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### ► **To cite this version:**

Bernhard Statzner, Christophe J Douady, Lara Konecny-Dupré, Sylvain Dolédec. Unravelling phylogenetic relationships among regionally co-existing species: Hydropsyche species (Trichoptera: Hydropsychidae) in the Loire River. *Zootaxa*, 2010, 2556 (1), pp.51-68. <10.11646/zootaxa.2556.1.3>. <halsde-00521281>

**HAL Id: halsde-00521281**

**<https://hal.science/halsde-00521281v1>**

Submitted on 22 Mar 2024

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# Unravelling phylogenetic relationships among regionally co-existing species: *Hydropsyche* species (Trichoptera: Hydropsychidae) in the Loire River

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## Abstract

Taxonomy is undoubtedly complementary to other fields in biology such as ecology, and both ecologists and taxonomists increasingly acknowledge that they can profit from phylogenetic ecology or ecological phylogeny, respectively. However, such mutual relations between these disciplines are constrained by traditional focuses on different operational scales: taxonomists are more familiar with large scales (e.g., global, continental, many species of a given clade), whereas ecologists are more familiar with small scales (regional, ecosystems, habitats, few species of a given clade). To foster mutually fruitful, 2-way exchanges between taxonomy and ecology at such smaller scales requires assessments of the small-scale performance of taxonomic practices so far used at larger scales. Such assessments are the objective of this study. To combine quantified ecology and phylogeny at the smaller scale, we designed a research project using 9 species of *Hydropsyche* (Trichoptera) from the Loire River (i.e., we focus on the regional scale). Here, we tried to unravel the phylogenetic relationships of this regional set of species using (1) many different characters (molecular and morphological characters of larvae and adults), (2) taxonomic congruence instead of total evidence (as the former provides more opportunities for future research on links between different data sets), and (3) an explicit data matrix and analysis methods that are commonly recommended for phylogenetic studies (e.g., maximum parsimony, maximum likelihood, bootstrapping), and we also included traditional, parsimonious, phylogenetic reasoning. Combining these elements, we obtained the following information for the regional *Hydropsyche* representatives from the Loire: (1) phylogenetic clusters of the species, (2) phylogenetic distances among the species, and (3) phylogenetic polarities of characters (plesio- to apomorphies) in the species tree. For our future ecological studies, this will enable (1) establishment of priorities in species selections for experimental approaches, (2) establishment of relationships between ecological and phylogenetic distances, and (3) interpretation of ecological response gradients across the species in the context of evolutionary processes such as adaptation, niche conservatism, or fitness. These future ecological studies will provide elements that in turn should be useful for taxonomists wishing to include small-scale ecological data into their analyses.

**Key words:** cladistics, molecular data, morphological data, phylogenetic distance, phylogenetic polarity

## Introduction

Particularly in its larger sense (including phylogenetic systematics), taxonomy is undoubtedly complementary to other fields in biology such as ecology or developmental biology (Wheeler 2007), and proponents of these other fields acknowledge fruitful 2-way exchanges with taxonomy (Minelli 2007). For example, ecologists are increasingly aware that evolutionary processes and thus phylogenetic relationships underlie many ecological patterns (e.g., Ackerly & Donoghue 1995, Poff *et al.* 2006, Webb *et al.* 2002, 2006, Agrawal *et al.* 2009, Cavender-Bares *et al.* 2009). Likewise, taxonomists make increasingly use of ecological data to complement phylogenetic analyses (e.g., Freeman 2000, Nylin *et al.* 2001, Kaila & Stahls 2006, Sharkey 2007, Pauls *et al.*

2008). Thus, ecologists and taxonomists recognize that they can profit from phylogenetic ecology or ecological phylogeny, respectively (e.g., Westoby 2006).

Prerequisite for such mutual relations between ecology and taxonomy is (at least) a basic understanding of the key controversies in the other discipline. For example, in ecology there is debate about historical versus actual or deterministic versus neutral processes (e.g., Ricklefs 2006, Cavender-Bares *et al.* 2009), which in turn have implications for taxonomists making use of ecology. Likewise, in taxonomy there is debate about the use of characters (molecular versus morphology) and analytical methods (taxonomic congruence versus total evidence, abduction versus parsimony versus likelihood) (Ernissse & Kluge 1993, Littlewood *et al.* 2004, Fitzhugh 2006, Yeates *et al.* 2007, Wheeler 2007), which in turn have implications for ecologists making use of taxonomy.

Furthermore, the operational scale constrains research that would progress both ecology and taxonomy. Operating at large spatial scales (e.g., global, continental) would involve many taxa, a well-resolved phylogeny of those taxa, and knowledge of ecological traits of these taxa (e.g., Westoby 2006). Such large-scale approaches are familiar to taxonomists, who often study as many taxa as possible to obtain a picture as complete as possible of the clade of interest (e.g., Nylin *et al.* 2001, Kaila & Stahls 2006, Yeates *et al.* 2007). In contrast, such large-scale approaches are less familiar to ecologists, who only begin to order relatively fuzzy information on multiple ecological and biological traits for many taxa in large databases (e.g., Lips *et al.* 2003, Ezenwa *et al.* 2006, Statzner *et al.* 2007) or limit their contribution to a few, better-known traits that are often related to feeding ecology (e.g., Freeman 2000, Kaila & Stahls 2006, Pauls *et al.* 2008) or case-building biology (e.g., Stuart & Currie 2001, Bucheli *et al.* 2002). Thus, at larger scales, experience, tools, and knowledge in taxonomy are more advanced than in ecology. These positions are inverted when studying smaller scales (e.g., regions, ecosystems, habitats). These are familiar scales to ecologists, who often quantify ecological patterns and processes in detailed comparative studies. Such studies would profit from knowledge of the phylogenetic relationships and particularly the phylogenetic distances and polarities among the species in these smaller systems, i.e., comparative ecology of co-existing species would be significantly enriched by phylogenetic considerations (e.g., Ackerly & Donoghue 1995, Cavender-Bares *et al.* 2009). Because species occurring at these smaller spatial scales typically represent a relatively small proportion of a given clade, such scales are obviously of limited interest for taxonomists. This is unfortunate, as they miss the opportunity to include detailed quantitative ecological information in their phylogenetic analyses. To apply such small-scale approaches, however, requires assessments of the small-scale performance of taxonomic practices so far used at larger scales. Such assessments are the objective of this study.

To combine quantified ecology and phylogeny at the smaller scale, we designed a research project using species of *Hydropsyche* (Trichoptera) from the Loire River (i.e., we focus on the regional scale here). Large European rivers (including the Loire) have relatively many *Hydropsyche* species that differ more or less in their longitudinal distribution, including co-existence in river sites or habitats (e.g., Hildrew & Edington 1979, Higler & Tolkamp 1983, Tachet *et al.* 1992, Ivol *et al.* 1997). Species living in different river sections differ in traits such as velocity preference, temperature dependence as related to respiration, or filter net-building (rate, mesh size) in correspondence with generally occurring downstream gradients in the environment (e.g., Hildrew & Edington 1979, Pierrot 1984, Roux *et al.* 1992, Tachet *et al.* 1992, Guinand *et al.* 1994). Thus, research on *Hydropsyche* from the Loire could provide multiple, detailed quantified ecological distances among the species.

Concerning the quantified phylogenetic distances among the species, we note that morphology-based and molecular-based phylogenies of caddisflies in general as well as of hydroptychids in particular typically have considered deeper relationships (e.g., Morse 1997, Kjer *et al.* 2001, Geraci *et al.* 2005, Scheffer 2005, Holzenthal *et al.* 2007), i.e., assessments of interspecific relationships within a genus are rare in this order (e.g., Espeland *et al.* 2008, Pauls *et al.* 2008; see Morse 1997 for a summary of older studies). Depending on the status (genus, subgenus) of the *Ceratopsyche* group, ~300–400 *Hydropsyche* species are currently known (Holzenthal *et al.* 2007, Morse 2009). Thus, we will wait a long time for the Utopia of a well-resolved phylogenetic tree of the species of this genus. Consequently, we tried to solve the task for the Loire species using (1) many different characters (molecular and morphological characters of larvae and adults),

corresponding to recommendations by, e.g., Grandcolas *et al.* (2001), Kjer *et al.* (2001), Stuart & Currie (2001) and Kaila & Stahls (2006), (2) taxonomic congruence instead of total evidence (as the former provides more opportunities for future research on links between different data sets, see Eernisse & Kluge 1993), and (3) an explicit data matrix and analysis methods that are commonly recommended for phylogenetic studies (e.g., bootstrapping and maximum likelihood: Felsenstein 1985, Yeates *et al.* 2007; maximum parsimony: Hennig 1969, Swofford 2002).

Combining these elements, we hoped to obtain the following information for the regional set of *Hydropsyche* from the Loire: (1) phylogenetic clusters of the species, (2) phylogenetic distances among the species, and (3) phylogenetic polarity of characters (plesiomorphies to apomorphies) in the species tree. For our future ecological studies, this would enable (1) establishment of priorities in species selections for experimental approaches, (2) establishment of relationships between ecological and phylogenetic distances, and (3) interpretation of ecological response gradients across the species in the context of evolutionary processes such as adaptation, niche conservatism, or fitness. As outlined above, these future ecological studies will provide elements that in turn should be useful for taxonomists wishing to include small-scale ecological data into their analyses.

## Material and methods

For West and Central Europe, 19 species of the genus *Hydropsyche* are known (e.g., Edington & Hildrew 1981, Waringer & Graf 1997, Neu & Tobias 2004). Among them is 1 species (*H. silfvenii*) belonging to the separate species group *Ceratopsyche* (see Statzner & Mondy 2009).

**Sampling, identifications, and the studied species.** To obtain material including both larvae and pupae (pupae with mature genitalia facilitate species identifications, see below), the entire Loire was sampled during 2 periods in 2005: at the end of May/beginning of June in its middle and lower section (>220 km distance from the source) and the end of June/beginning of July in its upper section (<220 km). At these occasions, we obtained larvae and pupae of hydropsychids by manually collecting typically larger stones in strong currents for ~20 min. We brushed the larvae from the stones and cut the silk of the pupal cases attaching them to the stones using sharp, thin knives. We added 95% ethanol to the samples, which was drained and replaced by fresh alcohol in the laboratory.

Overall, we sampled ~10,000 specimens of *Hydropsyche* and ~1000 specimens of *Cheumatopsyche* (the latter serving as outgroup in our phylogenetic analysis). These were identified using larval keys (also for immature pupae, based on their larval exuviae) by Pitsch (1993), Waringer & Graf (1997), Neu & Tobias (2004), and Higler (2005), or, if mature pupae (with well-developed genitalia) were available, using also the adult key by Neu & Tobias (2004). Use of the latter was not possible for rare species. Expectedly, the number of individuals available per species varied considerably (Table 1), and 1 species (likely *H. pellucidula*) was too rare to be included in our study.

**Character assessment. Molecules.** Prior to DNA extraction, we removed the larval digestive tract to avoid contamination, as we found ingested congeners in several larvae. Genomic DNA was extracted using either the DNeasy kit (Qiagen, Inc., Courtaboeuf, France) following manufacturer's recommendations or using a protocol derived from standard salt-chloroform based techniques (Mullenbach *et al.* 1989). Samples were incubated overnight in 200  $\mu$ l TNES buffer (0.05 M Tris HCl, 0.1 M NaCl, 0.01 M EDTA, 0.5% SDS) with 5  $\mu$ l proteinase K (15 mg ml<sup>-1</sup>), individually ground with a pestle at room temperature, incubated 1 h at 56°C, and centrifuged at 13200 rpm for 5 min to precipitate cellular fragments. The supernatant was vigorously shaken with 250  $\mu$ l NaCl (2.6 M) and centrifuged 15 min at 13200 rpm. The resulting supernatant was removed, vigorously mixed with 400  $\mu$ l chloroform for 15 s, and centrifuged 5 min at 13200 rpm. The resulting supernatant was collected, vigorously mixed with 2  $\mu$ l glycogen (20 mg ml<sup>-1</sup>), gently mixed with 600  $\mu$ l absolute ethanol (-20°C), and incubated 30 min at -80°C. Then, the DNA was precipitated at 13200 rpm and 4°C for 20 min. DNA pellets were washed using 300  $\mu$ l 70% ethanol (-20°C), dried for at least 2 h at room temperature, and then re-suspended in 50 ml TE (0.01 M Tris HCl, 0.01 M EDTA).

Initially, we used standard primer sets to PCR-amplify COI and 16S fragments: LCO1490 and HCO2198 (Folmer *et al.* 1994) for COI and 16Sar and 16Sbr (Palumbi 1996) for 16S. We could not amplify all species with these primers. Thus, we used hydropsyche-specific primers derived from our initial sequences (see Appendix 1 for primer sequences and target loci). PCRs were performed in 35  $\mu\text{l}$  reactions containing 0.17  $\mu\text{M}$   $\mu\text{l}^{-1}$  dNTPs, 1.5  $\mu\text{M}$   $\mu\text{l}^{-1}$   $\text{MgCl}_2$ , 0.1  $\mu\text{g}$   $\mu\text{l}^{-1}$  BSA, 0.16  $\mu\text{M}$   $\mu\text{l}^{-1}$  primers, 0.04 U  $\mu\text{l}^{-1}$  of Taq Polymerase (Invitrogen), 1 X PCR Buffer (Invitrogen, Cergy Pontoise, France), and ~50 ng of genomic DNA. Amplifications were performed in a PTC-200 thermocycler (MJ Research, GMI, Inc., Ramsey, Minnesota, USA) using the following settings: (1) 35 cycles of 30 s at 94°C, 30 s at the lowest primer's  $T_m$ , 30 s at 72°C, and (2) one step of 1 min at 72°C. Finally, PCR products were purified and sequenced at GATC biotech (Konstanz, Germany) or at the unit "Développements Techniques pour l'Analyse Moléculaire de la Biodiversité" (CNRS-Univ. Lyon 1).

Morphologically cryptic diversity has been reported for several freshwater invertebrate taxa (e.g., Jackson & Resh 1998, Lefébure *et al.* 2006). To account for this possibility, we thus analyzed 2 individuals each per species from the most-upstream and the most-downstream sites of its distribution.

*Morphology.* To obtain phylogenetic clusters and polarity among the *Hydropsyche* species, one would ignore all characters with an autapomorphic state (i.e., occurring in only 1 *Hydropsyche* species) or that are not present in the outgroup. However, needing also phylogenetic distances among the *Hydropsyche* species for future ecological analyses, such characters are particularly informative (imagine 3 species with plenty of synapomorphic character states, among them 1 species with plenty of autapomorphic character states). Thus, we included such otherwise ignored morphological characters in our analysis. Furthermore, we tried to assess numerous morphological characters, which would help to distribute the 9 *Hydropsyche* species over a greater range of phylogenetic distances.

We started by screening larval keys used to identify our material (see above) and other hydropsyche larval keys (e.g., Lepneva 1970, Schuster & Etnier 1978, Edington & Hildrew 1981, Statzner 1984, Scheffer & Wiggins 1986) for phylogenetically informative characters. Furthermore, we searched for additional phylogenetically informative characters when analyzing our larval material. These analyses included only characters that could be clearly measured in a repeatable manner with 50x magnification (dissecting microscope) or 400x magnification (compound microscope). For example, we were unable to resolve the chaetotaxal character states of the secondary setae as detailed in SEM studies (e.g., Scheffer & Wiggins 1986) and thus used a simpler coding (type-0, type-1, etc.). For qualitative characters or for semi-quantitative characters of the larvae, we checked well-sclerotized 5th instars from many sites along the river.

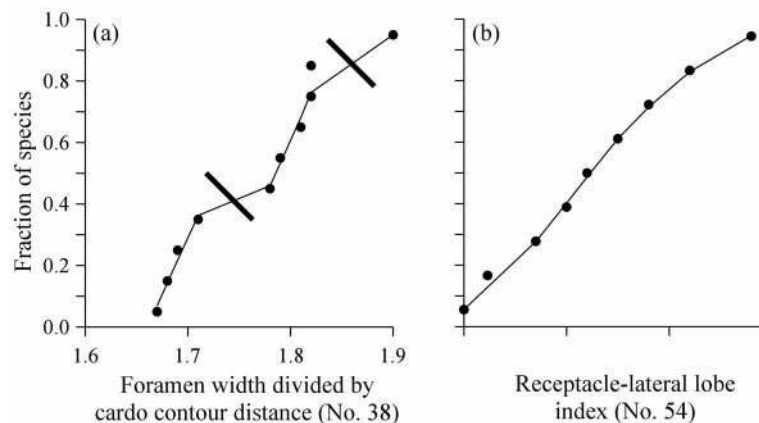
For measured quantitative characters, we started to analyze 2 larvae each per species from upstream and downstream of its distribution along the river. One upstream-downstream pair was dissected in 95% alcohol (the conservation medium) to obtain measurements on deformable structures. The second pair was macerated in NaOH, cleaned, and mounted in glycerol gelatine for compound microscope analysis and photography (photographs were used for image analysis of more complicated measures such as angles). Expectedly, distances on the larval head and mouthparts exhibited strong allometries across the 10 species. For example, the exterior cardo distance (between the points where the lateral cardo contours join the head capsule) increased significantly ( $P < 10^{-8}$ ,  $R^2 = 0.989$ ) with the submentum width, and the anterior, ventral head foramen width increased significantly ( $P < 10^{-7}$ ,  $R^2 = 0.969$ ) with the exterior cardo distance (see Appendix 2 for a detailed description of these and other characters). Therefore, we transformed such quantitative measures into relations between them.

Overall, we found 37 qualitative characters that discriminated among the larvae of the 10 species in our material that, at least for the *Hydropsyche* species, had *a priori* no relation to the environmental gradients prevailing along the Loire and other large European rivers. In addition, we retained 6 quantitative characters; most of these considered relations of distances from the larval head that indicated complex morphological changes (see below).

Given that adult material was not consistently available for us, we obtained all descriptions of adult characters from Neu & Tobias (2004). The illustrations (including photos) in the work by Neu & Tobias are of excellent quality and the positioning of the different parts illustrated by them is perfectly standardized.

Therefore, beyond using qualitative characters from that publication, we were confident that including quantitative characters (relations and angles) measured by us on their genitalia illustrations would provide useful additional information for our analyses. Focusing on characters used in the adult key of Neu & Tobias (2004), we retained 23 qualitative morphological characters of females and males (21 from the genitalia). In addition, we included 7 quantitative descriptions of the male and female genitalia that were indicative of possible prezygotic isolation (Tobias 1972, Statzner 1974).

Measurements and ratios of measurements are inherently continuous and should be coded according to data gaps. These gaps are not necessarily absolute differences between neighboring values because character states should be delimited by discontinuities in the data distribution (Stevens 1991). We used graphic software in Systat®10 (LOWESS smoothing) to identify discontinuities at larger gaps in the data (Fig. 1a) or to delete characters that did not collapse into groupings separated by clear discontinuities (Fig. 1b).



**FIGURE 1.** Coding of character states of measured data, using graphic software (see text) to identify discontinuities at larger gaps in the data (a) or to delete characters that did not collapse into groupings separated by clear discontinuities (b) (see Appendix 2 for details on characters).

**Phylogenetic analyses. Molecular analysis.** Identical sequences (in our case, these were the specific upstream and downstream replicates) were collapsed into haplotypes using Collapse 1.2 (Posada 2004). We estimated saturation levels in both phylogenetic loci using the graphical approach proposed by Philippe & Forterre (1999). The inferred substitution numbers for each codon position (COI) or for all positions (16S) were estimated from patristic distances on the most likely tree and compared to the observed number of transitions and transversions. This initial phylogenetic inference was performed with phym1\_v2.4.4 under a GTR model of evolution starting from a BIONJ tree, where the proportion of invariant sites and the gamma distribution parameter of 6 rate categories were estimated during the inference.

COI and 16S datasets were analyzed together and independently. Optimal trees were constructed using Bayesian and maximum likelihood (ML) approaches. Bayesian phylogenies were generated using MrBayes 3.12 (Ronquist & Huelsenbeck 2003) under the best-fit model as selected by AIC in MrModeltest 2.2 (Nylander 2004). Two sets of 4 chains sampled every 100 generations were run until the average standard deviation of split frequencies between the 2 sets fell below the default critical value of 0.01 using a burn-in fraction of 25% (i.e., we used 75% of the samples recorded up to the point when the split frequency reached 0.01). Bayesian posterior probabilities were finally recorded even if their significance, in terms of robustness, remains an open question (e.g., Douady *et al.* 2003b). For each dataset, we also determined the best-fitting model of substitution and its parameters using Modeltest and the AIC criterion, and used these values to infer ML trees with PAUP\* 4b10 (Swofford 2002) using the heuristic default search option. In addition to posterior probabilities, we assessed the strength of the phylogenetic signal using a nonparametric bootstrap (Felsenstein 1985) using 100 pseudoreplicates here as well as in the subsequent analysis of morphological characters (using parameters, models, and algorithms associated with each individual analysis).

*Analysis of morphological characters.* The morphological characters of larvae and adults that are *a priori* not changing along rivers were analyzed in a maximum parsimony framework. We searched the most parsimonious tree using a Branch & Bound algorithm [implemented in PAUP\* 4b10 (Swofford 2002); see above (Molecular analysis) for bootstrapping procedure].

## Phylogenetic analyses

**Molecular analysis.** The saturation plot analysis (not shown) suggested that transitions at the third codon position of the COI fragment (551 base pairs) were mostly saturated. This was also observed for transitions at the first codon position of the COI fragment, but only when comparing ingroup versus outgroup. The 16S (278 base pairs) analysis was less clear but also suggested some saturation of the transition. Thus, all datasets were further analyzed with and without outgroup and with and without transitions (only at the third codon positions when considering the COI dataset).

Considering the unrooted dataset (i.e., without *lep*), we found strong evidence for a grouping of *inc+din* and of *bul+orn* (see Table 1 for species acronyms). The *inc+din* relation was supported by posterior probabilities (PPs) of 100% for all unrooted partitions and by bootstrap values (BVs) ranging from 92 to 100%. The *bul+orn* association was supported by PPs of 100% and BVs ranging from 87 to 100%. Except for the 16S partition, we also found evidence to join *ins+sil* (99% < PP < 100% and 88% < BV < 94%). At a deeper level, we also found a strong support for a split between *con+exo+orn+bul* against *ins+sil+din+inc+ang*.

Rooting trees is often considered as the most problematic step in phylogenetic inferences (Swofford *et al.* 1996), and our specific case seemingly follows this rule. Indeed, depending on data partitions (and the inclusion or exclusion of transitions), the very long branch leading to *lep* connected the *Hydropsyche* tree in rather different positions. Most of our reconstructions suggested a rooting as shown in Fig. 2, although support for several nodes was weak and no phylogenetic polarity in the regional set of *Hydropsyche* was recognizable.

**TABLE 1.** Number of individuals (larvae and pupae) of *Hydropsyche* and the outgroup *Cheumatopsyche lepida* collected at 34 sites from the Loire that had easily accessible hydropsychid habitat in 2005 (in addition, the material included 711 first instar larvae that we could not identify as species).

Species	Acronym <sup>1</sup>	Individuals
<i>H. angustipennis</i> (Curtis 1834)	<i>ang</i>	33
<i>H. bulgaromanorum</i> Malicky 1977	<i>bul</i>	141
<i>H. contubernalis</i> McLachlan 1865	<i>con</i>	2910
<i>H. dinarica</i> Marinkovic 1979	<i>din</i>	115
<i>H. exocellata</i> Dufour 1841	<i>exo</i>	4517
<i>H. incognita</i> Pitsch 1993	<i>inc</i>	1110
<i>H. instabilis</i> (Curtis 1834)	<i>ins</i>	34
<i>H. ornatula</i> McLachlan 1878	<i>orn</i>	33
<i>H. siltalai</i> Döhler 1963	<i>sil</i>	1445
<i>H. sp.</i> <sup>2</sup>	---	2
<i>C. lepida</i> (Pictet 1834)	<i>lep</i>	1299
		Σ 11639

<sup>1</sup>Used consistently in subsequent sections; <sup>2</sup>Likely *H. pellucidula* (Curtis 1834), but too rare to be included in our study.

**Analysis of morphological characters.** All our *Hydropsyche* species share 2 character states with almost all other European species of the genus: (1) the same basic colour pattern on the larval frontoclypeus (6

contrasting light patches on a dark background) that varies intraspecifically across a certain range of colour contrasts and (2) a similar, relatively simple phallus shape (Statzner & Mondy 2009). In contrast, 2 European and many North American species of the genus (from the *Ceratopsyche* group) have a pattern of 7 light patches on the larval frontoclypeus that also varies intraspecifically; males of these species have a similar, relatively complicated phallus shape (Statzner & Mondy 2009).

The computerized maximum parsimony analysis provided a tree with generally good bootstrap support ( $BVs > 70\%$ ) for all nodes in the regional set of *Hydropsyche*, except nodes 4 and 5 (Fig. 3). The tree in Fig. 3 has few reversals of character states. Clearly, the re-appearance of a round contour of the posterior, ventral phallus apex in *bul* (67/1) would be such a reversal, and the occurrence of a high stridulation ridge density in larval *ang* from the Loire [but not in southern Finland (see fig. 3 in Jansson & Vuoristo 1979), i.e., *ang* scored 22/1] would perhaps be such a reversal. Two other reversals are less obvious, as the re-appearance of the states 25/1 (no type-4 setae on pronotum) and 31/1 (prosternites typically unfused but sometimes fused) in *exo* (at node 7) would be irrelevant, assuming that the character states 25/0 (type-4 setae on pronotum) and 31/2 (prosternites fused) evolved as convergences in *ang* and toward nodes 8 and 9 (Fig. 3).

Several morphological characters have been often illustrated for other hydroptychids so that we could compare them among more than our 10 Loire species. For example, the triangular submentum shape (character state 7/0) of larval *ins*, *sil*, and *lep* is also occurring in the *Ceratopsyche* group with the 7-spot colouration pattern (see above), in other North American *Hydropsyche* and *Cheumatopsyche* (Wiggins 1977, Schuster & Etnier 1978), in the North American hydroptychids Diplelectroninae and Macronematinae (Wiggins 1977), in European *Diplelectrona* (Neu & Tobias 2004), and in African Diplelectroninae, Hydroptychinae, and Macronematinae (Marlier 1961, Jacquemart & Statzner 1981, Scott 1983). Thus, we considered this character state as symplesiomorphic and the laterally elongated submentum lobes (7/1) as synapomorphic. In our material, a triangular submentum shape was exclusively associated with the existence of apical, long, rod-shaped appendices on the male abdominal segment X (62/0); these appendices are transformed cerci and a very old character state of caddisflies (evidenced by fossils; Ivanov 2005), providing another argument that *ins*, *sil*, and *lep* share a symplesiomorphic character state (in the male genitalia). Thus, the absence of apical, long rod-shaped appendices on the male abdominal segment X is another synapomorphic character state. Elongated larval submentum lobes and absence of apical appendices in the male genitalia are typically associated with a V-shaped larval frontoclypeus (14/1; note, however, that *din* has a U-shaped larval frontoclypeus), and a low stridulation ridge density on the ventral larval head [22/2, not occurring in *ang* from the Loire (but occurring in southern Finland, see above)].

Among other West and Central European *Hydropsyche* having a 6-spot derivation of the larval head colouration pattern (see above) and apical, long, rod-shaped appendices on the male abdominal segment X (*H. saxonica*, *H. tenuis*), and/or a triangular larval submentum shape (*H. bulbifera*, *H. fulvipes*, *H. saxonica*, *H. tenuis*), species have either U- or V-shaped larval frontoclypei as well as low or high stridulation ridge densities (see Pitsch 1993, Neu & Tobias 2004), suggesting that the group diverged into several lineages in an early period. Like *ins* and *sil*, however, *H. bulbifera*, *H. fulvipes*, *H. saxonica*, and *H. tenuis* have a straight anterior larval frontoclypeus contour (8/0, also occurring in *lep*), a darkly pigmented median part of the larval prosternites (32/2), a ventral lateral lobe that is similarly broad in its dorsal and ventral half on female abdominal segment IX (53/0), and a U-shaped posterior gap between the crests on male abdominal segment X (60/0) (see Pitsch 1993, Neu & Tobias 2004). Beyond these character states that could be compared to other species, the triangular larval submentum shape of *ins*, *sil*, and *lep* is exclusively associated with a flat lateral cardo area (3/0) and an angular latero-proximal cardo contour (4/0). Among these characters, 7 are present in ingroup and outgroup, thereby indicating polarity among our species. The synapomorphic states of these characters would be (Fig. 3): above node 4, dorsad-curved lateral cardo area (3/1), round latero-proximal cardo contour (4/1), elongated lateral submentum lobes (7/1), variable or low stridulation ridge density (22/1, 22/2), and absence of apical, long, rod-shaped appendices on male abdominal segment X (62/1); above node 5: V-shaped frontoclypeus (14/1); above node 7: convex anterior frontoclypeus contour (8/1, 8/2).

The importance of these characters (i.e., the complexity of changes involved) differed in our phylogenetic reasoning. Re-organization of the submentum, cardo, and frontoclypeus shape suggests major re-organization

of the muscular arrangement in the larval head capsule (Lepneva 1970). Similarly important should be re-organizations in the female and male genitalia, as different characters of the sexes assure the male-female locking mechanism in caddisflies (Ivanov 2005). For example, the occurrence or absence of apical, long, rod-shaped appendices on male abdominal segment X is associated with differences in the contact between male and female genitalia, and there are many more (in addition to these appendices) such key-lock structures on the genitalia of Hydropsychinae [e.g., occurrence (*Hydropsyche*; 50/1) or absence (*Cheumatopsyche*; 50/0) of dorsolateral lobes on female abdominal segment IX] (Tobias 1972, fig. 4; Statzner 1974, fig. 14). In comparison, the importance of a changing stridulation ridge density is currently not well understood: larvae stridulate in intraspecific or interspecific aggressive encounters during territorial defence, but relations between stridulation ridge structure and stridulation signal have not been established (Jansson & Vuoristo 1979, Silver 1980). Likewise, the importance of a changing shape of the anterior frontoclypeus contour is currently unclear.

Other characters provide more support for phylogenetic polarities in the tree of Fig. 3. From node 5 to 6 and at higher nodes, there appear several secondary setae types not shared by the other *Hydropsyche* species (in ascending order in the tree: 24/1, 25/0, 27/1, 18/1). Appearance and diversity of larval secondary setae would indicate syn- or autapomorphies, as they appear during the ontogeny of a species (first instar larvae lack such setae) and supposedly with progressing evolution (they are less developed in basal groups than in highly specialized groups; Lepneva 1970), suggesting successively hierarchically restrictive synapomorphies. Furthermore, the pigmentation (19, 20, 28, 32, 33) in the regional set of *Hydropsyche* is darker below node 7, intermediate in *exo*, and lighter above node 8. The synthesis of melanin represents a cost for insects (Stoehr 2006, Ma *et al.* 2008) that was perhaps reduced during evolution, thus also suggesting successively hierarchically restrictive synapomorphies. Finally, it is interesting that species with an intermediate position in the regional set of *Hydropsyche* have characters that vary between 2 states (*inc*: 21/1, 31/1; *ang*: 22/1; *exo*: 31/1), but that are invariant at lower or higher hierarchical levels in the phylogeny.

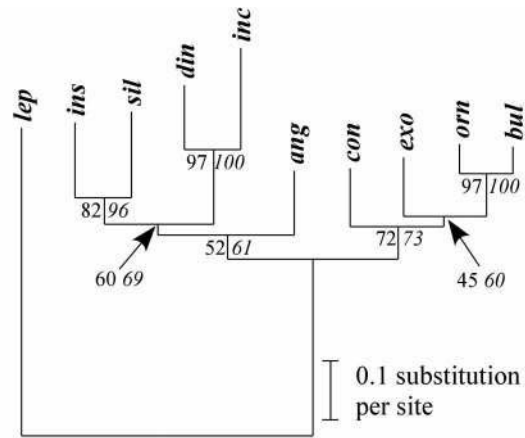
The morphological characters suggest that the species pair *ins+sil* should be placed at the base of the Loire *Hydropsyche* cladogram [with *ins* being nearer to node 2 than *sil*] and the pair *orn+bul* arose most recently [having 7 synapomorphic character states: wide lateral mandible wings (1/1), distinct hemispherical submentum hump (6/2), symmetrically convex anterior frontoclypeus contour (8/1), indistinct indentions of anterior frontoclypeus contour (9/1), ventral head length shorter than head width (42/1), dorsal half of ventral lateral lobe (not present in *lep*) pointed (53/2), and small receptacle-lateral lobe angle (not present in *lep*) (55/0); with *bul* being more distant from node 2 than *orn*]. Likewise, other larval characters suggest sister-group relationships for *ins+sil* and *orn+bul* (e.g., the shape of the stridulation hump on the foreleg), but they change so gradually along our tree that we decided to exclude them from our phylogenetic analysis.

Overall, *ang* is the most difficult to place in our tree: it combines character states from higher and lower positions in the tree, has several character states in female and male genitalia that likely are extremely autapomorphic (47/1, 55/2, 56/0, 60/2, 69/1), and its position in the tree requires several convergences (48/1, 58/1, perhaps also 25/0, 31/2, see above) and, at least for the Loire population, a reversal (22/1, see above). Thus, we are unsure about the position of *ang* in our tree, as it likely originates from a lineage from which species that might provide better links to our tree no longer exist in the Loire or Europe.

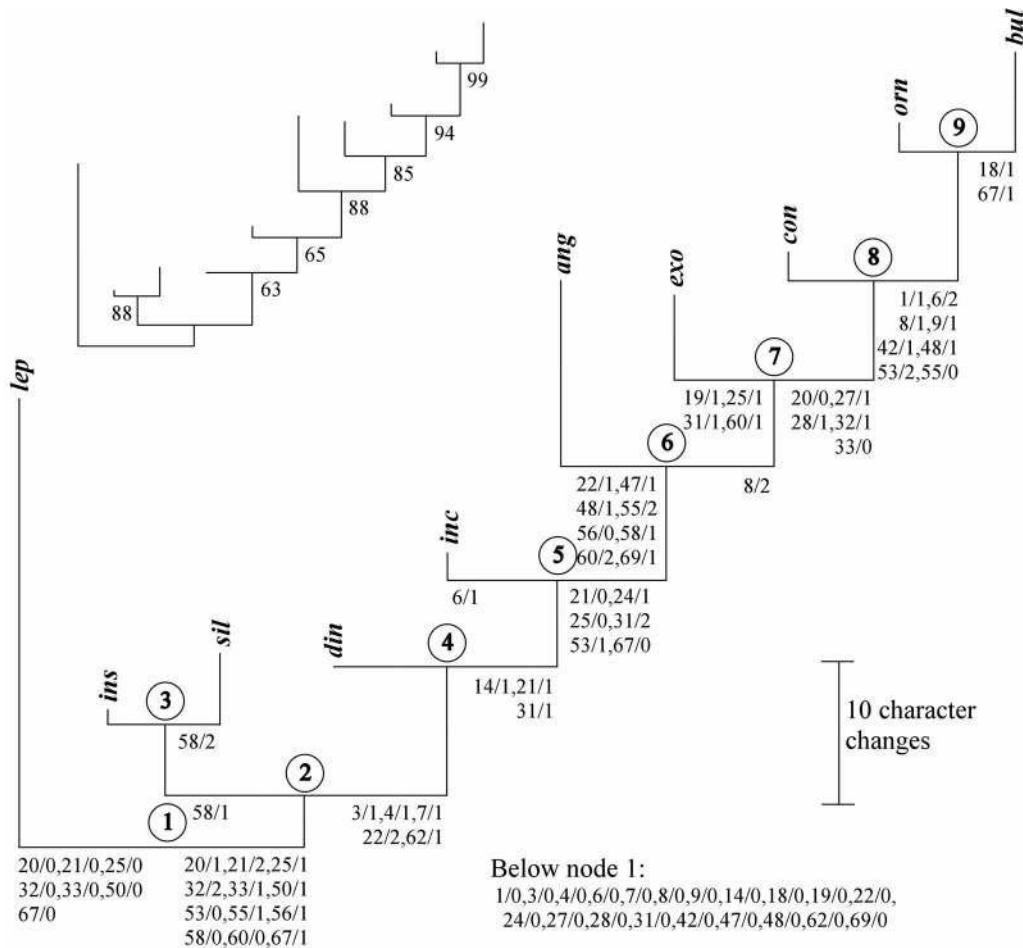
## Discussion

We obtained 2 trees with a similar general topology, one that is a molecular analysis (Fig. 2) and one that is an analysis of morphological characters of larvae and adults (Fig. 3). Tree rooting is sometimes the weakest point of a phylogeny and indeed our trees mostly differ from each other in the location of the connection of *lep* with the regional set of *Hydropsyche*. Considering unrooted topologies, both trees support *ins+sil* and *orn+bul* as pairs of sister species (Figs 2 and 3). Furthermore, both trees provide support for the group *exo*, *con*, and *orn+bul*, an unresolved, trichomous relationship. Next, *ang* appears in both trees as the potential sister taxon of the grouping *exo+con+orn+bul*, although support for this location is relatively weak. Depending on the

dataset, these 5 species perhaps group with the pair *inc+din* or *inc* followed by *din*. Finally, at the other end of the regional set of *Hydropsyche* in both trees, *ins+sil* are potential sister species.



**FIGURE 2.** Phylogenetic tree (maximum parsimony topology) based on analysis of fragments of cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S), with bootstrap values (BV) in % in normal font and posterior probabilities (PP) in % in italics (see Table 1 for species acronyms).



**FIGURE 3.** Phylogenetic tree (maximum likelihood topology) based on qualitative morphological characters, indicating node numbers in circles and character states that we discuss in the text (see Appendix 2 for details on all characters and Table 1 for species acronyms). BVs (in %) are indicated on the inset. Tree length: 122; consistency index: 0.797; retention index: 0.598; rescaled consistency index: 0.598; homoplasy index: 0.203.

Considering the root location, the analysis of molecular loci suggests rooting between *ins+sil+din+inc+ang* and *exo+con+orn+bul* (Fig. 2). In contrast, arguments supported by the analysis of morphological characters of larvae and adults suggest a root location between *ins+sil* and all other *Hydropsyche* (Fig. 3). Overall, our trees support the intuitive (not arguing about characters and phylogenies when defining the groupings) view of Pitsch (1993) that our *Hydropsyche* from the Loire belong to 4 groups: (1) *H. instabilis*-Group, represented in the Loire by *ins* and *sil*; (2) *H. pellucidula*-Group, represented in the Loire by the possibly paraphyletic grade *din* and *inc*; (3) *H. guttata*-Group, represented in the Loire by *con*, *exo*, *orn*, and *bul*; and (4) the isolated taxon *ang*. Designing future ecological experiments for *Hydropsyche* species from the Loire, our trees suggest selecting *ang* and 1 species from each of the pairs *ins+sil*, *din+inc*, *con+exo*, and *orn+bul* to assess species that are the most representative of the entire regional species set.

Beyond our search for phylogenetic clusters among our *Hydropsyche* species, we searched for a relative phylogenetic distance and polarity across regionally co-existing representatives of a genus that can be linked to gradients in ecological response patterns of these species. Obviously, the relative branch lengths within and among the trees in Figs 2 and 3 do not indicate a unifying phylogenetic distance. When needing approximate values of such distances for subsequent ecological analyses, one could normalize the distance of branch lengths from the likely most plesiomorphic species (being nearest to the root location, set to 0) to the likely most apomorphic species (being most distant from the root location, set to 1) in each tree. Scaled this way, one could statistically relate molecular and morphological phylogenetic distances to ecological distances among the species such as their spatial distribution along the river or their differences in ecological traits (e.g., velocity preference). Significance and slope of such relations would provide elements for further discussion of the phylogeny of the regional set of *Hydropsyche* species.

Although the phylogeny of Loire *Hydropsyche* species is incompletely resolved, some phylogenetic polarity is indicated. Consistent information provided by our 2 approaches is that there are 2 species sister groups *ins+sil* and *orn+bul* and that *con* and *exo* and the *orn+bul* lineage form a monophyletic clade with unresolved relationships. To support the phylogenetic polarity (from *ins+sil* to *orn+bul*) required supplementing the computerized maximum parsimony analysis of morphological characters with arguments using Hennig's (1969) approach through discussion of character states provided by the literature. Relating this phylogenetic polarity in future studies to ecological polarities among the species (e.g., headwater versus large river, fast versus slow velocity preference) should provide insights in evolutionary processes such as adaptation, niche conservatism, or fitness.

Our phylogenetic analyses of *Hydropsyche* species from the Loire River support the view that multiple approaches and characters should be included in cladistic analyses (e.g., Grandcolas *et al.* 2001, Kjer *et al.* 2001, Stuart & Currie 2001, Kaila & Stahls 2006). This point is particularly important when searching for phylogenetic distances and polarity across a regional species pool (representing a subsample of a larger clade) to interpret ecological patterns such as distribution along a river (e.g., Hildrew & Edington 1979, Higler & Tolkamp 1983, Tachet *et al.* 1992, Ivol *et al.* 1997) and associated gradients in respiratory or net-building traits (e.g., Hildrew & Edington 1979, Pierrot 1984, Roux *et al.* 1992, Tachet *et al.* 1992, Guinand *et al.* 1994).

In this context, the accuracy of phylogenetic hypotheses obtained by various approaches depends in part on the group examined. For cladistic studies of relatively few species, larval and adult caddisflies offer so many clearly varying morphological characters among the species that one has to analyze more than our 2 gene fragments to find 1 or a few genes providing a similarly accurate description of their phylogeny (see Kjer *et al.* 2001 for a discussion of the phylogenetic relevance of molecular versus morphological characters in this group). In contrast, mammals of a given clade have so few clearly varying morphological characters that gene fragments are likely a more accurate base for phylogenetic inferences (e.g., Murphy *et al.* 2001, Douady *et al.* 2003a). Obviously, such differences among taxa will decrease when the number of species included in the analyses increases (i.e., when the number of varying morphological characters is much lower than the number of species). Given that phylogenetic ecology typically relies on phylogenetic hypotheses considering fewer species that co-exist at smaller spatial scales, such differences among taxa should be acknowledged in future analyses.

## Acknowledgements

Marie-Noëlle Truchet sorted the samples, Nathalie Mondy made photographs and drew our attention to potential costs of melanin synthesis in insects, and François Bourgeot participated in the molecular analysis. The program “Bonus Qualité Recherche” (Univ. Lyon 1) supported CJD. Two anonymous reviewers and John Morse provided substantial comments that improved the manuscript. We gratefully acknowledge all this help.

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**APPENDIX 1.** Target loci and primer sequences of hydropsychid-specific primers derived from our initial sequences that were used for species we could not amplify with standard primers.

Locus	Name	5'	3'
16S	F1	AGTCAAATCTGCCACTGAATT	
16S	F2	AATTTTAACTGTACAAAGGTAGCATAATAA	
16S	R1	CATCTTAATCCAACATCGAGGTC	
16S	R2	TAACGCTGTTATCCCTTAGGTAATT	
COI	F1552	GATCTGGACTAATTGGATCCTCTATAAG	
COI	F1564	AATTGGATCCTCTATAAGACTAATTATTTCG	
COI	F1579	AAGACTAATTATTCGTATAGAATTAAGAAC	
COI	R2145	TGTCCAAAAAATCAAAATAAGTGTTG	
COI	R2088	GTCAAAAAAAGATGTATTTAGATTTCG	

**APPENDIX 2.** Details on the morphological characters (labeled by character number) included in the analysis and, if possible, references to published illustrations of the characters and their states. For general descriptions of the morphology of caddisfly larvae see, for example, works by Lepneva (1970), Wiggins (1977), Waringer & Graf (1997), Higler (2005); for adults, we used the terminology of Neu & Tobias (2004).

Larvae (well-sclerotized 5th instar)

- 1) Lateral mandible wings (situated at the dorsal part of the outer mandible surface): narrow (Neu & Tobias 2004, fig. 9a, i.e., most of the ventrally inserted setae are visible; 1/0) or wide (Neu & Tobias 2004, fig. 21a, i.e., only the longest of the ventrally inserted setae are visible; 1/1).
- 2) Proximal contour of proximal tooth of right mandible: curved basad (Neu & Tobias 2004, fig. 8d; 2/0) or almost rectangular (Neu & Tobias 2004, fig. 24a; 2/1).
- 3) Lateral cardo area (ventral area of cardo near its lateral contour, Waringer & Graf 1997, Morphology fig. 7): almost flat (3/0) or curved dorsad (3/1).
- 4) Latero-proximal cardo contour (where the lateral cardo contour joins the head capsule, Waringer & Graf 1997, Morphology fig. 7): angular (Pitsch 1993, photo 131; 4/0) or round (Pitsch 1993, photo 122; 4/1).
- 5) Distal contour of median mentum channel: angular (Edington & Hildrew 1981, figs 117, 118; 5/0) or round (Edington & Hildrew 1981, figs 119, 120; 5/1).
- 6) Submentum surface: flat (Waringer & Graf 1997, Hydropsychidae fig. 22; 6/0); or with indistinct, triangular hump, with almost flat ventral surface (Waringer & Graf 1997, Hydropsychidae fig. 7; 6/1); or with distinct, hemispherical hump, with round ventral surface (Waringer & Graf 1997, Hydropsychidae fig. 6; 6/2).
- 7) Submentum shape: almost triangular (Neu & Tobias 2004, fig. 25d; 7/0) or with laterally elongated lobes (Neu & Tobias 2004, fig. 12e; 7/1).
- 8) Anterior frontoclypeus contour: straight (Waringer & Graf 1997, Hydropsychidae fig. 11; 8/0); or symmetrically convex (Waringer & Graf 1997, Hydropsychidae fig. 8; 8/1); or asymmetrically convex (Waringer & Graf 1997, Hydropsychidae fig. 10; 8/2).
- 9) Indentations of anterior frontoclypeus contour (Neu & Tobias 2004, fig. 8b): indentations distinct ( $>0.003$  mm; 9/0) or indentations indistinct ( $<0.0013$  mm; 9/1).
- 10) Insertion of velocity sensor organs near indentations of anterior frontoclypeus contour (character 9, viewed dorsally, Kaiser 1965, fig. 15): in indentations (10/0) or in and besides indentations (10/1; note that this invariant character in the Loire River *Hydropsyche* species may be functionally important).
- 11) Density of velocity sensor organs in line near anterior frontoclypeus contour (in organs  $0.25$  mm<sup>-1</sup>; Kaiser 1965, fig. 15): ~10 organs (11/0) or 20–30 organs (11/1; note that this invariant character in the Loire River *Hydropsyche* species may be functionally important).
- 12) Frons and clypeus at epistomal suture near tentorial pit (Waringer & Graf 1997, Hydropsychidae fig. 28): flat, frons in same plane as clypeus (12/0) or stepped, frons recessed below plane of clypeus (12/1).
- 13) Approximate setal density on posterior frontoclypeus (in setae mm<sup>-2</sup>): high, 31–45 setae (13/0); or intermediate, 16–30 setae (13/1); or low, 0–15 setae (13/2).
- 14) Frontoclypeus shape: U-shaped (Edington & Hildrew 1981, figs 122, 123, 124; 14/0) or V-shaped (Edington & Hildrew 1981, figs 121, 125, 126; 14/1).
- 15) Frontoclypeus colouration: without 6-spot pattern (15/0) or with some version of 6-spot pattern (Statzner & Mondy

2009; 15/1).

- 16) Type-1 setae (cylindrical setae with surface stripes and blunt apices, Pitsch 1993, photo 118) on posterior frontoclypeus and/or adjacent head surface: absent (16/0) or present (16/1).
  - 17) Type-2 setae (long setae ending in single tip, Neu & Tobias 2004, fig. 8a) on posterior frontoclypeus and/or adjacent head surface: present (17/0) or absent (17/1).
  - 18) Type-3 setae (long setae ending in multiple tips, Pitsch 1993, photo 115) on posterior frontoclypeus and/or adjacent head surface: absent (18/0) or present (18/1).
  - 19) Light spots on posterior frontoclypeus and adjacent head surface: absent (Waringer & Graf 1997, Hydropsychidae fig. 28; 19/0) or present (Waringer & Graf 1997, Hydropsychidae fig. 14; 19/1).
  - 20) Dark muscle-insertion marks on posterolateral parts of head: absent (20/0) or present (20/1).
  - 21) Shape of lobe near ventral mandible articulation (this lobe situated laterally of submentum at anterior limit of head capsule): round (Pitsch 1993, photo 121; 21/0); or typically angular but sometimes with rounder contours (*H. incognita*; 21/1); or angular (Pitsch 1993, photo 126; 21/2).
  - 22) Stridulation ridge density (in ridges  $0.2 \text{ mm}^{-1}$ , measured parallel to ventral median suture in centre of stridulation ridge field): high (19–22 ridges, Pitsch 1993, photo 132; 22/0); or variable (*H. angustipennis*, with high ridge densities in Loire River but low ones in southern Finland (Jansson & Vuoristo 1979, fig. 3a); 22/1); or low (11–13 ridges, Pitsch 1993, photo 129; 22/2).
  - 23) Type-2 setae (character 17) on anterior pronotum margin: present (23/0) or absent (23/1).
  - 24) Type-4 setae (short setae ending in multiple tips, Pitsch 1993, photo 118) on anterior pronotum margin: sparse (24/0) or very abundant (24/1).
  - 25) Type-4 setae (character 24) on pronotum posterior to anterior margin: present (25/0) or absent (25/1).
  - 26) Anterodorsal area of pronotum: concolorous with anterolateral area (Waringer & Graf 1997, Hydropsychidae fig. 33; 26/0) or darker than anterolateral area (Waringer & Graf 1997, Hydropsychidae fig. 14, not visible in extremely light *H. contubernalis*; 26/1).
  - 27) Type-4 setae (character 24) on meso- and metanotum: absent (27/0) or present (27/1).
  - 28) Muscle insertion marks outside lateral wrinkle of meso- and metanotum: clearly visible, dark brown rings with lighter centers (28/0) or almost invisible, light yellow (28/1).
  - 29) Type-1 setae (character 16) on thoracic nota: absent (29/0) or present (29/1).
  - 30) Lateral and median part of posterior prosternites: pale (Neu & Tobias 2004, fig. 8e; 30/0) or distinctly pigmented (Neu & Tobias 2004, figs 9e and 17d; 30/1).
  - 31) Fusion of lateral and median part of posterior prosternites: not fused (with different colour where adjoining parts come in contact, Neu & Tobias 2004, fig. 15d; 31/0); or variable, typically unfused but sometimes fused (*H. incognita* and *H. exocellata*; 31/1); or fused (with similar colour where adjoining parts come in contact, Neu & Tobias 2004, figs 9e and 17d; 31/2).
  - 32) Pigment colour of median parts of posterior prosternites: no pigmentation (Neu & Tobias 2004, fig. 8e; 32/0); or light yellow (Neu & Tobias 2004, fig. 21e; 32/1); or brown (Neu & Tobias 2004, fig. 9e; 32/2).
  - 33) Posterior mesosternites with dark patches (Neu & Tobias 2004, fig. 9f): absent (33/0) or present (33/1).
  - 34) Number of tufted gills on metasternum: 3 gills (34/0) or 4 gills (34/1).
  - 35) Type-5 setae (scale-like setae, larger distally than proximally, without surface stripes) dorsally on abdomen: absent (35/0) or present (35/1).
  - 36) Ventral gills on abdominal segment VII: present (Waringer & Graf 1997, Hydropsychidae fig. 16b; 36/0) or absent (Waringer & Graf 1997, Hydropsychidae fig. 16a; 36/1).
  - 37) Sclerotization of lateral setal zone on abdominal segment IX: weakly sclerotized (37/0) or well sclerotized (37/1).
  - 38) Anterior, ventral head foramen width divided by distance between points where lateral cardo contours join head capsule (Waringer & Graf 1997, Morphology fig. 7): 1.67–1.71 (38/0); or 1.78–1.82 (38/1); or 1.90 (38/2).
  - 39) Distance between points where lateral cardo contours join head capsule (character 38) divided by submentum width: 1.30–1.32 (39/0) or 1.36–1.38 (39/1).
  - 40) Anterior head foramen width divided by distance between lateral edges of tentorial pits (character 12): 2.17–2.23 (40/0); or 2.03 (40/1); or 2.34 (38/2); or 2.41–2.49 (40/3); or 2.67 (40/4).
  - 41) Angle between the lines connecting posterior submentum tip with outer contours of lateral submentum lobes divided by angle between lines connecting posterior frontoclypeus tip with outer contour of tentorial pits (character 12): 2.46–2.67 (41/0); or 2.07–2.16 (41/1); or 2.31 (41/2).
  - 42) Ventral head length (as submentum is fused with head capsule, measured on median towards anterior contour of submentum) divided by maximum head width: 0.55–0.65 (42/0) or 0.45–0.47 (42/1).
  - 43) Approximate extension of black marginal ribbons on thoracic nota, estimated as summed proportion along anterior, lateral, and posterior margins of all thoracic nota (Waringer & Graf 1997, Family Key fig. 25): 0.55–0.70 (43/0) or 0.85–0.90 (43/1).
- Adults (male and female)
- 44) Median cell in hind wing: open (44/0) or closed (Neu & Tobias 2004, fig. 3; 44/1).

- 45) Oblique black lines on basal antennal segments: absent (45/0) or present (Neu & Tobias 2004, fig. 6a; 45/1).
- Female genitalia (abdominal segment IX)
- 46) Relative lengths of anterior and posterior margins of clasper receptacle opening: anterior>>posterior (Neu & Tobias 2004, fig. 28f; 46/0) or anterior≈posterior (Neu & Tobias 2004, figs 29d, 32h; 46/1).
- 47) Clasper receptacle and receptacle opening: large (Neu & Tobias 2004, fig. 32h; 47/0) or tiny (running over <10% of segment width; Neu & Tobias 2004, fig 29d; 47/1).
- 48) Tooth on margin of receptacle opening: present (Neu & Tobias 2004, fig. 36g; 48/0) or absent (Neu & Tobias 2004, fig. 32h; 48/1).
- 49) Tooth position on margin of receptacle opening: dorsal (Neu & Tobias 2004, fig. 28f; 49/0); or anterior (Neu & Tobias 2004, fig. 36g; 49/1); or posterior (Neu & Tobias 2004, fig. 40e; 49/2).
- 50) Dorsal lateral lobe: absent (Neu & Tobias 2004, fig. 28e; 50/0) or present (Neu & Tobias 2004, fig. 35g; 50/1).
- 51) Ventral contour of dorsal lateral lobe: not protruding ventrad (Neu & Tobias 2004, fig. 35g; 51/0) or protruding ventrad (Neu & Tobias 2004, fig. 36f; 51/1).
- 52) Ventral lateral lobe: absent (Neu & Tobias 2004, fig. 28e; 52/0) or present (Neu & Tobias 2004, fig. 35g; 52/1).
- 53) Shape of ventral lateral lobe: dorsal and ventral half similarly broad (Neu & Tobias 2004, fig. 35g; 53/0); or dorsal half broadest (Neu & Tobias 2004, fig. 36f; 53/1); or dorsal half broadest and pointed (Neu & Tobias 2004, fig. 32h; 53/2).
- 54) Receptacle-lateral lobe index (viewed laterally); relating 2 distances ( $a b^{-1}$ ) measured from the intersection of a line (receptacle line) across the clasper receptacle and the clasper channel with the dorsal receptacle limit (Neu & Tobias 2004, fig. 39h) towards (a) the posterior contour of the dorsal lateral lobe at its broadest part and (b) the most posterior contour of the ventral lateral lobe. Because this index did not collapse into groupings separated by clear discontinuities in the data (Fig. 1b) we deleted it from the analysis.
- 55) Receptacle-lateral lobe angle ( $^{\circ}$ ), situated between lines a and b (character 54): 3–7 (55/0); or 12–18 (55/1); or 25 (55/2).
- 56) Receptacle channel-dorsal lateral lobe angle ( $^{\circ}$ ), situated between receptacle line and line a (character 54): -10 (56/0) or 12–27 (56/1).
- Male genitalia (abdominal segments IX, X, and appendices)
- 57) Dorso-median keel on abdominal segment IX: absent (Neu & Tobias 2004, fig. 28b; 57/0) or present (Neu & Tobias 2004, fig. 39b; 57/1).
- 58) Shape of dorso-median keel on abdominal segment IX (viewed dorsally): contours continuously converging (Neu & Tobias 2004, fig. 39b; 58/0); or contours almost parallel (Neu & Tobias 2004, fig. 40b; 58/1); or contours diverging and then converging (Neu & Tobias 2004, fig. 46b; 53/2).
- 59) Dorsal crests on abdominal segment X: absent (Neu & Tobias 2004, fig. 28a; 59/0) or present (Neu & Tobias 2004, fig. 35a; 57/1).
- 60) Shape of posterior gap between dorsal crests on abdominal segment X (viewed dorsally): U-shaped (Neu & Tobias 2004, fig. 35b; 60/0); or V-shaped (Neu & Tobias 2004, fig. 36b; 60/1); or W-shaped (Neu & Tobias 2004, fig. 29c; 60/2).
- 61) Shape of posterior dorsal contour of dorsal crests on abdominal segment X (viewed laterally): round (Neu & Tobias 2004, fig. 35a; 61/0); or round and protruding dorsad (Neu & Tobias 2004, fig. 42a; 61/1); or pointed and protruding dorsad (Neu & Tobias 2004, fig. 33a; 61/2).
- 62) Apical, long, rod-shaped appendices on abdominal segment X: present (Neu & Tobias 2004, fig. 40a; 62/0) or absent (Neu & Tobias 2004, fig. 35a; 62/1).
- 63) Median part of phallus (viewed ventrally): not extended (Neu & Tobias 2004, fig. 39c; 63/0) or extended (Neu & Tobias 2004, figs 36d, 33g; 63/1).
- 64) Two "teeth" proximal of phallus apex (viewed ventrally): absent (Neu & Tobias 2004, fig. 28d; 64/0) or present (Neu & Tobias 2004, fig. 39e; 64/1). Neu & Tobias (2004) interpreted the lateral curvatures near the phallic apex of *H. ornatula* (their fig. 42d) as modifications of the larger, pointed, phallic teeth found in other *Hydropsyche* species (e.g., their fig. 39e); with this interpretation, all our *Hydropsyche* species would have such teeth (sometimes very indistinct, see their fig. 33g, showing *H. contubernalis*; note that the subspecies *H. contubernalis masovica* has distinct teeth, their fig. 34c).
- 65) Shape of two "teeth" proximal of phallus apex (viewed ventrally): pointed (Neu & Tobias 2004, fig. 39e; 65/0) or round (Neu & Tobias 2004, fig. 42d; 65/1).
- 66) Direction of apical, lateral phallic lobes (viewed laterally): directed ventrad (Neu & Tobias 2004, fig. 28c; 66/0) or directed caudad (Neu & Tobias 2004, fig. 39d; 66/1).
- 67) Contour of posterior, ventral phallus apex (viewed laterally): angular (Neu & Tobias 2004, fig. 36a; 67/0) or round (Neu & Tobias 2004, fig. 39d; 67/1).
- 68) Curvature direction of distal clasper segment (viewed ventrally): laterad (Neu & Tobias 2004, fig. 28d; 68/0) or mesad (Neu & Tobias 2004, fig. 33c; 68/1).
- 69) Distal clasper contour: round (Neu & Tobias 2004, fig. 32d; 69/0) or with a tiny hook (Neu & Tobias 2004, fig. 29b;

69/1).

- 70) Phallic apex index (viewed ventrally), length of apical phallic lobes (character 66) divided by phallic width measured at the proximal end of the phallic lobes: 1.15–1.25 (70/0); or 0.86–0.93 (70/1); or 0.57–0.67 (70/2); or 0.29–0.42 (70/3).
- 71) Clasper index (viewed ventrally), length of distal clasper segment divided by length of proximal clasper segment (see Neu & Tobias 2004, fig. 4, for ventral view of entire clasper): 0.25–0.33 (71/0); or 0.41–0.50 (71/1); or 0.63 (71/2).
- 72) Abdominal segment X-clasper index (viewed laterally), distance from proximal clasper end to dorso-posterior contour of abdominal segment X divided by clasper length (see Neu & Tobias 2004, fig. 4, for lateral view of male genitalia): 0.77 (72/0); or 0.85–0.89 (72/1); or 0.93–0.96 (72/2).
- 73) Setal zone X-clasper index (viewed laterally): distance from proximal clasper end to centre of lateral setal zone on abdominal segment X divided by clasper length (see Neu & Tobias 2004, fig. 4, for lateral view of male genitalia): 0.58 (73/0); or 0.67–0.70 (73/1); or 0.76 (73/2).
- 74) Phallic apex-clasper index (viewed laterally), distance from proximal clasper end to ejaculatory cave at phallic apex divided by clasper length (see Neu & Tobias 2004, fig. 4, for lateral view of male genitalia). Note that the basal part of the phallus is fixed through a joint to the basal end of the claspers (Statzner 1974, fig. 14): 0.50–0.52 (74/0); or 0.40 (74/1); or 0.56–0.61 (74/2).

Character Nos and coding assignments\* for the 10 species studied by us.

No	ang	bul	con	din	exo	inc	ins	orn	sil	lep
1	0	1	0	0	0	0	0	1	0	0
2	1	1	1	1	1	1	1	1	1	0
3	1	1	1	1	1	1	0	1	0	0
4	1	1	1	1	1	1	0	1	0	0
5	0	0	0	0	0	0	1	0	1	0
6	0	2	0	0	0	1	0	2	0	0
7	1	1	1	1	1	1	0	1	0	0
8	0	1	2	0	2	0	0	1	0	0
9	0	1	0	0	0	0	0	1	0	0
10	1	1	1	1	1	1	1	1	1	0
11	1	1	1	1	1	1	1	1	1	0
12	1	1	1	1	1	1	1	1	1	0
13	2	0	2	2	2	2	1	2	1	0
14	1	1	1	0	1	1	0	1	0	0
15	1	1	1	1	1	1	1	1	1	0
16	1	1	1	1	1	1	1	1	1	0
17	1	1	1	1	1	1	1	1	1	0
18	0	1	0	0	0	0	0	0	0	0
19	0	0	0	0	1	0	0	0	0	0
20	1	0	0	1	1	1	1	0	1	0
21	0	0	0	2	0	1	2	0	2	0
22	1	2	2	2	2	2	0	2	0	0
23	1	1	1	1	1	1	1	1	1	0
24	1	1	1	0	1	0	0	1	0	0
25	0	0	0	1	1	1	1	0	1	0
26	0	1	1	0	1	0	0	1	0	0
27	0	1	1	0	0	0	0	1	0	0
28	0	1	1	0	0	0	0	1	0	0
29	1	1	1	1	1	1	1	1	1	0
30	1	1	1	1	1	1	1	1	1	0

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Continued.

No	ang	bul	con	din	exo	inc	ins	orn	sil	lep
31	2	2	2	0	1	1	0	2	0	0
32	2	1	1	2	2	2	2	1	2	0
33	1	0	0	1	1	1	1	0	1	0
34	1	1	1	1	1	1	1	1	1	0
35	1	1	1	1	1	1	1	1	1	0
36	0	0	0	0	0	0	0	0	1	0
37	1	0	0	1	0	1	1	0	1	0
38	1	1	1	0	2	0	1	1	0	0
39	1	0	1	1	0	1	1	1	1	0
40	2	4	0	0	3	3	1	3	1	0
41	0	0	2	1	0	0	1	0	1	0
42	0	1	0	0	0	0	0	1	0	0
43	0	0	0	0	0	1	1	0	1	0
44	1	1	1	1	1	1	1	1	1	0
45	1	1	1	1	1	1	1	1	1	0
46	1	1	1	1	1	1	1	1	1	0
47	1	0	0	0	0	0	0	0	0	0
48	1	1	0	0	0	0	0	1	0	0
49	9999	9999	1	1	1	1	2	9999	2	0
50	1	1	1	1	1	1	1	1	1	0
51	0	1	1	0	1	0	0	1	0	9999
52	1	1	1	1	1	1	1	1	1	0
53	1	2	1	0	1	0	0	2	0	9999
54	Excluded from analysis (see Fig. 1b)									9999
55	2	0	1	1	1	1	1	0	1	9999
56	0	1	1	1	1	1	1	1	1	9999
57	1	1	1	1	1	1	1	1	1	0
58	1	0	0	0	0	0	1	0	2	9999
59	1	1	1	1	1	1	1	1	1	0
60	2	0	0	0	1	0	0	0	0	9999
61	0	2	2	0	2	0	0	1	0	9999
62	1	1	1	1	1	1	0	1	0	0
63	1	1	1	0	1	0	0	1	0	0
64	1	1	1	1	1	1	1	1	1	0
65	1	1	1	0	1	0	0	1	0	9999
66	1	1	1	1	1	1	1	1	1	0
67	0	1	0	1	0	1	1	0	1	0
68	1	1	1	1	1	1	1	1	1	0
69	1	0	0	0	0	0	0	0	0	0
70	2	3	3	1	3	0	1	3	2	0
71	0	2	1	1	1	1	0	1	1	0
72	1	1	2	1	1	1	1	2	1	0
73	1	1	1	1	1	1	1	2	1	0
74	2	2	0	99999	1	0	0	2	2	0

\*9999 = character non-existent; \*99999= missing value.