CHAPTER 4

ASSOCIATION OF LARVAL AND ADULT OF HYDROPSYCHID SPECIES USING MITOCHONDRIAL DNA SEQUENCING

Introduction

The caddisfly family Hydropsychidae is widely distributed and ubiquitous in running water, especially in rivers and streams. Hydropsychid larvae are of fundamental importance in aquatic food webs where they serve primarily in nutrient processing and cycling (Resh and Rosenberg, 1984). Adults, which are terrestrial and usually short lived, often go unnoticed, but it is this stage on which the taxonomy of the order is based. Only adult males can be reliably identified, whereas, most females, larvae, pupae, and eggs are unidentifiable. Knowledge of the taxonomy and ecology of the hydropsychid species in temperate regions, especially in North America and Europe, are well known. Larvae have proven valuable in biomonitoring programs because of the very different susceptibility of the various species to pollutants and other types of environmental disturbance (Rosenberg and Resh, 1993). Together with the orders Ephemeroptera (mayflies) and Plecoptera (stoneflies), Trichoptera are considered primary indicator species in the monitoring of water quality, collectively constituting an “EPT” index, one of a number of biotic indices using Trichoptera (Rosenberg and Resh, 1993). These indices depend on at least family level determinations, but more often on species level identification of aquatic stages (Resh and Unzicker, 1975). However, immature stages cannot be determined

until positive associations have been made with the identifiable adult male through rearing or other methods of associating immatures and adults (e.g., the "metamorphotype" method in Trichoptera; Wiggins, 1996). However, the limitations of the metamorphotype method are that it requires collecting the pupa at precisely the appropriate time of year, a few hours before adult emergence, and generally it requires capture of a male pupa. A process for rearing the adult from larva and pupa in the laboratory may be difficult for hydropsychids because particular current and temperature regimes are often difficult to simulate. Further, field rearing is subject to unpredictable problems associated with weather, shifting substrate, and changing water levels.

Molecular genetic techniques can overcome some of the problems associated with other methods to associate larvae with adults. Insect samples can be collected and stored in alcohol (Sperling *et al*., 1994) and DNA characteristics are independent of life stage or environment. DNA extracted from small amounts of tissue can be used for molecular analysis using Polymerase Chain Reaction (PCR) (Crozier, 1993). Therefore, tissue such as genitalia can be saved as morphological vouchers.

Mitochondrial DNA has been successfully employed in studying phylogenetic and phylogeographic relationships in numerous insect taxa (e.g. Roderick, 1996; Caterino and Sperling, 1999; Caterino *et al*., 2001; Mardulyn, 2001; Lin and Danforth, 2004). In higher-level phylogenetic studies on caddisflies, mitochondrial sequence markers revealed a high amount of divergence and were only of limited use in resolving deeper lineages, for example, between suborders (Kjer *et al*., 2001; Dreesmann and Wichard, 2002). Myers and Sperling (2002) showed that

mtDNA sequences were suitable for studying the classification of *Lepidostoma* species. Myers *et al*. (2001) were able to differentiate genetically isolated populations of two limnephilid species. Geraci *et al*. (2005) showed preliminary data regarding subfamilial and generic relationships of the family Hydropsychidae based on morphological attributes as well as a mitochondrial COI gene fragment, nuclear ribosomal RNA, and EF-1 alpha sequence from Kjer *et al*. (2001-2002). Combined data support the monophyly of the Arctopsychinae, Smicrideinae and Macronematinae, but that of the Hydropsychinae and Diplectroninae remains unresolved.

In comparison to rearing and the metamorphotype method, studies of DNA properties are more often successful and more accurate and sensitive for identifications at the species level. Associations of known and unknown stages of insects have been accomplished using molecular techniques for a wide variety of insect taxa [e.g., Diptera (Toma *et al*., 2000; Wells *et al*., 2001; Sharley *et al*., 2004), Hymenoptera (Sipes, 1999), Hemiptera (Stern *et al*., 1997), Coleoptera (Szalanki and Powers, 1996; Miller *et al*., 2005; Caterino *et al*. 2006), Lepidoptera (Roehrdanz, 1997), and Odonata (Zloty *et al*., 1993)]. These methods usually include procedure such as PCR, sequencing, aligning, and sequence matching of different life stages/sexes. Because of its greater specificity, use of nucleic acid sequences has been preferred, and with its decreasing costs, sequence analysis is now much more common. Direct sequence matching is a straightforward approach. Once an unknown larvae have been sequenced, its sequence can be matched with that of the identifiable adult stage, associating the unknown larvae and known adult forms, and facilitating description using morphological diagnostic features of the previously unknown

larvae. No one has yet used molecular techniques to associate unknown larvae with identifiable males of the Trichoptera.

Here, I used a molecular technique which involved DNA sequencing to match the identity of hydropsychid larvae and adults which were collected together in the same localities. If sequences from a larva and an adult match, this pair was then associated as conspecific.

Materials and methods

Specimens examined

In order to avoid confusion concerning species boundaries and intraspecific/geographic variations, we used only larvae and adults that were collected from same localities for molecular analyses. Twenty-three associated hydropsychid species for which larvae and adults had been associated (Chapter 3) were used to confirm association with molecular data. Larval and adult specimens were preserved in 95% and 80% etOH.

DNA extraction

Whole genomic DNA was extracted from the preserved organisms using DNeasy Tissue Kits (Qiagen) following the manufacturer's protocol for insects. The larvae and adults were dissected under a stereomicroscope in 95% etOH. Either the thorax or abdominal segments 1-7 were used. The head, thorax and abdominal segments 8-10 were always kept as specimen vouchers. The digestive tract was

removed to avoid contamination by food remains. The prepared thorax or abdomen was placed into a 1.5 ml sterile reaction tube with 180 µl ATL buffer. The tissue was ground using a sterile micro pestle. 20 µl of Proteinase K and 200 µl of lysis buffer AL were added to the solution, mixed thoroughly using a vortex and incubated for 10 to 12 min in a water bath at 70^{o}C. The mixture was vortexed once during the incubation period and directly thereafter. The solution was then loaded onto the supplied DNA spin columns and centrifuged at 8,000 rpm for 2 min. In this step, the DNA was bound to a membrane at the bottom of the spin columns. Bound DNA was washed with buffers in two subsequent steps: First, 600 µl of buffer AW1 were loaded and the sample centrifuged for 2 min at 8,000 rpm. Then, 200 µl of buffer AW2 were loaded and the sample centrifuged at 14,000 rpm for 4 min. If samples still appeared moist, they were centrifuged again for 2 min at 14,000 rpm to ensure that etOH residue from buffers AW1 and AW2 was removed, as etOH may inhibit subsequent PCR reaction. DNA was eluted in 100 µl 60^{o}C PCR-grade water and incubated for 2 min at room temperature, before it was centrifuged in a 1.5 ml reaction tube at 12,000 rpm for 2 min. The elution step was repeated to obtain a second, less concentrated DNA solution as “back-up” for each individual. Samples were generally stored at 4^{o}C until PCR reactions were finished and then frozen at -20^{o}C for long-term storage. Repeated freezing and thawing of samples was avoided.

DNA amplification

Polymerase chain reaction (PCR) was used to amplify approximately 257 bp of the mitochondrial dehydrogenase subunit 4 (*ND4*) gene with primers N4-J-8052: 5'-GTAGGAGGAGCTGCTATATTAG-3' and N4-N-8718:5'-

GCTTATTCATCGGTTGCTCA-3' (Simon *et al*., 1994), and approximately 710 bp of cytochrome oxidase I (*COI*) gene with primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al*., 1994). The PCR reactions were performed on a variety of different thermocycles at the Molecular Unit, Scientific Centre, Prince of Songkla University. PCR reactions were carried out with 2 to 5 µl of DNA template, 10.5 µl Taq polymerase using TaqMaster system (Eppendorf; Hamburg, Germany), 1 µl of each primer (10mM), 2.5-3.5 µl of $MgCl_2$, and de-ionized water were added to reach a final reaction volume of 25 µl. The PCR reactions were run on the GeneAmp PCR system 9700 (PE Applied Biosystems, U.S.A.) using the following program: 35 cycles of 94^{o}C for 45s, 45^{o}C for 1 min, and 72^{o}C for 1.5 min. Product was stored at 4^{o}C. Initial PCR reaction of *ND4* was performed following the procedure outlined in Szalanski and Powers (1996). The PCR reaction for *COI* was run using the following program:

Initial denaturation	1 cycle	94^{o}C for 5 min
Amplification	35 cycles	94^{o}C for 30 sec
		40^{o}C for 30 sec
		72^{o}C for 1 min
Final extension	1 cycle	68^{o}C for 5 min

Initial PCR reaction for *COI* was performed following the procedure outlined in Folmer *et al*. (1994).

Agarose gel electrophoresis

After the PCR products were amplified, they were analyzed using

agarose gel electrophoresis. In this study, gel electrophoresis was used to determine the size of DNA of interest. For the gel, 1.5% (w/v) of agarose gel in 1x TBE buffer (40 mM Tris-borate, 1 mM EDTA) was melted and poured in a plastic tray, and a comb was placed in the gel. After the agarose gel was completely set (15-20 min at room temperature), the comb was carefully removed and the gel was installed on a platform in the electrophoresis tank containing 1x TBE buffer. The PCR products were mixed with 20% (v/v) gel-loading buffer (40% Sucrose, 0.25% Bromophenol Blue) and slowly loaded into the slots of the submerged gel using an automatic micropipette. Electrophoresis was carried out at a constant 90 V for 35 min. Then, the gel was stained with 2.5 ug/ml of Ethidium Bromide (EtBr) solution for 15 min and de-stained with distilled water for 15 min. Finally, the DNA fragments were observed in a UV light box (Gel Doc, model 1000, BIO-RAD, USA).

Automated DNA sequencing

Preparation of 5% Acrylamide gel/6M Urea

Urea (18 g) and 50% acrylamide stock solution (5 ml) were combined in a 125 ml Erlenmeyer flask. De-ionized water was added to bring the volume up to 40 ml. The solution was stirred until the crystals began to dissolve. 5 ml of filtered 10 X TBE stock solution (40 mM Tris-borate, 1 mM EDTA) and de-ionized water were added to bring the volume to 50 ml. The acrylamide solution was vacuum filtered by using 0.2 µm unit for 10 min. 250 µl of 10% APS were added into the filtered gel solution and swirled for a few seconds. 25 µl of TEMED was added into the acrylamide solution and gently swirled for a few seconds. Then the solution was injected between the glass plates until the solution was up to the notch. The gel was

polymerized at room temperature for at least 2 hr.

Preparation of the DNA samples for sequence analysis

The ABI PRISM^{TM} BigDye Terminator Cycle Sequencing Kit was used to prepare the DNA samples for sequence analysis. The principle of the protocol is based on fluorescent-labeled terminator cycle sequencing. The PCR reaction was performed in a 10 µl reaction mixture containing a terminator ready reaction mix [A-dye terminator labeled with dichloro (R6G), C-dye terminator labeled with dichloro (ROX), G-dye terminator labeled with dichloro (R110), T-dye terminator labeled with dichloro (TAMR10), deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP), Tris-HCl pH 9.0, Amplitaq DNA polymerase], 200-500 ng of plasmid DNA template, 1.6 pmol of primer and sterile water. The amplification was carried out in a DNA thermal cycler (GeneAmp System 9700, Perkin Elmer) with the following condition: 25 cycles of 96^{o}C for 10s, 45^{o}C for 5s and 70^{o}C for 1 min. The PCR-bases sequencing products were precipitated by ethanol after amplification and the dye-labeled DNA was analysed on 5% Long Ranger/6M Urea Acrylamide Gel (Applied Biosystems 377 sequencer, Perkin-Elmer, Norwalk CT, USA).

Precipitation of sequencing products

10 µl of dye-labeled DNA in 0.2 ml PCR tube was pipetted to 1.5 ml microcentrifuge tubes and 25 µl of 100% ethanol, 2 µl of 3 M Sodium acetate, 2 µl of 125 MM EDTA were added. The tubes were closed and then vortexed briefly. The reaction was incubated at room temperature for 15 min to precipitate the PCR products. The tubes were placed in a microcentrifuge and their orientations marked,

followed by spinning for 30 min at maximum speed. The supernatants were aspirated and discarded by a separate pipette tip for each sample. 35 µl of 70% ethanol was added to the tubes and vortexed briefly again. The tubes were placed in the microcentrifuge in the same orientation as the first time and spun for 5 min at maximum speed. The supernatants were aspirated carefully. The pellet was dried in a 95^{o}C heating block for 1 min.

Preparing and loading the samples

6 µl of loading buffer (5 µl of deionized formamide, 1 µl of 25 mM EDTA pH 8 with 50 mg/ml blue dextran) was added to the dry pellet. This was vigorously vortexed and spun to dissolve the pellet. This solution was heated at 95^{o}C for 2 min to denature the nucleotide. Then the tubes were placed on ice until ready to load on polyacrylamide gel.

DNA sequence analysis

Sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) as implemented in BioEdit (Hall, 1999). Because gaps and length variants were not to be expected in the studied mitochondrial sequences, gaps were removed manually after rechecking the electropherogram in SEQMAN for the individuals and positions in question. Sequences were trimmed to length using Sequencher, resulting in 178 bp for *ND4* gene and 523 bp for *COI* gene. Pairwise numbers of nucleotide difference were calculated with the program MEGA 2.1 (Kumar *et al*., 2001) using the 'Calculate distances' option and 'Nucleotide: *p*-distance' model option for distances. The *p*-distance, the proportion of sites in which two sequences differ, was calculated

by dividing the number of nucleotide differences by the total number of nucleotides (Kumar *et al*., 2001).

Results

In this study, four universal primer pairs were used to amplify DNA from the specimens. Of 23 hydropsychid species belonging to 9 genera, the selected universal primer pairs successfully amplified DNA from eight species. The estimated lengths of the sequences were 257 bp in *ND4* gene and 710 bp in *COI* gene after 1.5 % agarose gel electrophoresis (Figs. 4.1 and 4.2). The two regions obtained from amplification were compared with the GenBank database.

To confirm the association between larvae and adult, the sequence of each stage was compared. The obtained sequences of *Hydatomanicus klanklini*, *Diplectrona gombak*, *Cheumatopsyche tramota*, *Hydromanicus serubabel*, *Macrostemum dohrni*, *M. hestia*, *Pseudoleptonema quinquefasciatum*, and *P. supalak* were compared using the BLASTN program in the GenBank database (www.ncbi.nih.nlm.gov/BLAST). *Hydatomanicus klanklini* showed 87% identity with NH4 gene of *Drosophila obscuroides* (Accession number: AY958414, e-value = -13), *Diplectrona gombak* showed 92% identity with ND4 gene of *Lucilia porphyrina* (Accession number: AY842716, e-value = -04), *Cheumatopsyche tramota* showed 84% identity with mitochondrial complete genome of *Coreana raphaelis* (Accession number: DQ102703, e-value = -13), *Hydromanicus serubabel* showed 91% identity with *COI* gene of *Callithomia lenea* (Accession number: DQ069232, e-value = -06), *Macrostemum dohrni* showed 85% identity with COI gene of *Arctopsyche grandis*

(Accession number: AF241379, e-value = -89), *M. hestia* showed 83% identity with *COI* gene of *Eupyrrhoglossum sagra* (Accession number: DQ276221, e-value = -57), and *Pseudoleptonema quinquefasciatum* showed 84% identity with *COI* gene of *Onycholyda amplecta* (Accession number: EF032223, e-value = -60).

Table 4.1 Species of Hydropsychidae for which DNA was successfully amplified. Stage is given as L = larva; A = adult male. Mal. & Chan. = Malicky and Chantaramongkol

Species	Gene region	Stage
Diplectrona gombak Oläh	*ND4*	L, A
Hydatomanicus klanklini Mal. & Chan.	*ND4*	L, A
Cheumatopsyche tramota Mal. & Chan.	*ND4*	A
Hydromanicus serubabel Mal. & Chan.	*COI*	A
Macrostemum dohrni Ulmer	*COI*	A
Macrostemum hestia Mal. & Chan.	*COI*	L, A
Pseudoleptonema quinquefasciatum	*COI*	A
Pseudoleptonema supalak Mal. & Chan.	*COI*	A

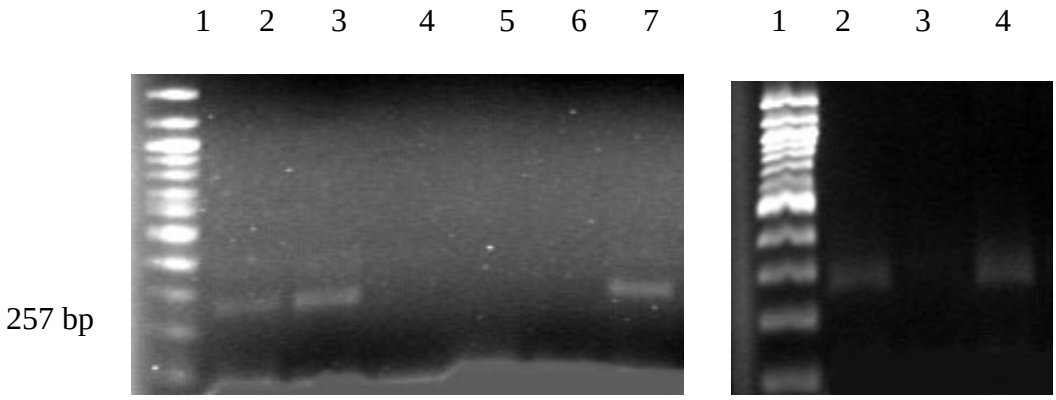

Figure 4.1 Ethidium bromide stained agarose gel of PCR products from *ND4* in Hydropsychidae species. Left column, lane 1: DNA ladder, lanes 2, 3, and 7: DNA fragments amplified by the primer sets. Lanes 2 and 3: larva and adult *Hydatomanicus klanklini*; lane7: adult *Cheumatopsyche tramota*; lanes 4, 5, and 6: no DNA template control. Right column, lanes 2 and 4: larva and adult *Diplectrona gombak*; lane 3: no DNA template control.

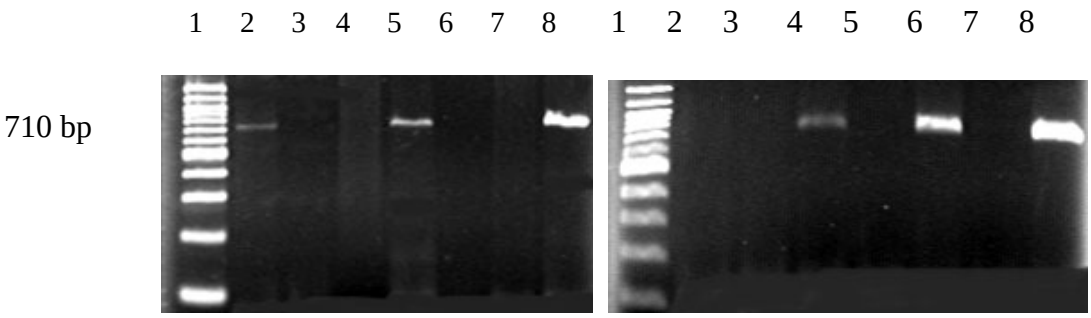

Figure 4.2 Ethidium bromide stained agarose gel of PCR products from *COI* in species of Hydropsychidae. Left column, lane 1: DNA ladder: lanes 2, 5, and 8: DNA fragments amplified by the primer sets. Lane 2: adult *Macrostemum hestia*; lane 5: adult *Hydromanicus serubabel*; lane 8: adult *M. dohrni*; lanes 3, 4, 6, and 7: no DNA template control. Right column, lane 4: larva *M. hestia*; lane 6: adult *Pseudoleptonema quinquefasciatum*; lane 8: adult *P. supalak*; lanes 2, 3, 5, and 7: no DNA template control.

The complete nucleotide sequence of *Hydatomanicus klanklini*, *Diplectrona gombak*, and *Cheumatopsyche tramota* are composed of 178 bp in length and the complete nucleotide sequence of *Hydromanicus serubabel*, *Macrostemum dohrni*, *M. hestia*, *Pseudoleptonema quinquefasciatum*, and *P. supalak* are composed of 523 bp in length. The nucleotide sequence alignments of the *ND4* gene and *COI* gene were shown in Figures 4.3 and 4.4.

Figure 4.3 Nucleotide sequence alignments of the *ND4* gene in *Hydatomanicus klanklini*, *Diplectrona gombak*, and *Cheumatopsyche tramota*.

Hydatomanicus klanklini Adult male

CCTTTATTAATTATTAACCTTCGTCTTCCTAACCGTTCATAAATTAAATTAATTAAAACAAA
TAAACCAGAAGAACATAATCCATGCCCAACTATTAAAATTAACCTACCTGTAAATCCAAA
AAAAGTTAAAGTCATAATACCTCCAATTACTAAAGCTATATGAGCAACCGATGAAT

Hydatomanicus klanklini Larva

CCGTTTTTATTTATTAGCCGTCGTCGTCCTACGCGCTCCTGGATTATATGAATTAATACAAA
TAAACCTGGAGACGGTTAGCCAGGCGCAGCTATTTAAATTAACCTGGCTGTTTATCCTTAT
AACGTTAAAGTCCTTTTACCTACAATTACTAAAGCTATATGAGCAACCGATGACC

Diplectrona gombak Adult male

CCCTTATTAATCATTAATCTACGTCTGCCCGACCGTTCATAAACTAAATTAACCAAAGCAA
ATAGCCCTGATGAACACAAGCCATGACCAATTATTAAAATATAACTCCCCAAAACCCCTA
GATAATTCAAAGACATTAGCCCCCCTAATACTAAAGCTATATGAGCAACCGATGAAT

Figure 4.3 Continued.

Diplectrona gombak Larva

CCCTTATTAATCAATAATCGACGTCGGCCCGACCGTTCATAAACTAAATAAACCAAAGCA
AATAGCCCTGATGAACACAAGCCATGACCAATTATTAAAATATAACTCCCCAAAACCCCT
AGATGATTCAAAGACATTAGCCCCCCTAATACTAAAGCTATATGAGCAACCGATGAAT

Cheumatopsyche tramota Adult male

CCCTTATTAACTAATATACTTCGTCTTCCCAGGCGATCGTACATAAAACCAATTAATACGA
ATAATCCAGAAGAACATAGGCCATGTCCAATTATTAAAACCAATCCACCTATAAACCCTC
AATAATTCAAGGATATAATCCCTCCAATAACTAAACTTATATGAGCAACCGATGAAT

Figure 4.4 Nucleotide sequence alignments of the *COI* genes in *Hydromanicus serubabel*, *Macrostemum dohrni*, *M. hestia*, *Pseudoleptonema quinquefasciatum* and *P. supalak*.

Hydromanicus serubabel Adult male

CAGTTAATAATATTGTAATTGCTCCTGCAAGAACAGGTAAAGAAAGAAGAAGTAAAACTG
CTGTAATTAAAACAGATCAAATAAATAAAGGAAGTTTATCTGATGTTAAATTGAAACTTTT
TATGTTTAAAATTGTTGAAATAAAATTAATGGCTCCAAGAATAGAAAAAATACCTGCAAG
ATGTAAAGAAAAAATTGTTAAATCAACAGCATTGCCTCTATGTGATAAATTTGATGATAAT
GGAGGATAAACAGTTCATCCTGTTCCTGCTCCTATATTGGTTAATCTTCTAATAATAAGGA
AAATTAATGAAGGAGGGAGTAATCAAAATCTTATGTTATTTATTCGTGGGAATGCTATATC
AGGGGCTCCTAATATTAATGGAACAAGTCAGTTTCCAAATCCTCCAATTATAATTGGTATT
ACTATAAAAAAAATTATGATAAAAGCATGAGCTGTAACTGTTACATTATAAATTTGATCAT
TACCAATTATTCTGAAAGGGGAAGATAATTCAATACG

Figure 4.4 Continued.

Macrostemum dohrni Adult male

CAGTTAATAATATGGTAATAGCTCCTGCTAAGACAGGAAGTGATAATAATAGAAGGATAG
TTGTAATTAAAATTGATCAGATAAATAAGGGAAGTTTATCAAATTTTAAATTATTAATTTT
TATGTTTATAATAGTGGTAATAAAATTAATAGCTCCAAGAATAGAAGAAATTCCTGCTAA
ATGAAGAGAAAAAATAGTAATATCTACTGATCTTCCTATATGGGAAAGGTTAGAAGATAG
AGGAGGGTAAACAGTTCATCCTGTACCAGCTCCATTATTTATTAGTCTTCTGAAGATTAAG
AAGGTAAGGGAGGGGGGTAGTAATCAAAATCTTATATTATTTATTCGAGGGAAGGCTATA
TCAGGGGCTCCTAATATTAAGGGAACTAATCAATTTCCAAATCCCCCGATTATAATGGGTA
TAACTATAAAGAAAATTATAATAAAGGCATGAGCTGTAACGATAACGTTATATATTTGAT
CATTTCCAATTAAAGAGTTAGGTGTTCTTAATTCAATTCG

Macrostemum hestia Adult male

CAGTTAGTAATATTGTAATAGCTCCCGATAATACTGGTAGAGAAAAAAAAAGAAGAATTG
TAATAATTAAGATTGATCATACAAATAGGGGAAGTTTATCTATTCTAAGGTTATTAACCTT
TATATTTAAAATTGTAGTAATAAAATTAATTGATCCAAGAATAGATGAGATACCCGCTAA
ATGGAGTGAAAAAATTGTTAAATCAACTGATCTTCCTATGTGGGATAGATTTGAAGATAG
GGGTGGGTAAACAGTTCAACCAGTTCCTGCTCCCTTATTTAAAAGTTTTCTGAAAAGAAGG
AAGGTTAGTGAGGGCGGTAAAAGTCAAAATCTTATATTATTTATTCGAGGAAAGGCTATA
TCTGGGGCGCCTAGTATTAGAGGTACTAATCAATTACCAAATCCTCCGATTATGAATGGAA
TAACTATAAAGAAAATTATAATGAAAGCATGAGCTGTCCCAATTACGTTATATATTTGATC
ATTACCGATAGGGGAGGATGGTGTTCCTTAATTCAATTC

Macrostemum hestia Larva

ACGAATTGGGAAAGGGACCCTCTGGTGGGTGGGGGGATCATATTTGCTGTGGCCGAAGAA
AGGTTTGTCATGAAGTTCTATCTCACATATGGACTAGTCTCGCTTATCAAGGGTGGGTTCG
TACAACCGAGCTACCATGCGATAGTATACTGAGATACTCTGTGTATATGAGTCAGAGGCT
CATGCTAGTCAGAGAGATACTTCTAGTCTCGTACCCTCGGAGATCAAACTATTTACTGAAT
ACGATTAGCAGAAAGAGTTCTGCACAAGGTAGAATGTTCAATGCATGATGGGAATAGAAC
AGCTACGGCTACTCAAAAATATACCAAAAGCTTTCAATAATGACAGTTGAGACAAGAAGA
GCTGAGAGACATGATGATTTAAATGGAGTAGGCATCGAAGAAGAGTACAGTATGTCGGTC
TTATATTTGTAGTTTTTTTATTTAGTTTCATACTAGACACTAATATGTAGTGTATTTGTGAG
GGTCGGGGCGTGCATATTCAGTTAATTTTTTTCGCACTT

Figure 4.4 Continued.

Pseudoleptonema quinquefasciatum Adult male

CAGTTAATAATATTGTAATTGCTCCTGCAAGAACAGGTAAAGAAAGAAGAAGTAAAACTG
CTGTAATTAAAACAGATCAAATAAATAAAGGAAGTTTATCTGATGTTAAATTGAAACTTTT
TATGTTTAAAATTGTTGAAATAAAATTAATGGCTCCAAGAATAGAAAAAATACCTGCAAG
ATGTAAAGAAAAAATTGTTAAATCAACAGCATTGCCTCTATGTGATAAATTTGATGATAAT
GGAGGATAAACAGTTCATCCTGTTCCTGCTCCTATATTGGTTAATCTTCTAATAATAAGGA
AAATTAATGAAGGAGGGAGTAATCAAAATCTTATGTTATTTATTCGTGGGAATGCTATATC
AGGGGCTCCTAATATTAATGGAACAAGTCAGTTTCCAAATCCTCCAATTATAATTGGTATT
ACTATAAAAAAAATTATGATAAAAGCATGAGCTGTAACTGTTACATTATAAATTTGATCAT
TACCAATTATTCTGAAAGGGGAAGATAATTCAATACG

Pseudoleptonema supalak Adult male

ATACTTTCTCTTTGGTATTTGATCAGGGTGTCTTGGATCCCTCATTAAGAATACTAATTCGT
TATTGAATTATCAACTCCTCAAACATTAATTGGCAATGATCAAATCTATAATACAATTGTA
ACAGCTCATGCGTTTATTATAATTTTTTTTATAGTTATACCAATTATAATTGGGGGATTTGG
AAATTGATTAGTCCCATTAATATTAGGAGCTCCTGATATGGCTTTCCCTCGAATAAATAAT
ATAAGATTTTGATTATTACCCCCTTCATTAACCTTTCTTATTATCGGTAGCCTTACAAATAG
GGGTGCAGGAACAGGCTGAACAGTCTACCCCCCACTCTCATCTAATTTATCACATTCTGGG
AGATCAGTAAATTTAACTATTTTTTCCCTTCACTTAGCTGGAATCTCTTCAATTTTAGGGGC
TATCAACTTCATCTCAACAATTATTAACATAAAAATTAATAACTTAAATCTTGATAAAATC
CCTCTTTTTGTTTGATCAGTTCTAATCACAG

The *p*-distance between larva and adult *Hydatomanicus klanklini* was 0.225 % and larva and adult *Diplectrona gombak* was 0.028 % (Table 4.2). The *p*-distance between larva and adult *Macrostemum hestia* was 0.724 % (Table 4.3).

Table 4.2 Pairwise distances between taxa expressed as a percentage of nucleotide differences of *ND4* (*p*-distances) (L = larva, A = adult male).

	H. klanklini A	*H. klanklini* L	*D. gombak* A	*D. gombak* L
H. klanklini A				
H. klanklini L	0.225			
D. gombak A	0.225	0.393		
D. gombak L	0.253	0.393	0.028	
C. tramota A 0	.219	0.354	0.281	0.292

Table 4.3 Pairwise distances between taxa expressed as a percentage of nucleotide differences of *COI* (*p*-distances) (L = larva, A = adult male).

	1	2	3	4	5
P. quinquefasciatum A					
M. hestia A	0.602				
M. hestia L	0.718	0.724			
P. supalak A	0.715	0.711	0.703		
H. serubabel A	0.674	0.684	0.728	0.747	
M. dohrni A	0.619	0.701	0.701	0.695	0.684

1, *P. quinquefasciatum* A; 2, *M. hestia* A; 3, *M. hestia* L; 4, *P. supalak* A; 5, *H. serubabel* A.

Discussion

The use of DNA sequence data to associate morphologically divergent larvae and adults has the potential to dramatically alter our rate of acquisition of knowledge of this character-rich life stage (Miller *et al*., 2005). In this study, the successful association between larvae and adults of three species of Hydropsychidae (*Diplectrona gombak, Hydatomanicus klanklini,* and *Macrostemum hestia*) were achieved by PCR amplification and DNA sequences matching.

Species-specific primer is the most important factor for successful amplification using PCR (Hoogendoorn and Heimpel, 2001). *Diplectrona gombak* and *Hydatomanicus klanklini* were associated based on the *ND4* gene and *Macrostemum hestia* were associated based on the *COI* gene. In addition, these selected primers amplified DNA from the adult of *Cheumatopsyche tramota, Pseudoleptonema supalak, P. quinquefasciatum, M. dohrni,* and *Hydromanicus serubabel* (Table 4.2). For the others species of Hydropsychidae in this study, these primers were not able to amplify DNA.

This was the first attempt to associate larvae and adults of Hydropsychidae using sequence data to work backward to find diagnostic features in the in the morphology. Because this project was limited in time and funding particular sets of primers, as mentioned above, were selected for PCR amplification and sequencing on *COI* and *ND4*. Unfortunately, these sets of primers were not appropriate for many species of Hydropsychidae. For the *COI* gene, the larva and adult *Macrostemum hestia* were successfully amplified, however the band corresponding to the target PCR products was not evident. When these genes were

sequenced and aligned, the length of sequence data of two products were ambiguous and varied. The pairwise numbers of nucleotide differences between the larva and adult *M. hestia* was 0.724%. Based on this, the larva and adult *M. hestia* may not assigned to same species. However, the larva and adult *M. hestia* were associated using the metamorphotype method.

Life stage associations in the Trichoptera using molecular data might be further explored as a method for identifying larvae and pupae. This approach has not been developed to date in immature stages, but represents a powerful new tool in larval taxonomy. Molecular species assignment to associate adult and immature specimens could be an interesting alternative to classical methods.

To use the database of *ND4* and *COI* under various specimen conditions, species-specific primers should be applied for different stages and specimens preserved for various times (Chen *et al*., 2003). In addition, the PCR should be optimized for future studies. The success or failure of PCR depends on many factors, such as the primer concentration, DNA template concentration, concentration of dNTPs, GC content of DNA template, annealing temperature, cycle hold timers, and number of cycles (Sambrook *et al*., 1989).