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Associating larvae and adults of Chinese Hydropsychidae caddisflies (Insecta:Trichoptera) using DNA sequences

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Abstract. The utility of hydropsychid (Trichoptera:Hydropsychidae) caddisfly larvae for freshwater biomonitoring has been demonstrated, but the major impediment to its implementation has been the lack of species-level larval descriptions and illustrations. A rapid and reliable molecular protocol that also uses morphology is proposed because conventional approaches to associating undescribed larvae with adults have been slow and problematic. Male adults were identified before DNA sequence analyses were used. These identifications established morphospecies boundaries that were mapped on phylograms constructed from 2 independent gene fragments: mitochondrial cytochrome c oxidase subunit I (COI) and large subunit (28S) nuclear ribosomal DNA expansion fragment D2 (D2). Species boundaries were confirmed if they were monophyletic on both molecular phylograms. Larval associations were made with reference to the phylogenetic analyses under 2 criteria: sequence identity across both genes or nested placement within a reference species boundary. A total of 133 individuals belonging to Chinese *Hydropsyche sensu lato* group (including *Hydropsyche* [*Hydropsyche*], *Hydropsyche* [*Occutanspsyche*], *Ceratopsyche*, *Mexipsyche*, *Hydatomanicus*, and *Herbertorossia*) were included in our study to test the new protocol. D2 sequences (all individuals) and COI sequences (101 individuals) were obtained, and 2 independent phylograms were constructed using neighbor joining. Both fragments provided enough nucleotide changes to differentiate independently most *Hydropsyche sensu lato* species, with ambiguity in only a few species that eventually could be resolved with additional sequences and specimens. COI diverges significantly within some species, suggesting a need for caution when applying typical genetic divergence thresholds in species diagnoses. The study enabled us to establish a procedure for delimiting species boundaries and associating larvae and adults using DNA sequences and morphological evidence. Ideal sampling strategies for larval–adult association are suggested. Associating larvae and adults of hydropsychids using DNA sequences appears to be promising in terms of both reliability and speed.

Key words: Hydropsychidae, species boundary, larval–adult association, 28S ribosomal DNA, mitochondrial COI, species barcodes, freshwater biomonitoring.

Water pollution is rapidly depleting potable water resources in China (Stockholm Environment Institute and United Nations Development Programme [UNDP] China 2002, UNDP 2006]. Freshwater biomonitoring, which involves identifying the species inhabiting an ecosystem to provide an ongoing assessment of water quality, promises to be an efficient and cost-effective method for helping China to manage its valuable water resources (Morse et al. 2007). Caddisfly larvae are used widely in freshwater biomonitoring because of their great abundance and the wide range of pollution tolerances among their species. Hydropsychid caddis-

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TABLE 1. Known Hydropsychidae genera[a] and associated larvae of Chinese species.

Genera	No. of species recorded	No. of larvae described in previous studies[b]
Subfamily Arctopsychinae		
Arctopsyche	8	1
Parapsyche	8	0
Subfamily Diplectroninae		
Diplectrona	6	0
Subfamily Hydropsychinae		
Cheumatopsyche	19	1
Potamyia	10	0
Hydromanicus	12	0
Hydatopsyche	2	0
Hydropsyche	10	2
Ceratopsyche	23	3
Mexipsyche	13	0
Herbertorossia	1	1
Hydatomanicus	1	0
Subfamily Macronematinae		
Macrostemum	10	2
Amphipsyche	4	1
Oestropsyche[c]	1	0
Trichomacronema[c]	3	0
Aethaloptera	1	1
Polymorphanisus	4	1

[a] Checklist compiled from Yang et al. 2005, where *Hydropsyche*, *Ceratopsyche*, and *Mexipsyche* were treated as subgenera of *Hydropsyche sensu lato*; *Arctopsyche* and *Parapsyche* were in Family Arctopsychidae

[b] Including 4 species illustrated by Dudgeon (1999), but without further description

[c] *Oestropsyche* and *Trichomacronema* are newly discovered in China. Descriptions will be published elsewhere by other authors. One new *Trichomacronema* species, *Trichomacronema anthodeum* is being described by C. Sun, Nanjing Agriculture University, and XZ (unpublished data)

flies are among the most frequently encountered macroinvertebrates in freshwater habitats. Hydropsychids have a dramatically wide range of tolerance values (Lenat 1993). However, their application in biomonitoring has been greatly impeded by the lack of identified and illustrated larvae, especially in countries such as China, where there has been limited research on larval identification. Thus, species identification has become a prerequisite for biomonitoring.

The identification of caddisfly larvae also is important to studies of higher-level trichopteran phylogenetics (Scott 1975, 1983, Schuster 1977, 1984, Schuster and Etnier 1978, Wiggins 1981, 1996, Schefter and Wiggins 1986, Frania and Wiggins 1997). The ability to associate larvae with adults and to identify Chinese hydropsychid larvae will help us to understand the phylogenetic status of Oriental caddisfly groups, such as *Hydromanicus* Brauer, 1865; *Hydatopsyche* Ulmer, 1926; *Hydatomanicus* Ulmer, 1951; *Trichomacronema* Schmid, 1964; and others.

Most caddisfly species are identified from adult males because male genitalia are complex, relatively invariant within species, and diagnostic among species. Therefore, taxonomy in caddisflies is based on morphological characters of adult males. Larvae must be associated with identified adults (usually males) to be described and illustrated at the species level. Conventional approaches to larval association usually involve rearing larvae or morphological identification of metamorphotypes (mature pharate adult, larval sclerites, and pupal exuviae in the same pupal case) (Milne 1938, Wiggins 1996). Both approaches work well when adequate resources and expertise are applied (Resh 1972, Floyd 1995, Glover 1996). However, both approaches have limitations. Larvae that develop into adults no longer exist as larvae, and descriptions must be made from similar (deemed identical) individuals. In addition, larval rearing is complicated by our imperfect understanding of species-specific microhabitat and water-chemistry requirements, particularly for some groups, such as hydropsychids. Metamorphotypes are relatively rare because that portion of the life cycle occurs for a short time only, which means that chance encounters play a significant role in metamorphotype associations. These factors might explain why a large portion of the caddisfly fauna remains unassociated, although the problem is also undoubtedly linked to a lack of resources, expertise, and effort applied to larval association, using either traditional or molecular methods.

To date, 1603 hydropsychid species have been described worldwide (Morse 2006). Yang et al. (2005) recorded 136 hydropsychid species, including Arctopsychinae, which is treated as a separate family by some workers (Schmid 1968, Nimmo 1987, Mey 1997, Gui and Yang 2000) from China. Formal descriptions do not exist yet for most Chinese caddisfly larvae. Only 13 of the known Chinese hydropsychid species, mostly from foreign populations, have been associated and described (or illustrated), whereas most endemic larvae remain unknown (Table 1). Among the 18 Chinese hydropsychid genera (Table 1), the larvae of *Hydatopsyche* and *Hydatomanicus*, both of which are distributed mainly in the Oriental region, remain undescribed. Considering the facts that a significant portion of Chinese hydropsychid species are still unknown and that natural habitats are disappearing rapidly in China, more resources should be applied to associating larvae and adults of Chinese caddisflies, especially hydropsychids, and new approaches should be developed as soon as possible. The molecular method for larval association, discussed below, could

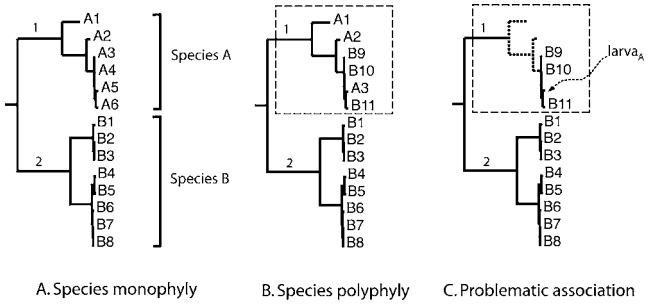

FIG. 1. Hypothetical species boundaries and problematic larval–adult association. Adults from species A and B are identified based on morphology before a phylogenetic analysis. Species boundaries are then mapped on the gene phylogram constructed from a DNA fragment. A.—Species boundaries without paraphyly/polyphyly: both species A and B are well delimited. B.—Species boundaries with paraphyly/polyphyly: adult individuals from both species mixed together in clade 1. C.—Problematic association: if adults of species A were not sufficiently sampled, a larval specimen of species A ($larva_A$) will be associated incorrectly with species B.

significantly accelerate the process of larval descriptions for a poorly known caddisfly fauna.

A clear statement of the species concept used and a method for delimiting species are critical in interpreting species boundaries using DNA sequences. A history-based phylogenetic species concept (Baum and Donoghue 1995) was used in our study because the DNA sequence for a particular gene is identical for all life stages and phylogenetic relationships among DNA sequences can be inferred. Under this species concept, conspecifics (individuals of the same species) are considered more closely related to each other than to members of any other species. Fewer changes in nucleotide sequences are expected among individuals within a species than between those individuals and members of other species. However, delimiting species boundaries based solely on estimated mean genetic divergence can be arbitrary and, therefore, is problematic.

Some success in associating life stages in some invertebrates has been realized through the use of DNA sequences, but few such studies have been done with insects (Sperling et al. 1994, Aoki et al. 1997, Wells et al. 2001, Shan et al. 2004a, b, Miller et al. 2005). In most studies of insects, a single DNA sequence (or linked sequences with dependent histories) taken from identifiable individuals of a certain life stage, such as adult males or late-instar larvae (e.g., some mosquitoes and mayflies), has been used as a reference. Once an individual of the alternative life stage is sequenced, a comparison of this test sequence to the reference sequence(s) can provide an association under certain criteria of species delimitation. Species boundaries often are delimited by the overall genetic similarity of the test sequence to the reference sequences, although the boundaries are not always explicitly expressed. Some researchers have proposed that some typical range of genetic divergence (*threshold*) exists among and within species. However, one should be cautious when applying these typical thresholds to other taxa because genetic divergence among species varies across taxa; a set genetic distance that typically defines species boundaries in one group might not be applicable to others. This caveat is particularly true for taxa that evolved rapidly, forming diversified cryptic species complexes, while other lineages remained less species diverse. Hence, species boundaries based on genetic distance must be defined specifically for various taxa, and rate changes can occur at any point in time.

Some researchers have applied a threshold generated from the data set used within their study (e.g., Miller et al. 2005). However, this approach has disadvantages. The average interspecific divergence can be reduced greatly by extensive sampling of taxa. Moreover, if the focal species is paraphyletic or polyphyletic, intraspecific divergence is dependent on the degree of polyphyly (Funk and Omland 2003). Paraphyly or polyphyly typically might not be detected if only a single gene is used in the study. More important, the logic of using only within-study data is circular because species boundaries and the distance criterion used to set them are determined from the same data. The species boundary should be defined or supported by sources other than the DNA sequences themselves, e.g., independent genes or morphology or both. Furthermore, the genetic divergence of a single gene might not provide enough resolution to differentiate closely related taxa, especially the youngest sister species (Hebert et al. 2003, Hebert and Gregory 2005). The potential for random lineage sorting of ancestral polymorphisms and introgressive hybridization can complicate diagnoses even further (Sota et al. 2001). Occasional sharing of mitochondrial sequences across species boundaries might not severely limit the utility of the sequences for providing species diagnoses in a large-scale project, such as the DNA barcoding initiative (Hebert and Gregory 2005), but it can lead to incorrect larval–adult association, particularly in closely related species (Fig. 1A–C).

Gene choice

Sequencing multiple independent genes might permit detection of species-level polyphyly. Moreover, no gene is ideal for all purposes, and the information provided by several independent genes might be

complementary. Nevertheless, most existing work on life-stage association has relied on a single gene or linked genes with dependent histories (e.g., mitochondrial cytochrome c oxidase subunit I [COI]/cytochrome c oxidase subunit II/transfer RNA (tRNA); but see Caterino et al. 2006), which should be treated as a single line of evidence for tracing gene histories. We propose using 2 independent gene fragments, one from mitochondrial COI and a second from large subunit (28S) nuclear ribosomal DNA (nrDNA; the nuclear gene that codes for ribosomal RNA), to construct phylogenetic trees from which species boundaries and the association of larvae and adults are made.

Mitochondrial genes (mtDNA) are used most frequently in species-level work. A rapid coalescence rate, high copy number, lack of introns, and the availability of universal primers for most animals are the major advantages of using mtDNA rather than other genetic markers for species diagnosis and phylogenetic studies. Mitochondria are rarely affected by recombination and paralogy (where genes without strict orthologous relationships are being compared) because they are maternally inherited. However, recombination of mtDNA has been discussed for some animals (e.g., Ladoukakis and Zouros 2001, Smith and Smith 2002), and the presence of mitochondrial pseudogenes in the nucleus (Simon et al. 1994, Villegas et al. 2002, Hay et al. 2004, Antunes and Ramos 2005, Schmitz et al. 2005) sometimes could result in incorrect phylogeny reconstructions. Like most protein-coding genes, mitochondrial protein-coding genes have few insertions and deletions (usually none among closely related species), reducing alignment problems. Furthermore, mtDNA haplotypes have smaller effective population size (N_e) than nuclear genes. In theory, their coalescence time is only ¼ that of nuclear genes (Palumbi et al. 2001, but see Hudson and Turelli 2003). Mitochondrial genomes are present in multiple copies, so they are much easier to amplify by polymerase chain reaction (PCR) than single-copy nuclear protein-coding genes. Numerous mtDNA primers have been described (e.g., Simon et al. 1994). The COI gene is, perhaps, the most sampled mitochondrial protein-coding gene and was selected in the DNA barcoding project for its robust primers and relatively conservative amino acid composition (Hebert et al. 2003). Moreover, a fragment of COI sequence close to the 5′ end is easily amplified with primers developed specifically for caddisflies by Kjer et al. (2001).

One of the major difficulties when using independent genes is finding a nuclear gene that is easily amplified and sufficiently variable to use in addition to the widely adopted COI. Small subunit (18S) nrDNA was used for associating larvae of Hetaeriinae hister beetles (Caterino et al. 2006). However, 18S nrDNA failed to provide sufficient characters to diagnose some species, even though the relevant species were from different genera. Thus, the low variation in the 18S nrDNA sequence within this subfamily has seriously limited its usefulness as an independent gene marker for species diagnosis.

We used the D2 region of 28S nrDNA as an independent DNA fragment. Nuclear ribosomal DNA belongs to a multigene family, where hundreds to thousands of copies of the nrDNA unit appear in tandem along the chromosome. Unlike most nuclear genes, which are present as single (or a few repeating) copies, the numerous copies of an nrDNA unit become homogenized very rapidly by molecular drive (Dover 1984). Therefore, this concerted evolutionary process minimizes the effects of paralogy.

The D2 expansion fragment of 28S ribosomal RNA (rRNA) (the "545 region" of Schnare et al. 1996; hereafter D2) is one of the most highly variable regions in eukaryote rRNA. The length and nucleotide composition of this fragment is highly variable among insects (Gillespie et al. 2004). These significant variations limited the utility of D2 in deep-level phylogenetics because of difficulties in alignment, although universally conserved RNA secondary structures have provided solutions for some taxa (e.g., Gillespie et al. 2004). However, length variation is not as severe a problem among closely related hydropsychid species. Large insertions or deletions potentially could be encountered at any level, including between sister species. However, our preliminary results in caddisflies indicate that the length of D2 is very conservative within all Chinese *Hydropsyche sensu lato* group genera (hereafter *Hydropsyche* group) that we have sampled. The changes in the hypervariable regions provide an opportunity to differentiate closely related species, even when they cannot be aligned across distantly related taxa. In fact, D2 provided sufficient genetic variation to distinguish 2 species of *Encarsia* wasps that could not be distinguished morphologically (Babcock and Heraty 2000), and we will show that D2 can distinguish closely related species in the most species diverse Hydropsychidae subfamily, Hydopsychinae. Furthermore, the highly conservative core segments that flank D2 serve as ideal anchor points for primers.

Delimiting species boundary based on phylogenetic congruence

In our study, the species boundary is defined both morphologically and phylogenetically. Morphological characters are mapped upon phylogenies constructed from D2 and COI data collected from identified adult males. All individuals that share the same genital

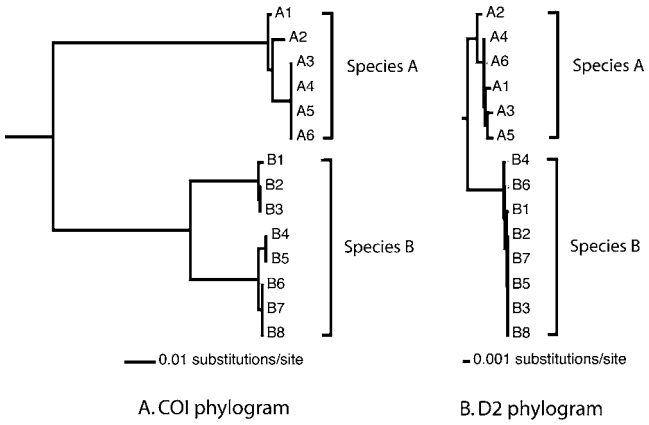

FIG. 2. Delimiting species boundary based on gene and morphology congruence. Species A and B are identified based on morphology before phylogenetic analysis. Species boundaries are, in turn, confirmed by gene congruence across independent mitochondrial cytochrome c oxidase subunit I (COI) (A) and large subunit nuclear ribosomal 28S expansion fragment D2 (D2) (B) sequences.

structures and are part of a monophyletic group on the phylogram are putatively considered to be members of the same species. If these putative, morphologically identified species boundaries are the same on the 2 independent gene phylograms, a working species boundary is established.

The application of morphology to confirm the species boundary is critical because: 1) the genes may not be able to reveal the real history of speciation because of the properties discussed previously, 2) mapping species boundaries on the DNA phylogram using morphology avoids the problem of defining species by arbitrary genetic divergence values, and 3) morphology provides a 3rd independent reference to species boundaries. In fact, morphological characters can be assumed to represent multiple molecular loci and, thus, are more representative of the *species tree*, than are individual *gene trees*.

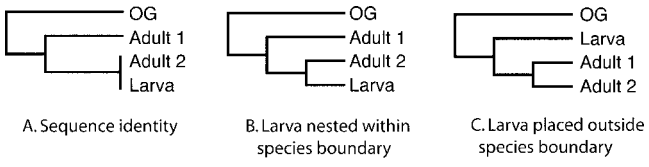

FIG. 3. Larval–adult association criteria. A.—A larva is considered associated if the sequences are identical across both cytochrome c oxidase subunit I (COI) and large subunit nuclear ribosomal 28S expansion fragment D2 sequences. B.—A larva is considered associated when it nests inside a clade of identified adults. C.—Additional adult sampling is required until the larva satisfies 1 of the 2 association criteria. OG = outgroup.

TABLE 2. *Hydropsyche sensu lato* species recognized in our study. Data are the total number of species in the genus/number of putative species (species that are probably new and are not yet assigned scientific names) that are counted in the total number of total species. Numbers after species names are group codes.

Genus	No. of species/no. of putative species in the genus	Species and group codes
Hydropsyche	4/1	*Hydropsyche hedini* (1)
		Hydropsyche (Occutanspsyche) polyacantha (10)
		Hydropsyche 20060328_02 (11a)
		Hydropsyche formosana (11b)
Ceratopsyche	17/7	*Ceratopsyche* n sp d15 (4)
		Ceratopsyche kozhantschikovi (8)
		Ceratopsyche 20060320_01 (19)
		Ceratopsyche gautamittra (20)
		Ceratopsyche 20060315_01 (21)
		Ceratopsyche 20060314_01 (22)
		Ceratopsyche fukienensis (23)
		Ceratopsyche conoidea (24)
		Ceratopsyche compressa (25)
		Ceratopsyche 20060316_01 (26)
		Ceratopsyche CR09 (27)
		Ceratopsyche simulata (28)
		Ceratopsyche penicillata (29)
		Ceratopsyche sp118 (30)
		Ceratopsyche tetrachotoma (31)
		Ceratopsyche columnata (32)
		Ceratopsyche serpentine (33)
Mexipsyche	11/8	*Mexipsyche rhomboana* (5)
		Mexipsyche 20060406_01 (6)
		Mexipsyche 20060406_02 (7)
		Mexipsyche 20060413_01 (9)
		Mexipsyche grahami c1 (12)
		Mexipsyche n sp 2005_01 (13)
		Mexipsyche n sp *2005_02* (14)
		Mexipsyche 20060414_01 (15)
		Mexipsyche furcula (16)
		Mexipsyche grahami (17)
		Mexipsyche grahami c10 (18)
Herbertorossia	1/0	*Herbertorossia quadrata* (3)
Hydatomanicus	1/0	*Hydatomanicus ovatus* (2)
Total	34/16	

We propose the following molecular approach, integrated with morphology, to delimit the species boundary:

1) Construct phylogenetic trees based on independent analyses of both D2 and COI gene fragments collected from adult males.
2) On the phylograms, delimit tentative species boundaries based on male genital morphology.
3) Compare the 2 gene trees. If a tentative species boundary, defined by morphology, proves to be monophyletic on both trees, the species boundary

is delimited (Fig. 2A, B). If polyphyly appears on one or both of these trees within a tentative species boundary (Fig. 1B), the species delimitation cannot be determined at this time. More complete sampling is required to clarify the specific cause of the polyphyly, but a growing database of DNA sequences, to which additional samples can easily be added, is established.

Association criteria

Once species boundaries (based on male adults) are delimited, larval sequences can be placed into the analysis. The association is made from the resultant phylograms. The criteria for associating larvae and adults are established based on the placement of the unknown larvae in the trees, relative to the placement of identified adults. In the following schemes (Fig. 3A–C), the reference species boundary is represented by adult individual 1 and adult individual 2, the representatives of the most distant individuals in the species clade (e.g., A1 and A6 in Fig. 2A or A2 and A5 in Fig. 2B). Many other adults could nest within individuals 1 and 2. We expect 3 different scenarios: 1) 1 or more larvae are identical to 1 or more of the sequenced adults across both genes (sequence identity; Fig. 3A), 2) a larval sequence nests within the reference species (Fig. 3B), or 3) the larval sequence is placed outside of a reference species (Fig. 3C). Scenarios 1 and 2 are both successful associations. More individuals are needed if larval sequences do not nest within a reference species (scenario 3; Fig. 3C). In most cases, the desired additional taxa needed to resolve the problem would be adult males with the same genital morphology from a wider geographical range.

*Taxa (*Hydropsyche *group)*

We tested species boundaries using taxa from 5 Chinese genera: *Hydropsyche* [including *Hydropsyche* (*Hydropsyche*) and *Hydropsyche* (*Occutanspsyche*)], *Ceratopsyche*, *Mexipsyche*, *Herbertorossia*, and *Hydatomanicus*. Among these genera, *Ceratopsyche* and *Mexipsyche* were sometimes considered subgenera of *Hydropsyche* (Tian et al. 1996) and are morphologically very similar. The generic assignments in the subfamily Hydropsychinae are far from universally accepted (Ross and Unzicker 1977, Schmid 1979, Schuster 1984, Schefter 1996, 2005, Malicky and Chantaramongkol 2000), but these 5 groups are treated as a *Hydropsyche* group in our study because they share a nearly identical secondary structure in D2. Preliminary likelihood analysis using Bayesian inferences has revealed that the *Hydropsyche* group is a monophyletic group and several groups currently treated as genera (such as *Herbertorossia*, *Mexipsyche*, and *Hydatomanicus*) should be combined into the genus *Hydropsyche* (C. J. Geraci, Clemson University, and XZ, unpublished data). However, our study is not attempting to solve the phylogenetic problems in the subfamily Hydropsychinae.

TABLE 3. Polymerase chain reaction primers used most often in our study.

Primer	Sequence (5′ to 3′)
D2up4	GAGTTCAAGAGTACGTGAAACCG
D2dnB	CCTTGGTCCGTGTTTCAAGAC
COI 1709Fs	TAATTGGAGGATTTGGAAATTG
COI 1709Fg	TAATTGGAGGATTTGGWAAYTG
COI 1751F	GGATCACCTGATATAGCATTCCC
COI 2191R	CCYGGTAAAATTAAAATATAAACTTC
COI 2209R	GAGAAATTATTCCAAATCCRGGTAA

The Chinese *Hydropsyche* group, including 48 known species, represents the most diversified subgroup of Chinese Hydropsychidae (~35% of total hydropsychids; Table 1). The *Hydropsyche* group provides a good opportunity to test species boundaries across independent genes because it contains a number of very closely related species, especially in *Ceratopsyche* and *Mexipsyche*. Temporary taxon codes are assigned to some putative species, many of which are new, because a significant part of the Chinese caddisfly fauna remains unknown. DNA was extracted from 133 *Hydropsyche* group specimens, including 83 adults, 40 larvae, and 10 pharate adults (Appendices 1, 2). Among the adult males, 34 species, including putative new species, have been recognized (Table 2, Fig. 4). In our collection, ~½ of the *Hydropsyche* group species are probably new species (16 of 34). Ten representatives of the 3 Hydropsychidae subfamilies, Diplectroninae (*Diplectrona*), Macronematinae (*Macrostemum*, *Trichomacronema*, *Oestropsyche*, *Amphipsyche*, and *Polymorphanisus*), and Hydropsychinae other than the *Hydropsyche* group (*Hydatopsyche*, *Hydromanicus*,

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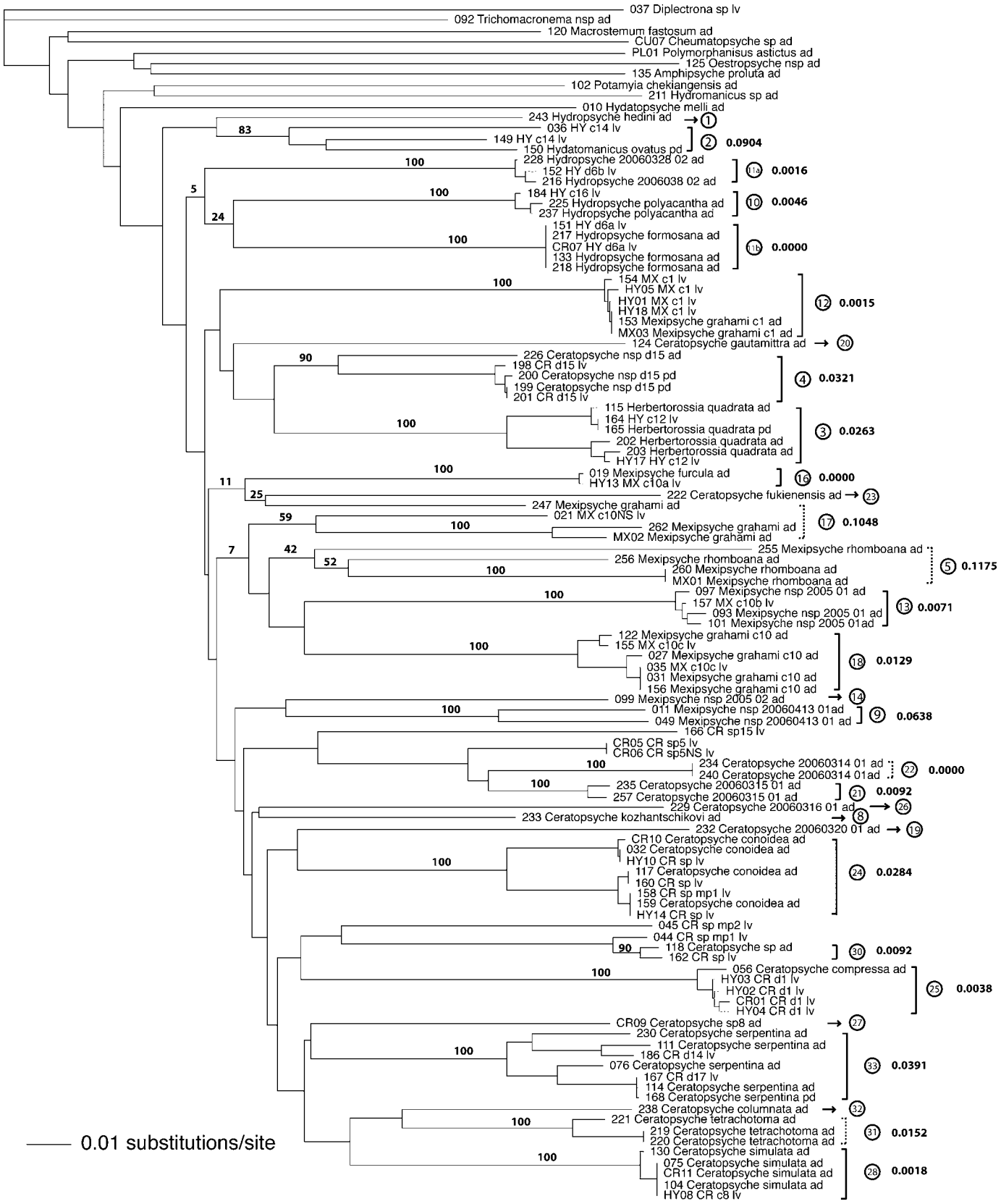
FIG. 4. Large subunit nuclear ribosomal 28S expansion fragment D2 phylogram using neighbor-joining. Putative species boundaries (morphospecies) are labeled with group codes corresponding to those in Table 2 and Fig. 5. Species boundaries that appear to be incongruent between D2 and mitochondrial cytochrome c oxidase subunit I (COI) trees or are paraphyletic/polyphyletic on either tree are marked with dashed brackets. Numbers on the internodes represent the bootstrap values using neighbor-joining/1000 replicates.



Potamyia, and *Cheumatopsyche*), are included as outgroups (Appendices 1, 2).

Adult specimens of each species were selected based on the widest available phenotypic variation and geographic distribution. However, only a few specimens (very often only a single specimen) of rare species were available for extraction. We chose to start with larval specimens that co-occurred with the selected adult specimens. Larval morphospecies were given taxon codes based largely on their head marking patterns. The tentative taxon codes for larval specimens might not necessarily indicate real species boundaries because hydropsychid larvae can share similar head markings across species and the head markings of intraspecific populations of a given species can differ significantly (Schuster and Etnier 1978, Smith and Lehmkuhl 1980). Thus, larval specimens with the same code might be grouped into different species boundaries, and different larval morphotypes might be clustered together. In both cases, more specimens of these problematic larval morphotypes should be added to clarify the associations, and other morphological characters, such as setation, should be analyzed to differentiate these similar larvae. The goal of our article, however, is only to specify the association method using DNA sequences.

Objectives

Larval descriptions of associated species will be published separately. Here, we focus on the following: 1) Can D2 provide good enough resolution to differentiate closely related species in the Chinese *Hydropsyche* group? 2) Does within-species genetic divergence in COI show some typical threshold in the Chinese *Hydropsyche* group? 3) Can we delimit species boundaries and associate larvae with adults with the acquired sequences of both genetic markers (D2 and COI)? 4) Given the limitations of current data, how might we improve species delimitation and larval–adult association?

Methods

Molecular protocols

Most larval and adult specimens used in our study were collected from 6 provinces in China—Guangdong, Guangxi, Jiangxi, Sichuan, Yunnan, and Beijing—from 2001 to 2005. Larval and adult specimens were sorted cursorily after collection and preserved in 95% ethanol. Larvae and adults were sorted into morphospecies as soon as possible (typically within 6 wk), and individuals were preserved separately. Voucher larval and adult specimens will be deposited permanently in Nanjing Agricultural University, Nanjing, China. Abdominal segments III–VI or legs of larvae and adults were used for DNA extraction. Larval intestines and gut contents were removed carefully to reduce the potential for contaminants. The rest of the specimen was preserved in ethanol for morphological study. Each individual was given a unique process code, which was linked to a Genbank accession number.

Genomic DNA was extracted using Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany), which uses silica to bind DNA. The PCR mix was preheated at 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 60°C (with D2up4/D2dnB) or 53°C (with COI 1709Fs/2191R) for 45 s, and 72°C for 60 s. After 10 min of final extension at 72°C, the products were maintained at 4°C. The most frequently used PCR primers are provided in Table 3. PCR products were purified with the Qiagen QIAquick PCR Purification Kit and then sequenced. Each individual DNA fragment was sequenced from both directions.

Sequences from both directions were aligned and proofread with the program ChromasPro (version 1.2, Windows; Technelysium Pty Ltd, Tewantin, Queensland, Australia) or ABI Prism Sequence Navigator (version 1.0.1, Mac OS; Applied Biosystems, Foster City, California). Any conflict or ambiguous reading was given one of the appropriate International Union of Biochemistry symbols (Y, R, S, W, K, M, or N). Use of these ambiguity codes usually does not indicate a real polymorphism, but rather, problems with reading the peaks on the chromatograph unambiguously. Therefore, when an ambiguity was encountered in 1 taxon and a defined nucleotide existed in another taxon that nested within the ambiguity code, the sequences were considered identical.

COI sequences were aligned using ClustalX (version 1.83; Thompson et al. 1997) and MacClade (version 4.08; Maddison and Maddison 2005). D2 sequences were aligned manually in Microsoft Word according to

←

FIG. 5. Mitochondrial cytochrome c oxidase subunit I (COI) phylogram using neighbor joining. The putative species boundaries (morphospecies) are labeled with group codes (numbers in circles) corresponding to those in Table 2 and Fig. 4. Species boundaries that appear to be incongruent between large subunit nuclear ribosomal 28S expansion fragment D2 and COI trees or are paraphyletic/polyphyletic on either tree are marked with dashed brackets. Numbers on the internodes represent the bootstrap values using neighbor-joining/1000 replicates. Numbers after the group codes represent average within-group *p*-distances (% of nucleotide changes of a particular putative species) calculated in MEGA v3.1 (Kumar et al. 2004).

the secondary structure (following Kjer 1995). Manual alignment was done to serve our other purpose of higher-level phylogenetics. However, it was not necessary for closely related species, where the secondary structure of D2 sequences is nearly identical. Multiple D2 sequences can be aligned first in ClustalX and then adjusted by eye. The D2 alignment is available from the authors and on KMK's web site (http://www.rci.rutgers.edu/~insects/indexpersonnel.htm). Primer regions were eliminated from the final sequences (COI: 439 base pairs [bp], D2: ~430 bp). D2 has shown significant length variation in the family Hydropsychidae but little length variation within the *Hydropsyche* group. Unalignable regions in D2 sequences of outgroups were excluded from the analysis because these regions did not improve the resolution of the phylogenetic relationships among ingroup individuals.

Phylogenetic analysis

Phylograms were constructed independently from D2 and COI sequences using distance and neighbor-joining in PAUP*4.0b10 (Swofford 2003). Distance parameters were obtained as follows: 1) DNA distances were estimated using the Kimura-2-parameter (K2P) model, 2) missing and ambiguous data were ignored for pairwise comparisons, 3) all substitutions were estimated or counted, and 4) the distance criterion was set to minimum evolution. The K2P model was used to take into account transition and transversion changes. Pairwise distances and within- and between-species divergences of COI nucleotides were calculated in MEGA v3.1 (Kumar et al. 2004) to provide comparisons to other species diagnosis works. Bootstrap values were calculated in PAUP*4.0b10 using neighbor-joining for 1000 replicates; groups with a frequency >50% were retained.

Results

Species boundaries in the Hydropsyche *group across D2 and COI*

D2 phylogram.—D2 sequences were collected from 143 individuals, including 10 outgroup taxa and 133 *Hydropsyche* group specimens. The D2 length of the *Hydropsyche* group ranges from 421 to 427 bp, with only minor length heterogeneity among species. In contrast to mitochondrial protein-coding genes, D2 is cytosine/guanine (C/G)-rich, with the average nucleotide composition of C and G at 30.3% and 35.9%, respectively. A similar pattern was observed in the 10 outgroup taxa, with the nucleotide composition of C and G at 29.8% and 35.3%, respectively. Thirty-four putative *Hydropsyche* group species, delimited based on male genitalia, are listed in Table 2 and marked on the D2 phylogram (Fig. 4). Species boundaries that appear to be paraphyletic or polyphyletic on the D2 tree and boundaries that are incongruent between COI and D2 trees are marked with dashed parentheses.

D2 successfully delimited 15 species with multiple specimens: *Hydatomanicus ovatus* Li, Tian, and Dudgeon (species 2), *Herbertorossia quadrata* (species 3), *Ceratopsyche* n sp d15 (species 4), *Mexipsyche* n sp 20060413_01 (species 9), *Hydropsyche polyacantha* Li and Tian (species 10), *Mexipsyche grahami* Banks c1 (species 12), *Mexipsyche* n sp 2005_01 (species 13), *Mexipsyche* n sp 20060414_01 (species 15), *Mexipsyche furcula* Tian and Li (species 16), *M. grahami* Banks c10 (species 18), *Ceratopsyche* 20060315_01 (species 21), *Ceratopsyche conoidea* Li and Tian (species 24), *Ceratopsyche compressa* Li and Tian (species 25), *Ceratopsyche simulata* Mosely (species 28), and *Ceratopsyche serpentina* (Schmid) (species 33). In addition, 10 species, each represented by a single specimen [*Hydropsyche hedini* Forsslund, *Ceratopsyche kozhantschikovi* (Martynov), *Mexipsyche* n sp 2005_02, *Ceratopsyche* 20060320_01, *Ceratopsyche gautamittra* (Schmid), *Ceratopsyche fukienensis* (Schmid), *Ceratopsyche* 20060316_01, *Ceratopsyche* CR09, *Ceratopsyche penicillata* (Martynov), and *Ceratopsyche columnata* (Martynov)] all fell outside the delimited species boundaries listed above. However, the number of nucleotide changes in D2 can be very small between some closely related species, especially in *Ceratopsyche*. Consequently, bootstrap support for most *Ceratopsyche* species is very low (Fig. 4). Nevertheless, in all but a few species (see below), the changes provided enough characters to cluster the individuals of the same species together and to exclude individuals of different species from the species boundary.

D2 failed to yield monophyly in 2 species groups—the *Mexipsyche rhomboana* (Martynov) group (species 5, 6, 7) and the *Hydropsyche formosana* Ulmer group (11a, 11b, 11c)—and in 2 other species (22 and 31). Species 22 and 31 (*Ceratopsyche* 20060314_01 and *Ceratopsyche tetrachotoma* Li and Tian, respectively) are paraphyletic on the D2 phylogram but monophyletic on the COI phylogram. At this time, species delimitations in the 2 problematic species groups cannot be assured because of the lack of COI sequences and sufficient specimens for some morphospecies (see below).

Mexipsyche rhomboana group (5, 6, and 7; Fig. 4) contains 3 morphospecies—*M.* 20060406_01, *M.* 20060406_02, and *M. rhomboana*. Six *M. rhomboana* specimens (245/251/255/256/260/MX01) are mixed together with *M.* 20060406_01 (252/254) and *M.* 20060406_02 (261). The intraspecific genetic divergence of *M. rhomboana* appears to be much greater than that

of the other 2 morphospecies, whereas 2 individuals of different morphospecies (254 and 261) share identical D2 sequences. At this time, COI sequences have been acquired from only 4 *M. rhomboana* specimens (255/256/260/MX01), which are monophyletic on the COI phylogram (Fig. 5). The morphological differences among these 3 morphospecies in the *M. rhomboana* group are very subtle. All specimens in this species group were collected from localities in close proximity to each other (Appendices 1, 2). Morphological variation also was observed among local populations. The paraphyletic pattern of D2 among these focal morphospecies suggests the existence of gene flow among local populations. It is certainly possible that the morphological differences are merely intraspecific and all specimens of the *M. rhomboana* group are actually variants of a single species (*M. rhomboana*). Lineage sorting and hybridization between distinctive species, however, cannot be excluded because of the lack of COI sequences in morphospecies 6 and 7. The acquisition of the COI sequences and additional specimens would help to clarify the ambiguity.

The *H. formosana* group consists of 3 exclusive clades: 11a, 11b, and 11c. Individuals of 11a have distinctive male genitalia. In addition, sympatric specimens 228/216 (11a) and 217 (11b) that differ in genital structures were clustered into distinctive clades on both D2 and COI, suggesting genetic isolation between local populations. This evidence indicates that 11a is a valid species that is different from *H. formosana*. The remaining individuals of the *H. formosana* group could not be differentiated easily by morphology although the dorsal projection on tergum X in 11c is not as protruding as in 11b. Thus, these individuals (085/087/133/217/218) all were identified as *H. formosana*, which formed 2 paraphyletic groups with clade 11a nested between them. Among others, specimens 085 and 087 were collected from a distinctive site that is isolated from all other collecting sites (Appendices 1, 2). Depending on species definition, it is possible that 11c represents a cryptic species that could not be differentiated solely by morphology from *H. formosana*. Evidence from an independent gene marker is required to solve the problem. COI amplifications, however, were not successful for 085 and 087, leaving its status an open question.

The D2 fragment provided enough changes to differentiate most *Hydropsyche* group species, with some ambiguity in a few species. However, these problems could be the result of imperfect taxonomy, which could be confirmed with additional sequences and additional specimens.

COI phylogram.—COI sequences were collected from all outgroup taxa and 101 ingroup individuals. A total of 439 bp are included in the phylogenetic analysis. No length variation was observed across taxa. The average nucleotide composition of COI sequences in the *Hydropsyche* group showed an adenine/thymine (A/T)-rich pattern, with the nucleotide composition of A and T at 28.9% and 39.1%, respectively. Outgroup taxa did not differ significantly in nucleotide composition (A and T were 29.9% and 38.6%, respectively). The corresponding group codes are labeled in Fig. 5. Species boundaries that appear to be paraphyletic or polyphyletic on the COI tree and boundaries that are incongruent between COI and D2 trees are marked with dashed brackets. Branch supports (both bootstrap and posterior possibility values [not shown]) typically were high in the COI tree on most of the putative species nodes including *Ceratopsyche*, whose branch supports generally were low on the D2 phylogram. This difference is probably because COI sequences

TABLE 4. Intraspecific *p*-distance (number of nucleotide changes divided by total number of nucleotides) of mitochondrial cytochrome c oxidase subunit I (COI) sequences in *Hydropsyche sensu lato* species. Species with intraspecific divergence that was significantly higher than the typical species threshold implied by other studies (e.g., 1% in Wells et al. 2001) are marked in bold. – indicates *p*-distance not available because only 1 specimen was sequenced.

Group code	Species	Within-group *p*-distance
1	*HY hedini*	–
2	*HT ovatus*	**0.0904**
3	*HB quadrata*	0.0263
4	*CR* n sp d15	0.0321
5	*MX rhomboana*	**0.1175**
8	*CR kozhantschikovi*	–
9	*MX* n sp 20060413_01	**0.0638**
10	*HY polyacantha*	0.0046
11a	*HY* 20060328_02	0.0016
11b	*HY formosana*	0.0000
12	*MX grahami* c1	0.0015
13	*MX* n sp 2005_01	0.0071
14	*MX* n sp 2005_02	–
16	*MX furcula*	0.0000
17	*MX grahami*	**0.1048**
18	*MX grahami* c10	0.0129
19	*CR* 20060320_01	–
20	*CR gautamittra*	–
21	*CR* 20060315_01	0.0092
22	*CR* 20060314_01	0.0000
23	*CR fukienensis*	–
24	*CR conoidea*	0.0284
25	*CR compressa*	0.0038
26	*CR* 20060316_01	–
27	CR09	–
28	*CR simulata*	0.0018
30	*CR* sp118	0.0092
31	*CR tetrachotoma*	0.0152
32	*CR columnata*	–
33	*CR serpentina*	0.0391

TABLE 5. Interspecific *p*-distance (number of nucleotide changes divided by total number of nucleotides) of mitochondrial cytochrome c oxidase subunit I (COI) sequences between *Hydropsyche sensu lato* species.

Group code	9	16	17	18	24	2	25	28	33	13	14	3	30
16	0.1522												
17	0.1666	0.1454											
18	0.1672	0.1612	0.1392										
24	0.1586	0.1705	0.1469	0.1558									
2	0.1640	0.1547	0.1572	0.1713	0.1689								
25	0.1846	0.1575	0.1891	0.1564	0.1571	0.1822							
28	0.1711	0.1585	0.1641	0.1579	0.1378	0.1746	0.1558						
33	0.1602	0.1597	0.1604	0.1437	0.1392	0.1753	0.1569	0.1407					
13	0.1568	0.1717	0.1625	0.1467	0.1552	0.1904	0.1645	0.1559	0.1608				
14	0.1390	0.1517	0.1560	0.1560	0.1623	0.1579	0.1705	0.1595	0.1589	0.1549			
3	0.1680	0.1537	0.1620	0.1513	0.1647	0.1357	0.1775	0.1637	0.1640	0.1969	0.1588		
30	0.1751	0.1569	0.1688	0.1911	0.1608	0.1754	0.1445	0.1483	0.1654	0.1661	0.1762	0.1747	
20	0.1708	0.1574	0.1521	0.1716	0.1586	0.1617	0.1836	0.1722	0.1690	0.1846	0.1800	0.1539	0.1762
11b	0.1606	0.1653	0.1481	0.1689	0.1566	0.1344	0.1870	0.1954	0.1693	0.1788	0.1754	0.1436	0.1556
11a	0.1615	0.1399	0.1464	0.1494	0.1481	0.1391	0.1819	0.1507	0.1285	0.1678	0.1683	0.1534	0.1748
12	0.1731	0.1573	0.1578	0.1693	0.1754	0.1593	0.1815	0.1781	0.1708	0.1823	0.1541	0.1538	0.1732
10	0.1519	0.1471	0.1467	0.1640	0.1599	0.1440	0.1732	0.1836	0.1759	0.1811	0.1511	0.1531	0.1724
4	0.1543	0.1319	0.1404	0.1477	0.1472	0.1424	0.1573	0.1636	0.1335	0.1759	0.1422	0.1161	0.1555
31	0.1701	0.1688	0.1693	0.1610	0.1390	0.1878	0.1492	0.1168	0.1320	0.1434	0.1762	0.1752	0.1350
23	0.1821	0.1489	0.1545	0.1611	0.1760	0.1675	0.1775	0.1882	0.1667	0.1952	0.1538	0.1474	0.1804
26	0.1526	0.1710	0.1577	0.1803	0.1546	0.1853	0.1686	0.1472	0.1462	0.1706	0.1617	0.1821	0.1533
19	0.1697	0.1859	0.1623	0.1712	0.1464	0.1663	0.1688	0.1699	0.1635	0.1898	0.1549	0.1771	0.1579
8	0.1481	0.1402	0.1281	0.1477	0.1270	0.1519	0.1486	0.1358	0.1293	0.1544	0.1207	0.1505	0.1304
22	0.1651	0.1585	0.1765	0.1868	0.1580	0.1815	0.1706	0.1563	0.1784	0.1753	0.1640	0.1668	0.1613
21	0.1451	0.1476	0.1643	0.1661	0.1486	0.1642	0.1654	0.1340	0.1639	0.1580	0.1463	0.1431	0.1424
32	0.1765	0.1448	0.1577	0.1507	0.1353	0.1898	0.1375	0.1221	0.1299	0.1684	0.1595	0.1695	0.1304
1	0.1651	0.1550	0.1588	0.1682	0.1614	0.1246	0.1901	0.1711	0.1603	0.1593	0.1537	0.1634	0.1659
5	0.1570	0.1754	0.1634	0.1579	0.1635	0.1696	0.1765	0.1758	0.1821	0.1691	0.1561	0.1769	0.1700
27	0.1560	0.1425	0.1657	0.1572	0.1452	0.1701	0.1506	0.1358	0.1276	0.1840	0.1481	0.1497	0.1396

possess more changes than D2 sequences. A similar pattern also was observed in water beetles and dung beetles, where total tree support was always higher for COI than 28S (Monaghan et al. 2005). The intraspecific divergence of COI was significant, and divergence values ranged from 0.0000 to 0.1175. Among *Hydropsyche* group species, species 2 (*H. ovatus*), species 5 (*M. rhomboana*), species 9 (*Mexipsyche* n sp 20060413_01), and species 17 (*M. grahami* [Banks]) showed greater intraspecific divergence than the typical species threshold (0.0904, 0.1175, 0.0638, and 0.1048, respectively; Table 4) and could be confused with the interspecific divergence (Table 5). Thus, no typical divergence threshold was observed that can assure species delimitation in *Hydropsyche* group species.

In *H. ovatus* (species 2), the COI sequence was acquired from only 1 pharate adult specimen because no mature adult specimen was available in our collection. COI amplification from pharate adults has been difficult. However, the larvae of this species have been associated with adults by examining the morphology of metamorphotypes. Therefore, larval specimens were used when calculating the intraspecific *p*-distance for this species.

Species of the Chinese *M. grahami* group, including morphospecies 12/13/14/15/16/17/18, possess extremely subtle morphological variation in their male genital structures. Even so, D2 and COI sequences have both provided strong support for their species delimitations except in morphospecies 17 (COI amplification has not yet been successful for morphospecies 15). Morphospecies 17 (*M. grahami*) was paraphyletic on the COI phylogram—specimen 247 was sister to 222 (*C. fukienensis*)—but with very low branch support. Morphospecies 22 and 31, both of which were paraphyletic on the D2 phylogram, yielded monophyletic taxa on the COI phylogram.

Examples of successful association

In addition to *H. ovatus*, whose larvae were associated through metamorphotype morphology, larvae of 8 other species were successfully associated with their adults using association criterion 1 or 2 (Fig.

TABLE 5. Extended.

20	11b	11a	12	10	4	31	23	26	19	8	22	21	32	1	5
0.1640															
0.1502	0.1249														
0.1590	0.1442	0.1623													
0.1458	0.1268	0.1415	0.1548												
0.1413	0.1253	0.1324	0.1412	0.1515											
0.1587	0.1731	0.1653	0.1622	0.1754	0.1500										
0.1692	0.1692	0.1711	0.1698	0.1547	0.1503	0.1940									
0.1754	0.1845	0.1594	0.1594	0.1716	0.1600	0.1716	0.1821								
0.1731	0.1640	0.1832	0.1709	0.1617	0.1613	0.1412	0.1744	0.1731							
0.1572	0.1572	0.1266	0.1438	0.1329	0.1240	0.1306	0.1590	0.1344	0.1458						
0.1822	0.1617	0.1611	0.1663	0.1602	0.1545	0.1549	0.2000	0.1708	0.1640	0.1458					
0.1486	0.1463	0.1514	0.1439	0.1508	0.1347	0.1432	0.1840	0.1611	0.1646	0.1337	0.0686				
0.1617	0.1708	0.1602	0.1667	0.1686	0.1477	0.0964	0.1718	0.1617	0.1481	0.1253	0.1503	0.1474			
0.1812	0.1560	0.1363	0.1582	0.1460	0.1418	0.1758	0.1783	0.1743	0.1560	0.1399	0.1881	0.1657	0.1858		
0.1726	0.1715	0.1702	0.1704	0.1519	0.1551	0.1679	0.1897	0.1664	0.1766	0.1607	0.1846	0.1804	0.1766	0.1687	
0.1481	0.1663	0.1509	0.1499	0.1488	0.1335	0.1238	0.1718	0.1526	0.1686	0.1276	0.1481	0.1303	0.1230	0.1881	0.1681

3A, B): *H. quadrata* (species 3), *C.* n sp d15 (species 4), *H.* 20060328_02 (species 11a), *M. grahami* c1 (species 12), *M. grahami* c10 (species 18), *C. conoidea* (species 24), *C. simulata* (species 28), and *C. serpentina* (species 33). Meanwhile, *H. formosana* (species 11b) was tentatively associated (leaving clade 11c [specimens 085 and 087]) aside. Further work, including morphological analysis and addition of COI sequences, on local population 11c is expected to confirm the association.

Two species, *H. polyacantha* (species 10) and *M.* n sp 2005 01 (species 13) fulfill association criterion 1 (sequence identity) in only 1 of 2 gene markers. Their larvae fall outside the reference species boundaries on the alternative gene phylogram. They are very probably larvae of the species, but additional specimens (adults or larvae) are required to confirm the association. We also expect the acquisition of the COI sequence of individual 241 to assure the association in *C. compressa* (species 25).

Larval specimens of several species (5/6/7/9/15/17/21/22/31) are not available in the collection or not yet sequenced. The addition of larval specimens might lead to successful associations. Rare species, for which only a single adult male specimen was available, could be associated only through sequence identity across both genes (association criterion 1) until larval specimens are collected or until additional adult specimens are added to the data set.

Discussion

Species recognition method

Larval association should not be confused with phylogenetic analysis. The goal of larval association is to find the closest identified sequence to that of the unknown larva. Phylogenetic systems that use only synapomorphies as data to infer relatedness assume bifurcation of noninterbreeding terminal taxa, an assumption that is invalidated at the intraspecific level. Our neighbor-joining method also produces a bifurcating tree, but it is more likely to link haplotypes that are very similar. For the most closely related taxa, it might be advantageous to cluster individuals that share similar haplotypes. The bifurcating phylogenetic

pattern in interspecific relationships is not expected at the intraspecific level because ancestral haplotypes are rarely extinct in the population and "a single ancestral haplotype may give rise to multiple, descendent haplotypes" (Crandall and Templeton 1996). On the other hand, if sequence divergence has even a coarse relationship to time, then the individuals that share a more recent common ancestor, regardless of their network breeding relationships, should possess fewer nucleotide changes when they are compared to each other than when they are compared to other individuals. It is not coincidental that species recognized from adult males with traditional morphological methods are nearly identical to species boundaries revealed in the neighbor-joining trees. Where they differ, the neighbor-joining trees revealed species that had not yet been diagnosed with morphological methods alone. We do not advocate distance methods for phylogenetic analysis, but we find that neighbor-joining is adequate for the larval association work we propose. In addition, we predict that as the databases grow to thousands or tens of thousands of individuals, the speed of neighbor-joining will be increasingly important. None of our conclusions are dependent on our choice of optimality criterion, however, so those who favor parsimony or likelihood alternatives would be free to use them.

Species delimitation using DNA and morphology

Monaghan et al. (2005) proposed that the congruence between nuclear 28S and mitochondrial COI could be used to delimit putative species boundaries for poorly known tropical beetles. Furthermore, independent DNA sequences alone were believed to be sufficient to define unknown species. We agree that independent lines of DNA evidence might delimit tentative boundaries for species found in poorly known faunas. However, we believe that morphological studies are crucial to reinforce the hypothesized species boundaries and to provide meaningful species diagnoses.

Species boundaries in our study were confirmed by comparing independent gene trees, but we are not attempting to delimit species solely on the basis of DNA sequences. In fact, species boundaries are always proposed with morphological characters *prior* to DNA analysis (but see Huber 2003). Individuals are considered conspecifics only if they have essentially the same genital structure. Because our method relies heavily on adult morphology, 2 potential problems could affect a species delimitation and association. First, in our study, sibling species whose morphological characters are indistinguishable, but that are reproductively isolated, will be considered populations of the same species. However, if both gene markers indicate that these populations are highly exclusive (i.e., each of them forms a monophyletic group), larvae of each monophyletic group will be associated independently no matter how the species was first defined. The 2 paraphyletic clades (11b and 11c) in *H. formosana* are probably an example of sibling species, although additional COI sequences and specimens are needed to support this hypothesis. Of course, it is possible that their larval or adult or both forms might not be differentiable based on morphology because sibling species often are thought to be the result of recent speciation.

Second, polymorphisms in male genitalia will cause oversplitting in species delimitation, and various morphs of a given species would be treated as different species. Larvae can be associated independently for each morphospecies if each of them forms an exclusive clade, which could be the case if various morphospecies were collected in allopatry. However, if individuals of various morphospecies are mixed together on a DNA phylogram, such as in the case of the *M. rhomboana* group, a carefully designed sampling program should be undertaken. In the meantime, one should be cautious about any larval association made for these morphospecies.

The purpose of our study was to use DNA to associate larvae and adults. A thorough investigation of species taxonomy of Chinese Hydropsychidae is beyond the scope of our paper. Nevertheless, an ideal species taxonomy should consider male genital morphology and a combination of other independent characters, such as morphology of the different life stages, independent molecular data sets, geographic information, etc. In addition, associated larvae can be used to support species delimitation of the adults in holometabolous taxa like caddisflies. For example, the adults of 2 *Mexipsyche* species, *M. grahami* c1 (species 12) and *M. grahami* c10 (species 18), have only minor differences in their male genitalia. In contrast, their larvae differ significantly in their head markings—larvae of *M. grahami* c1 have a uniformly darkened head capsule, whereas larvae of *M. grahami* c10 have the typical A-shaped markings shared by most *M. grahami* group members. Combining evidence from the male morphology, geographic distribution, larval morphology, and independent molecular markers, we infer that these 2 phenotypes are different species.

Larval–adult associations using traditional techniques are critical when specimens are scarce and DNA sequences are difficult to amplify, such as in the case of *H. ovatus* (species 2). Limited specimen sources are not uncommon in explorations of a poorly known

fauna where the collecting effort is condensed into a short period of time. In most cases, researchers prefer visiting a variety of locations rather than thoroughly investigating the fauna at any particular site. Moreover, larval rearing operations are rarely attempted. Thus, we emphasize that a combination of approaches for associating larvae and adults will improve the chance of successful associations.

Sampling strategy

Thorough sampling is critical in larval–adult association. Incomplete sampling can lead to an incorrect association because of potential species-level polyphyly. Good sampling should include as many closely related species as possible, and DNA should be extracted from multiple individuals of each species when specimens are available. The widest range of intraspecific morphological differentiation from the widest geographic distribution should be included when choosing the particular individuals for DNA analysis. Adult specimens with intraspecific variations of genital structures or other morphological characters should be included in the analysis. Larval morphs that are putatively considered to be of the same species in a priori DNA analysis also should be included. Specimens of different species collected sympatrically can be used to test for potential hybridization.

Among the association criteria, sequence identity (criterion 1; Fig. 3A) is more likely to be obtained when larval and adult specimens are collected from the same site. Thus, to associate a particular species, the best collecting strategy is to collect repeatedly at the same site over different seasons. If collecting must be done intensively in a short period, or if a significant part of the fauna is unknown, the best strategy is to collect samples from geographic regions that are as far apart from each other as possible. In the latter case, criterion 2 (Fig. 3B) is more likely to meet the objective of associating larvae and adults.

Feasibility and prospects

As in the DNA method, conventional association approaches also can be affected by species-level polyphyly, which can obscure species boundaries. Larvae of different species can possess similar morphology because of hybridization or retention of ancestral polymorphisms. These obscured species boundaries might be clarified by examining a greater number of metamorphotypes or by rearing many larvae of various morphotypes. Unfortunately, associations by conventional approaches were beyond the scope of our work, because in our collecting experience, metamorphotypes were rare, and we were neither prepared nor equipped to undertake large-scale larval rearing. Thus, it is much easier to acquire a great number of DNA sequences from multiple individuals of a particular species. The number of DNA sequences is crucial in population-level studies.

In addition, minute differences in larval morphologies can be overlooked easily when the disassociated larval sclerites from a pupal case are being examined (although a careful taxonomist with good optics probably will succeed when a metamorphotype is available). If metamorphotypes were available for only 1 of these closely related species, or if larval rearing were only successful in only 1 of these species, all larvae that possessed similar morphological characters probably would be assigned to this 1 species. The *M. grahami* group (morphospecies 12/13/14/15/16/17/18) provides a good example. Larval specimens #157/HY13/021/155/035 originally were identified as the same species because the differences in their head markings were very small. It became clear that they actually belong to different species when additional adult specimens of these closely related *Mexipsyche* species were added to the phylogram. In this case, we would have missed the opportunity to differentiate these similar larval specimens had we examined only their larval sclerites. Thus, the DNA method provides potentially finer resolution in differentiating larvae with similar morphological characters than the morphological method by providing a finite number of potential species hypotheses the morphologist should consider.

In addition to its reliability, the DNA method is expected to be an efficient way to associate the entire Chinese caddisfly fauna. Molecular characterization of an entire fauna might seem to be a daunting task at the onset, but progress will accelerate the work. The more complete the DNA database, the easier future associations will become. The chance of obtaining successful associations might not be very high at the beginning of the work (for example, only 11 out of 34 recognized putative *Hydropsyche* group species were associated in this initial effort), but the number is expected to increase at a faster pace as DNA sequences accumulate with time.

To date, ~30 Chinese hydropsychid species, including all Chinese genera of Hydropsychidae except *Oestropsyche* and *Aethaloptera*, have been associated. As we gain knowledge in larval taxonomy, studies on the ecology and biology of the species can begin. In particular, tolerance values can be assigned to specific species, and the opportunity to use hydropsychid larvae in biomonitoring of China's aquatic habitats will be greatly improved.

Last, our work provides a fundamental framework

for solving similar problems in other taxa, including many of the economically or medically important groups, such as aphids and mosquitoes. And of course, our work will facilitate identifications in other unknown life stages (e.g., eggs, pupae, early larval instars) and various seasonal, geographic, or caste morphotypes.

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APPENDIX 1. Specimen information and GenBank accession numbers. A = adult, L = larva, PD = pharate adult, D2 = large subunit nuclear ribosomal 28S expansion fragment D2, COI = mitochondrial cytochrome c oxidase subunit I, – = GenBank accession number is not available for specimens with unsuccessful COI amplification, Prov. = province. Collection details are in Appendix 2.

Specimen ID	Species[a]	Life stage	Collection code	Collection locality	GenBank accession number (D2)	GenBank accession number (COI)
	Outgroup					
PL01	*Polymorphanisus astictus*	A	GNK030726	Guangdong Prov.: Nan-kun-shan	EF513890	EF513884
CU07	*Cheumatopsyche* sp.	A	GNL030723	Guangdong Prov.: Nan-ling	EF513894	EF513883
010	*Hydatopsyche melli*	A	CN040516-02	Guangdong Prov.: Nan-kun-shan	EF513891	EF513875
037	*Diplectrona* sp.	L	CN040518-05	Guangdong Prov.: Nan-ling	EF513885	EF513876
092	*Trichomacronema* n.sp.	A	CN040518-05	Guangdong Prov.: Nan-ling	EF513886	EF513877
102	*Potamyia nr. chekiangensis*	A	CN040609-03	Guangxi Prov.: Cen-wang-lao-shan	EF513892	EF513878
120	*Macrostemum fastosum*	A	CN040531-02	Guangdong Prov.: Huang-you-bi	EF513887	EF513879
125	*Oestropsyche* n.sp.	A	CN040612-01	Guangxi Prov.: Nan-pan-jiang	EF513888	EF513880
135	*Amphipsyche proluta*	A	Hunan An-hua	Hunan Prov.: An-hua	EF513889	EF513881
211	*Hydromanicus* sp.	A	CN050706-04	Sichuan Prov.: Da-feng-ding	EF513893	EF513882
	Ingroup					
011	*Mexipsyche* 20060413_01	A	CN040516-02	Guangdong Prov.: Nan-kun-shan	EF513895	EF513774
019	*Mexipsyche furcula*	A	CN040518-01	Guangdong Prov.: Nan-ling	EF513896	EF513775
021	*Mexipsyche* sp.	L	CN040518-02	Guangdong Prov.: Nan-ling	EF513897	EF513776
027	*Mexipsyche grahami* c10	A	CN040518-02	Guangdong Prov.: Nan-ling	EF513898	EF513777
031	*Mexipsyche grahami* c10	A	CN040518-03	Guangdong Prov.: Nan-ling	EF513899	EF513778
032	*Ceratopsyche conoidea*	A	CN040518-03	Guangdong Prov.: Nan-ling	EF513900	EF513779
035	*Mexipsyche* c10c	L	CN040518-05	Guangdong Prov.: Nan-ling	EF513901	EF513780
036	*Hydatomanicus* sp.	L	CN040518-05	Guangdong Prov.: Nan-ling	EF513902	EF513781
044	*Ceratopsyche* sp.	L	CN040520-01	Guangdong Prov.: Che-ba-ling	EF513903	EF513782
045	*Ceratopsyche* sp.	L	CN040520-01	Guangdong Prov.: Che-ba-ling	EF513904	EF513783
049	*Mexipsyche* 20060413_01	A	CN040520-01	Guangdong Prov.: Che-ba-ling	EF513905	EF513784
056	*Ceratopsyche compressa*	A	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF513906	EF513785
075	*Ceratopsyche simulata*	A	CN040606-01	Guangxi Prov.: Shi-wan-da-shan	EF513907	EF513786
076	*Ceratopsyche serpentina*	A	CN040601-02	Guangdong Prov.: Luo-fu-shan	EF513908	EF513787
085	*Hydropsyche formosana*	A	CN040605-03	Guangxi Prov.: Shi-wan-da-shan	EF513909	–
087	*Hydropsyche formosana*	A	CN040605-01	Guangxi Prov.: Shi-wan-da-shan	EF513910	–
093	*Mexipsyche* n.sp. 2005_01	A	CN040608-01	Guangxi Prov.: Cen-wang-lao-shan	EF513911	EF513788
097	*Mexipsyche* n.sp. 2005_01	A	CN040609-01	Guangxi Prov.: Cen-wang-lao-shan	EF513912	EF513789
099	*Mexipsyche* n.sp. 2005_02	A	CN040609-03	Guangxi Prov.: Cen-wang-lao-shan	EF513913	EF513790
101	*Mexipsyche* n.sp. 2005_01	A	CN040609-02	Guangxi Prov.: Cen-wang-lao-shan	EF513914	EF513791
104	*Ceratopsyche simulata*	A	CN040610-01	Guangxi Prov.: Jin-zhong-shan	EF513915	EF513792
111	*Ceratopsyche serpentina*	A	CN040612-01	Guangxi Prov.: Nan-pan-jiang	EF513916	EF513793
114	*Ceratopsyche serpentina*	A	CN040527-01	Guangdong Prov.: Yang-chun	EF513917	EF513794
115	*Herbertorossia quadrata*	A	CN040527-01	Guangdong Prov.: Yang-chun	EF513918	EF513795
117	*Ceratopsyche conoidea*	A	CN040526-02	Guangdong Prov.: Da-wu-ling	EF513919	EF513796
118	*Ceratopsyche* sp118.	A	CN040529-01	Guangdong Prov.: Ye-qu-gou	EF513920	EF513797
122	*Mexipsyche grahami* c10	A	CN040531-02	Guangdong Prov.: Huang-you-bi	EF513921	EF513798
124	*Ceratopsyche gautamittra*	A	CN040612-01	Guangxi Prov.: Nan-pan-jiang	EF513922	EF513799
129	*Ceratopsyche conoidea*	A	CN040615-03	Guangxi Prov.: Jiu-wan-da-shan	EF513923	–
130	*Ceratopsyche simulata*	A	CN040616-02	Guangxi Prov.: Xing-an	EF513924	EF513800
133	*Hydropsyche formosana*	A	CN040618-01	Guangxi Prov.: Yang-shuo	EF513925	EF513801
148	*Hydatomanicus ovatus*	PD	CN040525-01	Guangdong Prov.: Ding-hu-shan	EF513926	–
149	*Hydatomanicus* c14	L	CN040525-01	Guangdong Prov.: Ding-hu-shan	EF513927	EF513802
150	*Hydatomanicus ovatus*	PD	CN040615-01	Guangxi Prov.: Jiu-wan-da-shan	EF513928	EF513803
151	*Hydropsyche* d6a	L	CN040618-01	Guangxi Prov.: Yang-shuo	EF513929	EF513804
152	*Hydropsyche* d6b	L	CN040613-01	Guangxi Prov.: Mo-li	EF513930	EF513805
153	*Mexipsyche grahami* c1	A	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF513931	EF513806
154	*Mexipsyche* c1	L	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF513932	EF513807
155	*Mexipsyche* c10c	L	CN040521-01	Guangdong Prov.: Nan-ling	EF513933	EF513808
156	*Mexipsyche grahami* c10	A	CN040521-01	Guangdong Prov.: Nan-ling	EF513934	EF513809
157	*Mexipsyche* c10b	L	CN040609-02	Guangxi Prov.: Cen-wang-lao-shan	EF513935	EF513810
158	*Ceratopsyche* sp.	L	GNL030723	Guangdong Prov.: Nan-ling	EF513936	EF513811
159	*Ceratopsyche conoidea*	A	GNL030723	Guangdong Prov.: Nan-ling	EF513937	EF513812

APPENDIX 1. Continued.

Specimen ID	Species[a]	Life stage	Collection code	Collection locality	GenBank accession number (D2)	GenBank accession number (COI)
160	*Ceratopsyche* sp.	L	CN040526-01	Guangdong Prov.: Da-wu-ling	EF513938	EF513813
161	*Ceratopsyche* sp.	L	CN040611-01	Guangxi Prov.: Jin-zhong-shan	EF513939	–
162	*Ceratopsyche* sp.	L	CN040527-01	Guangdong Prov.: Yang-chun	EF513940	EF513814
163	*Hydatomanicus ovatus*	PD	CN040605-03	Guangxi Prov.: Shi-wan-da-shan	EF513941	–
164	*Herbertorossia* c12	L	CN040527-02	Guangdong Prov.: Yang-chun	EF513942	EF513815
165	*Herbertorossia quadrata*	PD	CN040527-02	Guangdong Prov.: Yang-chun	EF513943	EF513816
166	*Ceratopsyche* sp.	L	CN040610-02	Guangxi Prov.: Jin-zhong-shan	EF513944	EF513817
167	*Ceratopsyche* d17	L	CN040527-02	Guangdong Prov.: Yang-chun	EF513945	EF513818
168	*Ceratopsyche serpentina*	PD	CN040527-02	Guangdong Prov.: Yang-chun	EF513946	EF513819
180	*Herbertorossia quadrata*	PD	CN040527-01	Guangdong Prov.: Yang-chun	EF513947	–
182	*Mexipsyche grahami* c1	PD	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF513948	–
183	*Mexipsyche grahami* c1	PD	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF513949	–
184	*Hydropsyche* c16	L	CN040527-01	Guangdong Prov.: Yang-chun	EF513950	EF513820
186	*Ceratopsyche* d14	L	CN040613-01	Guangxi Prov.: Mo-li	EF513951	EF513821
198	*Ceratopsyche* d15	L	CN050605-01	Jiangxi Prov.: Li-tou-jian	EF513952	EF513822
199	*Ceratopsyche* n.sp. d15	PD	CN050605-01	Jiangxi Prov.: Li-tou-jian	EF513953	EF513823
200	*Ceratopsyche* n.sp. d15	PD	CN050605-01	Jiangxi Prov.: Li-tou-jian	EF513954	EF513824
201	*Ceratopsyche* d15	L	CN050605-01	Jiangxi Prov.: Li-tou-jian	EF513955	EF513825
202	*Herbertorossia quadrata*	A	CN050604-03	Jiangxi Prov.: Lei-gu-ling	EF513956	EF513826
203	*Herbertorossia quadrata*	A	CN050605-02	Jiangxi Prov.: Li-tou-jian	EF513957	EF513827
216	*Hydropsyche formosana*	A	CN050605-01	Jiangxi Prov.: Li-tou-jian	EF513958	EF513828
217	*Hydropsyche formosana*	A	CN050605-02	Jiangxi Prov.: Li-tou-jian	EF513959	EF513829
218	*Hydropsyche formosana*	A	CN050604-03	Jiangxi Prov.: Lei-gu-ling	EF513960	EF513830
219	*Ceratopsyche tetrachotoma*	A	CN050630-02	Sichuan Prov.: Li-zi-ping	EF513961	EF513831
220	*Ceratopsyche tetrachotoma*	A	CN050706-01	Sichuan Prov.: Da-feng-ding	EF513962	EF513832
221	*Ceratopsyche tetrachotoma*	A	CN050707-01	Sichuan Prov.: Ma-bian	EF513963	EF513833
222	*Ceratopsyche fukienensis*	A	CN050601-01	Jiangxi Prov.: Tong-mu-he	EF513964	EF513834
223	*Ceratopsyche* n.sp. d15	A	CN050604-01	Jiangxi Prov.: Lei-gu-ling	EF513965	–
224	*Hydropsyche* (*Occutanspsyche*) *polyacantha*	A	CN050604-01	Jiangxi Prov.: Lei-gu-ling	EF513966	–
225	*Hydropsyche* (*Occutanspsyche*) *polyacantha*	A	CN050604-01	Jiangxi Prov.: Lei-gu-ling	EF513967	EF513835
226	*Ceratopsyche* n.sp. d15	A	CN050604-01	Jiangxi Prov.: Lei-gu-ling	EF513968	EF513836
227	*Ceratopsyche* n.sp. d15	A	CN050604-01	Jiangxi Prov.: Lei-gu-ling	EF513969	–
228	*Hydropsyche formosana*	A	CN050605-02	Jiangxi Prov.: Li-tou-jian	EF513970	EF513837
229	*Ceratopsyche* 20060316_01	A	CN050603-03	Jiangxi Prov.: Tong-mu-he	EF513971	EF513838
230	*Ceratopsyche serpentina*	A	CN050609-04	Jiangxi Prov.: Huang-niu-shi	EF513972	EF513839
231	*Ceratopsyche serpentina*	A	CN050610-04	Jiangxi Prov.: Da-qiu-tian	EF513973	–
232	*Ceratopsyche* 20060320_01	A	CN050619-01	Beijing: Song-shan	EF513974	EF513840
233	*Ceratopsyche kozhantschikovi*	A	CN050619-01	Beijing: Song-shan	EF513975	EF513841
234	*Ceratopsyche* 20060314_01	A	CN050702-01	Sichuan Prov.: Zhang-hu-he	EF513976	EF513842
235	*Ceratopsyche* 20060315_01	A	CN050704-02	Sichuan Prov.: Zhao-jue	EF513977	EF513843
236	*Ceratopsyche* 20060315_01	A	CN050706-01	Sichuan Prov.: Da-feng-ding	EF513978	–
237	*Hydropsyche* (*Occutanspsyche*) *polyacantha*	A	CN050604-02	Jiangxi Prov.: Lei-gu-ling	EF513979	EF513844
238	*Ceratopsyche columnata*	A	CN050619-01	Beijing: Song-shan	EF513980	EF513845
239	*Ceratopsyche penicillata*	A	CN050619-01	Beijing: Song-shan	EF513981	–
240	*Ceratopsyche* 20060314_01	A	CN050702-02	Sichuan Prov.: Zhang-hu-he	EF513982	EF513846
241	*Ceratopsyche compressa*	A	CN050704-02	Sichuan Prov.: Zhao-jue	EF513983	–
242	*Ceratopsyche* 20060315_01	A	CN050705-01	Sichuan Prov.: Mei-gu	EF513984	–
243	*Hydropsyche hedini*	A	CN050630-01	Sichuan Prov.: Li-zi-ping	EF513985	EF513847
245	*Mexipsyche rhomboana*	A	CN050627-04	Sichuan Prov.: Feng-tong-zhai	EF513986	–
247	*Mexipsyche grahami*	A	CN050602-01	Jiangxi Prov.: Tong-mu-he	EF513987	EF513848
248	*Mexipsyche furcula*	A	CN050602-01	Jiangxi Prov.: Tong-mu-he	EF513988	–
249	*Mexipsyche furcula*	A	CN050601-02	Jiangxi Prov.: Tong-mu-he	EF513989	–
250	*Mexipsyche furcula*	A	CN050603-01	Jiangxi Prov.: Tong-mu-he	EF513990	–
251	*Mexipsyche rhomboana*	A	CN050629-03	Sichuan Prov.: Kang-ding	EF513991	–
252	*Mexipsyche* 20060406_01	A	CN050629-03	Sichuan Prov.: Kang-ding	EF513992	–

APPENDIX 1. Continued.

Specimen ID	Species[a]	Life stage	Collection code	Collection locality	GenBank accession number (D2)	GenBank accession number (COI)
254	*Mexipsyche* 20060406_01	A	CN050629-04	Sichuan Prov.: Kang-ding	EF513993	–
255	*Mexipsyche rhomboana*	A	CN050701-02	Sichuan Prov.: Li-zi-ping	EF513994	EF513849
256	*Mexipsyche rhomboana*	A	CN050701-03	Sichuan Prov.: Li-zi-ping	EF513995	EF513850
257	*Ceratopsyche* 20060315_01	A	CN050705-02	Sichuan Prov.: Mei-gu	EF513996	EF513851
258	*Mexipsyche* 20060414_01	A	CN050706-03	Sichuan Prov.: Da-feng-ding	EF513997	–
259	*Mexipsyche* 20060414_01	A	CN050710-02	Sichuan Prov.: Qing-cheng-shan	EF513998	–
260	*Mexipsyche rhomboana*	A	CN050628-03	Sichuan Prov.: Tian-quan	EF513999	EF513852
261	*Mexipsyche* 20060406_02	A	CN050629-02	Sichuan Prov.: Kang-ding	EF514000	–
262	*Mexipsyche grahami*	A	CN050703-01	Sichuan Prov.: Mian-ning	EF514001	EF513853
263	*Mexipsyche* 20060414_01	A	CN050709-01	Sichuan Prov.: San-jiang	EF514002	–
CR01	*Ceratopsyche* d1	L	YHL021202	Yunnan Prov.: Hei-long-tan	EF514003	EF513854
CR02	*Ceratopsyche* d1	L	YHL021202	Yunnan Prov.: Hei-long-tan	EF514004	–
CR05	*Ceratopsyche* sp.	L	SFT030819	Sichuan Prov.: Feng-tong-zhai	EF514005	EF513855
CR06	*Ceratopsyche* sp.	L	SFT030819	Sichuan Prov.: Feng-tong-zhai	EF514006	EF513856
CR07	*Hydropsyche* d6a	L	GNL030721	Guangdong Prov.: Nan-ling	EF514007	EF513857
CR08	*Ceratopsyche simulata*	A	GNK030726	Guangdong Prov.: Nan-kun-shan	EF514008	–
CR09	*Ceratopsyche* CR09	A	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF514009	EF513858
CR10	*Ceratopsyche conoidea*	A	GNL030723	Guangdong Prov.: Nan-ling	EF514010	EF513859
CR11	*Ceratopsyche simulata*	A	GNL030723	Guangdong Prov.: Nan-ling	EF514011	EF513860
HY01	*Mexipsyche* c1	L	YYS021203	Yunnan Prov.: Yu-shui-zhai	EF514012	EF513861
HY02	*Ceratopsyche* d1	L	YHL021202	Yunnan Prov.: Hei-long-tan	EF514013	EF513862
HY03	*Ceratopsyche* d1	L	YHL021202	Yunnan Prov.: Hei-long-tan	EF514014	EF513863
HY04	*Ceratopsyche* d1	L	YHL021202	Yunnan Prov.: Hei-long-tan	EF514015	EF513864
HY05	*Mexipsyche* c1	L	YYS021203	Yunnan Prov.: Yu-shui-zhai	EF514016	EF513865
HY06	*Mexipsyche* c1	L	YYS021203	Yunnan Prov.: Yu-shui-zhai	EF514017	–
HY08	*Ceratopsyche* c8	L	GNL030721	Guangdong Prov.: Nan-ling	EF514018	EF513866
HY10	*Ceratopsyche* sp.	L	GNL030721	Guangdong Prov.: Nan-ling	EF514019	EF513867
HY13	*Mexipsyche* c10a	L	GNL030723	Guangdong Prov.: Nan-ling	EF514020	EF513868
HY14	*Ceratopsyche* sp.	L	GNL030723	Guangdong Prov.: Nan-ling	EF514021	EF513869
HY17	*Herbertorossia* c12	L	GNK030726	Guangdong Prov.: Nan-kun-shan	EF514022	EF513870
HY18	*Mexipsyche* c1	L	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF514023	EF513871
HY19	*Mexipsyche* c1	L	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF514024	–
MX01	*Mexipsyche rhomboana*	A	SFT030819	Sichuan Prov.: Feng-tong-zhai	EF514025	EF513872
MX02	*Mexipsyche grahami*	A	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF514026	EF513873
MX03	*Mexipsyche grahami* c1	A	YYS030801	Yunnan Prov.: Yu-shui-zhai	EF514027	EF513874

[a] Specimen was given a putative species name if it is a new species or identified with uncertainty

APPENDIX 2. Collection locality details. Prov. = province, Co. = county, elev. = elevation, Mt = mountain, Rt = route, coll. = collector. Collection codes are given in Appendix 1.

Collection code	Collection locality details
CN040516-02	CHINA: Guangdong Prov., Long-men Co., Nan-kun Shan Provincial Nature Preserve, Tian Tang Ding He stream, N23.64370°, E113.84729°, elev. 542 m, 16 May 2004, Coll. J. Morse, L. Yang, X. Tong, X. Zhou, C. Sun, C. J. Geraci
CN040518-01	CHINA: Guangdong Prov., Ru-yuan Co., Nan-ling National Nature Preserve, Lao-peng-keng Field Station, Lao-peng Keng, Rt X327, marker 21.5 km, N24.92918°, E113.01584°, elev. 1010 m, 18 May 2002, Coll. J. Morse, L. Yang, X. Tong, C. Sun, X. Zhou, C.J. Geraci
CN040518-02	CHINA: Hunan Prov., Yi-zhang Co., Mang Shan National Nature Preserve, Xiang-si Keng near bridge ~200 m S of Nature Preserve gate, N24.95113°, E112.98470°, elev. 1332 m, 18 May 2004, Coll. J. Morse, L. Yang, X. Tong, C. Sun, X. Zhou, C. J. Geraci
CN040518-03	CHINA: Guangdong Prov., Ru-yuan Co., Nan-ling National Nature Preserve, Lao-peng Keng at cascading tributary, Rt X327, marker 22.5 km, N24.93433°, E113.00953°, elev. 1110 m, 18–19 May 2004, Coll. J. Morse, X. Tong, X. Zhou
CN040518-05	CHINA: Guangdong Prov., Ru-yuan Co., Nan-ling National Nature Preserve, Xiao-huang-shan, Shi-keng scenic spot, on Rt Z210 between 6–7 km, 18 May 2004, Coll. J. Morse, X. Tong, L. Yang, C. J. Geraci, C. Sun, X. Zhou
CN040520-01	CHINA: Guangdong Prov., Shi-xing Co., Che-ba-ling National Nature Preserve, fork of Che-ba-ling He headwaters, 10 km upstream of Preserve headquarters, N24°42′09″, E114°10′35″, elev. 496 m, 20–21 May 2004, Coll. X. Tong, J. Morse, C. Sun, X. Zhou
CN040521-01	CHINA: Guangdong Prov., Ru-yuan Co., Nan-ling National Nature Preserve, unnamed tributary of Lao-peng-keng, Rt X327, marker 17.45 km, N24.91276°, E113.03421°, elev. 935 m, 21–22 May 2004, Coll. J. Morse, C. Sun
CN040525-01	CHINA: Guangdong Prov., Zhao-qing City, Ding-hu District, Ding-hu-shan Forest Ecosystem, Research Station, Academia Sinica, Xi Gou at trail crossing, N23.17322°, E112.53537°, elev. 334 m, 25 May 2004, Coll. L. Yang, C. J. Geraci, J. Morse, C. Sun, Tang, Xu
CN040526-01	CHINA: Guangdong Prov., Xin-yi Co., Da-cheng town, Da-wu-ling Nature Reserve, stream inside entrance of Reserve, N22°16′25″, E111°11′38″, elev. 1021 m, 26 May 2004, Coll. X. Zhou, Tang
CN040526-02	CHINA: Guangdong Prov., Xin-yi Co., Da-cheng town, Da-wu-ling Nature Reserve, upstream of the stream at the entrance of Reserve, N22°16′08″, E111°11′48″, elev. 1110 m, 26 May 2004, Coll. C. Sun
CN040527-01	CHINA: Guangdong Prov., Yang-chun Co., Xin-he village, 16 km NW of Yong-ning town, He-cang stream, N22°20′04″, E111°30′25″, elev. 436 m, 27 May 2004, Coll. J. Morse, C. Sun, Tang
CN040527-02	CHINA: Guangdong Prov., Yang-chun Co., Xin-he village, 16 km NW of Yong-ning town, He-cang Stream, N22.32889°, E111.50315°, elev. 393 m, 27 May 2004, Coll. L. Yang, X. Zhou, C. J. Geraci
CN040529-01	CHINA: Guangdong Prov., Luo-fu-shan Mt, Gui-shan, Ye-qu-gou, 18 km SW of He-yuan city, 100 m downstream of Xiang-shui-ping Falls, N23°42′10″, E114°36′43″, elev. 302 m, 29 May 2004, Coll. L. Yang, C. J. Geraci, Tang
CN040531-02	CHINA: Guangdong Prov., Jiao-ling Co., Huang-you-bi Nature Reserve, Guan-keng-zi Creek, ~4.5 km beyond Reserve station, N24.75032°, E116.26217°, elev. 531 m, 31 May 2004, Coll. L. Yang, J. Morse, X. Zhou, C. J. Geraci
CN040601-02	CHINA: Guangdong Prov., Bo-luo Co., Luo-fu-shan Mt, unnamed stream, 400 m on trail to Shan-bei-shui scienic spot, trailhead 3.2 km W of ridge of Cha-shan, N23.31900°, E114.01157°, elev. 290 m, 01 Jun 2004, Coll. J. Morse, X. Zhou, C. J. Geraci
CN040605-01	CHINA: Guangxi Prov., Shang-si Co., Na-lin-he stream, tributary of Ming-jiang River, 2.0 km NW of main entrance to Shi-wan-da-shan National Forest Park, N21.90700°, E107.89668°, elev. 281 m, 05 Jun 2004, Coll. J. Morse, C. Sun
CN040605-03	CHINA: Guangxi Prov., Shang-si Co., Shi-wan-da-shan National Forest Park, Shi-tou-he Stream, tributary of Ming-jiang river, 1.35 km SW of main entrance to Park, N21.90221°, E107.90460°, elev. 300 m, 05 Jun 2004, Coll. L. Yang, C. J. Geraci
CN040606-01	CHINA: Guangxi Prov., Shang-si Co., Shi-wan-da-shan National Forest Park, 1st tributary of Shi-tou-he stream, Zhu-jiang-yuan Waterfall, ~4 km SW of main entrance to Park, elev. 485 m, 06 Jun 2004, Coll. X. Zhou, K. Kjer
CN040608-01	CHINA: Guangxi Prov., Tian-lin Co., Cen-wang-lao-shan Provincial Forest Preserve, Headwaters of Bu-liu-he river, Co. Road 794 marker 38.9 km, N24.42097°, E106.38340°, elev. 1247 m, 08 Jun 2004, Coll. X. Zhou, K. Kjer
CN040609-01	CHINA: Guangxi Prov., Tian-lin Co., Cen-wang-lao-shan Provincial Forest Preserve, Yao-shan-gou, tributary of Bu-liu-he, Co. Road 794 marker 52.7 km, N24.47080°, E106.35784°, elev. 1223 m, 09 Jun 2004, Coll. L. Yang, C. J. Geraci
CN040609-02	CHINA: Guangxi Prov., Tian-lin Co., Cen-wang-lao-shan Provincial Forest Preserve, Yang-cun-he stream, tributary of Bu-liu-he river, trailhead at An-jia-ping village, Co. Road 794 marker 43.2 km, ~2.5 km trail, elev. 1155 m, 09 Jun 2004, Coll. X. Zhou, K. Kjer
CN040609-03	CHINA: Guangxi Prov., Tian-lin Co., Cen-wang-lao-shan Forest Preserve, unnamed tributary of Ban-cun-he River, jeep trailhead at Co. Road 794 marker 46.9 km, ~7.0 km on lower jeep trail and 1.0 km on foot trail, N24.45559°, E106.31970°, elev. 1035 m, 09 Jun 2004, Coll. J. Morse, C. Sun

APPENDIX 2. Continued.

Collection code	Collection locality details
CN040610-01	CHINA: Guangxi Prov., Long-lin Co., Jin-zhong-shan Provincial Forest Preserve, waterfall of Wu-chong-gou, 800 m W of Wu-chong village, N24.67178°, E104.87846°, elev. 775 m, 10 Jun 2004, Coll. J. Morse, C. Sun
CN040610-02	CHINA: Guangxi Prov., Long-lin Co., Jin-zhong-shan Forest Preserve, Ping-liu-cun village in Jin-zhong-shan town, Ping-liu-he Stream, ~100 m upstream of Lou-fang-gou, elev. 895 m, 10 Jun 2004, Coll. X. Zhou, K. Kjer
CN040611-01	CHINA: Guangxi Prov., Long-lin Co., Jin-zhong-shan Provincial Forest Preserve, Nong-heng-gou, ~1.3 km N of Xi-she village, N24.57867°, E104.91399°, elev. 1140 m, 11 Jun 2004, Coll. L. Yang, X. Zhou, C. J. Geraci, K. Kjer
CN040612-01	CHINA: Guangxi Prov., Long-lin Co., east bank of Nan-pan-jiang River at Wei-le town, ~1.0 km downstream of Ping-ban Hydropower Station, N23.81129°, E105.49690°, elev. 398 m, 12 Jun 2004, Coll. L. Yang, J. Morse, C. Sun, X. Zhou, C. J. Geraci, K. Kjer
CN040613-01	CHINA: Guangxi Prov., Le-ye Co., Bu-liu-he River 500 m upstream of Muo-li town, N24.66877°, E106.71723°, elev. 427 m, 13 Jun 2004, Coll. L. Yang, J. Morse, C. Sun, X. Zhou, C. J. Geraci, K. Kjer
CN040615-01	CHINA: Guangxi Prov., Hua-jiang Co., Jiu-wan-da-shan Provincial Nature Preserve, Jiu-ren Station, Nei-chang Xi, jeep trailhead at Co. Road 5309 marker 125.2 km, 4.0 km on jeep trail, N25.21611°, E108.64043°, elev. 1144 m, 15 Jun 2004, Coll. C. Sun, X. Zhou, K. Kjer
CN040615-03	CHINA: Guangxi Prov., Hua-jiang Co., Jiu-wan-da-shan Provincial Nature Preserve, unnamed tributary of Yang-mei-au stream, 50 m upstream of Co. Road 5309 marker 123.2 km, N25.19538°, E108.65986°, elev. 1148 m, 15 Jun 2004, Coll. L. Yang
CN040616-02	CHINA: Guangxi Prov., Xing-an Co., Liu-dong-he River and Hua-jiang-he River confluence, ~1 km S of Hua-jiang town, N25.76573°, E110.48203°, elev. 262 m, 16 Jun 2004, Coll. L. Yang, J. Morse, C. Sun, C. J. Geraci
CN040618-01	CHINA: Guangxi Prov., Yang-shuo Co., Jin-bao-he River 1.6 km upstream of Jin-bao town, N24.79562°, E110.31092°, elev. 192 m, 18 Jun 2004, Coll. C. Sun, X. Zhou
CN050601-01	CHINA: Jiangxi Prov., Wu-Yi-shan National Nature Reserve, Tong-mu-he River upstream of Wu-Yi-shan Station, N27°49′62″, E117°43′10″, elev. 989 m, 01 June 2005, Coll. C. Sun
CN050601-02	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Reserve, Tong-mu-he River at Wu-yi-shan Station, N27°50′43″, E117°43′37″, elev. 900 m, 01 June 2005, Coll. L. Yang
CN050602-01	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Reserve, unnamed tributary of Tong-mu-he River, 23.8 km upstream of Wu-yi-shan Station, N27.83820°, E117.75736°, elev. 1790 m, 02 June 2005, Coll. X. Zhou, C. J. Geraci
CN050603-01	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Preserve, unnamed tributary of X. Tong-mu-he River, N27.89694°, E117.72255°, elev. 930 m, 03 June 2005, Coll. C. Sun
CN050603-03	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Preserve, unnamed tributary of X. Tong-mu-he River, N27°50′57″, E11743′53″, elev. 877 m, 03 June 2005, Coll. L. Yang
CN050604-01	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Preserve, Lei-gu-ling stream, N27°58′56″, E117°53′57″, 04 June 2005, Coll. C. Sun
CN050604-02	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Preserve, Lei-gu-ling stream, N27.99142°, E117.89111°, elev. 424 m, 04 June 2005, Coll. L. Yang, C. J. Geraci
CN050604-03	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Preserve, Lei-gu-ling Stream, N28.00453°, E117.88145°, elev. 344 m, 04 June 2005, Coll. X. Zhou, C. Zhou
CN050605-01	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Preserve, Li-tou-jian Stream, 500–900 m upstream of protected area marker, N27°58′49″, E117°51′43″, elev. 375–404 m, 05 June 2005, Coll. C. Sun, C. Zhou, X. Zhou
CN050605-02	CHINA: Jiangxi Prov., Wu-yi-shan National Nature Preserve, Li-tou-jian Stream, 100 m upstream of protected area marker, N27.98627°, E117.85617°, elev. 342 m, 05 June 2005, Coll. L. Yang, C. J. Geraci
CN050609-04	CHINA: Jiangxi Prov., Jiu-lian-shan National Nature Reserve, Dun-tou-gou stream at San-dui-qiao bridge, 500 m SE of Dun-tou village, N24°32′03″, E114°25′19″, elev. 480 m, 09 June 2005, Coll. L. Yang, C. J. Geraci
CN050610-04	CHINA: Jiangxi Prov., Jiu-lian-shan National Nature Reserve, Da-qiu-tian scenic spot, 13.2 km NW of Jiu-lian-shan Nature Reserve, Xia-gong-tang Station, Mei-hua-luo-di, main river, N24°35′25″, E114°27′17″, elev. 377 m, 10 Jun 2005, Coll. L. Yang
CN050619-01	CHINA: Beijing city, Song-shan Mt National Nature Reserve, small stream beside Da-zhuang-ke village, elev. ~1000 m, 19 June 2005, Coll. X. Zhou
CN050627-04	CHINA: Sichuan Prov., Bao-xing Co., Feng- tong-zhai National Nature Preserve, Da-shui-gou Stream, Da-shui-gou Station, Rt S210 at 257.7 km marker, N30.57915°, E102.87560°, elev. 1580 m, 27 Jun 2005, Coll. C. Sun
CN050628-03	CHINA: Sichuan Prov., Tian-quan Co., Tian-quan River at mouth of Xiao-yu-xi Stream, State Rt G318 at 2693.5 stone marker, N30°01′30″, E102°33′36″, elev. 996 m, 28 Jun 2005, Coll. C. Sun, J. Morse
CN050629-02	CHINA: Sichuan Prov., Kang-ding Co., Gu-za-zhen Town, Da-du-he River, Wa-si-gou, at suspension footbridge across river from State Rt G318 at 2819.9 km stone marker, N30.07551°, E102.16013°, elev. 1425 m, 29 Jun 2005, Coll. X. Zhou, C. J. Geraci
CN050629-03	CHINA: Sichuan Prov., Lu-ding Co., Leng-zhu-guan village, Leng-zhu-guan stream, 100–200 m upstream of State Rt G318 at 2815.2 km stone marker, N30.05196°, E102.15760°, elev. 1430 m, 29 Jun 2005, Coll. C. Zhou

APPENDIX 2. Continued.

Collection code	Collection locality details
CN050629–04	CHINA: Sichuan Prov., Lu-ding Co., Da-ba-cun village, La-zi-gou Stream, tributary of Da-du-he River, 100 m upstream of State Rt G318 at 2788.7 km stone marker, N29.86180°, E102.22346°, elev. 1515 m, 29 Jun 2005, Coll. C. Sun
CN050630-01	CHINA: Sichuan Prov., Shi-mian Co., Li-zi-ping Nature Preserve, Ca-luo-xiang town, Hai-zi-gou Stream,3rd-level Hydropower Station, 4.3 km S of State Rt G108 from 2600.8 km stone marker, N29.13947°, E102.36948°, elev. 1390 m, 30 Jun 2005, Coll. X. Zhou, J. Morse
CN050630-02	CHINA: Sichuan Prov., Shi-mian Co., Li-zi-ping Nature Preserve, Ca-luo-xiang town, unnamed tributary of Hai-zi-gou Stream, 200 m W of 3rd-level Hydropower Station, 4.3 km S of State Rt G108 from 2600.8 km stone marker, N29°08′27.7′′, E102°22′08.9′′, elev. 1384 m, 30 Jun 2005, Coll. C. Sun, C. Zhou, C. J. Geraci
CN050701-02	CHINA: Sichuan Prov., Shi-mian Co., Li-zi-ping Nature Preserve, Zi-ma-he Station, tributary of Zi-ma-he Stream, 3.7 km from unnamed paved road at 3.8 km stone marker, N29.01089°, E102.28135°, elev. 2158 m, 01 Jul 2005, Coll. C. Sun
CN050701-03	CHINA: Sichuan Prov., Shi-mian Co., Li-zi-ping Nature Preserve, Zi-ma-he Station, Zi-ma-he Stream at power station, 2.8 km from unnamed paved road at 3.8 km stone marker, N29.00621°, E102.28369°, elev. 2090 m, 01 Jul 2005, Coll. C. J. Geraci, J. Morse
CN050702-01	CHINA: Sichuan Prov., Mian-ning Co., Hui-an town, southern braid of Zhang-mu-gou Stream, 3.1 km W of Zhang-mu-gou Bridge, N28.62900°, E102.15925°, elev. 1901 m, 2 Jul 2005, Coll. X. Zhou, C. J. Geraci
CN050702-02	CHINA: Sichuan Prov., Mian-ning Co., Hui-an town, Zhang-mu-gou Stream, 100 m W of Zhang-mu-gou Bridge, N28.61820°, E102.18356°, elev. 1849 m, 02 Jul 2005, Coll. J. Morse
CN050703-01	CHINA: Sichuan Prov., Mian-ning Co., Da-jia-cun village, Yang-jia-gou Stream, 100 m downstream of S215 at 409.6 km stone marker, N28.36013°, E101.99158°, elev. 2420 m, 03 Jul 2005, Coll. C. J. Geraci, J. Morse
CN050704-02	CHINA: Sichuan Prov., Zhao-jue Co., Shang-you-cun village, Long-yan-ri-da Stream, beside S307 at 546.3 km stone marker, N27.89487°, E102.59136°, elev. 2624 m, 04 Jul 2005, Coll. C. J. Geraci, J. Morse
CN050705-01	CHINA: Sichuan Prov., Mei-gu Co., unnamed tributary of Mei-gu River at Te-xi village, dirt road from Mei-gu at 521.7 km stone marker, N28.38691°, E103.20153°, elev. 2255 m, 05 Jul 2005, Coll. X. Zhou, J. Morse
CN050705-02	CHINA: Sichuan Prov., Mei-gu Co., unnamed tributary of Mei-gu River, dirt road between,Mei-gu and Te-xi at 532.6 km stone marker, N28.37937°, E103.19186°, elev. 2189 m, 05 Jul 2005, Coll. C. J. Geraci, C. Zhou
CN050706-01	CHINA: Sichuan Prov., Mei-gu Co., Mei-gu Da-feng-ding National Nature Preserve, Long-wo-xiang village, Wo-qi-wo Stream, 3.7 km E of Long-wo, N28.77269°, E103.20991°, elev. 1700 m, 06 Jul 2005, Coll. C. Zhou
CN050706-03	CHINA: Sichuan Prov., Mei-gu Co., Mei-gu Da-feng-ding National Nature Preserve, Shu-wo-xiang village, Cha-cha-kou stream, 9.0 km E of Long-wo, N28.76082°, E103.25356°, elev. 1650 m, 06 Jul 2005, Coll. C. J. Geraci, J. Morse
CN050706-04	CHINA: Sichuan Prov., Mei-gu Co., Mei-gu Da-feng-ding National Nature Preserve, Shu-wo-xiang village, Gong-fan-yi Stream, 9.5 km E of Long-wo, N28.76059°, E103.25813°, elev. 1653 m, 06 Jul 2005, Coll. X. Zhou
CN050707-01	CHINA: Sichuan Prov., Ma-bian Co., Tian-xing village, Zhong-shan-gou stream, 4.9 km W of bridge in Ma-bian, N28.84924°, E103.50916°, elev. 597 m, 07 Jul 2005, Coll. X. Zhou, C. Sun, C Zhou, J. Morse
CN050709-01	CHINA: Sichuan Prov., Wen-chuan Co.,San-jiang scenic area, An-jia-ping-gou stream, 13.5 km NW San-jiang town, 7.7 km NW gate 400 m upstream of bridge, N30.96362°, E103.30141°, elev. 1740 m, 09 Jul 2005, Coll. C. Zhou
CN050710-02	CHINA: Sichuan Prov., Du-jiang-yan Co., Qing-cheng-hou-shan scenic area, beside road to Hong-yan-cun Forest Station, E tributary of unnamed stream 11.5 km SE main gate, N30.8962°, E103.47073°, elev. 1155 m, 10 Jul 2005, Coll. X. Zhou
GNK030726	CHINA: Guangdong Prov., Guang-zhou City, Nan-kun-shan Mt Nature Reserve, Xia-ping, small river, N23.64584°, E113.88322°, 26 Jul 2003, Coll. X. Zhou
GNL030721	CHINA: Guangdong Prov., Shao-guan Co., Ru-yang Town, 100-200 m upstream Hydropower Station, elev. ~510 m, ~N24.92214°, E113.08069°, 21 Jul 2003, Coll. X. Zhou
GNL030723	CHINA: Guangdong Prov., Shao-guan Co., Nan-ling Nature Reserve, Lao-peng-keng stream near station, elev. ~1000 m, N24.92865°, E113.01663°, 23-24 Jul 2003, Coll. X. Zhou
Hunan An-hua	CHINA: Hunan Prov., An-hua Co., Tuo-xi Reservoir, 05 Sep 2002
SFT030819	CHINA: Sichuan Prov., Bao-xing Co., Feng-tong-zhai Nature Reserve, small stream beside Da-shui-gou Station, N30.57188°, E102.88286°, elev. 1560 m, 19 Aug 2003, Coll. X. Zhou
YHL021202	CHINA: Yunnan Prov., Li-jiang Co./Town, Hei-long-tan Park, upstream creek, N26°53.527′, E100°13.911′, elev. 2422 m, 02 Dec 2002, Coll. X. Zhou, K. Li
YYS021203	CHINA: Yunnan Prov., Li-jiang Co., Hei-shui-he stream, bridge at main road from Li-jiang to Yu-long Snow Mt., elev. 2806 m, 03 Dec 2002, Coll. X. Zhou, K. Li
YYS030801	CHINA: Yunnan Prov., Li-jiang Co., Yu-shui-zhai Park, small creek, 100 m NW to the front park gate, N27°00.004′, E100°11.997′, elev. 3150 m, 01 Aug 2003, Coll. K. Li