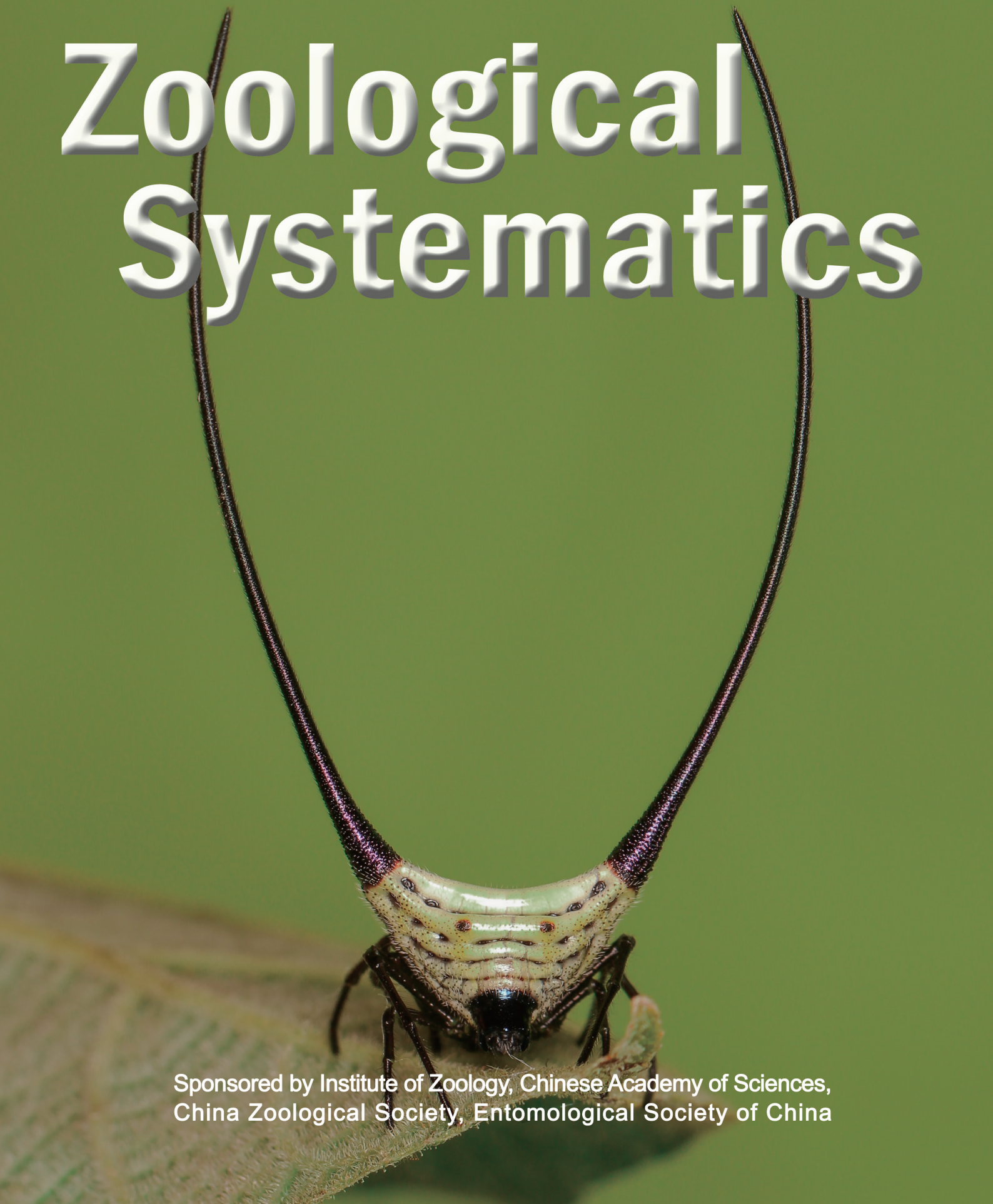
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ORIGINAL ARTICLE

# Identifying PCR primers to facilitate molecular phylogenetics in Caddisflies (Trichoptera)

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**Abstract** The molecular phylogenetics of the Lepidoptera (butterflies and moths) is well studied, but that of Trichoptera (caddisflies), the sister clade of Lepidoptera, is less studied. The PCR primer libraries developed for lepidopteran phylogenetics might work in Trichoptera. DNA from 8 caddisfly species (*Asynarchus nigriculus* (Banks, 1908), *Grammotaulius lorettae* Denning, 1941, *Hesperophylax occidentalis* (Banks, 1908), *Limnephilus externus* Hagen, 1861, *Limnephilus picturatus* McLachlan, 1875, *Limnephilus secludens* Banks, 1914, *Limnephilus sublunatus* Provancher, 1877 and *Agrypnia deflata* (Milne, 1931)) was used to screen for amplification. 107 primer pairs for 45 nuclear and 3 mitochondrial genes were tested. Primers for 1 new gene (*40S ribosomal protein S2* (*RPS2*)) and 8 genes previously used in Trichopteran phylogenetics were recovered (*16S rRNA, 18S rRNA, carbamoyl-phosphate synthetase* (*CAD*), *cytochrome oxidase I* (*COI*), *cytochrome oxidase II* (*COII*), *elongation factor-1 alpha* (*EF-1 alpha*), *isocitrate dehydrogenase* (*IDH*), and *RNA polymerase-II* (*POL-II*)). New primer pairs extended the genomic region sampled for many genes. Evolution rates among loci varied by 2 orders of magnitude. Differences among evolution rates and modes of inheritance offer flexible tools for resolving phylogenetic questions and examining genome evolution in the Trichoptera. Screening libraries of PCR primers is a useful approach for identifying PCR primers in related taxa with limited molecular genetic resources.

**Key words** Trichoptera, molecular phylogenetics, mosaic genome evolution, rates of sequence evolution, PCR primer library.

## 1 Introduction

The order Trichoptera (caddisflies) is a holometabolous insect order with aquatic larvae and terrestrial adults capable of flight. They are globally distributed with 14291 known extant species (Holzenthal, 2011). Caddisflies are well studied morphologically and phylogenies of this order based on morphological characters have been established (Morse, 1997). However, to test phylogenetic hypotheses based on morphology, acquiring independent data sets based on other characters, such as DNA sequence data, is often very informative (Marcus & McCune, 1999; deQueiroz, 1996).

The most common way to construct a molecular phylogeny is to amplify and sequence a series of individual genes using PCR appropriate primers. *Cytochrome C oxidase* subunit 1 (*COI*) is a mitochondrial gene that is frequently used in molecular phylogenetics. It is the standard gene used in the animal Barcode of Life project (Hebert *et al.*, 2003) due to the

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availability of primers that work in diverse organisms (Folmer *et al.*, 1994) including caddisflies (Wickson *et al.*, 2014). However, beyond using universal primers, other primers have to be designed for the specific taxa being studied. The inclusion of multiple genes helps guard against any inherent bias introduced by any individual locus because different loci can have distinctly different phylogenetic signals that may or may not reflect the evolution of the species as a whole (Nosenko *et al.*, 2013). The inclusion of sequences of multiple genes also allows for the identification of phenomena such as hybridization, lateral transfer, and introgression (Wahlberg *et al.*, 2009; Anderson *et al.*, 2009; Borchers & Marcus, 2014). Including both protein and RNA coding genes, as well as nuclear and mitochondrial genes, can give complementary information about the organisms being studied because they differ with respect to rates of sequence evolution, constraints produced by the structural requirements of their gene products (Nosenko *et al.*, 2013), and mode of inheritance (biparental vs. maternal) (Borchers & Marcus, 2014). The inclusion of multiple genes can be difficult in non-model systems where DNA sequence information for developing primers is often very limited. Primers for use in systems without well established molecular tools are often based on primers used in closely related, but more thoroughly studied taxa that may share conserved regions of the genome.

In Trichoptera, there have been several degenerate PCR primers designed or modified based on sequences from other insect groups (Kjer *et al.*, 2002; Johanson & Malm, 2010; Johanson *et al.*, 2012; Geerts *et al.*, 2001; Malm & Johanson, 2008; Kjer *et al.*, 2001), especially the Lepidoptera (moths and butterflies), which are thought to be the sister order to the Trichoptera on the basis of morphology (Kristensen & Skalski, 1999; Wang *et al.*, 2014). Molecular phylogenetic analysis has supported this sister-clade relationship using both traditional (Malm *et al.*, 2013) and phylogenomic (Misof *et al.*, 2014) approaches. Depending on the data set and analysis methods used, estimates for the timing of the divergence between the Trichoptera and the Lepidoptera vary from 210 to 234 Ma (Misof *et al.*, 2014; Malm *et al.*, 2013). There have been many molecular phylogenetic studies of the Lepidoptera (e.g. Mutanen *et al.*, 2010, Regier *et al.*, 2013) and there are several large libraries of degenerate Lepidoptera primers that might also be effective in related groups. To date, however, there have been no reports of systematic testing of PCR primers from the Lepidoptera or other insects to determine if any of these reagents could also be used in the Trichoptera.

This screen was performed to expand the number of genes and primer sets available for use in Trichopteran molecular phylogenetics. In this screen, several primer pairs already in use for Trichopteran systematics were re-identified. Several additional primer pairs that are very effective in caddisflies, but have not been used previously in molecular phylogenetic studies of the Trichoptera were identified.

## 2 Materials and methods

Larval caddisflies of 5 species were collected with a D-shaped benthos net from aquatic habitats in the vicinity of the Rocky Mountain Biological Laboratory (Gunnison County, Colorado, USA) in June 2013, an area with a well-studied caddisfly fauna (Wissinger *et al.*, 1999; Wissinger *et al.*, 2006; Wissinger *et al.*, 2003). Two species, *Agrypnia deflata* (Milne, 1931) and *Hesperophylax occidentalis* (Banks, 1908), were collected from ponds 1 and 18, respectively, in the Mexican Cut Nature Preserve (see maps in Wissinger *et al.* 1999, Wissinger *et al.*, 2003; 39.028785ºN, 107.065114ºW); two species, *Limnephilus externus* Hagen, 1861 and *L. picturatus* McLachlan, 1875, were collected from a temporary sedge marsh near the outlet of Emerald Lake (39.007440ºN, 107.039086ºW), and one species, *Asynarchus nigriculus* (Banks, 1908), was collected from both of these habitats. In June 2014, 3 additional species of caddisflies, *L. secludens* Banks, 1914, *L. sublunatus* Provancher, 1877 and *Grammotaulius lorettae* Denning, 1941, were collected in a shallow kettle south of the Rocky Mountain Biological Laboratory ("Kettlepond 1"; 38.943252ºN, 106.976714ºW). *Agrypnia deflata* is in the trichopteran family Phryganeidae while the remaining species are in the trichopteran family Limnephilidae.

The specimens were identified to species level on the basis of associated larval and adult morphology (Ruiter, 1995). Species identifications were later verified by comparing *COI* DNA barcode sequences from specimens collected for this study with sequences from specimens from prior studies (Ratnasingham & Hebert, 2007; Ruiter *et al.*, 2013). The specimens were stored in their cases in either 95% ethanol or RNALater (Qiagen, Düsseldorf, Germany) at -20°C. DNA was prepared from individual specimens of each species using the Qiagen DNEasy Blood and Tissue kit as described previously (Gemmell & Marcus, 2015). Each specimen was dissected from its case before being ground, using a ceramic mortar and pestle, in 180 μL of tissue lysis buffer ATL. Once homogenized, 20 μL of protein kinase K (Qiagen, 600 mU/mL)

**Table 1. Genes, primer pairs, and reaction conditions for PCR primers used successfully in the Trichoptera.**

| Gene | Primer | Conditions |
|---|---|---|
| *16S ribosomal rRNA (16S)* | ARL (f)/BRH (r) (Johanson & Malm, 2010)<br>ARL (f): CGCCTGTTTATCAAAAACAT<br>BRH (r): CCGGTCTGAACTCAGATCACGT | 95°C for 5 min,<br>40x (95°C for 30 sec,<br>50°C for 30 sec,<br>72°C for 50 sec),<br>72°C for 8 min,<br>4°C hold |
| | LeptoF/LeptoR (Johanson & Malm, 2010)<br>LeptoF: TAAGTGTGCAAAGGTAGC<br>LeptoR: TTAATCCAACATCGAGGTC | Same as *16S*: ARL/BRH |
| *18S ribosomal RNA (18S)* | 18S rRNA (f)/18S rRNA (r) (Medlin *et al.*, 1988)[1]<br>18S rRNA (f): AACCTGGTTGATCCTGCCAGT<br>18S rRNA (r): TGATCCTTCTGCAGGTTCACCTAC | 95°C for 5 min,<br>35x (95°C for 30 sec,<br>55°C for 1 min,<br>72°C for 2 min),<br>72°C for 10 min,<br>4°C hold |
| *Carbamoyl-phosphate synthetase (CAD)* | CAD743nF/CADmidR (Wahlberg & Wheat, 2008)<br>CAD743nF: TAATACGACTCACTATAGGGGGNGTNACNACNGCNTGYTTYGARCC<br>CADmidR: ATTAACCCTCACTAAAGCATTCWGCKGCWACTGTATC | 95°C for 5 min,<br>40x (94°C for 30 sec,<br>55°C for 30 sec,<br>72°C for 1 min 30 sec),<br>72°C for 10 min,<br>4°C hold |
| | CAD743nF-ino/CAD1028R-ino (Johanson & Malm, 2010)<br>CAD743nF-ino: GGIGTIACIACIGCITGYTTYGARCC<br>CAD1028R-ino: TTRTTIGGIARYTGICCICCCAT | 95°C for 5 min,<br>40x (95°C for 30 sec,<br>53°C for 30 sec,<br>72°C for 50 sec),<br>72°C for 8 min,<br>4°C hold |
| *Cytochrome c oxidase subunit I (COI)* | LCO1490 (f)/HCO2198 (r) (Folmer *et al.*, 1994)<br>LCO1490 (f): GGTCAACAAATCATAAAGATATTGG<br>HCO2198 (r): TAAACTTCAGGGTGACCAAAAAATCA | 95°C for 5 min,<br>40x (95°C for 1 min,<br>46°C for 1 min,<br>72°C for 1 min 30 sec),<br>72°C for 5 min,<br>4°C hold |
| | LCO1490 (f)/Nancy (r) (Folmer *et al.*, 1994; Monteiro & Pierce, 2001)[1]<br>LCO1490 (f): GGTCAACAAATCATAAAGATATTGG | Same as COI: LCO1490 /HCO2198 |

**Table 1 (continued)**

| Gene | Primer | Conditions |
|---|---|---|
| | Nancy (r): CCCGGTAAAATTAAAATATAAACTTC | |
| | Ron (f)/Hobbes (r) (Monteiro & Pierce, 2001)[1]<br>Ron (f): GGATCACCTGATATA GCATTCCC<br>Hobbes (r): AAATGTTGNGGRAAAAATGTTA | Same as COI: LCO1490 /HCO2198 |
| | Jerry (f)/Pat (r) (Blum *et al*., 2003)[1]<br>Jerry (f): CAACATTTATTTTGATTTTTTGG<br>Pat (r): TCCAATGCACTAATCTGCCATATTA | Same as COI: LCO1490 /HCO2198 |
| | Tonya (f)/Hobbes (r) (Monteiro & Pierce, 2001)[1]<br>Tonya (f): GAAGTTTATATTTTAATTTTACCGGG<br>Hobbes (r): AAATGTTGNGGRAAAAATGTTA | Same as COI: LCO1490 /HCO2198 |
| *Cytochrome c oxidase subunit II (COII)* | George (f)/Phyllis (r) (Monteiro & Pierce, 2001)[1]<br>George (f): ATACCTCGACGTTATTCAGA<br>Phyllis (r): GTAATAGCNGGTAARATAGTTCA | Same as COI: LCO1490 /HCO2198 |
| | Strom (f)/BtLys (r) (Monteiro & Pierce, 2001)[1]<br>Strom (f): TAATTTGAACTATYTTACCIGC<br>BtLys (r): GTTTAAGAGACCAGTACTTG | Same as COI: LCO1490 /HCO2198 |
| | Strom (f)/Eva (r) (Monteiro & Pierce, 2001)[1]<br>Strom (f): TAATTTGAACTATYTTACCIGC<br>Eva (r): GAGACCATTACTTGCTTTCAGTCATCT | Same as COI: LCO1490 /HCO2198 |
| *Elongation Factor-1 Alpha (EF-1 alpha)* | HybStarsky/HybLuke (Wahlberg & Wheat, 2008)[1]<br>HybStarskyF: TAATACGACTCACTATAGGGCACATYAACATTGTCGTSATYGG<br>HybLuke: ATTAACCCTCACTAAAGCATRTTGTCKCCGTGCCAKCC | 95°C for 5 min,<br>40x (95°C for 1 min,<br>50°C for 1 min,<br>72°C for 1 min 30 sec),<br>72°C for 10 min,<br>4°C hold |
| | HybStarsky (f)/HybMonica (r) (Wahlberg & Wheat, 2008)[1]<br>HybStarsky (f): TAATACGACTCACTATAGGGCACATYAACATTGTCGTSATYGG<br>HybMonicaR (r): ATTAACCCTCACTAAAGCATRTTGTCKCCGTGCCARCC | Same as EF-1 alpha: HybStarsky/HybLuke |
| | EF44 (f)/EF51R (r) (Monteiro & Pierce, 2001)[1]<br>EF44 (f): GCYGARCGYGARCGTGGTATYAC<br>EF51r (r): CATGTTGTCGCCGTGCCAAC | Same as EF-1 alpha: HybStarsky/HybLuke |
| | HybCho (f)/HybVerdi (r) (Wahlberg & Wheat, 2008)[1]<br>HybCho (f): TAATACGACTCACTATAGGGGTCACCATCATYGACGC<br>HybVerdi (r): ATTAACCCTCACTAAAGGACACCAGTTTCIACTCTGCC | Same as EF-1 alpha: HybStarsky/HybLuke |

**Table 1 (continued)**

| Gene | Primer | Conditions |
|---|---|---|
| | HybA1F/EFrcM4 (Monteiro & Pierce, 2001)[1]<br>HybAlF: TAATACGACTCACTATAGGGGAGGAAATYAARAARGAAG<br>HybEFrcM4: ATTAACCCTCACTAAAGACAGCVACKGTYTGYCTCATRTC | Same as EF-1 alpha: HybStarsky/HybLuke |
| | HybEF51.9 (f)/EFrcM4 (r) (Monteiro & Pierce, 2001)[1]<br>HybEF51.9 (f): CARGACGTATACAAAATCGG<br>HybEFrcM4 (r): ACAGCVACKGTYTGYCTCATRTC | Same as EF-1 alpha: HybStarsky/HybLuke |
| *Isocitrate dehydrogenase (IDH)* | IDHdeg27F-ino/IDHdegR-ino (Wahlberg & Wheat, 2008; Malm & Johanson, 2011)<br>IDHdeg27F-ino: GGWGAYGARATGACIAGRATHATHTGG<br>IDHdegR-ino: TTYTTRCAIGCCCAIACRAAICCICC | 95°C for 5 min,<br>40x (95°C for 30 sec,<br>52°C for 30 sec,<br>72°C for 50 sec),<br>72°C for 8 min,<br>4°C hold |
| | IDHdeg27F/IDHdegR (Wahlberg & Wheat, 2008)<br>IDHdeg27F: TAATACGACTCACTATAGGGGGWGAYGARATGACNAGRATHATHTGG<br>IDHdegR: ATTAACCCTCACTAAAGTTYTTRCAIGCCCANACRAANCCNCC | 95°C for 5 min,<br>40x (94°C for 30 sec,<br>55°C for 30 sec,<br>72°C for 1 min 30 sec),<br>72°C for 10 min,<br>4°C hold |
| *RNA polymerase II (POL-II)* | POLFOR2 (f)/POLREV2 (r) (Danforth *et al.*, 2006)<br>POLFOR2 (f): TGGGAYGSYAAAATGCCKCAACC<br>POLREV2 (r): TYYACAGCAGTATCRATRAGACCTTC | 95°C for 5 min,<br>40x (95°C for 30 sec,<br>53°C for 30 sec,<br>72°C for 50 sec),<br>72°C for 8 min,<br>4°C hold |
| | LeptoF-ino (f)/POLREV2 (r) (Danforth *et al.*, 2006; Johanson & Malm, 2010)<br>LeptoF-ino (f): TRAARCCIAARCCIYTITGGAC<br>POLREV2 (r): TYYACAGCAGTATCRATRAGACCTTC | Same as POL-II: POLFOR2/POLREV2 |
| *40S ribosomal protein S2 (RPS2)* | RPS2_nF/RPS2_nR (Wahlberg & Wheat, 2008)[1]<br>RPS2_nF: TAATACGACTCACTATAGGGATCWCGYGGTGGYGATAGAG<br>RPS2_nR: ATTAACCCTCACTAAAGATGRGGCTTKCCRATCTTGT | 95°C for 5 min,<br>40x (94°C for 30 sec,<br>55°C for 30 sec,<br>72°C for 1 min 30 sec),<br>72°C for 10 min,<br>4°C hold |

[1]Primer pairs not previously used in the Trichoptera.

was added and the mixture was incubated for one hour in a 55°C water bath until the tissue was lysed. Samples were then loaded into a Qiagen QiaCube extraction robot and processed using the standard animal tissue extraction protocol for total DNA. DNA yield and quality was checked on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). Extracted DNA was stored at -20°C.

The 5 species of caddisflies collected in 2013 were used to screen sets of PCR primers originally designed for use in the Lepidoptera. Additional species collected in 2014 were only tested with primers that amplified successfully in the 2013 samples. Primer sets were drawn from Kronforst (2005), Regier (2006), Wahlberg and Wheat (2008), Monteiro and Pierce (2001), Reed and Nagy (2005), and Brower and DeSalle (1998). Several primer pairs that were designed to amplify genes from an even broader range of organisms, such as from all animals (*COI*, *18S*) (Folmer *et al.*, 1994; Medlin *et al.*, 1988) were also included.

Finally, several other primer pairs were identified that had been designed specifically for use in caddisflies. These included primers for the mitochondrial *16S rRNA* (Johanson & Malm, 2010), *Carbamoyl-phosphate synthetase* (Johanson & Malm, 2010), *Isocitrate dehydrogenase* (Wahlberg & Wheat, 2008; Malm & Johanson, 2011), and *RNA polymerase II* (Danforth *et al.*, 2006; Johanson & Malm, 2010) (Table 1).

Each primer was tested on 5 caddisfly species with a *Junonia* butterfly positive control (Borchers & Marcus, 2014) and a distilled deionized water negative control using the recommended PCR reaction conditions by the designers of each primer pair. PCR reactions were conducted in a total volume of 25 µL consisting of 1 µL template, 1 µL Forward primer (1 µmol/L), 1 µL Reverse primer (1 µmol/L), 9.5 µL distilled deionized water, and either 12.5 µL NEB 2X Quickload MasterMix (New England Biolabs, Ipswich, Massachusetts, USA) or 12.5 µL Top-Taq DNA Polymerase Mastermix (Qiagen). PCR amplifications were performed in a BioRad MyCycler, C1000, or a S1000 Thermal Cycler (BioRad, Hercules, California, USA). Successful PCR primers were then used to amplify the 3 remaining caddisfly species collected in 2014.

The primers that were originally designed for use in Trichoptera (16S, CAD-ino, IDH-ino, and POL-II (Johanson & Malm, 2010; Malm & Johanson, 2011)) were most successful in amplifying DNA when used with puReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), which contains dNTPs, BSA, stabilizers, reaction buffer, and puReTaq DNA polymerase. PCR reactions took place in manufacturer provided PCR tubes each containing a PCR bead. The total reaction volume was 25 µL, consisting of 1 µL template, 1 µL Forward primer, 1 µL Reverse primer, and 22 µL distilled deionized water.

PCR product sizes were verified by agarose gel electrophoresis (1% agarose slab gels in TAE buffer or on a Qiagen QiAxcel capillary electrophoresis instrument). PCR products of the correct size were prepared for sequencing in both directions using Sanger dideoxy sequencing using ABI Big Dye V3.1 Dye Termination sequencing chemistry (Applied Biosystems, Carlsbad, California, USA) as previously described (Borchers & Marcus, 2014). Sequencing reactions were analyzed on an Applied Biosystems 3730xl automated sequencer. Sequences were edited in Sequencher 5.1 software (GeneCodes Corporation, Ann Arbor, Michigan, USA) before being aligned in CLUSTAL W (Thompson *et al.*, 1994). Sequence alignments were converted to NEXUS format and then analyzed phylogenetically using an exhaustive search with the maximum likelihood algorithm (HKY model, tree bisection and reconnection branch swapping algorithm) of PAUP* 4.0b8/4.0d78 (Swofford, 2002). Sequences in FASTA format were also analyzed using MEGA 6 Alignment Explorer (Tamura *et al.*, 2013) using the Align DNA option of CLUSTAL W within MEGA. Pairwise distances were calculated in MEGA using the Kimura 2-parameter model with default settings. The Kimura 2-parameter model was chosen to allow protein and RNA encoding genes to be compared with each other using the same distance metric. Distances within the genus *Limnephilus*, within family Limnephilidae, and between families Limnephilidae and Phryganeidae were recorded for each gene.

## 3 Results

A total of 107 primer pairs corresponding to 45 nuclear genes and 3 mitochondrial genes were tested. Of the primers tested, 24 primer pairs for 9 different genes (*16S, 18S, CAD, COI, COII, EF-1 alpha, IDH, POL-II,* and *RPS2*) produced PCR amplification products of the expected size and DNA sequences homologous to the intended target genes (Table 2). The full names of the successfully amplified genes, primer pairs, and reaction conditions for each primer pair are provided in Table 1.

**Table 2. Location of caddisfly sequence products in corresponding *Bombyx mori* reference sequences.**

| Gene | Caddisfly sequence length (bp) | Position within *B. mori* reference sequence | GenBank accession for reference sequences |
|---|---|---|---|
| *16S* | 449 | 481-956 | KP192478.1 |
| *18S* | 1780 | 56-1806 | DQ347470.1 |
| *CAD* | 850 | 2298-3147 | XM_004924807 |
| *COI* | 1220 | 39-1258 | KM875545.1 |
| *COII* | 966 | 1317 (*COI*) - 682 (*COII*)[1] | KM875545.1 |
| *EF-1 alpha* | 1240 | 1584-2823 | JQ638952.1 |
| *IDH* | 720 | 254-964 | GU270852.1 |
| *POL-II* | 772 | 75-917 | DQ443209.1 |
| *RPS2* | 429 | 89-512 | AY769315 |

[1]The George primer originates near the 3' end of the *COI* coding sequence and the sequenced region extends across *tRNA-Leu* to include the entire *COII* coding sequence.

DNA sequences from this project were submitted to GenBank (Accession numbers KM463941–KM463997). Between 3 205 bp–7 938 bp were recovered from up to 9 genes for each caddisfly species. For the 5 caddisfly species collected in 2013 that were the subject of our intensive PCR screen, usable DNA sequences were recovered for all 9 genes except *RPS2* for *A. deflata* and *IDH* for *L. picturatus*.

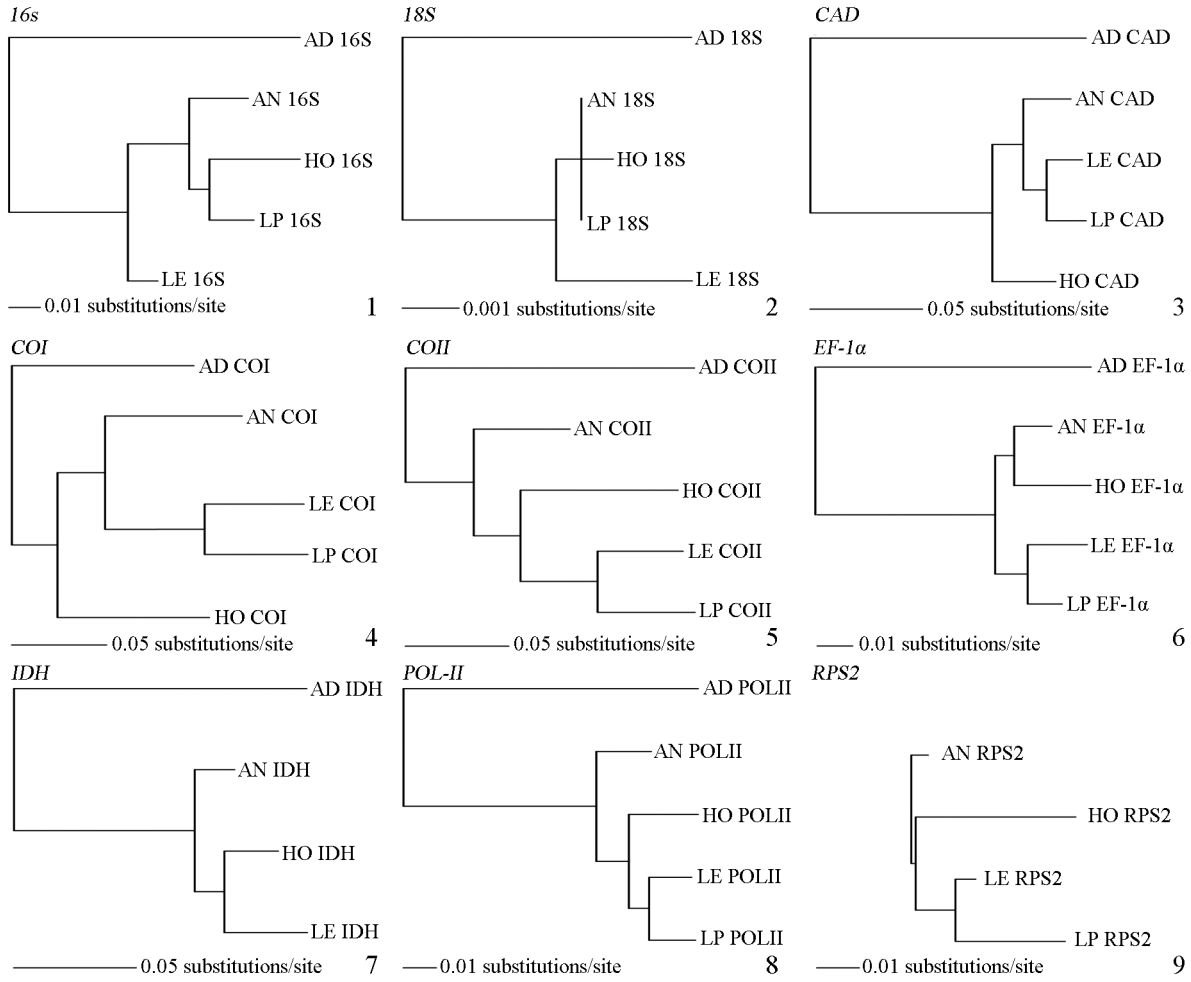
In most cases, constructing sequence alignments appropriate for phylogenetic analysis was trivial because the amplified products were primarily from protein-coding sequences with essential biological functions and so were subject to few or no insertions and deletions. For the portions of the 16S and 18S rRNAs that we amplified, there were similarly few ambiguous regions within the alignment. The only non-trivial region in the rRNA alignments was a 9 bp ambiguous interval in the 16s rRNA caused by the close proximity of a 5 bp deletion in the *A. deflata* sequence and a 3 bp insertion in the *H. occidentalis* sequence that has no effect on the resulting phylogenetic trees. Maximum likelihood phylogenetic trees for the 5 species for each of the 9 genes are shown in Fig. 1. *Agrypnia deflata* sequences were used as the outgroup. The ingroup species belong to the family Limnephilidae. Sequences from the genus *Limnephilus* most frequently form a monophyletic group, as expected. The phylogenetic trees generated from *16S* and *18S* sequences are the most different from expected results, given that *Limnephilus* is not monophyletic in these trees. The positions of *H. occidentalis* and *A. nigriculus* tend to vary between the different genes sequenced. The RPS2 tree shows a monophyletic *Limnephilus*, but no sequence from this gene was recovered for *A. deflata*.

The results from MEGA analysis using the Kimura 2-parameter model (Table 3) show the amount of change present for each gene within the genus *Limnephilus*, within the family Limnephilidae, and between families (Phryganeidae vs. Limnephilidae). The *18S* rRNA is the slowest evolving gene, with a between-family Kimura 2-parameter distance of 0.002 to 0.006. By contrast, the *COI* and *COII* mitochondrial genes evolve most quickly, with a Kimura 2- parameter distances of 0.09 to 0.143 just within the genus *Limnephilus.* The nuclear protein coding genes *CAD, EF-1A*, *IDH*, *POL-II*, and *RPS2*, evolve at intermediate rates between these two extremes. The mitochondrial *16S* rRNA gene evolves at a rate similar to these nuclear protein coding genes.

**Table 3. Kimura 2-parameter pairwise distances for genes analyzed in MEGA (Tamura *et al.*, 2013).**

| Distance | *16S* | *18S* | *CAD* | *COI* | *COII* | *EF-1 A* | *IDH* | *POL-II* | *RPS2* |
|---|---|---|---|---|---|---|---|---|---|
| Within genus | 0.052 | 0.002 | 0.025–0.060 | 0.101–0.143 | 0.09 | 0.022–0.054 | 0.053 | 0.016–0.029 | 0.031 |
| Within family | 0.035–0.057 | 0.001–0.005 | 0.033–0.058 | 0.102–0.180 | 0.114–0.146 | 0.022–0.061 | 0.033–0.062 | 0.019–0.043 | 0.019–0.054 |
| Between families | 0.145–0.167 | 0.002–0.006 | 0.207–0.227 | 0.180–0.203 | 0.213–0.239 | 0.122–0.144 | 0.203–0.212 | 0.161–0.175 | - |

In Fig. 2, a maximum likelihood phylogenetic tree combining all 9 genes for all 8 Trichoptera species is shown with bootstrap values indicated for each node. The genus *Limnephilus* is monophyletic as expected, but only has 47% bootstrap support. *Grammotaulius* is the sister group to *Limnephilus* in our tree with bootstrap support of 67%. The relative positions of *Hesopherophylax occidentalis* and *A. nigriculus* change in different gene trees (Fig. 1), and the topology in the




Figs 1–9. Results of maximum likelihood analysis in PAUP* (Swofford, 2002). 1. *16s rRNA*. 2. *18s rRNA*. 3. *CAD*. 4. *COI*. 5. *COII*. 6. *EF-1α*. 7. *IDH*. 8. *POL-II*. 9. *RPS2*. All trees were rooted with *A. deflata* except for *RPS2* for which no *A. deflata* sequence was recovered. Species names are abbreviated as: AD=*Agrypnia deflata*, AN=*Asynarchus nigriculus*, HO=*Hesperophylax occidentalis*, LE=*Limnephilus externus*, LP=*Limnephilus picturatus*.

combined analysis of all 9 genes has bootstrap support of 51%. Additional taxon sampling should help to reconstruct a more robust phylogeny and resolve the relationships among the more basal lineages of the family Limnephilidae, but that is outside the scope of this study.

## 4 Discussion

The purpose of this study was to identify new PCR primers that could be used in Trichopteran molecular phylogenetics. A variety of primer sets originally designed for use in the Lepidoptera were tested (Kronforst, 2005; Regier, 2006; Wahlberg & Wheat, 2008; Monteiro & Pierce, 2001; Reed & Nagy, 2005; Brower & DeSalle, 1998) along with some well-established universal primers (Folmer *et al.*, 1994; Medlin *et al.*, 1988). This method of screening primer sets proved to be productive as it recovered 24 primer pairs amplifying portions of 9 different genes (*16S*, *18S*, *CAD*, *COI*, *COII*, *EF-1 A*, *IDH*, *POL-II*, *RPS2*) in the Trichoptera. A majority of these primer pairs (15/24) have not been used previously in the Trichoptera (Table 1), extending the sequences available from these loci for phylogenetic analysis. In the most extreme case, the sequence available from the *18S* locus was extended from 228 bp (Geerts *et al.*, 2001) to 1 780 bp. One entirely new locus (*RPS2*) that has not been used previously for Trichopteran phylogenetics was also identified.

Given its success in this study, this screening method may be helpful for identifying PCR primers for use in other

groups without extensive genomic data or large numbers of established genetic markers from which to design PCR primers. Sampling of Trichoptera in this study was limited, including species in only 2 of the 42 extant families of Trichoptera (Holzenthal, 2011). However, primer binding sequences that are conserved between the Trichopteran species studied and Lepidopteran species are likely to be broadly conserved in most Trichoptera and may be good candidates for use in other orders of holometabolous insects as well.

Some of these genes, such as *18S* and *COI*, were amplified from primers that are considered universal. The Lepidopteran primers that were most successful in this study were specifically designed to amplify coding regions within genomic DNA that do not include introns (Wahlberg & Wheat, 2008; Monteiro & Pierce, 2001). Many of the primers previously used successfully in the Trichoptera (Johanson & Malm, 2010; Malm & Johanson, 2011) were modified from Lepidopteran primers designed using this approach (Wahlberg & Wheat, 2008). Not surprisingly, primer sets that were designed to span Lepidopteran introns (Kronforst, 2005) or to produce amplification products from cDNA (Regier, 2006) were much less successful in the screen, perhaps because large introns in the Trichoptera prevented successful amplification. It would be interesting to test cDNA from Trichoptera to see if these primer sets would be more successful from templates lacking introns. For some primer pairs from one of the sets tested (Regier, 2006), the primers produce robust PCR amplification products from Trichopteran cDNA (Regier *et al.*, 2013).

Recovery of both protein and RNA coding genes from the mitochondrial and nuclear genomes of Trichoptera should facilitate addressing many questions related to lateral transfer and mosaic evolution within caddisfly genomes. The sequences and trees generated by this study show that some genes may be more useful for exploring specific phylogenetic questions than others. The *18S* rRNA gene evolves slowly, making it most useful for addressing deep phylogenetic questions such as determining the relationships among Trichopteran families or examining relationships among insect orders (Nosenko *et al.*, 2013). Genes that evolve more quickly, such as mitochondrial *COI* and *COII*, are likely more useful for addressing shallower phylogenetic questions related to more recent divergences within genera or between closely related genera. Comparing the evolutionary history of these mitochondrial genes with nuclear genes can aid in detecting patterns of hybridization among closely related species (Borchers & Marcus, 2014; Wahlberg *et al.*, 2009). The nuclear protein coding sequences and the mitochondrial *16S* sequences that were recovered from caddisflies evolve at intermediate rates and may be appropriate for examining either deep or shallow divergences in the Trichoptera. By studying several different loci simultaneously, it is possible to detect distinct evolutionary rates and histories in different parts of the genome (Soltis & Soltis, 1998; Nosenko *et al.*, 2013). The availability of additional loci for molecular phylogenetic studies in caddisflies facilitates the study of mosaic evolution in the genomes of Trichoptera and potentially also in the genomes of other insects.

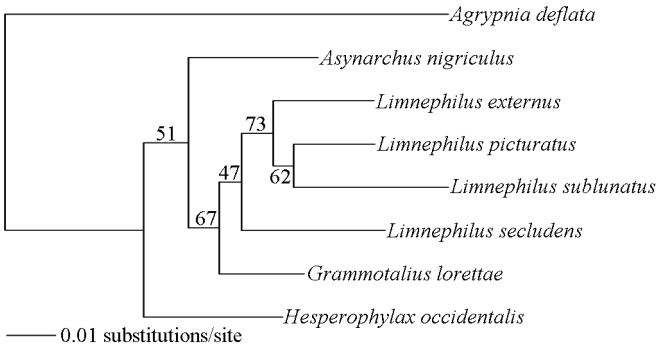



Fig. 10. Results of maximum likelihood analysis in PAUP* (Swofford, 2002) using sequences from all 8 species. Bootstrap values are labeled for each node.

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