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DNA-based association and description of the larval stage of *Apatania helvetica* Schmid 1954 (Trichoptera, Apataniidae) with notes on ecology and zoogeography

JOHANN WARINGER^{1,*}, VERENA LUBINI², FELICITAS HOPPELER³, and STEFFEN U. PAULS³

¹Department of Limnology and Bio-Ocenaography, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

²Gewässerökologie, Eichhalde 14, CH-8053 Zürich, Switzerland. lubini@sunrise.ch

³Senckenberg Biodiversity and Climate Research Centre (BiK-F), Frankfurt a.M., Germany

Abstract

This paper describes the hitherto unknown larva of *Apatania helvetica* Schmid 1954. Sequence data from the mitochondrial cytochrome *c* oxidase region were used to associate adult females and larvae. Genetic data confirmed the autochthonous status of this taxon within the parthenogenetic *Apatania muliebris* complex ('sous groupe') *sensu* Schmid 1954. Information on the morphology of the larva is given, and the most important diagnostic features are illustrated. *Apatania helvetica* is morphologically close to *Apatania muliebris* McLachlan 1866 and *A. fimbriata* (Pictet 1834). In the context of Apataniidae, this trio of species can be separated by the presence of long tapering setae with flexuous tips at the anterior border of the pronotum, by a central gap within the transverse setal band on the 1st abdominal dorsum, by biometry of the frontoclypeal setation and by their distribution ranges in Europe.

With respect to European ecoregions (Graf *et al.* 2008), *Apatania muliebris* has been recorded from the Alps, the central plains and highlands, the Baltic province, Great Britain, Ireland and Scandinavia; *A. fimbriata* is known from the Alps, the western and central highlands, the western plains, the Hungarian lowlands and the Carpathians. *A. helvetica* is restricted to the Alps and has only been recorded in Switzerland.

Keywords

5th instar larva; description; identification; distribution; life stage association

Introduction

So far, 32 species of genus *Apatania* Kolenati 1848 are included in the "Atlas of European Trichoptera" of Malicky (2004, 2005). In the meantime, another two taxa were described:

*Corresponding author. johann.waringer@univie.ac.at.
Felicitas.Hoppeler@senckenberg.de; Steffen.Pauls@senckenberg.de

one taxon of the *Apatania muliebris* complex from France (Botosaneanu & Giudicelli 2004) and *A. szczesnyorum*, a taxon belonging to the *crassa* subgroup of the *complexa* complex *sensu* Schmid (1955) (Olah 2006). Graf *et al.* (2008) also included *Apatania crymophila* McLachlan 1880, yielding a total of 35 European taxa. However, we are aware of only 12 species which are known in the larval stage and were included in keys so far (Gislason 1979; Lepneva 1966; Solem 1985; Pitsch 1993; Wallace *et al.* 2003). Recently, however, V.L. managed to collect fifth instar larvae and many adult females of the parthenogenetic *A. helvetica* at two Swiss locations. The identified females could be fully associated with the collected larvae using standard molecular sequence data. This material enabled us to work out reliable diagnostic characters permitting integration of the species in existing keys.

Material and methods

Four larvae (fifth instars) of *A. helvetica* Schmid 1954 were collected by V. Lubini on 31 July 2014 using a hand net at spring Punt Periv, Zernez, Switzerland (46° 38.6' N, 10°11.2' E, 1660 m a.s.l.) and another three larvae (fifth instars) by the same collector on 5 June 2007 at a spring location at Netstal, Switzerland (47° 04.6' N, 09°03.1' E, 450 m a.s.l.). The material was preserved in pure ethanol. The larvae were studied and photographed using a Nikon SMZ 1500 binocular microscope with DS-Fi1 camera and NIS-elements D 3.1 image stacking software for combining 8–42 frames in one focussed image.

Species affiliation was based on sequence data from the mitochondrial cytochrome *c* oxidase (mtCO1) region to associate the larvae as this region has proven suitable to delimit species in *Apatania* (Salokannel *et al.* 2010) and for associating life stages in caddisflies (Graf *et al.* 2005, 2009; Zhou *et al.* 2007; Waringer *et al.* 2008). We extracted DNA of four larval and two adult specimens, as well as one female of *A. fimbriata* (Table 1) using the DNEasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. PCR reactions were set up in 10 µl reactions using the following recipe: 1 µl 10× Taq S-Buffer (Peqlab, Erlangen, Germany), 1 µl dNTPs (2 µM each, Carl Roth, Karlsruhe, Germany), 0.25 µl of each 10 µmol oligonucleotide primer (LCO1490 and HCO2198; both Folmer *et al.* 1994), 0.1 µl Hot Taq DNA Polymerase (Peqlab, Erlangen, Germany), and 6.4 µl of sterile H₂O. PCR settings were: 5' 95°C, 5 × (30" 95°C, 1' 44°C, 1' 72°C), 15 × (30" 95°C, 30" 48°C, 1' 72°C), 20 × (30" 95°C, 30" 50°C, 1' + (10" * n) 72°C), 5' 72°C. PCR products were sequenced on an ABI 3177XL capillary sequencer at the Senckenberg Biodiversity and Climate Research Laboratory Centre. Sequences were edited in Geneious vR7 (Biomatters). Sequences were aligned using the Muscle-plugin in Geneious vR7.

We inferred a Bayesian phylogeny for the adult females and putative larvae of *Apatania helvetica* as well as *A. fimbriata* (Pictet, 1834) and 15 additional previously published or publicly available *Apatania* species and five outgroup species from various subfamilies of the Limnephilidae (*Anisogamus waringeri* Graf & Vitecek 2015, *Drusus annulatus* (Stephens 1837), *Ecclisopteryx ivkae* Previšić, Graf & Vitecek 2014, *Limnephilus rhombicus* (Linnaeus 1758), *Melampophylax austriacus* Malicky 1990). We calculated a phylogenetic tree with a Bayesian/MCMC approach implemented in MrBayes v3.2.1 (Ronquist *et al.* 2012). Nucleotide substitution models were selected separately for each codon position using the Bayesian Information Criterion in the model test module of MEGA v5.2 (Tamura

et al. 2011). The B/MCMC analysis was based on 2 parallel runs with six chains each that explored tree space for 10 million generations. Phylogenetic trees were based on 10,002 trees (2×5001) following a 50% burn-in phase. We assessed the parameter files in Tracer to ensure that each run had reached stationarity. We used the average standard deviation of split frequencies between runs after 5000000 generations to assess if both runs reached the same optimality space (>0.01).

Deposition of voucher specimens: The seven 5th instar larvae of *A. helvetica* are deposited in the collection of J. Waringer, Vienna, Austria. Comparative material of *Apatania fimbriata*: eight 5th instar larvae (leg. W. Graf); *A. muliebris* McLachlan 1866: two 5th instar larvae; *A. zonella* (Zetterstedt 1840): two 5th instar larvae (leg. W. Graf) (collection of J. Waringer, Vienna, Austria).

Results

DNA-based life stage association

The best suited nucleotide substitution models were K2+G, HKY, and GTR+G for codon positions 1, 2, and 3, respectively. The average standard deviation of split frequencies after 5000000 generations was 0.006588 and decreased to 0.003586 after 10000000 generations. Chain swapping continued until the end of the run.

The two females and four larvae of *Apatania helvetica* all shared identical haplotypes. The Bayesian phylogenetic inference placed *A. helvetica* (pp= 0.99) as a highly supported sister of *A. muliebris* (pp= 1.0; Fig. 21). Together these results clearly support that the females and larvae from two sites in Switzerland are conspecific. Regarding the other taxa, *A. shoshone* Banks 1924, *A. stigmatella* (Zetterstedt 1840), *A. wallengreni* McLachlan 1871a, *A. incerta* (Banks 1897), *A. comosa* (Denning 1949), *A. copiosa* (McLachlan 1875), *A. forsslundi* Tobias 1981, and *A. auricula* (Forsslund 1930) represent highly supported clades (pp=1.0). *A. cypria* Tjeder 1952 and *A. fimbriata* were represented by a single specimen only and appear to be relatively isolated, whereas the single representative of *A. sorex* (Ross 1941) is close to *A. comosa*. Within the largely unresolved *A. zonella*-group *sensu* Salokannel *et al.* (2010) *A. hispida* (Forsslund 1930) and *A. nr. hispida sensu* Salokannel *et al.* (2010) all share the same haplotype, and are nested in a poorly supported clade with one group of *A. zonella* specimens (pp=0.6). As in Salokannel *et al.* (2010), *A. zonella* is paraphyletic, with a single specimen nested with in a highly supported *A. dalecarlica* (Forsslund 1942) (pp=0.99).

Description of the fifth instar larva of *Apatania helvetica*

Biometry—Body length of 5th instar larvae ranging from 4.7 to 7.7 mm, head width from 0.75 to 0.85 mm (n= 7).

Head: Head capsule distinctly granulated due to a dense cover of microspinules, roundish in shape and hypognathous (Figs. 1–4). Coloration predominantly medium brown with paler areas in center of frontoclypeus (Figs. 1, 3), at anterior border of parietalia (Fig. 4) and around foramen occipitale (Fig. 2). Along posterior half of frontoclypeal suture with dark to black brown bands and with dark brown anterior frontoclypeal corners (Fig. 1). Muscle

attachment spots on frontoclypeus and parietalia small, clearly defined, and paler than surrounding areas (Figs. 1, 4, 5). Narrow, yellowish ring present around the eyes (Fig. 3). Besides set of 18 pairs of primary setae (nomenclature by Wiggins 1998) head capsule with many short, pale and almost translucent secondary setae. Frontoclypeus bell-shaped, with narrow central constriction (Fig. 1). Antennae situated halfway between eye and anterior head margin (Fig. 3, arrow), short, each consisting of 1 short cylindrical base and 1 prominent lateral seta. At each parietal, 10 dorsal and 2 ventral primary setae present, with setae 9, 14 and 15 long and conspicuous (Fig. 3); seta 15 0.5× to 0.75× as long as seta 14. Each side of frontoclypeus with 6 primary setae, 3 of them along anterior border. Setal ratio (= median separation 'a' of frontoclypeal setae 5 divided by distance to frontoclypeal border 'b') is < 5.5 (Fig. 5). Labrum light brown, with setal brush at anterolateral corners originating from whitish pads of soft cuticle; primary setae 1-3 at anterolateral margins; dorsal area with primary setae 4-6 (Fig. 3). Submentum wedge-shaped, sclerite as long as wide, yellowish with medium brown pre-apical transverse band. Postgenal suture less than 17% of apotome length. Scraper-type mandibles reddish to black brown, blade-like, narrow, elongated and without terminal teeth along cutting edge (Figs. 3, 4); in addition, ridges are lacking in central concavity.

Thorax: Pronotum medium to reddish brown, sometimes with paler anterior border and with densely granulated surface; its posterior margins thickened and darkly striped (Figs. 6, 7). Pronotal transverse groove lacking, as typical for Apataniidae larvae (Figs. 2, 3). In profile, pronotum slightly rounded (Fig. 7). Pronotal surface covered by pale, translucent, tapering setae with flexuous tips, especially along the anterior border (Fig. 9); in addition 12–22 longer and darker setae present at each pronotal half (Fig. 6). Median ecdysial line not straight, but in zigzag-like pattern (Fig. 6). Pentangular prosternite densely covered by microspinules, very pale and indistinct in its anterior and lateral sections; along posterior border with brownish transverse band. Prosternal horn present. Mesonotum completely covered by 2 yellowish-brown sclerites; their anterior, lateral and postero-lateral margins darkly sclerotized; mesonotal surface with medium brown muscle attachment spots (Figs. 7, 8). Metanotum partially covered by only 1 pair of yellowish lateral sclerites (sa3) with anterior groups of approximately 10 setae per sclerite and dark brown posterior muscle attachment spots; anterior sclerites of setal area 1 (sa1, *sensu* Wiggins 1998) and posterior sclerites (setal area 2, sa2) completely lacking. Each setal area 1 with 9–15 setae, each setal area 2 with 10–13 setae (Figs. 8, 13). Legs yellowish to light brown with numerous setae on coxae, trochanters and femora; tibiae and tarsi with only a small number of setae (Figs. 10–12). Femora each with more than 1 proximodorsal seta. Coxa, femur and tibiae of each foreleg wider than those of mid- and hind legs. Additional setae present at anterior and posterior faces of all femora. Setae lacking at distal sections of trochanter on all legs. Each claw with long, pale seta originating from subbasal socket (Figs. 10–12).

Abdomen: 1st abdominal segment with 1 dorsal and 2 lateral fleshy protuberances densely covered by microspinules (Figs. 13, 14). In dorsal midline, setal areas sa 1 (*sensu* Wiggins 1998) distinctly separated by a gap created by the dorsal protuberance. At each body side, setal areas sa 1 and sa 2 fused, thereby creating continuous transverse row of 15–18 setae approximating lateral protuberance; the most ventral setae of this group situated ventrally of

dorsal presegmental gill insertion at 2nd segment (Fig. 13). Setal group posterior to the dorsal protuberance lacking (Fig. 3). Setal area *sa* 3 covering dorsal section of each lateral protuberance (Fig. 13). On 1st abdominal sternum, setal areas *sa* 1, 2 and 3 fused, creating continuous field of setae with basal sclerites minute and inconspicuous; setal number is 48–60 (Fig. 14).

8th abdominal dorsum with 14–18 posterodorsal setae. Mostly 1 posterolateral seta present on each half of 9th abdominal dorsum. All gills single filaments; however, 2 single filament gills may be present in close proximity at the dorsal presegmental position. Dorsal gills present at most from 2nd segment (presegmental position) to 4th segment (postsegmental position). Ventral gills ranging from 2nd (postsegmental) to 6th segment (postsegmental). Lateral gills lacking. Lateral fringe extending from end of anterior ¼ of 3rd to end of 7th abdominal segment. Ninth abdominal tergite with 28 setae, the pair of central and 2 lateral setae conspicuously enlarged.

Median brown sclerite on 9th abdominal segment semicircular, with darker muscle attachment spots along anterior border; with 26–33 setae, 4 long and the remainder short to medium in length (Fig. 15). Anal prolegs of the limnephilid type, medium brown and with black bar at anterodorsal border of claw base. Anal proleg lateral sclerite with 5 setae along posterior edge (Fig. 15). Anal claws dark brown, without accessory hook (Fig. 16).

Case: Fifth instar larval case 5.4–8.8 mm long (n= 7), curved, tapering posteriorly (width at anterior opening 1.8–3.4 mm and at posterior opening 1.0–1.5 mm), consisting of mineral particles of varying size, sometimes mixed with much larger particles which are mostly attached laterally, resulting in a rather rough outline (Fig. 17).

Morphological distinction of fifth instar larvae of *Apatania helvetica* from other European Trichoptera

Larvae of the family Apataniidae share the following set of morphological characters (Pitsch 1993; Solem 1985; Wallace *et al.* 2003; Waringer and Graf 2011; Wiggins 1998):

- with transportable case (Fig. 17);
- sclerites present on pro-, meso- and metanota (Fig. 8);
- no transverse rim at the anterior 3rd of the pronotum (Fig. 7);
- pronotum and mesonotum completely covered by 2 sclerites in close contact, separated by an unbranched longitudinal suture (Fig. 8);
- median and posterior metanotal sclerites reduced and represented only by setal groups (Fig. 13);
- prosternal horn present;
- antennae situated halfway between eye and anterior head margin (Fig. 3, arrow);
- scraper-type mandibles without terminal teeth along cutting edge (Figs. 3, 4);
- submentum wedge-shaped (Fig. 2);

- head with many secondary setae;
- fleshy protuberances present laterally and dorsally on the 1st abdominal segment (Figs. 13, 14);
- all gills consisting of single filaments.

Within Apataniidae, *Apatania helvetica* belongs to the species group where the pronotum is covered by long, tapering setae with flexuous tips (Fig. 9) as opposed to the short and dagger-shaped setae present in *Apatania wallengreni*, *A. auricula*, *A. crymophila* and *A. zonella* (Figs. 18, 19). In *A. helvetica* both setal areas *sa* 1 are distinctly separated by a gap created by the dorsal protuberance (Fig. 13) and the species originates from Central Europe. It thus keys together with *Apatania muliebris* and *A. fimbriata* (Pictet 1834). Within this group, the setal ratio (Fig. 5) is > 8 in *A. fimbriata* (Fig. 20) but < 5.5 in *A. helvetica* and *A. muliebris* (Fig. 5). The latter species pair cannot be separated morphologically; however, *A. helvetica* is hitherto only known from Switzerland where *A. muliebris* is lacking.

Key to the hitherto known European larvae of genus *Apatania*

The key is based on information given by Gíslason (1979), Lepneva (1966), Solem (1985), Pitsch (1993) and Wallace *et. al.* (2003). *Apatania helvetica*, *A. muliebris* and *A. zonella* are strictly parthenogenetic, although sometimes males are recorded in *A. zonella* and other species of the *Apatania zonella* group, including *A. dalecarlica* and *A. forsslundi* (Malicky 2005; Solem. 1985).

1	Setae at anterior edge of pronotum short and dagger-shaped.....	10
-	Setae at anterior edge of pronotum long, tapering and with flexuous tips.....	2
2	Setal transversal band at 1st abdominal dorsum interrupted at center.....	3
-	Setal transversal band at 1st abdominal dorsum continuous.....	<i>Apatania eatoniana</i> McLachlan 1880
3	Species from the Caucasus area.....	<i>Apatania subtilis</i> Martynov 1909
-	Species not from the Caucasus area.....	4
4	North European species from Scandinavia or Russian tundra and taiga.....	5
-	European species not from Scandinavia or Russian tundra and taiga.....	8
5	Seta 15 less than half length of seta 14; abdominal tergite 9 with 2 central and 2 lateral setae conspicuously enlarged.....	<i>Apatania muliebris</i>
-	Seta 15 about ¾ to equal length of seta 14; abdominal tergite 9 with 2 or 4 setae conspicuously enlarged.....	6
6	Abdominal tergite 9 with 2 central setae conspicuously enlarged.....	<i>Apatania stigmatella</i>
-	Abdominal tergite 9 with 2 central setae and 2 lateral setae conspicuously enlarged ...	7
7	Species from NW Russia.....	<i>Apatania majuscula</i> McLachlan 1872
-	Species from Scandinavia and Belarus.....	<i>Apatania hispida</i>
8	Setal ratio (= median separation 'a' of pair of frontoclypeal setae #5 divided by distance 'b' to next frontoclypeal border; Fig. 5) is > 8	<i>Apatania fimbriata</i>
-	Setal ratio is < 5.5	9
9	Species from Switzerland.....	<i>Apatania helvetica</i> *

- Species lacking in Switzerland.....*Apatania muliebris* *
- 10 Number of posterior edge setae on anal proleg lateral sclerite is 6–8; number of setae at 9th abdominal dorsum is 30.....*Apatania wallengreni*
- Number of posterior edge setae on anal proleg lateral sclerite is 5; number of setae at 9th abdominal dorsum is < 30..... 11
- 11 Gula almost as long as wide.....*Apatania zonella* **
- Gula narrow and oblong..... 12
- 12 With postero-dorsal gill on 1st abdominal segment.....*Apatania auricula* **
- Without postero-dorsal gill on 1st abdominal segment.....*Apatania crymophila* **

* Data according to Anonymous (2014), Cianficconi (2002), Lubini-Ferlin & Vicentini (2005), Malicky (2009), and Robert (2004).

** Data according to Lepneva (1966) and Wallace et al. (2003).

Discussion

The Apataniidae Wallengren 1886 was, for most of its history, considered a subfamily of Limnephilidae Kolenati 1848 (Holzenthal *et al.* 2007). Since the early nineties of the last century, however, the group is seen as a distinct family (Ivanov & Menshutkina 1996), and subsequent workers have increasingly accepted this designation and confirmed its validity by recent phylogenies (e.g., Holzenthal *et al.* 2007; Vshivkova *et al.* 2007).

In the European inventory there are only two genera of Apataniidae which belong to subfamily Apataniinae Wallengren 1886: the largest genus *Apatania* Kolenati 1848 with nearly 100 holarctic species and the palearctic and oriental genus *Apataniana* Mosely 1936 with only 6 European species (Holzenthal *et al.* 2007; Malicky 2004, 2005; Graf et al. 2008; Morse 2012).

As pointed out by Botosaneanu & Giudicelli (2004) and Malicky (2005), the description of *A. muliebris* by McLachlan (1866) attracted the attention of entomologists to an entirely parthenogenetic (thelytoke) Trichoptera species. In addition, facultative parthenogenesis has been observed in the *Apatania zonella* group where occasionally males can be observed (Solem 1985). In the forthcoming decades there was much debate whether the *muliebris* complex represents one large, very variable taxon (superspecies concept) or whether this complex consists of many distinct taxa with species status (see Botosaneanu & Giudicelli 2004 for further details). Malicky (2005) stated that the customary use of the species category does not work in this parthenogenetic group where currently 10 ‘species’ or ‘forms’ of the *muliebris* complex with distinctly different genitalia morphology are listed (Malicky 2005). Extrapolating this interpretation, parthenogenesis, like geographical barriers, may also be seen as an effective isolation mechanism with the potential for shaping and stabilizing species-analogous structures (Nielsen 1950; Botosaneanu & Giudicelli 2004).

In his seminal monography on subfamily Apataniinae, *Apatania helvetica* was described by Schmid (1954) as a distinct species of ‘sous-groupe de *muliebris*’, based on Swiss material from the Haslital in the Berner Oberland which was originally described as *A. muliebris* by McLachlan (‘J’ai vérifié la fausse identité des exemplaires signalés sous le nom de *muliebris*

du Haslital, par McLachlan.'; Schmid 1954). In addition, Schmid's description is based on 3 populations from the Swiss canton of Valais: Entremont valley in the vicinity of the Great St. Bernard at 2200 m a.s.l. (district of Entremont), Ferret valley near the Fenêtre lakes at 2600 m a.s.l. (border between Italy and Switzerland, on the southern side the Mont Blanc Massif), and Zinal, Anniviers valley (1800 m a.s.l.). In 1969, *A. helvetica* was again assigned the rank of a subspecies of *A. muliebris* by Nielsen, but Graf et al. (2008), Malicky (2005) and Morse (2012) consider *A. helvetica* again as a distinct and separate taxon.

Larvae of *A. helvetica* inhabit springs and small, cold, high-gradient springbrooks with high water current at higher altitudes in the Alps (Graf et al. 2008), although one of our larval sampling locations at a spring at Netstal was situated as low as 450 m a.s.l. Both habitats of *A. helvetica* sampled in the present study were rheocrenes with little discharge (0.5–30 l min⁻¹; Vetter 1992; Robinson 2008); most larvae were found under moss covered stones and pebbles within several meters downstream of the spring proper. Maximum water temperature at Punt Periv at the end of July was 7.3°C directly at the spring. Accompanying Trichoptera species at that spring were *Plectrocnemia geniculata* McLachlan 1871b, *P. conspersa* (Curtis 1834), *Philopotamus ludificatus* McLachlan 1878, *Rhyacophila bonaparti* Schmid 1947, *Drusus nigrescens* Meyer-Duer 1875, *D. chrysotus* (Rambur 1842), *D. discolor* (Rambur 1842) and *Pseudopsilopteryx zimmeri* (Kolenati 1860). Interestingly, Schmid (1954) states that he never found larvae directly in the springs, but only approximately 30 m further downstream.

The females are on the wing from the onset of June to the end of August, with a maximum in July. The winter is spent in the larval stage, and pupation starts in spring; pupae can be recorded until July (Schmid 1954). Icelandic populations of *A. zonella* are also univoltine, with the main emergence period taking place in July and August. Most larvae reach the 5th instar in September, and pupation takes place as early as April and May, but last until September (Gíslason & Sigfússon 1987). An interesting observation is that prepupae were recorded in September in Iceland and spend the winter in an inactive stage (Gíslason & Sigfússon 1987).

As in the other known larvae of *Apatania*, the mandibles in *A. helvetica* take the shape of scraper blades and lack terminal teeth along their cutting edges; larvae graze autotrophic biofilm and epilithic algae. Icelandic populations of *A. zonella* feed almost exclusively on Bacillariophyceae (mainly genera *Cocconeis*, *Fragilaria*, *Nitzschia*, *Rhoicosphenia*, *Achnanthes* and *Navicula*) throughout the year, with lower proportions of detrital particles; vascular plants are negligible food items (Gíslason & Sigfússon 1987).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figures 1–7.

Apatania helvetica Schmid 1954, 5th instar larva. **1** Head, dorsal. **2** Head, ventral. **3** Head and pronotum, frontal (arrow: antenna). **4** Head, lateral (arrow: antenna). **5** Detail of frontoclypeus (arrows: alveolae of frontoclypeal setae 5; a= median separation of alveolae; b= distance of alveola to nearest frontoclypeal margin). **6** Pronotum, dorsal. **7** Head and thorax, dorsolateral. Scale bars: 0.5 mm (except **5**: 0.25 mm).

Figures 8–13.

Apatania helvetica Schmid 1954, 5th instar larva. **8** Thorax and 1st abdominal segment, dorsal. **9** Detail of anterior pronotal margin. **10** Left foreleg, posterior face. **11** Left midleg, posterior face. **12** Left hind leg, posterior face. **13** Mesothorax, metathorax and 1st abdominal segment, dorsolateral. Scale bars: 0.5 mm (except **9**: 0.25 mm).

Figures 14–20.

Figures 14–17. *Apatania helvetica* Schmid 1954, 5th instar larva. **14** Sternum of 1st abdominal segment, ventral. **15** Dorsal sclerite of 9th abdominal segment, dorsal. **16** Anal sclerite and claw, posterolateral. **17** Case, right lateral. **Figures 18–19.** *Apatania zonella* (Zetterstedt 1840), 5th instar larva. **18** Pronotum, dorsal. **19** Detail of anterior pronotal margin. **Figure 20.** *Apatania fimbriata* (Pictet 1834), 5th instar larva. Detail of

frontoclypeus (arrows: alveolae of frontoclypeal setae 5). Scale bars: 0.5 mm (except **17**: 1 mm and **19, 20**: 0.25 mm).

Table 1Specimens used for the molecular life stage association of *A. helvetica* and differentiation from *A. fimbriata*.

Taxon / Locality	N (F/L)	Specimen Code	Collection ID Code	BOLD sequence ID	GenBank Accession*
<i>Apatania helvetica</i>					
Punt Periv spring, Zerne, Switzerland; July	2 / 2	Ahe0101F	SMFTRI00018075	SPAPA005-15	KT613189
31, 2014; 46°38.6'N, 10°11.2'E; 1660 m a.s.l.; leg. Lubini		Ahe0102F	SMFTRI00018076	SPAPA002-15	KT613192
		Ahe0103L	SMFTRI00018077	SPAPA003-15	KT613191
		Ahe0104L	SMFTRI00018078	SPAPA004-15	KT613190
Unnamed spring at Netstal, Switzerland;	0 / 2	Ahe0201L	SMFTRI00018079	SPAPA006-15	KT613188
June 5, 2007; 47°04.6'N, 09°03.1'E; 450 m a.s.l.; leg. Lubini		Ahe0202L	SMFTRI00018080	SPAPA007-15	KT613187
<i>Apatania fimbriata</i>					
St. Lorenzen, Austria, August 8, 1995, leg. Graf	1 / 0	Afim001		SPAPA008-15	KT613186