A taxonomic study of the caddisfly *Oxyethira falcata* Morton, 1893 (Trichoptera: Hydroptilidae) using genital morphology and DNA barcoding

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Taxonomists have had problems with the hydroptilid caddisfly, *Oxyethira falcata* Morton, in the past. Four described taxa have been synonymized with *O. falcata* due to considerable intra-specific morphological variation of the male genitalia, and due to misinterpretation of some of the structures of these. In the present study specimens resembling morphologically *O. boreella* Svensson & Tjeder, one of the synonymized taxa, were compared with the true *O. falcata* using DNA barcoding and studying the male genitalia. Further, a molecular examination of all the Fennoscandian *Oxyethira* species were carried out, including the rare *O. klingstedti* Nybom, *O. tamperensis* Malicky, and *O. ecornuta* Morton. The results support keeping *O. boreella* as a synonym of *O. falcata*. In addition, the DNA analyses showed the presence of monophyletic groups for all of the studied *Oxyethira* species.

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1. Introduction

Oxyethira falcata Morton, 1893 is a tiny hydroptilid species whose west Palaearctic distribution ranges from northern Fennoscandia to North Africa and from the Iberian Peninsula and the British Isles to Central Asia and Tibet (Schmid 1960, Ivanov 2011, Tobias & Tobias 2011). The species inhabits predominantly groundwater feed streams, including small sized rivers, small brooks and trickles. The larval stages of the species are not described, but larvae whose identity

Table 1. List of *Oxyethira* specimens examined and sampled for DNA. For the specimens without a Process ID only male genitalia were examined. In column Note, b refers to *boreella* form and ♀ refers to female specimen.

Process ID	Species	Note	Country	Region
TRIFI155-10	*O. distinctella*		Finland	Ka
TRIFI156-10	*O. distinctella*		Finland	Ka
TRIFI346-11	*O. ecornuta*		Finland	Kb
TRIFI347-11	*O. ecornuta*		Finland	Kb
TRIFI344-11	*O. falcata*		Spain	CyL
TRIFI345-11	*O. falcata*	b	Spain	CyL
TRIFI106-10	*O. falcata*	b	Finland	Tb
TRIFI109-10	*O. falcata*	b	Finland	Tb
TRIFI110-10	*O. falcata*	b	Finland	Tb
TRIFI111-10	*O. falcata*	b	Finland	Tb
TRIFI112-10	*O. falcata*	b	Finland	Tb
TRIFI113-10	*O. falcata*	b	Finland	Li
TRIFI114-10	*O. falcata*	b	Finland	Li
TRIFI118-10	*O. falcata*		Finland	Tb
TRIFI119-10	*O. falcata*		Finland	Tb
TRIFI125-10	*O. falcata*		Finland	Sb
TRIFI206-10	*O. falcata*		Finland	Le
TRIFI207-10	*O. falcata*		Finland	Le
TRIFI208-10	*O. falcata*		Finland	Le
TRIFI209-10	*O. falcata*		Finland	Le
TRIFI213-10	*O. falcata*		Finland	Li
TRIFI214-10	*O. falcata*		Finland	Li
TRIFI636-11	*O. falcata*	♀	Finland	Li
TRIFI637-11	*O. falcata*	♀	Finland	Li
TRIFI638-11	*O. falcata*	♀	Finland	Li
TRIFI639-11	*O. falcata*	♀	Finland	Li
TRIFI640-11	*O. falcata*	♀	Finland	Li
TRIFI159-10	*O. flavicornis*		Finland	Ok
TRIFI160-10	*O. flavicornis*		Finland	Li
TRIFI349-11	*O. frici*		Finland	Li
TRIFI350-11	*O. frici*		Finland	Li
TRIFI165-10	*O. klingstedti*		Finland	Li
TRIFI210-10	*O. klingstedti*		Finland	Le
TRIFI211-10	*O. klingstedti*		Finland	Le
TRIFI212-10	*O. klingstedti*		Finland	Le
TRIFI146-10	*O. mirabilis*		Finland	Li
TRIFI149-10	*O. mirabilis*		Finland	Li
TRIFI148-10	*O. mirabilis*		Finland	Tb
TRIFI154-10	*O. sagittifera*		Finland	Li
TRIFI215-10	*O. sagittifera*		Finland	Kb
TRIFI162-10	*O. simplex*		Finland	Tb
TRIFI163-10	*O. tamperensis*		Finland	Le
TRIFI216-10	*O. tamperensis*		Finland	Le
TRIFI348-11	*O. tristella*		Finland	Ab
TRIFI425-11	*O. tristella*		Finland	Ab
TRIFI424-11	*O. tristella*		Finland	Sb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb

Process ID	Species	Note	Country	Region
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Tb
	O. falcata	b	Finland	Li
	O. falcata		Finland	Li
	O. falcata		Finland	Li
	O. falcata		Finland	Li
	O. falcata		Finland	Li
	O. falcata		Finland	Li
	O. falcata		Finland	Li
	O. falcata		Finland	Li
	O. falcata		Finland	Le
	O. falcata		Finland	Ks
	O. falcata		Finland	Ks
	O. falcata		Finland	St
	O. falcata		Finland	St
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb
	O. falcata		Finland	Tb

was confirmed by rearing to adults have been found among submerged macrophytes (P. Wiberg-Larsen, pers. comm.).

Four taxa, *O. rhodani* Schmid 1947, *O. bidentata* Nybom 1948 nec Mosely, 1934 (= *O. dentata* Nybom, 1954 **nom. nov.),** *O. boreella* Svensson & Tjeder 1975, and *O. assia* Botosaneanu & Moubayed 1985 were described during the 20th century. All of them were later synonymized with *O. falcata* by Schmid (1960), Kelley (1984) and Malicky (2005, 2007). The descriptions of the new species originate from a peculiar morphological feature of *O. falcata*: a part of the male genitalia may occur in two positions, apparently causing the specimens to look like different species. The explanations for the synonyms in earlier literature are however very brief that they may not prevent new authors from repeating previous mistakes.

One of the synonymized taxa, *O. boreella*, has been reported from northern Europe: Sweden (Svensson & Tjeder 1975), Finland (Laasonen & Laasonen 2000) and north Asia (Morse 2012). Two specimens of a taxon resembling *O. boreella* have also been found in Spain. In this paper, we compare Spanish and Finnish specimens of *O. falcata* (including "*O. boreella*" specimens), based on the male genitalia and DNA barcodes (Hebert *et al.* 2003). The purpose is once and for all to confirm the synonymy of the two species. In addition, most other north European *Oxyethira* species, representing about half of the European species (Malicky 2005), were barcoded as references.

2. Material and methods

Specimens from which the DNA barcode was sequenced are given in Table 1. The sequenced *O. falcata* samples were collected from four locations in Finland and one location in Spain. "*Oxyethira boreella*" samples were collected from two locations in Finland and one location in Spain. Other sequenced *Oxyethira* material was collected from eight locations in Finland. The Finnish material was mostly caught with Malaise traps using 50% glycol and stored afterwards in 70% or 96% ethanol.

Total DNA was extracted using QIAgen's DNEasy extraction kit. The DNA barcode region (cytochrome oxidase subunit I) was amplified and sequenced from all specimens using universal primers LCO1490: 5'- GGG TCA ACA AAT CAT AAA GAT ATT GG-3' and HCO2198: 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer *et al.* 1994). All Polymerase chain reactions (PCR) were performed in 20 µl reaction volumes containing 1 µl of DNA extract, 12.5 µl ddH2O, 2.0 µl 10× buffer, 2.0 µl MgCl2, 1.0 µl primerF (LCO), 1.0 µl primerR (HCO), 0.4 µl dNTPs, and 0.1 µl AmpliTaq Gold polymerase. PCR was performed using following program: 95 °C for 5 min, 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1 min 30 sec, with a final extension period of 10 min at 72 °C. Successful PCR products were purified and sequenced by Macrogen Inc. (South Korea). Sequences were trimmed and aligned first using the software Geneious (Drummond *et al.* 2010) and then manually confirmed by eye.

The resulting sequences were uploaded to TRIFI project in the Barcode of Life Data System (Ratnasingham & Hebert 2007). The sequences were analyzed in BOLD using the standard neighbour-joining (NJ) method with pairwise distances modified using the K2P model. The tree was downloaded from BOLD server as newick tree and formatted using program TreeGraph2 (Stöver & Müller 2010). Sequence divergences were calculated on BOLD server.

Further specimens were studied based on the male genitalia alone. These are also presented in Table 1. The morphological study was based on examining the characters of the last segments of male specimens. The genitalia were examined in 70% or 96% ethanol or in some cases prepared for glass slides, using a magnification of 20–30×.

3. Results

3.1. Molecular results

In total 46 *Oxyethira* samples were successfully sequenced for their DNA barcode. All of the *Oxyethira* specimens, except *O. falcata* and "*O. boreella*", formed monophyletic groups in the DNA barcode tree (Fig. 1). *Oxyethira falcata* and

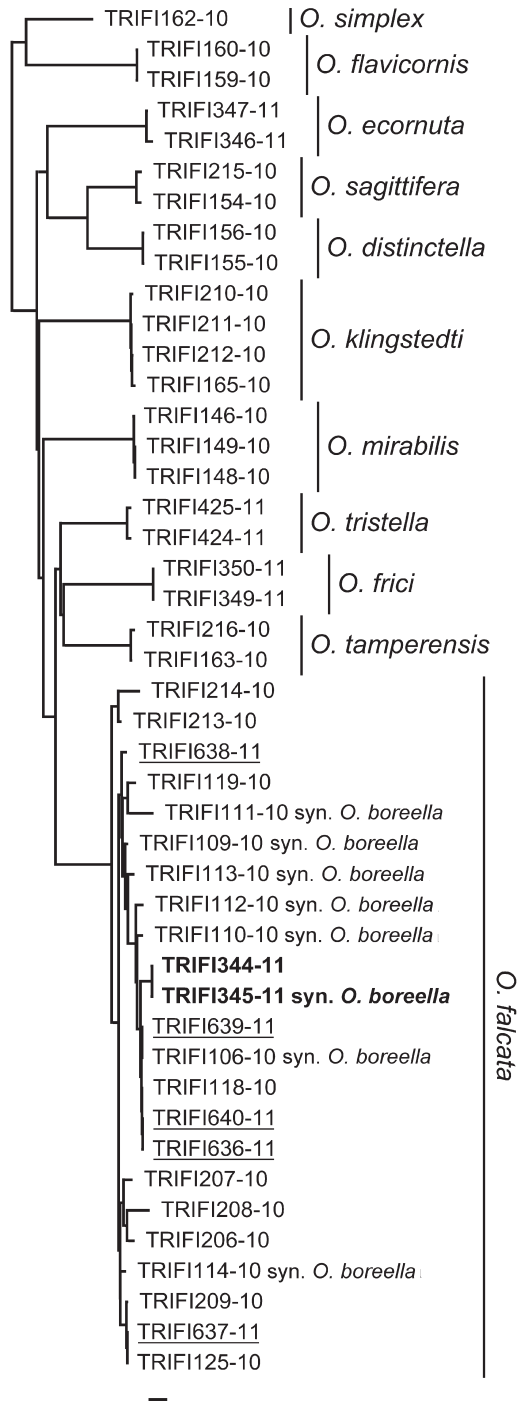

Fig. 1. Neighbour joining tree of partial COI (DNA barcode) gene sequences for *Oxyethira* species based on genetic distances calculated with the Kimura 2 parameter model. Specimens in bold were collected from Spain, underlined specimens are females. Details for specimens are in Table 1. Scale bar represents 2% nucleotide divergence.

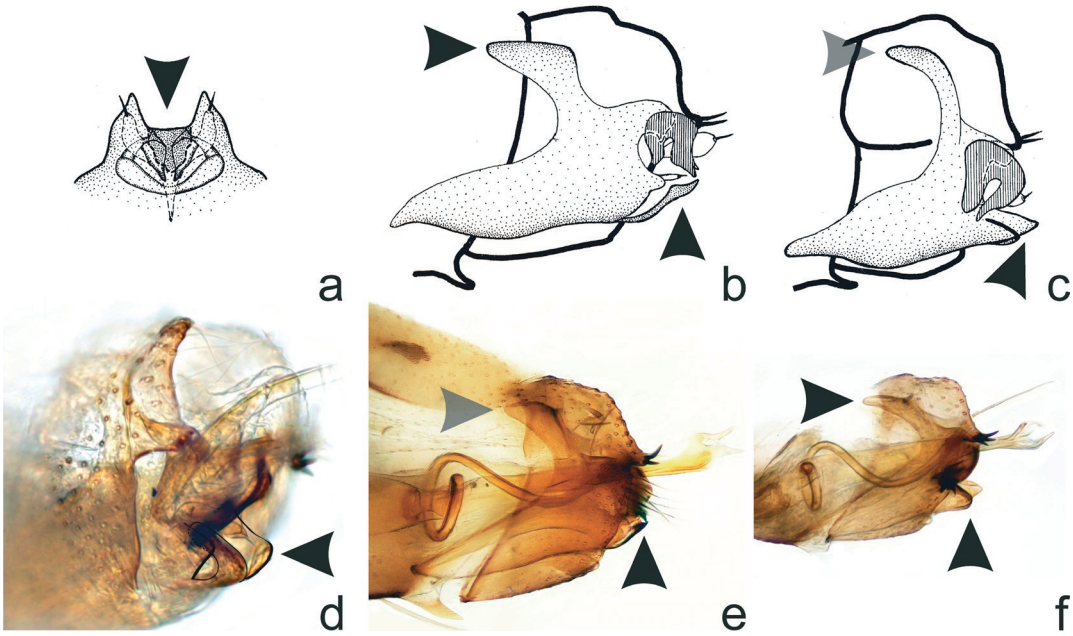

Fig. 2. Morphology of male genitalia of *Oxyethira falcata* and "*boreella*" specimens – a. *O. boreella*: inferior appendages, ventral view (after Kelley, 1985); arrow indicates the "lack" of central lobe. – b. *O. falcata*: lateral view (after Kelley, 1985); left arrow points to the wide dorsal part of 9th segment and the right arrow points to the inferior appendages of 9th segment. – c. *O. boreella*: lateral view (after Kelley, 1985); left arrow points to the narrow dorsal part of 9th segment and the right arrow points to the inferior appendages of 9th segment. – d. *O. falcata* ("*boreella*" specimen from Spain: Candelario, Salamanca): right latero-caudal view; arrow indicates the "lack" of the central lobe. – e. *O. falcata*, lateral view (specimen from Spain: Candelario, Salamanca); left arrow points to the medium size dorsal part of 9th segment and the right arrow points to the inferior appendages of 9th segment. – f. *O. falcata* ("*boreella*" specimen from Spain: Candelario, Salamanca): lateral view; left arrow points to the narrow dorsal part of 9th segment and the right arrow points to the inferior appendages of 9th segment.

"*O. boreella*" were clustered together in one clearly defined monophyletic group, where they could not be distinguished from each other. Indeed, several specimens of "*O. boreella*" had DNA haplotypes identical with those of *O. falcata*.

3.2. Morphological results

In addition to the molecular information, genitalia of 51 male samples of *Oxyethira falcata-boreella* were studied. The specimens were preliminarily identified as *O. falcata* (32 specimens) or "*O. boreella*" (19 specimens) due to the shape of the inferior appendages in the ventral and lateral view. In the ventral view, the inferior appendages of "*O. boreella*" specimens had large lateral projecting lobes, but no central process (Figs. 2a, d) (Svensson & Tjeder 1975), whereas *O. falcata* had such a central lobe (Fig. 3b). However, when studying the inferior appendages of "*O. boreella*" specimens more closely, it appears that it actually did have a central lobe.

4. Discussion

The molecular results of *O. falcata* group (including "*O. boreella*" specimens) showed moderate diversity and there was no apparent correlation between the groups of haplotypes and morphological characters. Also, the DNA barcode groups did not directly reflect a geographical pattern: e.g. the Spanish specimens are very similar to central and northern Finnish specimens.

Although according to Svensson and Tjeder (1975) it may be easy to recognize two *Oxyethira*

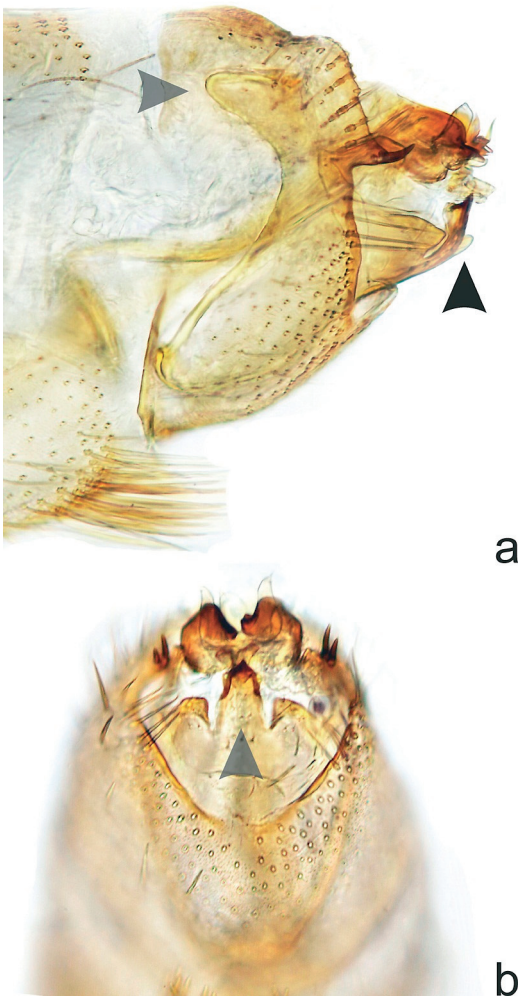

Fig. 3. *O. falcata* ("*boreella*" specimen from Finland: Toivakka, Ruostesuo): In this specimen the central lobe was turned after KOH treatment and a typical "*boreella*" was thereby transformed into a *falcata*. – a. Lateral view; left arrow points to the wide dorsal part of 9th segment and right arrow points to the inferior appendages of 9th segment. – b. Ventral view; arrow points to the visible central lobe.

species, *boreella* and *falcata*, using characters of the 9th segment and inferior appendages, the main difference between the two is in fact based on how this part of the male genitalia is positioned during preparation. Thus in specimens of "*O. boreella*" the apical process is strongly bent inwards (Figs. 2a, d), causing an illusion of genitalia different from those of *O. falcata*. The reason for the two different positions is however unknown. Our suggestion is that it may depend on whether or not a male has copulated, and that the two positions represent pre- or postmating stages. This could however not be confirmed by the present study. The recognition of two possible positions of the 9th segment in *falcata* has accordingly been observed by Schmid (1960, p. 99) studying some specimens of *O. rhodani* from Pakistan: "En étudiant mes *rhodani* pakistanais, j'ai eu l'idée d'introduire une épingle dans l'abdomen traité à la potasse et de pousser les pieces génitales vers l'extérieur. Mes *rhodani* se sont alors tous transformés en des *falcata* des plus orthodoxes" translated in English as: "While studying Pakistanese *rhodani*, I had the idea to introduce a pin in the abdomen after treating it with potassium hydroxide and to push the genitalia outwards. All *rhodani* then became most perfect *falcata*". Further, Malicky (2007) came to the same conclusion regarding *O. falcata* and *O. boreella*, but provided no extensive verbal documentation or illustrations.

According to Svensson and Tjeder (1975), the ventral part of the 9th segment (in lateral view) in *O. boreella* should be clearly shorter than in *O. falcata*. In the specimens examined by us, this character varied considerably within and between the two forms (Figs. 2b, c, e, f). Also, the dorsal part of the 9th segment (in lateral view) varied strongly between the two forms (Figs. 2b, c, e, f, and 3a). It seems evident that *O. boreella* is just a variation of *O. falcata*.

The results, both molecular and morphological, strongly support keeping *O. boreella* as a synonym for *O. falcata* with the reservation that the type specimens of both species were not studied. Altogether, the DNA barcoding results show monophyletic groups for all of the studied *Oxyethira* species, including the rare boreal species *O. ecornuta*, *O. klingstedti* and *O. tamperensis*. This result is similar to a recent study on the genus *Apatania* in Finland that found almost all species to be identifiable using DNA barcodes (Salokannel *et al.* 2010).

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