aug-96	C,G	X	(no data for *H. glacialis*); 2-4	
57	3	CAN	NU	Sarcpa Lk 14	68.402	-83.017	23-Aug-96	C	X	--	
58	3	CAN	NU	Sarcpa Lk 15	68.289	-82.824	23-Aug-96	G	X	2-1	
59	2	CAN	NT	Small Frog	67.382	-134.152	12-Aug-97	G	X	--	
60	1	CAN	NU	Steensby 2	69.891	-77.422	11-Aug-94	G		2-1	
61	1	CAN	NU	Steensby 3	69.851	-77.505	11-Aug-94	G		2-1	
62	2	CAN	NT	Stony Pond	67.148	-135.963	12-Aug-97	G	X	2-2	
63	4	CAN	ON	Bee	49.838	-93.826	25-Jul-97	C	X	--	
64	4	CAN	ON	Beech	45.083	-78.717	05-Jul-94	C	X*	1-12	
65	4	CAN	ON	Bigwind	45.058	-79.050	05-Jul-94	C	X*	--	
66	4	CAN	ON	Bird	45.067	-79.067	05-Jul-94	C	X*	--	
L		CAN	ON	Blue Chalk	45.200	-79.933	17-Oct-96	C	--	--	+
67	4	CAN	ON	Brady	45.083	-78.833	05-Jul-94	C	X*	--	
68	3	CAN	ON	Byng	48.250	-80.750	05-Jul-94	C	X*	--	
69	3	CAN	ON	Chub	45.217	-78.983	06-Jul-94	C	X*	--	
70	4	CAN	ON	Clear	45.042	-79.017	05-Jul-94	C	X*	--	
M		CAN	ON	Como	47.850	-83.567	28-Jun-92	C	--	--	+
71	4	CAN	ON	Echo	45.166	-79.066	05-Jul-94	C	X*	--	
72	4	CAN	ON	Fairy	45.333	-79.183	05-Jul-94	C	X*	--	
73	4	CAN	ON	Five Mile	47.000	-82.000	05-Jul-94	C	X*	--	
74	4	CAN	ON	Grandview	45.200	-79.050	05-Jul-94	C	X*	--	
75	4	CAN	ON	Gullfeather	45.100	-79.033	05-Jul-94	C	X*	--	
76	4	CAN	ON	Hall	45.117	-78.750	05-Jul-94	C	X*	--	
77	4	CAN	ON	Kenny	47.278	-84.560	24-Jul-97	C	X	--	
78	4	CAN	ON	Kushog	45.016	-78.816	05-Jul-94	C	X*	--	
79	3	CAN	ON	Lac des Milles Lacs	48.837	-90.470	25-Jul-97	C	X	1-2 (x3),1-10	
80	3	CAN	ON	Lk of the Woods	49.150	-94.417	09-Sep-97	C	X	1-1	
81	4	CAN	ON	McCormick	45.000	-79.000	05-Jul-94	C	X*	--	
82	3	CAN	ON	McNamara	49.333	-91.783	10-Sep-97	C	X	--	
83	4	CAN	ON	Paint 93	45.217	-78.950	17-Jun-93	C	X*	--	
84	4	CAN	ON	Paint 97	45.217	-78.950	15-Jun-97	C	X	--	
85	4	CAN	ON	Plastic	45.183	-78.833	06-Jul-94	C	X	--	
86	4	CAN	ON	Prospect 93	44.992	-79.133	17-Jun-93	C	X*	--	
87	4	CAN	ON	Prospect 97	44.992	-79.133	21-Sep-97	C	X	1-1	
88	3	CAN	ON	Radisson	48.000	-81.000	06-Jul-94	C	X*	--	

89	4	CAN	ON	Ril	45.168	-79.000	06-Jul-94	C	X*	--	
90	4	CAN	ON	Shoe	45.225	-78.917	06-Jul-94	C	X*	--	
91	4	CAN	ON	Spring	46.000	-78.000	06-Jul-94	C	X*	1-11	
92	4	CAN	ON	St.Nora	45.133	-78.833	06-Jul-94	C	X*	--	
93	3	CAN	ON	White	48.730	-85.632	24-Jul-97	C	X	1-6	
94	4	CAN	ON	Wolfkin	45.233	-78.950	06-Jul-94	C	X*	--	
95	4	CAN	ON	Wren	45.183	-78.866	06-Jul-94	C	X*	1-21	X
96	4	CAN	QC	Archambault	46.313	-74.245	20-Jul-93	C	X*	--	
97	4	CAN	QC	Beland	46.200	-74.200	20-Jul-93	C	X*	--	
98	4	CAN	QC	Chibougamau	49.833	-74.250	29-Jun-96	C	X*	1-12	
99	4	CAN	QC	Rond	46.200	-74.250	20-Jul-93	C	X*	--	
100	4	CAN	QC	St.Francois	45.950	-71.183	21-Jul-96	C	X*	--	
101	4	CAN	QC	St.Jean	48.600	-76.033	28-Jun-96	C	X*	--	
102	3	CAN	SK	Amisk	54.687	-102.079	28-Jul-97	C	X	1-4	
103	3	CAN	SK	Lac La Ronge 93	55.000	-104.000	10-Jun-93	C	X	--	
104	3	CAN	SK	Lac La Ronge 97	55.000	-104.000	29-Jul-97	C	X	1-2,1-7	
105	2	CAN	YK	Herschel Island 7	69.138	-138.195	21-Aug-97	G	X	2-2(x2)	
106	2	CAN	YK	Herschel Island 8	69.092	-138.173	21-Aug-97	G	X	--	
107	2	CAN	YK	Old Crow 3	68.197	-138.673	15-Aug-97	G	X	--	
108	2	CAN	YK	Old Crow 10	67.923	-139.647	15-Aug-97	G	X	2-11	
109	2	CAN	YK	Shingle Pt 4	68.823	-137.303	21-Aug-97	G	X	2-3	
110	2	CAN	YK	Shingle Pt 7	68.769	-137.858	06-Aug-93	G	X	--	
111	2	CAN	YK	Shingle Pt 8	68.790	-138.045	06-Aug-93	G	X	--	
112	2	CAN	YK	Shingle Pt 11	68.737	-137.938	06-Aug-93	G	X	--	
113	2	CAN	YK	Shingle Pt 12	68.755	-137.855	06-Aug-93	G	X	--	
114	2	CAN	YK	Shingle Pt 13	68.715	-137.385	06-Aug-93	G	X	--	
115	2	CAN	YK	Shingle Pt 16	69.030	-137.160	21-Aug-97	G	X	2-2	
116	2	CAN	YK	Shingle Pt 17	68.942	-137.576	21-Aug-97	G	X	2-2,2-10(x3)	
117	2	CAN	YK	Shingle Pt 18	69.030	-137.874	21-Aug-97	G	X	2-2(x2),2-10	
118	--	CZE	--	Fláje	~ 50.7	~ 13.6	03-Jun-94	G		2-6	
119	--	NOR	--	Bergen P2	~ 60.3	~ 5.3	15-Jun-95	G		2-5	X
120	--	POL	--	Nizny Toporowy Staw (lake near Zakopane)	~ 49.3	~ 19.9	28-Sep-95	G		2-12	
121	6	USA	CA	Ellery	37.936	-119.234	01-Aug-98	C	X	1-2	
122	6	USA	CA	George	37.604	-119.010	01-Aug-98	C	X	1-3	
123	6	USA	CA	Grass	38.881	-120.097	31-Jul-98	C	X	--	
124	6	USA	CA	Mary	37.605	-119.003	01-Aug-98	C	X	--	
125	6	USA	CA	Susie	38.883	-120.125	31-Jul-98	C	X	--	
126	7	USA	CO	Yankee Doodle	39.933	-105.650	04-Aug-98	C	X	--	
127	11	USA	FL	Dorr	29.000	-81.625	22-Jun-96	A	X*	--	X

128	11	USA	FL	Santa Fe	29.738	-82.075	16-Dec-92	A	X*	--	
129	11	USA	GA	Banks	31.017	-83.138	23-Jun-96	A	X*	3-2	
130	10	USA	GA	Blue Ridge	34.883	-84.279	23-Jun-96	A	X*	3-3	
131	9	USA	ME	Boyd	45.175	-68.920	04-Jun-91	A	X*	--	
132	9	USA	ME	Crowell	44.583	-70.133	04-Jun-91	A	X*	3-6	
133	9	USA	ME	E. Grand	45.700	-69.817	12-Jun-93	C		1-16	
134	9	USA	ME	Flying Pond	44.533	-70.000	04-Jun-91	A	X*	--	
135	9	USA	ME	Hebron	45.282	-69.531	04-Jun-91	C	X*	--	
136	9	USA	ME	Long	44.483	-69.917	04-Jun-91	A	X*	--	
137	9	USA	ME	Maranacook	44.350	-69.967	04-Jun-91	C	X*	--	
138	9	USA	ME	Messalongskee	44.483	-69.783	04-Jun-91	A	X*	--	
139	9	USA	ME	Moosehead	45.633	-69.683	04-Jun-91	A	X*	3-4	X
140	9	USA	ME	Patrick	44.875	-67.383	17-Jun-94	A	X*	--	
141	9	USA	ME	Torsey	44.417	-70.000	04-Jun-91	A	X*	3-4	
142	9	USA	MI	Soldier Pond[2]	46.333	-84.883	31-May-97	L	X	4-2	X
143	8	USA	MI	Thousand Islands	46.217	-89.417	10-May-91	C	X*	1-14	
144	8	USA	MN	Maple	44.870	-93.793	07-May-91	C	X*	--	
145	8	USA	MN	Pughole	44.850	-93.767	07-May-91	C	X*	--	
146	10	USA	NC	Hiwassee	35.153	-84.178	24-Jun-96	A	X*	3-1	X
147	10	USA	NC	James	35.750	-81.867	24-Jun-96	A	X*	--	X
148	10	USA	NC	Santeetlah	35.376	-83.876	24-Jun-96	A	X*	--	
149	9	USA	NY	Salmon River	43.545	-75.920	18-Apr-91	C		1-13	
150	5	USA	OR	Blue River	44.173	-122.278	16-Jun-97	C	X	--	
151	5	USA	OR	Florence 3	43.817	-124.167	15-Apr-93	L		4-6	
152	5	USA	OR	Foster Reservoir	44.414	-122.671	15-Jun-97	C	X	--	
153	5	USA	OR	Link	44.394	-121.801	15-Jun-97	C	X	1-1	
154	5	USA	OR	Lost	44.000	-121.913	15-Jun-97	C	X	1-5	
155	5	USA	OR	Marie	43.660	-124.198	17-Jun-97	L	X	4-5	
156	5	USA	OR	Saunders Lk	43.530	-124.217	17-Jun-97	L	X	4-5	X
157	5	USA	OR	Saunders Pond	43.530	-124.217	17-Jun-97	L	X	4-3,4-4	
158	5	USA	OR	Unnamed Pond	44.393	-121.801	15-Jun-97	C	X	1-5	
159	8	USA	PA	Corilla	41.529	-75.086	13-May-91	C	X*	--	
160	8	USA	PA	Forest	40.126	-79.450	13-May-91	C	X*	--	
161	8	USA	PA	Lacawac	41.383	-75.291	13-May-91	C	X*	--	
162	5	USA	WA	Keechelus	47.396	-121.391	13-Jun-97	C		1-24	
163	5	USA	WA	Ki	48.154	-122.263	13-Jun-97	C	X	1-23	
164	5	USA	WA	Samish	48.671	-122.412	13-Jun-97	C	X	1-22	
165	5	USA	WA	Spada	47.971	-121.683	13-Jun-97	C	X	1-25	
166	8	USA	WI	Big Frank Bog	46.017	-89.565	01-Jun-97	C	X	1-3	
167	8	USA	WI	Big Muskellunge	46.020	-89.613	01-Jun-97	C	X	1-17,1-20	

168	8	USA	WI	Crystal Bog	46.002	-89.613	11-May-91	C	X*	--	
169	8	USA	WI	Day	46.063	-89.704	11-May-91	C	X*	--	
170	8	USA	WI	Lynx	46.198	-89.674	11-May-91	C	X*	--	
171	8	USA	WI	Merrill	45.185	-89.671	11-May-91	C	X*	--	
172	8	USA	WI	Mud	45.983	-89.342	01-Jun-97	C	X*	1-15	
173	8	USA	WI	Mystery	46.058	-89.565	01-Jun-97	C	X	1-2	
174	8	USA	WI	Nebish	46.052	-89.591	11-May-91	C	X*	--	
175	8	USA	WI	North Turtle	46.238	-89.883	11-May-91	C	X*	--	
176	8	USA	WI	Rest 93	46.142	-89.877	02-Jun-93	C	X*	--	
177	8	USA	WI	Rest 97	46.142	-89.877	01-Jun-97	C	X	1-1	X
178	8	USA	WI	Spruce	46.053	-89.567	01-Jun-97	C	X	1-2	
179	8	USA	WI	Wood	46.158	-90.358	11-May-91	C	X*	--	
180	7	USA	WY	Lookout	41.342	-106.322	06-Aug-98	C	X	--	
Totals:				**193 sites in total**					**164 A**	**77 D**	**15 M**

[1] Great Slave Lake is in the Yukon-MacKenzie biogeographic province (BP 2) but is included in Hudson Bay (3) for F-statistic calculations (see Results).
[2] Soldier Pond is in the Great Lakes (4) BP but has been included in N. Appalachian (9) for F-statistic calculations (see Results).

Appendix B. Discriminating species of *Holopedium*

Allozyme key

All allelic mobilities employed in this key are relative to the dominant allele in *H. glacialis* from the reference site Prospect Lake, Ontario (see Methods), with allozymes analyzed on cellulose acetate gels in a Tris glycine (pH 8.5) buffer. An R_f value of 100 thus indicates an allozyme product with the same mobility as that of the reference population, while 113 indicates an allele having 13% greater mobility. Note that *H. amazonicum* was not included in the allozyme analysis and is therefore excluded from this key.

1a *sMdh* with faster mobility (R_f = 113) than standard..... **2** (*amazonicum* complex)
1b *sMdh* with same mobility as standard or slower ..... **3** (*gibberum* complex)
2a *Pgm* 85 .....*H. atlanticum*
2b *Pgm* 90 ..... *H. acidophilum*
3a *Gpi* 114..... *H. gibberum*
3b *Gpi* 119 or slower than 114 ..... *H. glacialis*

Morphological/ distributional key

Species within complexes may be morphologically indistinguishable. It is advisable to use morphology (see Fig. 5) in combination with distributional and genetic evidence in order to reliably identify species.

1a Postabdominal claw without basal spine; anterior jelly curl arches toward the anterior portion of the jelly coat, and its lateral lobes are undivided ..... 2 (*H. amazonicum* complex)
1b Postabdominal claw with at least one basal spine; anterior jelly curl is relatively straight and ends in a short curve posteriorly toward the carapace and its lateral lobes are divided .....4 (*H. gibberum* complex)
2a Large size, with adult carapace mean length 1.10 mm (range 0.62–1.46 mm); greater anal spine number (mean 14.07; range 8–21); found in lowland acidic lakes and bogs in New Brunswick, Oregon, and Michigan (Fig. 4d) .... *H. acidophilum*
2b Smaller size, adult carapace mean length 0.74 mm (range 0.39–1.27 mm); lower number of anal spines (mean 8.36; range 5–12) 3
3a Found in the Amazon river basin (Fig. 4e) .....*H. amazonicum*
3b Found in parts of eastern North America, east of the Appalachians (Fig. 4c).....*H. atlanticum*
4a Found in Eurasia, the circumpolar high Arctic (north of 67° N in North America) and west coastal regions of Alaska and British Columbia (Fig. 4b)..... *H. gibberum*
4b Found over many of the formerly glaciated regions of North America, from the Coastal and Rocky Mountain ranges in the west through to the Atlantic coast in the east, excepting the central Great Plains. It occurs as far south as the Sierra Nevada mountains of California and the Rocky Mountains of Colorado, but in the east there are no genetically confirmed records south of New York State. Also present in the Canadian arctic, but not north of 68.5° N (Fig. 4a).. *H. glacialis*