

The cryptic origins of environment-indicating phantom midges (*Chaoborus*)

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Abstract

Fragile freshwater ecosystems have acted as important sentinels for global environmental change. A detailed understanding of the implications of global change in aquatic ecosystems relies on indicator species whose body parts are preserved in aquatic sediments for millennia. The phantom midge, *Chaoborus flavicans* (Meigen, 1830), for example, has well-preserved mandibles in aquatic sediments and may act as an indicator of boreal zone temperatures, lake depth, and the presence of fish. Nevertheless, we report that *Chaoborus* with *C. flavicans*-type mandibles are common in fishless tundra ponds above the tree line in western Alaska. We carried out genetic comparisons of nuclear and mitochondrial deoxyribonucleic acid (mtDNA) sequences in *Chaoborus* at a Holarctic scale to determine the origins of the Alaskan “*C. flavicans*”. Nuclear DNA, mtDNA, and morphological results indicated that the Alaskan *C. flavicans* represents a unique lineage of *Chaoborus*. The average mtDNA divergence (Kimura’s two-parameter) between the Palearctic *C. flavicans* and the Alaskan *C. flavicans* was >17%. mtDNA strongly supported *Chaoborus crystallinus* (De Geer, 1776) as the sister species to the Alaskan *C. flavicans*. Our present understanding of larval mandible morphology disagrees with the major ecological properties and evolutionary lineages of *Chaoborus*. We identify a new mandible character and highlight the value of validating the diagnostic morphologies of environmental indicator species with genetic studies.

There is now a consensus that during the last 30 yr the Arctic has undergone a dramatic warming, perhaps greater than any warming observed in the last 400 yr (Chapin et al. 2005). In some areas, such as Beringia, boreal forest and shrub tundra bands have expanded rapidly since 1850 (Serreze et al. 2000; Sturm et al. 2001). Moreover, pond surfaces have dramatically decreased from internal drainage and permafrost melting in the last century (Yoshikawa and Hinzman 2003; Ilyashuk et al. 2005; Smith et al. 2005). Little is known about how freshwater communities will be affected by climate change, but extensive changes have already occurred (Smol et al. 2005). Here we report that the boreal climate zone indicator species, *Chaoborus* cf. *flavicans* (Meigen, 1830), a phantom midge, is now common beyond the tree line in northwest Alaska (>150 km; Fig. 1). The historical distribution of *C. cf. flavicans* in Alaska is unknown, but the genus has colonized several tundra ponds on the Seward Peninsula since 2000. Johansen (1922) reported the coldwater specialist *Lepidurus*

arcticus (Crustacea: Branchiopoda) and unspecified dipterans from tundra ponds on the Seward Peninsula. However, *L. arcticus* has been undetected in recent surveys (2000–2005) of the same ponds (Taylor unpubl. data), suggesting that freshwater community changes related to warming may have occurred.

Chaoborus is an important invertebrate predator in temperate freshwater ecosystems, but it is rarely recorded in the Arctic. *Chaoborus trivittatus* is the only species that occurs well above the tree line (O’Brien et al. 2004). Indeed, temperature is an excellent predictor of the altitudinal and latitudinal distributions of *Chaoborus*, which are absent in waters with a midsummer surface water temperature <12°C (Lamontagne et al. 1994; Barley et al. 2006). *Chaoborus americanus* (Johanssen, 1903) needs a temperature cue to complete ecdysis (Quimet 2001) and will fail to emerge from pupation below 12°C. Walker and MacDonald (1995) proposed that the northern distributional limits of boreal *Chaoborus* species (i.e., the *Chaoborus* line) are likely better indicators of climate change than trees because the establishment of *Chaoborus* populations lacks the time lag of tree maturation. Moreover, unlike pollen, midge remains are produced in situ, which eliminates noise from long-distance dispersal in climate reconstruction efforts (Walker and MacDonald 1995; Fallu et al. 2005).

Historical reconstruction of global change is made possible by the preservation of *Chaoborus* mandibles in lacustrine sediments (Hofmann 1971; Walker and MacDonald 1995; Walker et al. 2003). The mandibles appear to possess diagnostic characters for species identification

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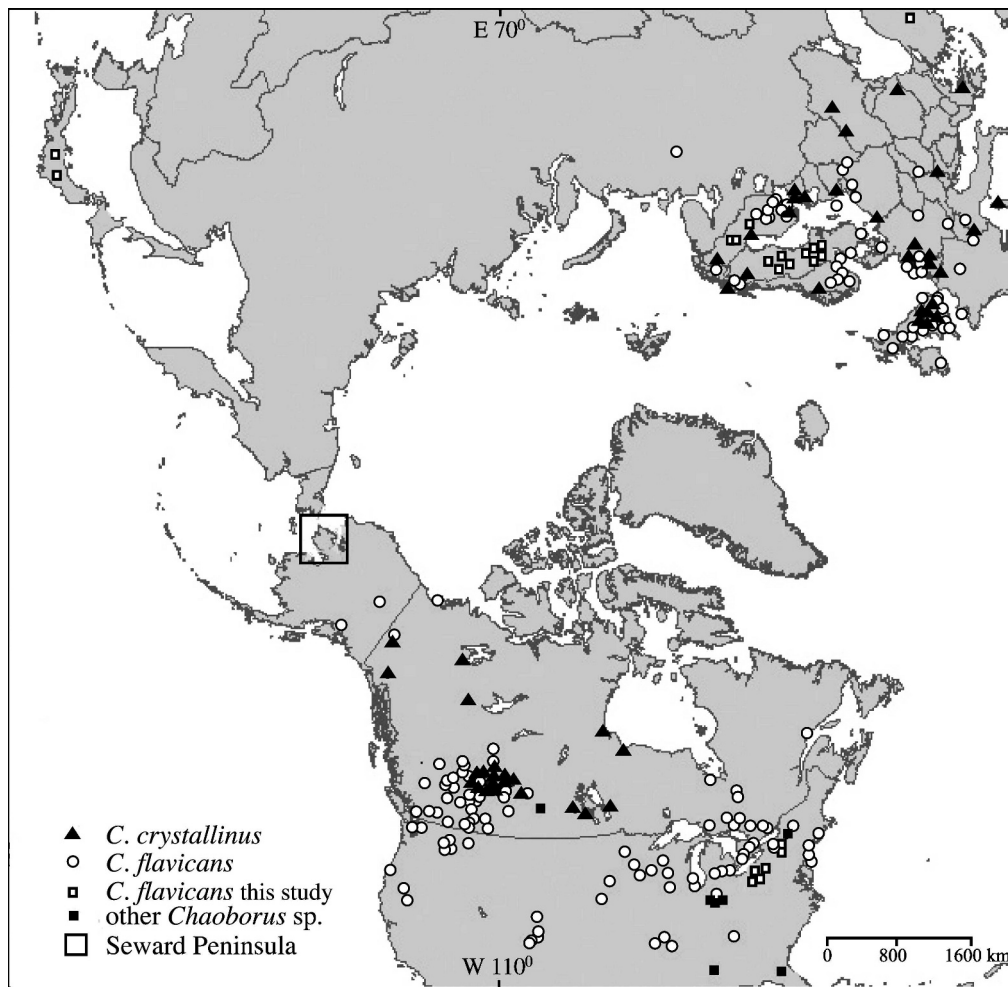


Fig. 1. A Holarctic map showing the distribution of *Chaoborus* sp. and the sample locations from the present study (see Web Appendix 1 for coordinates). Box symbols represent sites examined in the present study and triangle/circle symbols represent sites described by Borkent (1981). The black box in Alaska indicates study sites on the Seward Peninsula, a region beyond the known geographic range of *Chaoborus flavicans*.

(Saether 1970) that are consistent with mitochondrial deoxyribonucleic acid (mtDNA), habitat, and behavioral characters (Berendonk et al. 2003). *C. flavicans* is the only Holarctic species of *Chaoborus* in which the subordinate tooth of the mandible is located at the vertex between the posterior and medial teeth (Cook 1956; Saether 1970, 1997) (Fig. 2). *C. flavicans* is one of a few species of *Chaoborus* that has a habitat preference for lakes with fish (Borkent 1981) and undergoes diel vertical migration—a presumed adaptation that reduces visual predation by fish (Pope et al. 1973; Bass and Sweet 1984; Lamontagne et al. 1994). Several species including *Chaoborus crystallinus* and *C. americanus* tend to inhabit ponds without fish and lack diel vertical migration (Borkent 1981). The presence of *C. flavicans*-like mandibles in aquatic sediments has therefore been proposed or used as an indicator of boreal climate, water depth (Ilyashuk et al. 2005), and the presence of fish (Johnson et al. 1990; Uutala 1990; Lamontagne and Schindler 1994).

Despite a congruence of characters for some species, we lack a detailed understanding of *Chaoborus* species diversity and relationships. Part of the reason for the

uncertainty is that geographic sampling has been limited. Berendonk et al. (2003), for example, used only one population per species in their molecular phylogeny, when some species (*C. flavicans* and *C. crystallinus*) have near Holarctic distributions (the type locations of *C. flavicans* and *C. crystallinus* are in northern Europe). Wende et al. (2006) recently found that European and North American populations formed regional clades separated by 2% sequence divergence. Still, only the central section of North America has been sampled and the relationships of these clades to the proposed sister species, *C. crystallinus*, is unknown. It is also unknown if proposed complications of mtDNA-based phylogenies (e.g., selection) are acting in *Chaoborus* because no nuclear sequence phylogenies are available.

We found populations of *Chaoborus* in 15 small fish-free tundra ponds in northwestern Alaska, where *Chaoborus* was previously unknown (Fig. 1). The larval morphology (mandibles, palps, anal segments, and antennal seta) matched *C. flavicans* (Borkent pers. comm.), but the habitat character (small fishless ponds) agreed with *C.*

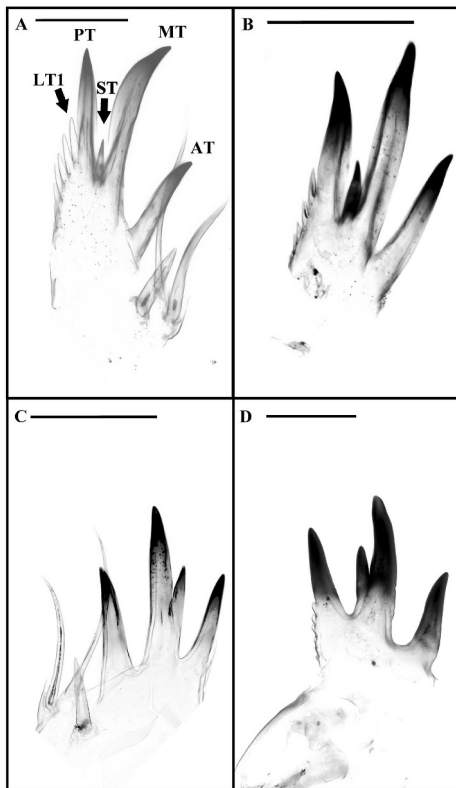


Fig. 2. Extended focus images of the mandibles of *Chaoborus* larvae showing potentially diagnostic morphologies. Abbreviations are: LT1, first lateral tooth; PT, posterior tooth; ST, subordinate tooth; MT, medial tooth; and AT, anterior tooth. (A) *Chaoborus flavicans* from a pond near Teller, Alaska showing a first lateral tooth and subordinate tooth of similar size; in other species the LT1 is much shorter than the ST (see also Uutala 1990); (B) *C. flavicans* from Sweden near the type location; (C) *C. americanus* from Council, Alaska near the tree line (oriented with lateral teeth on the right side); and (D) *C. crystallinus* from Sweden. Scale bar is 100 μm for all panels.

crystallinus. We carried out a Holarctic phylogeographic analysis of *Chaoborus* to determine the origins of the Alaskan tundra specimens. We collected both mtDNA and nuclear protein coding sequences and scored morphological characters. Our sampling focused on the proposed *C. flavicans*–*C. crystallinus* clade. The results indicate that the colonists of the tundra habitat are not *C. flavicans*, but a distantly related cryptic species. Thus, our current understanding of larval morphology (used in aquatic ecology, paleolimnology, and climatology) may be inadequate to distinguish even distantly related species of *Chaoborus*.

Methods

Sample collection—We collected *Chaoborus* larvae from 48 Holarctic lakes and ponds and identified the specimens according to Cook (1956) and Saether (1970, 1997) (see Web Appendix 1, www.aslo.org/lo/toc/vol_53/issue_1/0236a1.pdf). Those samples, usually with duplicates, were sequenced for *COI* and *HSP90*. Ten *Chaoborus* sequences

(Berendonk et al. 2003) and outgroup mosquito sequences were obtained from Genbank (see Web Appendix 1). We scored five distinct morphological characters between *C. flavicans* and *C. crystallinus*: antennal hair location, subordinate mandibular tooth placement, mandible coloration, dorsal projection shape, and anal tubule shape. The diagnostic features of the mandibles, which are preserved in lacustrine sediments (Uutala 1990), are shown in Fig. 2. After the genetic analysis, 20 additional individuals from the Alaskan (test) and Scandinavian (reference) groups were scored for the relative sizes of the first lateral tooth and subordinate teeth (Fig. 2).

Laboratory methods—We preserved specimens in 90% ethanol or acetone and extracted the DNA from the posterior half of the animals using DNA Quickextract (Epicentre). *Chaoborus*-specific polymerase chain reaction (PCR) primers were designed for a nuclear gene, *HSP90* (HSPF 5'-CCGATGCCCTCGATAAAATCC-3'; HSPR 5'-ACATCTTCAATTTTGGGTTTCATC-3') and a mitochondrial gene *COI* (COIF 5'-GAAGCTAAAATTCAATGCACTAGTCT-3'; COIR 5'-CTTATTTTACTTCAGCAATAATTA-3'). We carried out standard PCR amplification for each gene and sequenced 89 individuals from 43 populations for *COI* (mtDNA) and 65 individuals from 31 populations for *HSP90*. The *COI* primers amplified a segment of approximately 690 base pairs (bp) and the *HSP90* primers amplified a segment of approximately 620 bp. PCR amplifications contained 0.3 $\mu\text{mol L}^{-1}$ of each primer, 0.25 $\mu\text{mol L}^{-1}$ each of the four deoxynucleotide triphosphates, and 1 U of *Taq* polymerase in 10 \times PCR buffer. These preparations were placed in a thermocycler (Minicycler, MJ Research) for 40 cycles of the following program: 30 s at 95 $^{\circ}\text{C}$ for denaturation, 30 s at 48 $^{\circ}\text{C}$ for annealing, and then 90 s at 72 $^{\circ}\text{C}$ for elongation. PCR products were detected on a 1% agarose gel with ethidium bromide and 0.5 \times Tris–boric acid–EDTA buffer. Most specimens could be amplified with both nuclear and mtDNA primers, but several poorly preserved specimens failed to amplify with either the mtDNA or the *HSP90* primers. Upon verification of successful PCR, the products were purified and both strands were sequenced (Genaisance Pharmaceuticals).

Phylogenetic inferences—Sequences were aligned using ClustalX 1.81 (Higgins and Sharp 1988) and edited with Sequencher 4.2.2 (Genecodes). Unreliable sequences from the ends of the sequences were trimmed. For both the *COI* gene and the *HSP90* gene, all sites were aligned unambiguously with no indels in the final alignment.

Phylogenetic analyses were carried out with Bayesian analysis, maximum parsimony (MP), and neighbor-joining (NJ) with maximum likelihood distances. To assess clade reliability, we used Bayesian posterior probabilities and nonparametric bootstrapping (10,000 pseudoreplicates). Bayesian analysis was implemented in MrBayes (Huelsenbeck and Ronquist 2001) with partitioned codon positions and a model with six substitution sites and among site rate variation. For each run of the program, four chains were run for 1,000,000 generations to estimate the posterior

probabilities. Trees were sampled every 1000 generations. The likelihoods were graphed and trees were conservatively removed to ensure that the burn-in phase was removed. The trees were then imported into PAUP* (Swofford 2002) and a 50% majority-rule consensus tree was constructed.

Results

mtDNA and nuclear phylogenies of C. flavicans—Morphological assignment of larval specimens to *C. flavicans* or to *C. crystallinus* was ambiguous with the soft-tissue characters, but clear with the mandibular characters. The mtDNA phylogeny revealed that *C. flavicans*-like specimens had geographic structure with two divergent clades (Fig. 3). The Alaskan *C. flavicans* grouped with other Beringian specimens from Japan and western North America, and was the sister group to the Palearctic *C. crystallinus*. The remaining temperate North American specimens formed a closely related clade (Kimura's two-parameter distance or $K2 < 0.01$) that was the sister clade to the Palearctic *C. flavicans* (Scandinavia and Turkey). These nodes were well supported by posterior probabilities ($p = 1.0$) and MP bootstrap values but not NJ bootstrap values. The average K2 distance between the two divergent clades of *C. flavicans* was 17.6%.

The same divergent clades of *C. flavicans* were also found in the phylogram estimated for the nuclear *HSP90* gene (Fig. 4). Clade 1 was recovered with $p = 1.0$ posterior probability and high MP and NJ bootstrap values, whereas clade 2 had modest support (81–100) for monophyly. Although populations of species grouped with strong support using then nuclear *HSP90* gene, among-species relations were unresolved. After the genetic analyses, we re-examined the mandibles of clade 1 and clade 2 and noticed that the relative size of the first lateral tooth of the mandibles of clade 2 specimens from Alaska is unique for Holarctic *Chaoborus*. Specifically, the first lateral tooth is at least as large as the subordinate tooth of the mandible. In other *Chaoborus* species the first lateral tooth is much smaller than the subordinate tooth of the mandible (Fig. 2).

Discussion

Our study reveals that *C. flavicans*-type larvae likely represent at least two cryptic species. The concordance of mtDNA and nuclear DNA (*HSP*) makes alternative explanations such as incomplete lineage sorting, selection, and introgression unlikely. Moreover, the large mitochondrial genetic distance between the putative cryptic species (17.6%) makes hybridization and lineage sharing less likely. These groups also appear to have morphological differences (i.e., the relative lengths of the lateral teeth and subordinate teeth of the mandible; Fig. 2). Finally, the two major clades seem to group according to habitat (a lake-dwelling *C. flavicans* one clade and a pond-dwelling *C. crystallinus*: *C. flavicans* a second clade), providing further independent evidence for the reality of cryptic species.

The results highlight the value of a combined genetic and morphological diagnosis of species and of genetically verifying characters used in paleobiology. We had reference

COI sequences available for the Holarctic *Chaoborus*, but the genetic analysis alone failed to classify the tundra specimens because the new sequences were unique and divergent from the reference sequences in Genbank. Existing morphological keys also failed because the tundra larvae incorrectly keyed to *C. flavicans*. A post hoc assessment of the morphological differences of the divergent genetic clades allowed us to identify a potentially diagnostic morphological character for the tundra species (the relative size of the first lateral tooth and subordinate tooth).

Our results show that a cryptic species related to *C. crystallinus* has colonized the subarctic tundra ponds of northwest Alaska. It is likely that these populations advanced from boreal forest ponds or fishless lakes in Alaska, as near identical haplotypes were found in the boreal forests of Alberta and Alaska. The response to climate warming is expected to be more rapid in pond specialists than in lake specialists such as *C. flavicans* s.s. because subarctic ponds are expected to reach boreal-zone temperatures faster than subarctic lakes. Interestingly, our personal observations indicate that the Alaskan *C. flavicans* lack diel vertical migration, a behavior that is apparently innate in *C. flavicans*. It is unknown if *C. flavicans* s.s. has advanced beyond the tree line in Alaska. We did detect *C. americanus* near the tree line in Council, Alaska, but it was absent from the tundra and tundra-shrub ponds of the Seward Peninsula. The species' habitat difference is consistent with Lamontagne et al. (1994), who found that the midsummer temperature minimum of *C. flavicans*-containing lakes was 2°C cooler than the minimum for *C. americanus* in alpine Alberta.

Our results have some potential implications for environmental reconstruction in addition to the existence of cryptic species. In most of North America, the range of *C. flavicans* and all species of the genus *Chaoborus* sharply follows the tree line. In the tundra ponds where *Chaoborus* was abundant, the mid-July temperatures averaged 15.4°C, suggesting that there is nothing unusual about the temperature tolerances for the cryptic species we found. However, the results do confirm the prediction (Walker and MacDonald 1995) that *Chaoborus* ranges respond more rapidly to climate warming than some types of terrestrial vegetation used in paleobiology such as trees. Our findings also suggest that caution be applied when using *C. flavicans*-type mandibles in aquatic sediments as a proxy for the profundal zone, lake depth, or the presence of fish in the Nearctic. We found *C. flavicans*-like larvae to be common in shallow (<3 m) fishless tundra ponds, but we can't rule out their presence in larger bodies of water. More studies are needed to understand the ecological differences among the members of the *C. flavicans* complex.

Our phylogenetic analysis generally revealed strong support for shallow clades of *Chaoborus* and weak support for the deepest branches. Unlike previous proposals (Borkent 1981; Berendonk et al. 2003), we found no evidence that *C. crystallinus* and *C. flavicans* (sensu stricto from Europe) are closely related sister taxa. Indeed we found no support for the subgenus *Chaoborus* (*C. flavicans*, *C. crystallinus*, *C. americanus*, and *C. obscuripes* [Cook

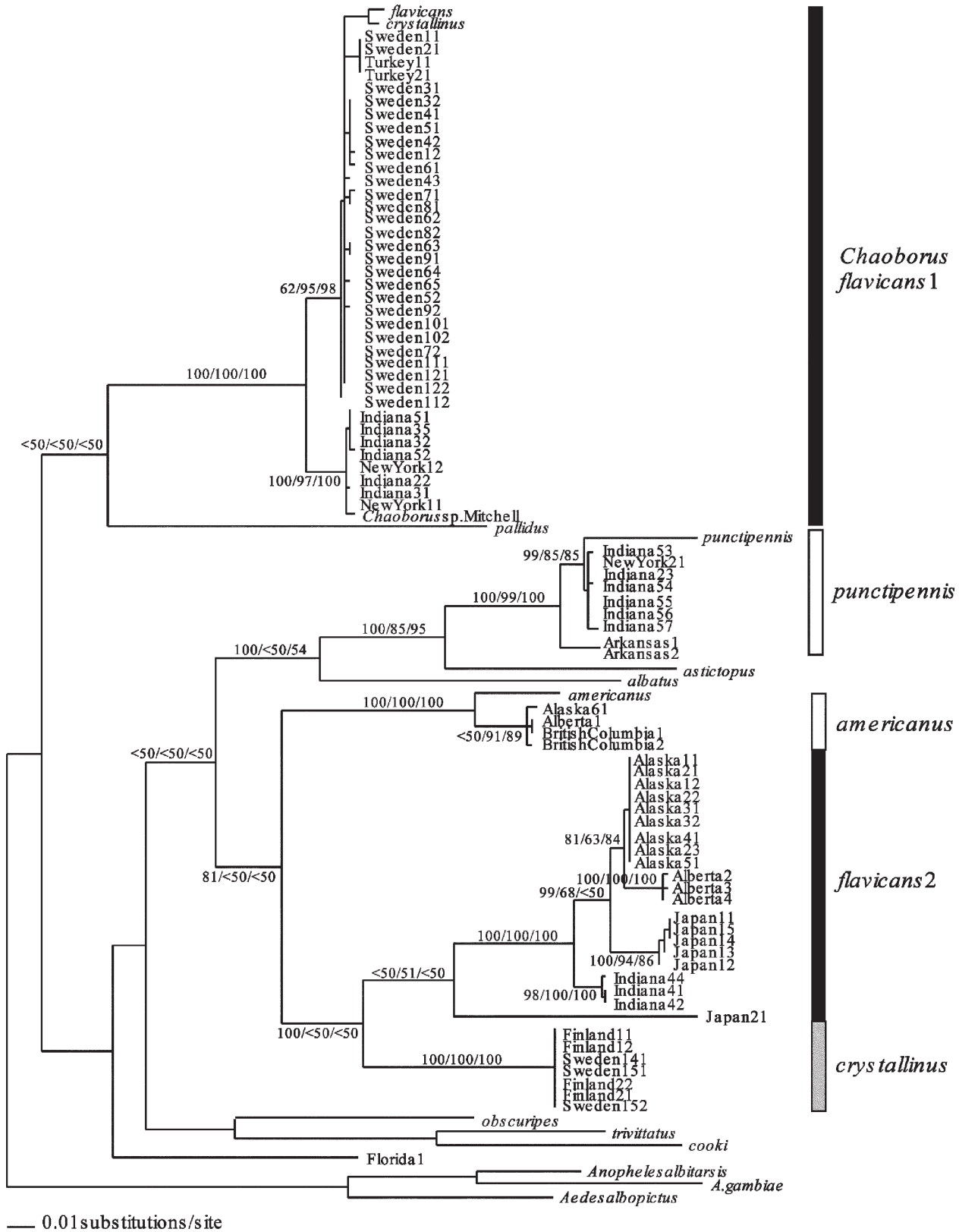


Fig. 3. Phylogram for *Chaoborus* estimated from partial mitochondrial *COI* nucleotide sequences. Branch support values are given above branches (Bayesian posterior probabilities/maximum parsimony bootstrap/neighbor-joining bootstrap). Vertical black bars represent larvae with a *Chaoborus flavicans*-like morphology. Open bars represent multiple populations of additional species of *Chaoborus*. Sequences with nongeographic (taxonomic) names and outgroup sequences of mosquitoes are from Genbank (see Web Appendix 1).

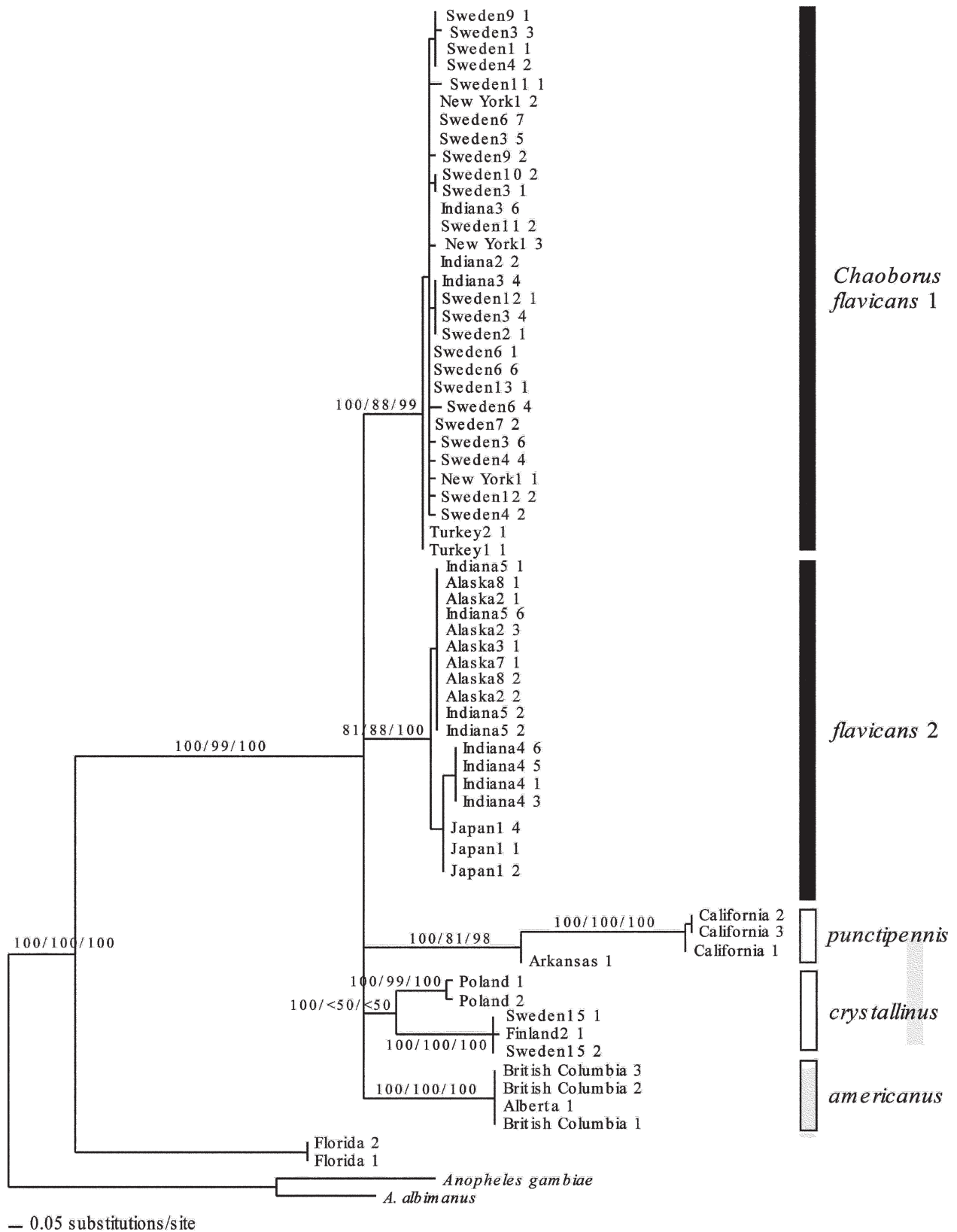


Fig. 4. Phylogram for *Chaoborus* estimated from partial nuclear *HSP90* nucleotide sequences. Branch support values are given above branches (Bayesian posterior probabilities/maximum parsimony bootstrap/neighbor-joining bootstrap). Vertical black bars represent larvae with a *Chaoborus flavicans*-like morphology. Open bars represent multiple populations of additional species of *Chaoborus*. Sequences with nongeographic (taxonomic) names and outgroup sequences of mosquitoes are from Genbank.

1956; Saether 1970]). Lack of informative sites is perhaps the most common cause of phylogenetic incongruence in empirical data (Taylor and Piel 2004). In the case of *Chaoborus*, genes with a less conserved amino acid substitution rate than *COI* and *HSP90* may better resolve higher-level relations.

The mtDNA results provide some insights into the phylogeography of *Chaoborus* in the Holarctic. Despite the ability to actively disperse with flight, we find some evidence that *C. flavicans* s.s. (i.e., our *flavicans* clade I) is subdivided at the continental level. This agrees with the recent findings of Wende et al. (2006). More geographic sampling is necessary within continents to understand how this division arose, but the near identity of geographically distant specimens (Turkey and Sweden; Indiana and New York) within a continent favors partitioning in separate glacial refugia over simple isolation by distance as an explanation. The phylogeographic structure of Holarctic *C. crystallinus* remains unknown because we failed to detect the species in our North American samples. It is possible that one of our pond-dwelling *C. flavicans*-type clades has been confused for North American *C. crystallinus* in prior studies. Still, there are mandible differences and pronounced nuclear DNA and mtDNA differences between *C. crystallinus* from near the type location in Scandinavia and the North American pond-dwelling species that we sampled. Thus the status of *C. crystallinus* in North America remains ambiguous.

In conclusion, we have provided evidence that two or more divergent cryptic species of *C. flavicans* exist. At least one of these species has colonized shallow tundra ponds in northwest Alaska. The use of *C. flavicans* as an indicator in paleoenvironment studies needs to be re-evaluated because the current mandible characters may perform poorly as indicators of depth and fish. Our results also provide evidence that combining genetic analysis with morphological analysis may provide new paleolimnological characters that can reduce noise introduced by the presence of cryptic species.

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