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Systematics and phylogeny of the West Palaearctic representatives of subfamily Baetinae (Insecta: Ephemeroptera): combined analysis of mitochondrial DNA sequences and morphology

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This study represents the first formal combined (morphological and molecular) phylogenetic analysis of the highly diversified subfamily Baetinae (*sensu* Kazlauskas 1972). Taxonomic sampling comprised the majority of different Palaearctic lineages within the subfamily recognised so far. The data set of 47 coded morphological characters was analysed together with the partial mitochondrial DNA cytochrome oxidase *c* subunit I gene (*COI*) sequences using parsimony and Bayesian inference. From the eight genera and species-groups investigated, three were supported as monophyletic in the analyses. The monophyly of another three genera/species-groups could not be tested because only a single species was included in the sampling. The remaining two subgenera/species-groups were recovered as paraphyletic at least using one methodological approach. A monophyletic group comprising the genera *Labiobaetis* + *Nigrobaetis* + *Alainites* was supported as a sister lineage to the genus *Baetis* s.str. Morphological characters were mapped on a cladogram, clade robustness was tested by multiple approaches and alternative views to the taxonomy of the subfamily were discussed. Intraspecific and interspecific divergences in the *COI* sequence were estimated for the species studied. The existence of a distinct 'barcoding gap' was not supported. *Baetis rhodani* and *Alainites muticus* exhibited unusually high values of intraspecific variability pointing to the possible existence of cryptic species.

Keywords: Baetidae; taxonomy; cladistics; barcoding

Introduction

Systematic concept of Baetinae and its recent development

The subfamily Baetinae, as defined by Kazlauskas (1972) exhibits an almost cosmopolitan distribution except for New Zealand (McCafferty and Waltz 1990). The subfamily probably originated as a clade in the northern hemisphere (Lugo-Ortiz and McCafferty 1998), where it often forms a dominant taxocoenosis of mayflies in running water; larvae being routinely used in aquatic biomonitoring (e.g. Fiałkowski, Kłonowska-Olejnik, Smith and Rainbow 2003).

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Kazlauskas (1972) defined Baetinae and Cloeoninae as the two subfamilies within Baetidae. The validity of these subfamilies was later supported also by Landa and Soldán (1985), Gillies (1991) and Kluge (1997). In the cladistic analyses of the order Ephemeroptera performed recently, the whole family Baetidae was recovered as one of the basal lineages within Ephemeroptera (Ogden and Whiting 2005; Ogden et al. 2009), with Baetinae supported as monophyletic (Gattolliat et al. 2008).

The mutual relationships between different lineages within Baetinae have been frequently revised but up to now there is no general agreement on the internal classification. All Western Palaearctic species included in the subfamily were originally viewed as belonging to the single genus *Baetis* Leach, 1815 s.l.

The first comprehensive revision of *Baetis* s.l. focused on the European fauna. It was published by Müller-Liebenau (1969), and the genus was divided into 11 'species-groups' (*Baetis alpinus*, *Baetis lutheri*, *Baetis pavidus*, *Baetis lapponicus*, *Baetis rhodani*, *Baetis vernus*, *Baetis fuscatus*, *Baetis buceratus*, *Baetis atrebatinus*, *Baetis niger* and *Baetis gracilis* species-group). This concept of 'species-groups' was widely accepted and formed the basis for all future classifications.

The concept of Müller-Liebenau (1969) was slightly modified by Novikova and Kluge (1987), who established six subgenera for the genus *Baetis* s.l. These subgenera in most cases corresponded with the species-groups of Müller-Liebenau (1969) and included *Nigrobaetis* (=*B. niger* and *B. gracilis* species-groups *sensu* Müller-Liebenau 1969), *Labiobaetis* (=*B. atrebatinus* species-group *sensu* Müller-Liebenau 1969) and *Acentrella* (=*B. lapponicus* species-group *sensu* Müller-Liebenau 1969). The remaining species-groups were retained as such and subsumed in the nominal subgenus *Baetis* s.str. Moreover, *Baetiella* and *Takobia* were added as new subgenera to *Baetis* s.l., both formerly considered outside the genus by Müller-Liebenau (1969).

For the Nearctic Region the first comprehensive study of *Baetis* s.l. was published by Morihara and McCafferty (1979). The authors adopted the species-groups defined by Müller-Liebenau (1969), and for related North American species where applicable. Several species were not, however, attributed to any particular species-group.

A series of papers deeply affecting the classification of mayflies attributed to *Baetis* s.l. in a global scale was published in the late 1980s and 1990s by the McCafferty school (Waltz and McCafferty 1987a,b, 1997; McCafferty and Waltz 1990; Waltz, McCafferty and Thomas 1994; Lugo-Ortiz and McCafferty 1996). Particularly important was the finding of the femoral villopore, a group of setae located at the base of the larval femora, described for the first time by Waltz and McCafferty (1987a). The presence of this character was considered to be a significant synapomorphy uniting a number of Baetidae taxa. This presumably monophyletic group was referred to as the '*Baetis* complex' (see Waltz et al. 1994; Lugo-Ortiz and McCafferty 1996; Waltz and McCafferty 1997) and included *Baetis sensu* Waltz et al. 1994 together with several related Holarctic and Oriental taxa viewed within this concept as genera (*Acentrella* Bengtsson, 1912; *Baetiella* Uéno, 1931; *Barbaetis* Waltz and McCafferty, 1985; *Cymulabaetis* McCafferty and Waltz, 1995; *Gratia* Thomas, 1992; *Heterocloeon* McDunnough, 1925; *Labiobaetis* Novikova and Kluge, 1987; *Liebebiella* Waltz and McCafferty, 1987; *Platybaetis* Müller-Liebenau, 1982).

On the other hand, the 'non-*Baetis* complex' assemblage was characterised by the absence of femoral villopore and included almost exclusively Baetidae species previously placed in the subgenus *Nigrobaetis sensu* Novikova and Kluge (1987), i.e. original *Baetis niger* and *Baetis gracilis* species-groups *sensu* Müller-Liebenau (1969).

According to the opinion of the McCafferty school, these *Nigrobaetis* species, formerly classified in *Baetis* s.l., but lacking the villopore and hence not considered as members of the *Baetis* complex, required reclassification to avoid a paraphyletic concept of the *Baetis* complex. This goal led to the erection of several new genera (*Alainites* Waltz and McCafferty, 1994; *Diphetor* Waltz and McCafferty, 1987; *Acerpenna* Waltz and McCafferty, 1987) mostly containing species of *Nigrobaetis sensu* Novikova and Kluge (1987). Other groups were raised to generic rank, such as *Takobia* Novikova and Kluge, 1987 in Waltz et al. (1994). The *Baetis* complex was subsequently found to be supported by more characters than solely the presence of the villopore, e.g. by the presence of flat-tipped sensillae mainly on antennal segments (Gaino and Rebora 1999).

Western Palaearctic representatives of both generic complexes (*Baetis* complex and non-*Baetis* complex) can be attributed to the common subfamily Baetinae *sensu* Kazlauskas (1972) according to its diagnostic characters, although the McCafferty school do not mention a division of Baetidae into subfamilies in the respective papers.

No further major revisionary effort concerning Palaearctic Baetinae has been undertaken, except for some minor changes and synonymies being published (e.g. five new subgenera of *Baetis* published by Kang et al. (1994), subsequently synonymised with already existing taxa by Waltz and McCafferty (1997). Regarding other biogeographical regions, Nieto (2010) performed an extensive cladistic analysis of the morphological characters of South American Baetidae and found most of the previously proposed higher taxa paraphyletic.

Nevertheless, the concept of the *Baetis* complex has not been generally accepted. In some studies of the European fauna authors have retained the conservative classification and have not adopted most of the recently described genera such as *Alainites* or *Diphetor* (Bauernfeind and Humpesch 2001; Jacob 2003; Haybach 2010; Bauernfeind and Soldán, in press). The scheme of the alternative classifications of internal branches within Baetinae as summarised above is presented in Figure 1.

For the purpose of the present study, all taxa studied are compiled into the subfamily Baetinae *sensu* Kazlauskas (1972). Regarding the generic placement of the individual species, the latest classification published is referred to, as used by Fujitani (2008), which is consistent with the McCafferty school. This classification is confronted with the results of the present study and alternative views are discussed herein.

Use of molecular data in the systematics of Baetidae

All systematic concepts of the subfamily Baetinae mentioned above are based solely on the investigation of morphological characteristics.

Except for morphology, the analysis of DNA sequences proved to be a useful tool for estimating relationships between insect taxa, especially when morphological characters are ambiguous. Moreover, a detailed taxonomic study of molecularly divergent lineages has already led to the description of new species (e.g. Handfield and Handfield 2006).

Several studies targeted at the mayfly family Baetidae, analysing individual species and their phylogenetic relationships by molecular methods, have already been published.

Afrotropical Baetidae were analysed by Gattolliat et al. (2008) using nuclear (18S) and mitochondrial (12S, 16S) gene regions from 65 species belonging to

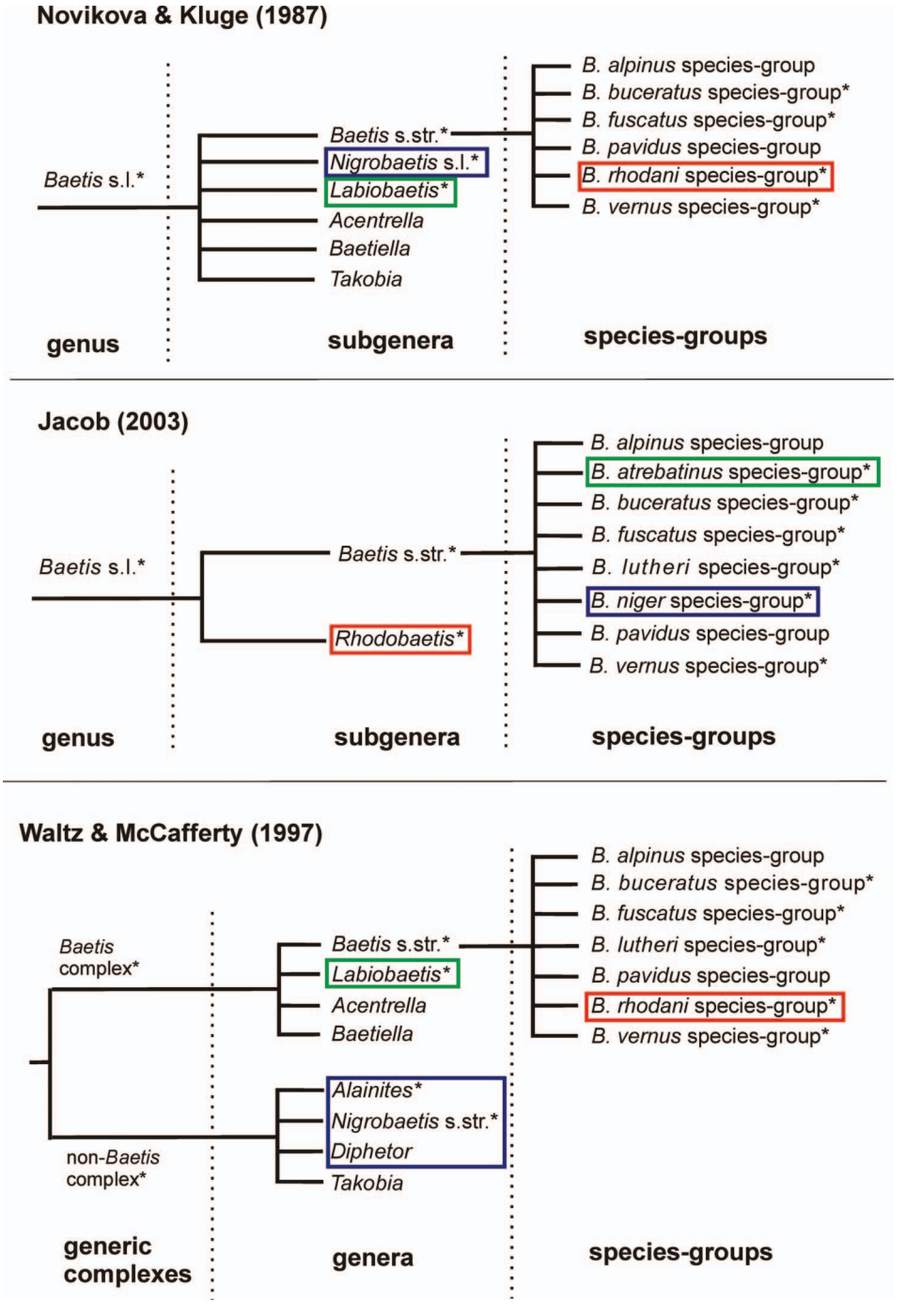


Figure 1. Scheme of alternative classifications of the internal lineages within Baetinae (only Western Palaearctic taxa are listed). Taxa treated differently by individual authors, but including the same species are marked in the frames of the same colour. Taxa analysed in the present study are marked with asterisk.

26 genera. The main goal of this study was to test the phylogenetic relevance of the recently described genera and to clarify suprageneric relationships.

Williams, Ormerod and Bruford (2006) studied the cryptic species complex *Baetis rhodani*. The study was aimed at estimating and evaluating differences between populations of *B. rhodani* collected from various localities in Western Europe. Phylogenies were constructed using data from the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene. The results provided strong evidence for cryptic species in the *B. rhodani* complex, although the taxonomic status and morphological characters of these cryptic species remained undefined.

COI sequences were also used by Ståhls and Savolainen (2007) for estimating species boundaries within the *Baetis vernus* species-group. Specimens from Finland were used in the study and the results provided evidence that *Baetis macani* Kimmins, 1957 and *Baetis vernus* Curtis, 1834 comprise morphologically cryptic but molecularly distinct taxa.

Gattolliat et al. (2008) published sequences from the mitochondrial cytochrome oxidase *b* (*cob*) gene for *Cloeon peregrinator* and *Baetis atlanticus*, based on specimens from the island of Madeira. The *cob* sequence was also published for *Baetis rhodani* from the type locality of the species (Gattolliat and Sartori 2008), as a part of the neotype designation. However, *cob* sequences of *C. peregrinator*, *B. atlanticus* and *B. rhodani* were described without subsequent analyses or comparison with related species.

The sequence from the mitochondrial *COI* gene generally proved to be very useful for separating species within mayfly genera and species-groups (Williams et al. 2006; Ståhls and Savolainen 2007). Moreover, comparison of a short section of the *COI* sequence has been proposed as a possible solution to some of the problems of traditional species identification in general (Hebert, Cywinska Ball and deWaard 2003). This approach, called 'DNA barcoding', has been pursued by the Consortium for the Bar Code of Life (CBOL) founded in September 2004. It intends to create a global biodiversity barcode database to subsequently facilitate routine automated species identifications (e.g. Ratnasingham and Hebert 2007). The ability to successfully identify species depends on the presence of a barcoding gap, a distinct difference between intraspecific and interspecific sequence divergences; in most taxa there is an order of magnitude difference between the two (Hebert et al. 2003; Ball, Hebert, Burian and Webb 2005). The applicability of the *COI* sequences ('barcodes') as a tool for mayfly species identification was tested by Ball et al. (2005), who created the reference sequence profile of 80 species. Through the use of these sequences they identified 70 additional specimens with a very high level of certainty. However, the reliability of barcoding and the existence of the barcoding gap has from the very start been the subject of fierce debate and has been challenged several times (see e.g. Wiemers and Fiedler 2007 and references therein).

In the present study the partial *COI* sequences were used together with the morphological data to estimate genetic distances and clarify phylogenetic relationships within selected taxa of the subfamily Baetinae from the Western Palaearctic region.

Main objectives of the present study

The main objectives of this study were aimed at: (1) providing *COI* sequences for a majority of Western Palaearctic genera and species-groups within Baetinae; (2) testing intraspecific and interspecific variability within Baetinae and revealing the

possibility of cryptic species; and (3) analysing the phylogenetic relationships of the genera/species-groups and individual species within Baetinae.

Materials and methods

Mayfly specimens were collected in 70–96% ethanol from the localities as specified in the Table 1. Determination was performed using keys by Bauernfeind and Humpesch (2001) and Müller-Liebenau (1969). Cuticular structures of the larvae were preserved and deposited in the Biology Centre CAS, Institute of Entomology as voucher specimens under registration numbers DNA No. 1 to DNA No. 137 (association with respective specimens in Table 1).

In all, 55 specimens belonging to 19 species were analysed. *Ameletus inopinatus* Eaton, 1887 (Ameletidae) and *Ephemerella ignita* (Poda, 1761) (Ephemerellidae) were used as outgroups. Within Baetinae, 17 species were sampled, representing nine genera and species-groups (approximately 60% of the currently recognised Palaearctic genera and species-groups within the subfamily).

The DNA quality displayed itself as a key issue in extraction and amplification procedures, because usually only fresh specimens collected into 96% ethanol allowed a successful DNA processing. Hence, the choice of specimens analysed was influenced by the availability of freshly collected material stored in pure ethanol. If possible, several specimens within one species from distant Western Palaearctic populations were included in the sampling.

Molecular data set

DNA was extracted from the whole body of mayflies using DNeasy® 96 Tissue Kit (Quiagen, Hilden, Germany) following the manufacturer's protocols.

The conserved primers C1-N-2191 (5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′) and C1-J-1718 (5′-GGA GGA TTT GGA AAT TGA TTA GTT CC-3′) from Simon et al. (1994) were used to amplify a region of *COI* mitochondrial DNA (mtDNA). Templates were amplified in 20-μl volume, 2 mM $MgCl_2$, 0.2 μM primers, 100 μM dNTPs and 0.5 U *Taq* polymerase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reation conditions were: initial activation step 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C and a final extension of 72°C for 5 min, using the Mastercycler® epgradient S (Eppendorf, Hamburg, Germany).

The polymerase chain reaction products purification and sequencing reaction was performed by Macrogen Inc. (Seoul, Korea). Sequencing was conducted under BigDye™ terminator cycling conditions. The reacted products were purified using ethanol precipitation and sequenced in forward and reverse directions using the Automatic 3730XL DNA Sequencer.

DNA sequences were aligned and edited using the program SEQSCAPE 2.5 (Applied Biosystems, Carlsbad, CA, USA), CLUSTALX (Larkin et al. 2007) and BIOEDIT (Hall 1999).

Morphological data set

For the cladistic analysis a matrix of 47 morphological characters was compiled, including 36 larval and 11 adult characters. Binary characters were coded 0 and 1,

Table 1. List of specimens studied.

Labelling used in analyses	Name of the species	Locality of collection	GenBank accession number	Specification of the voucher specimen
6_Alainites_muticus_GE	*Alainites muticus* (Linnaeus, 1758)	GEORGIA, Korkhistskali River, Akhalkalaki village, N41 27.461 E43 27.889, 28.viii.2009, Sroka leg.	JN164314	DNA No. 6
95_Alainites_muticus_BG	*Alainites muticus* (Linnaeus, 1758)	BULGARIA, Rodopy Mountains, Bujnovska River, Devin village, N41 42.29 E24 24.55, 27.v.2009, Sroka leg.	JN164319	DNA No. 95
100_Alainites_muticus_GE	*Alainites muticus* (Linnaeus, 1758)	GEORGIA, left side tributary of Machakhela River, Machakhela village, N41 30.620 E41 49.301, 20.viii.2009, Sroka leg.	JN164316	DNA No. 100
101_Alainites_muticus_GE	*Alainites muticus* (Linnaeus, 1758)	GEORGIA, Khanistskali River, Baghdati town, N42 01.498 E42 49.747, 24.viii.2009, Sroka leg.	JN164317	DNA No. 101
102_Alainites_muticus_GE	*Alainites muticus* (Linnaeus, 1758)	GEORGIA, small brook, Trebalo village, N42 34.091 E42 58.349, 26.viii.2009, Sroka leg.	JN164318	DNA No. 102
103_Alainites_muticus_GE	*Alainites muticus* (Linnaeus, 1758)	GEORGIA, Paravani River, Dzhigrasheni village, N41 20.183 E43 30.096, 27.viii.2009, Sroka leg.	JN164315	DNA No. 103
24_Baetis_baksan_RU	*Baetis baksan* Soldán, 1977	RUSSIA, SW Caucasus Mts, Belaia River, 1 km upstream Khamyshki village, N44 05.294 E40 09.312, 17.vii.2008, Sroka leg,	JN164290	DNA No. 24
25_Baetis_baksan_GE	*Baetis baksan* Soldán, 1977	GEORGIA, Bruzhi River, Ozurgeti town, N41 54.494 E42 03.874, 22.viii.2009, Sroka leg.	JN164288	DNA No. 25
134_Baetis_baksan_GE	*Baetis baksan* Soldán, 1977	GEORGIA, Gubazouri River, Nabeghlavi village, N41 56.917 E42 20.568, 23.viii.2009, Sroka leg.	JN164289	DNA No. 134
15_Baetis_braaschi_GE	*Baetis braaschi* Zimmermann, 1980	GEORGIA, Rioni River, Chala village, N42 32.948 E43 02.299, 26.viii.2009, Sroka leg.	JN164278	DNA No. 15

(*continued*)

Table 1. (*Continued*).

Labelling used in analyses	Name of the species	Locality of collection	GenBank accession number	Specification of the voucher specimen
18_Baetis_braaschi_UA	*Baetis braaschi* Zimmermann, 1980	UKRAINE, Lugansk District, Antracitovskyi region, near village Fashevka, stream–left tributary of Mius River, 24.x.2009, Martynov leg.	JN164282	DNA No. 18
19_Baetis_braaschi_UA	*Baetis braaschi* Zimmermann, 1980	UKRAINE, Donetsk city, stream–right tributary of Durnaya River, 19.x.2009, Martynov leg.	JN164280	DNA No. 19
20_Baetis_braaschi_UA	*Baetis braaschi* Zimmermann, 1980	UKRAINE, Donetsk city, stream in Putilovskyi park, 9.xi.2008, Martynov leg.	JN164283	DNA No. 20
21_Baetis_braaschi_UA	*Baetis braaschi* Zimmermann, 1980	UKRAINE, Donetsk District, Volodarskii region, near village Ukrainka, stream–left tributary of Karatisch River, 9.x.2009, Martynov leg.	JN164284	DNA No. 21
22_Baetis_braaschi_UA	*Baetis braaschi* Zimmermann, 1980	UKRAINE, Donetsk District, Yasinovatskyi region, stream near village Mineralnoe, 20.x.2009, Martynov leg.	JN164281	DNA No. 22
29_Baetis_braaschi_UA	*Baetis braaschi* Zimmermann, 1980	UKRAINE, Donetsk city, stream–right tributary of Durnaya River, 19.x.2009, Martynov leg.	JN164279	DNA No. 29
53_Baetis_buceratus_CZ	*Baetis buceratus* Eaton, 1870	CZECH REPUBLIC, Ohře River, Postoloprty village, 30.vii.2008, Soldán leg.	JN164306	DNA No. 53
62_Baetis_fuscatus_GE	*Baetis fuscatus* (Linnaeus, 1761)	GEORGIA, small brook, Lesa village, N42 04.130 E41 57.545, 22.viii.2009, Sroka leg.	JN164321	DNA No. 62
63_Baetis_fuscatus_BG	*Baetis fuscatus* (Linnaeus, 1761)	BULGARIA, Struma River, Rybnik village, N41 29.24 E23 15.43, 27.v.2009, Sroka leg.	JN164327	DNA No. 63
128_Baetis_fuscatus_GE	*Baetis fuscatus* (Linnaeus, 1761)	GEORGIA, Supsa River, Baileti village, N41 59.111 E41 58.818, 22.viii.2009, Sroka leg.	JN164322	DNA No. 128

(*continued*)

Table 1. (*Continued*).

Labelling used in analyses	Name of the species	Locality of collection	GenBank accession number	Specification of the voucher specimen
129_Baetis_fuscatus_GE	*Baetis fuscatus* (Linnaeus, 1761)	GEORGIA, Kura River, Aspindza village, N41 35.531 E43 10.528, 28.viii.2009, Sroka leg.	JN164324	DNA No. 129
130_Baetis_fuscatus_GE	*Baetis fuscatus* (Linnaeus, 1761)	GEORGIA, right side tributary of Chakvistskali River, N41 42.385 E41 47.863, 19.viii.2009, Sroka leg.	JN164323	DNA No. 130
131_Baetis_fuscatus_GE	*Baetis fuscatus* (Linnaeus, 1761)	GEORGIA, Paravani River, Dzhigrasheni village, N41 20.183 E43 30.096, 27.viii.2009, Sroka leg.	JN164326	DNA No. 131
132_Baetis_fuscatus_GE	*Baetis fuscatus* (Linnaeus, 1761)	GEORGIA, river, Citelchevi village, N42 06.180 E42 43.691, 23.viii.2009, Sroka leg.	JN164325	DNA No. 132
104_Baetis_cf_gadeai_GE	*Baetis* cf. *gadeai* Thomas, 1999	GEORGIA, Rikotula River, Tsakva village, N42 05.484 E43 27.335, 24.viii.2009, Sroka leg.	JN164291	DNA No. 104
105_Baetis_cf_gadeai_GE	*Baetis* cf. *gadeai* Thomas, 1999	GEORGIA, Korkhistskali River, Akhalkalaki village, N41 27.461 E43 27.889, 28.viii.2009, Sroka leg.	JN164293	DNA No. 105
106_Baetis_cf_gadeai_GE	*Baetis* cf. *gadeai* Thomas, 1999	GEORGIA, Kemerkobis River, Khala village, N41 42.145 E41 48.855, 18.viii.2009, Sroka leg.	JN164292	DNA No. 106
135_Baetis_ilex_GE	*Baetis ilex* Jacob & Zimmermann, 1978	GEORGIA, Khandostskali River, Akhalkalaki village, N41 28.887 E43 22.961, 28.viii.2009, Sroka leg.	JN164294	DNA No. 135
67_Baetis_lutheri_RU	*Baetis lutheri* Müller-Liebenau, 1967	RUSSIA, SW Caucasus Mts, Belaia River, 1 km upstream Khamyshki village, N44 05.294 E40 09.312, 17.vii.2008, Sroka leg.	JN164287	DNA No. 67
113_Baetis_lutheri_GE	*Baetis lutheri* Müller-Liebenau, 1967	GEORGIA, Rikotula River, Tsakva village, N42 05.484 E43 27.335, 24.viii.2009, Sroka leg.	JN164286	DNA No. 113

(*continued*)

Table 1. (*Continued*).

Labelling used in analyses	Name of the species	Locality of collection	GenBank accession number	Specification of the voucher specimen
114_Baetis_lutheri_GE	*Baetis lutheri* Müller-Liebenau, 1967	GEORGIA, Gubazouri River, Nabeghlavi village, N41 56.917 E42 20.568, 23.viii.2009, Sroka leg.	JN164285	DNA No. 114
77_Baetis_macani_FI	*Baetis macani* Kimmins, 1957	FINLAND, brook near Nilsiá town, N63 11.58 E28 18.32, 2.vii.2007, Savolainen leg.	JN164275	DNA No. 77
30_Baetis_rhodani_CZ	*Baetis rhodani* (Pictet, 1843)	CZECH REPUBLIC, Telnický brook, Adolfov village, 29.v. 2008, Soldán leg.	JN164305	DNA No. 30
31_Baetis_rhodani_BG	*Baetis rhodani* (Pictet, 1843)	BULGARIA, Rodopy Mountains, small brook, Sokolovci village, N41 38.20 E24 45.48, 26.v.2009, Sroka leg.	JN164303	DNA No. 31
108_Baetis_rhodani_GE	*Baetis rhodani* (Pictet, 1843)	GEORGIA, river, Mikadze village, N41 37.411 E43 02.701, 28.viii.2009, Sroka leg.	JN164301	DNA No. 108
110_Baetis_rhodani_GE	*Baetis rhodani* (Pictet, 1843)	GEORGIA, Kintrishi River, Kobuleti village, N41 48.156 E41 53.969, 18.viii.2009, Sroka leg.	JN164304	DNA No. 110
111_Baetis_rhodani_GE	*Baetis rhodani* (Pictet, 1843)	GEORGIA, Paravani River, Dzhigrasheni village, N41 20.183 E43 30.096, 27.viii.2009, Sroka leg.	JN164302	DNA No. 111
72_Baetis_vardarensis_GE	*Baetis vardarensis* Ikomonov, 1962	GEORGIA, Kura River, Aspindza village, N41 35.531 E43 10.528, 28.viii.2009, Sroka leg.	JN164299	DNA No. 72
120_Baetis_vardarensis_GE	*Baetis vardarensis* Ikomonov, 1962	GEORGIA, Supsa River, Baileti village, N41 59.111 E41 58.818, 22.viii.2009, Sroka leg.	JN164296	DNA No. 120
121_Baetis_vardarensis_GE	*Baetis vardarensis* Ikomonov, 1962	GEORGIA, Kvirila River, Shorapani village, N42 05.573 E43 07.808, 24.viii.2009, Sroka leg.	JN164298	DNA No. 121

(*continued*)

Table 1. (*Continued*).

Labelling used in analyses	Name of the species	Locality of collection	GenBank accession number	Specification of the voucher specimen
122_Baetis_vardarensis_GE	*Baetis vardarensis* Ikomonov, 1962	GEORGIA, small brook, Lesa village, N42 04.130 E41 57.545, 22.viii.2009, Sroka leg.	JN164297	DNA No. 122
124_Baetis_vardarensis_GE	*Baetis vardarensis* Ikomonov, 1962	GEORGIA, river, Mikadze village, N41 37.411 E43 02.701, 28.viii.2009, Sroka leg.	JN164300	DNA No. 124
127_Baetis_vardarensis_GE	*Baetis vardarensis* Ikomonov, 1962	GEORGIA, Paravani River, Dzhigrasheni village, N41 20.183 E43 30.096, 27.viii.2009, Sroka leg.	JN164295	DNA No. 127
79_Baetis_vernus_CZ	*Baetis vernus* Curtis, 1834	CZECH REPUBLIC, Žehrovka River, Březina village, 31.vii.2008, Soldán leg.	JN164276	DNA No. 79
80_Baetis_vernus_BG	*Baetis vernus* Curtis, 1834	BULGARIA, Strandzha National Reserve, Cerovska River, Krushevets village, N42 16.16 E27 29.56, 22.v.2009, Sroka leg.	JN164277	DNA No. 80
46_Labiobaetis_atrebatinus_BG	*Labiobaetis atrebatinus* (Tshernova, 1928)	BULGARIA, Strandzha National Reserve, Veleka River, Kosti village, N42 03.34 E27 46.34, 23.v.2009, Sroka leg.	JN164312	DNA No. 46
47_Labiobaetis_tricolor_HU	*Labiobaetis tricolor* (Tshernova, 1928)	HUNGARY, channel near Sonkád village, N48 03.41 E22 45.35, 1.vi.2009, Sroka leg.	JN164313	DNA No. 47
1_Nigrobaetis_digitatus_BG	*Nigrobaetis digitatus* (Bengtsson, 1912)	BULGARIA, Strandzha National Reserve, Fakijska River, Zidarovo village, N42 20.03 E27 24.15, 21.v.2009, Sroka leg.	JN164307	DNA No. 1
2_Nigrobaetis_digitatus_GE	*Nigrobaetis digitatus* (Bengtsson, 1912)	GEORGIA, small brook, Ozurgeti town, N41 56.906 E41 59.445, 22.viii.2009, Sroka leg.	JN164308	DNA No. 2
137_Nigrobaetis_digitatus_GE	*Nigrobaetis digitatus* (Bengtsson, 1912)	GEORGIA, small brook, Lesa village, N42 04.130 E41 57.545, 22.viii.2009, Sroka leg.	JN164309	DNA No. 137
13_Nigrobaetis_gracilis_GE	*Nigrobaetis gracilis* (Bogoescu & Tabacaru, 1957)	GEORGIA, Supsa River, Baileti village, N41 59.111 E41 58.818, 22.viii.2009, Sroka leg.	JN164320	DNA No. 13

(*continued*)

Table 1. (*Continued*).

Labelling used in analyses	Name of the species	Locality of collection	GenBank accession number	Specification of the voucher specimen
9_Nigrobaetis_niger_CZ	*Nigrobaetis niger* (Linnaeus, 1761)	CZECH REPUBLIC, Černá voda River, Kovářská village, 30.vii.2008, Soldán leg.	JN164310	DNA No. 9
10_Nigrobaetis_niger_RO	*Nigrobaetis niger* (Linnaeus, 1761)	ROMANIA, Timiş River, Brebu Nou village, 17.v.2009, Sroka leg.	JN164311	DNA No. 10
91_Ameletus_inopinatus_CZ	*Ameletus inopinatus* Eaton, 1887	CZECH REPUBLIC, small brook, Říčky village, 9.v.2007, Soldán leg.	JN164274	DNA No. 91
92_Ephemerella_ignita_CZ	*Ephemerella ignita* (Poda, 1761)	CZECH REPUBLIC, Ohře River, Postoloprty village, 30.vii.2008, Soldán leg.	JN164273	DNA No. 92

Table 2. Morphological characters states.

	1 5 10 15 20 25 30 35 40 45
Alainites muticus	0010 0 1010 1 1112 0 0020 1 0120 0 0000 0 1001 0 0001 0 1100 2 00
Baetis baksan	1100 0 0001 0 0111 1 0010 0 0000 1 1121 0 0010 0 0000 1 1100 2 00
Baetis braaschi	1100 0 0001 0 0111 1 0000 0 1000 1 1100 0 0010 0 1001 1 1100 2 00
Baetis buceratus	1000 0 0001 0 0111 0 0020 0 1010 1 0010 0 0000 0 0001 0 0111 2 00
Baetis fuscatus	1000 0 0001 0 0112 1 0020 0 1000 1 0000 0 0000 0 1001 1 0110 1 00
Baetis cf. gadeai	1100 0 0001 0 0111 1 0000 0 0000 1 1120 0 0011 0 0??? ? ???? ? ??
Baetis ilex	1100 0 1001 0 0211 0 0000 0 0000 1 1101 0 0011 0 0??? ? ???? ? ??
Baetis lutheri	1000 0 0001 0 0112 0 0001 0 2011 1 0010 0 0110 2 0001 1 1110 1 00
Baetis macani	1000 0 0001 0 0111 1 0010 0 0100 1 0000 0 0000 0 0001 1 0100 1 00
Baetis rhodani	1100 0 0001 0 0111 1 0000 0 P000 1 1101 0 0011 0 1001 1 1100 2 00
Baetis vardarensis	1000 0 0001 0 0112 0 0001 0 2011 1 0010 0 0100 0 0001 1 1110 1 00
Baetis vernus	1000 0 0001 0 0111 1 0020 0 1000 1 0000 0 0001 0 0001 1 1110 1 00
Nigrobaetis digitatus	0010 0 1000 0 1112 2 0020 1 0120 0 0000 1 0001 0 1000 0 0110 0 00
Nigrobaetis gracilis	0010 0 1010 0 1002 2 0020 1 1120 0 1000 0 0001 0 1001 0 0000 0 00
Nigrobaetis niger	0010 0 1000 0 1102 2 0020 1 0120 0 0000 0 0001 0 1000 0 0110 0 00
Labiobaetis atrebatinus	1011 0 0101 0 1120 2 0120 0 0120 0 0000 0 0001 0 0010 1 1000 2 01
Labiobaetis tricolor	1011 0 0001 0 1120 2 0120 0 0120 0 0000 0 0001 0 1010 1 0000 2 01
Ephemerella ignita	0000 1 0-1- 0 1- -- 0 1020 1 1100 1 0000 0 0-00 0 010- - 1110 1 10
Ameletus inopinatus	0000 1 1-1- 1 1- - - 0 1010 1 1100 1 0000 0 0-00 0 110- - 1100 1 10

Non-applicable characters in the outgroup taxa refers to the morphological structures present in the subfamily Baetinae only. Missing data in the adult characteristics refers to the species with imaginal stage unknown at present, P means polymorphism.

multistate characters were assigned different numbers. Characters no. 12, 13, 14, 35 and 45 were treated as ordered, other characters were viewed as unordered. Missing data and non-applicable characters were scored as ? and –, respectively. Character states were checked for each specimen separately when compiling the appropriate matrix for analyses. Nevertheless, character states did not differ within individual species (with an exception of a single character in a single species). Hence, in the publication only the species-matrix is presented (Table 2).

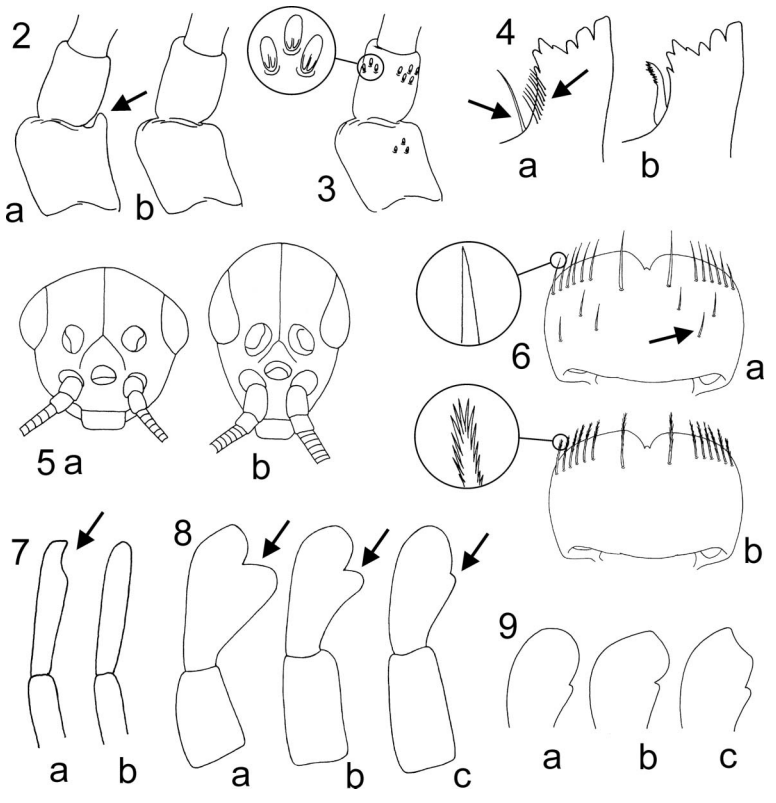
Larval characters

1. Antennal flat-tipped sensillae: (0) absent; (1) present.
2. Setae on scape and pedicel (Figure 3): (0) absent; (1) present.
3. Distance between antennal bases (Figure 5): (0) larger than scape base width; (1) as wide as scape base width or narrower.
4. Lateroapical protuberance on scape (Figure 2): (0) absent; (1) present.
5. Coronal suture: (0) proximal to lateral ocelli; (1) distal to lateral ocelli.
6. Labrum: (0) wider than long; (1) as wide as long.
7. Labral bristles of dorsal submarginal row (Figure 6): (0) pointed; (1) serrated.
8. Scattered labral bristles proximal to dorsal row (Figure 6): (0) absent; (1) present.
9. Number of labral bristles of the dorsal submarginal row on each side: (0) less than 1 + 4; (1) more than 1 + 4.

10. Right prostheca (Figure 4): (0) well developed; (1) bristle-like.
11. Row of hairs between right prostheca and incisors (Figure 4): (0) absent; (1) present.
12. Number of rows of setae on aboral side of paraglossa: (0) two; (1) three; (2) four.
13. Paraglossa: (0) as broad as glossa; (1) paraglossa at most 1.8 times broader; (2) paraglossa at least 2.5 times broader.
14. Medioapical protuberance on segment 2 of labial palp (Figure 8): (0) more pronounced than two-thirds of segment 3 width; (1) as wide as one-third to two-thirds; (2) less.
15. Segment 3 of labial palp (Figure 9): (0) symmetrically rounded; (1) asymmetrically rounded; (2) truncate (concave medially).
16. Maxillar palp: (0) two-segmented; (1) three-segmented.
17. Incurvation on distal segment of maxillar palp (Figure 7): (0) absent; (1) present.
18. Number of apical scales on maxillar palp: (0) one; (1) several; (2) absent.
19. Protuberances on thoracic sterna near coxae (Figure 10): (0) absent; (1) present.
20. Femoral villopore: (0) present; (1) absent.
21. Femoral setae apically: (0) pointed; (1) rounded; (2) serrated.
22. Femoral setae: (0) long; (1) short, spine-like.
23. Minor femoral setae (Figure 12): (0) simple; (1) serrated; (2) absent.
24. Subapical bristles of claw: (0) absent; (1) present.
25. Abdomen in cross-section: (0) compressed laterally; (1) circular.
26. Chagrined surface: (0) present; (1) absent.
27. Row of setae on posterior margin of terga (Figure 11): (0) absent; (1) present.
28. Projections on posterior margin of terga (Figure 11): (0) pointed; (1) rounded; (2) absent.
29. Thick, spine-like setae on gill margins: (0) absent; (1) present.
30. Inner margin of gill VII: (0) convex; (1) concave.
31. Paraproct with medioapical projection (Figure 13): (0) absent; (1) present.
32. Marginal teeth on paraproct: (0) pointed; (1) rounded.
33. Setae on paraproct (Figure 14): (0) absent; (1) present.
34. Scales on paraproct (Figure 14): (0) absent; (1) present.
35. Length of paracercus: (0) more than half of cerci; (1) less than half; (2) paracercus rudimental.
36. Dark band on caudal filaments: (0) absent; (1) present.

Adult characters

37. Free marginal intercalaries in the forewing: (0) present; (1) absent.
38. Processus costalis on hind wing: (0) present; (1) absent.
39. Number of longitudinal veins on hind wing: (0) two; (1) three.
40. Second longitudinal vein on hind wing: (0) bifurcate; (1) simple.
41. Medioapical protuberance on basal forceps segment (Figure 16): (0) present; (1) absent.
42. Medioapical protuberance on first forceps segment (Figure 16): (0) present; (1) absent.



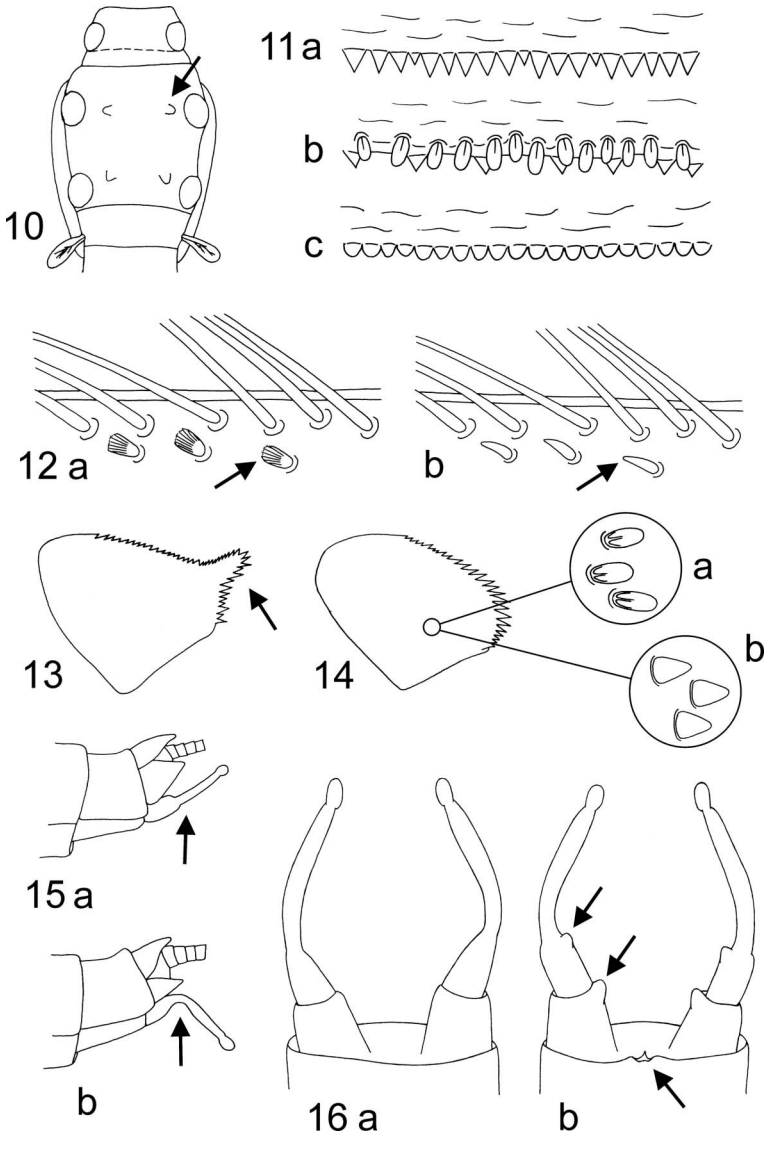
Figures 2–9. Selected morphological characters used in the combined matrix: (2) lateroapical protuberance on scape; (3) setae on scape and pedicel; (4) right prostheca and row of hairs between right prostheca and incisors; (5) distance between antennal bases; (6) labral bristles of dorsal submarginal row and scattered labral bristles proximal to dorsal row; (7) incurvation on distal segment of maxillar palp; (8) extent of medioapical protuberance on segment 2 of labial palp; (9) shape of segment 3 of labial palp. (Figures 2, 3, 4, 7, 8, 9 modified from Müller-Liebenau 1969, Figure 5 modified from Novikova and Kluge 1987.)

43. First forceps segment (Figure 16): (0) cylindrical (with parallel margins); (1) tapering.
44. Forceps (lateral view) (Figure 15): (0) straight; (1) bent ventrally.
45. Apical forceps segment: (0) strongly elongated, length/width ratio at least 2.5.; (1) slightly elongated, length/width ratio 1.1–2.5.; (2) rounded, length/width ratio approximately 1.
46. Penis: (0) not visible externally; (1) visible.
47. Penis cover (Figure 16): (0) absent; (1) well-developed.

Data analysis

Intraspecific variability

For estimating genetic variability within individual species, Kimura two-parameter (K2P, Kimura 1980) distances were calculated using MEGA4 (Tamura et al. 2007) under default parameters. The K2P model is appropriate when genetic distances are



Figures 10–16. Selected morphological characters used in the combined matrix: (10) protuberances on thoracic sterna near coxae; (11) projections and setae on posterior margin of terga; (12) minor femoral setae; (13) paraproct with medioapical projection; (14) structures on the paraproct surface, (a) setae, (b) scales; (15) forceps (lateral view), (a) straight, (b) bent ventrally; (16) medioapical protuberances on basal and first forceps segment (a) absent, (b) present, First forceps segment (a) tapering, (b) cylindrical (with parallel margins), Penis cover (a) absent, (b) well-developed. (Figures 10, 13, 14, 15, 16 modified from Bauernfeind and Humpesch 2001, Figure 12 modified from Müller-Liebenau 1969.)

low (Nei and Kumar 2000) and has previously been used in studies of mtDNA barcoding of mayflies (e.g. Hebert et al. 2003; Ball et al. 2005; Webb, Sun, McCafferty and Ferris 2007). This simple neighbour-joining algorithm was used to compare results of the present study with already published papers dealing with ranges of intraspecific and interspecific distances in mayflies and to test its reliability in distinguishing species.

Phylogeny

Phylogenetic relationships were estimated by several methodological approaches. Phylograms were visualised using TREEVIEW v1.2.2 (Page 1996).

A maximum parsimony (MP) analysis was conducted using methods implemented in PAUP* (Swofford 2002). Tree searches were performed using the heuristic search option with tree bisection and reconnection branch swapping. Branches collapsed (creating polytomies) if the maximum branch length was zero. Gaps were treated as missing data. A strict consensus tree was constructed based on the most-parsimonious trees. Bootstrap values were calculated with 1000 replicates. The effect of the individual data partitions was estimated using partitioned Bremer support indices (PBS) using TREEROT v.3 (Sorenson and Franzosa 2007).

Baysian inference was performed in MRBAYES (v3.1.2) (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The best-fit model (TrN+I+G) with parameters Nst = 6, and Rates = gamma was selected by Akaike's information criterion using MODELTEST 3.7 (Posada and Crandall 1998). In total, 3,000,000 generations, running on four chains, were sampled every 100 generations. The first 750,000 generations were excluded (as the burn in).

Results

Intraspecific and interspecific sequence divergence

Intraspecific divergences were computed for 11 Baetinae species, from which more than one specimen was included in the analysis. Divergence values varied considerably (0.000–0.239) according to the particular species; see Table 3 for detailed information.

The pattern of K2P distances mostly followed geographical distance between populations, with specimens from the distant regions exhibiting higher degree of divergences (e.g. *B. fuscatus*). On the other hand, some species were found to be very homogeneous even when comparisons of distant populations were made (e.g. *B. braaschi*). For the detailed analysis of the relationship between geographical and

Table 3. Mean values and ranges of intraspecific sequence divergences (Kimura two-parameter distance) of *COI* mitochondrial DNA partial sequences of 17 species of Western Palaearctic Baetinae for which more than one sequence was analysed.

Species	No. of specimens	Mean K2P distance	Range of K2P distance
Alainites muticus	6	0.085	0.000–0.239
Baetis baksan	3	0.003	0.000–0.005
Baetis braaschi	7	0.006	0.000–0.009
Baetis fuscatus	6	0.040	0.000–0.087
Baetis cf. *gadeai*	3	0.009	0.000–0.014
Baetis lutheri	3	0.015	0.011–0.018
Baetis rhodani	5	0.122	0.002–0.168
Baetis vardarensis	6	0.013	0.000–0.023
Baetis vernus	2	0.077	–
Nigrobaetis digitatus	3	0.085	0.007–0.124
Nigrobaetis niger	2	0.005	–

COI, cytochrome oxidase *c* subunit I gene; K2P, Kimura two-parameter.

genetic distances in individual species, the sample size was too small. The cases of most pronounced differences probably reflect a situation where morphologically undescribed, but molecularly distinct cryptic species occurr (*A. muticus*, *B. rhodani*, *N. digitatus*; see part of the Discussion dealing with individual taxa below). When these problematic taxa are excluded from the analysis intraspecific divergences ranged between 0.000 and 0.087 (mean 0.021).

Mean interspecific divergences computed for 17 species of Baetinae ranged from 0.120 to 0.318 (see Table 4). The lowest levels of interspecific divergence occurred between several species within genus *Labiobaetis* and the *B. rhodani* species-group. The mean of all interspecific divergences was computed as 0.256.

A histogram of all pairwise K2P distances is depicted in Figure 17.

Analysis of phylogenetic relationships

We obtained a molecular data set of 490 nucleotides for 55 specimens from 19 species (17 ingroup and two outgroup species), from which 199 were parsimony-informative. This molecular data set was augmented by the morphological data set of 47 characters.

Individual species were well defined; and in every species for which more than one specimen was available the species was monophyletic with high support in every tree. From the eight genera and species-groups investigated, three were supported as monophyletic under both MP and Bayesian criteria and their monophyly was relatively well supported (*Labiobaetis*, *B. lutheri* species-group and *B. rhodani* species-group). The monophyly of another three genera/species-groups could not be tested because only a single species was included in the sampling (*Alainites, B. fuscatus* species-group, *B. buceratus* species-group). The remaining two genera/

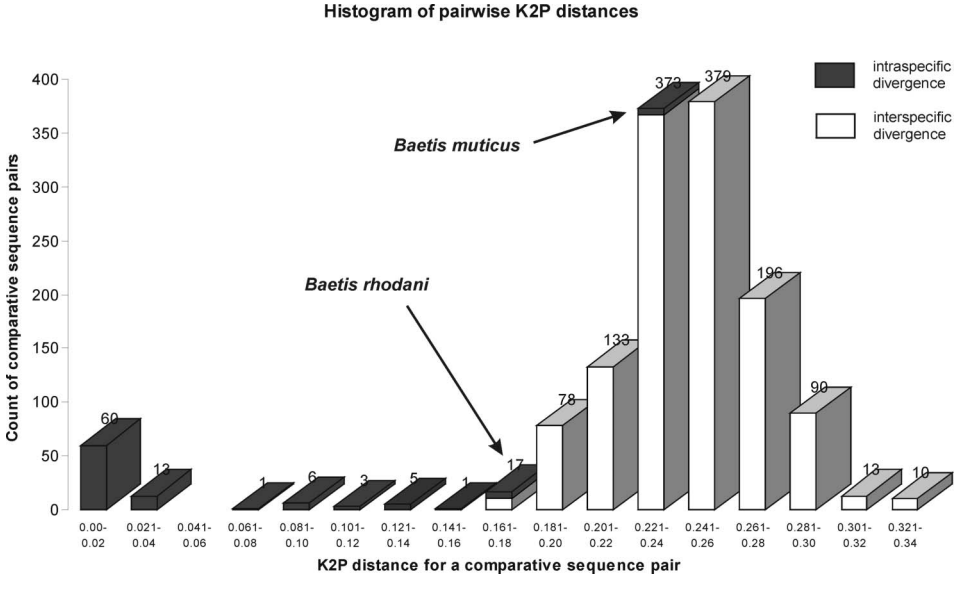


Figure 17. Histogram showing the number of intraspecific and interspecific pairwise Kimura two-parameter distances. Species with particularly high values of intraspecific Kimura two-parameter distances marked with arrows.

Table 4. Interspecific divergences (Kimura two-parameter distance) among *COI* mitochondrial DNA partial sequences of 17 species of Western Palaearctic Baetinae. If a comparative pair included at least one species for which multiple sequences were obtained, the mean value of distances between two sequences from the two species in a combination was used.

	Alainites muticus	*Baetis baksan*	*Baetis braaschi*	*Baetis buceratus*	*Baetis fuscatus*	*Baetis* cf. *gadeai*	*Baetis ilex*	*Baetis lutheri*	*Baetis macani*	*Baetis rhodani*	*Baetis vardarensis*	*Baetis vernus*	*Labiobaetis atrebatinus*	*Labiobaetis tricolor*	*Nigrobaetis digitatus*	*Nigrobaetis gracilis*
Baetis baksan	0.248															
Baetis braaschi	0.281	0.212														
Baetis buceratus	0.289	0.239	0.236													
Baetis fuscatus	0.271	0.232	0.250	0.242												
Baetis cf. *gadeai*	0.279	0.172	0.200	0.222	0.247											
Baetis ilex	0.271	0.212	0.223	0.262	0.267	0.199										
Baetis lutheri	0.318	0.236	0.236	0.259	0.272	0.265	0.237									
Baetis macani	0.289	0.242	0.212	0.237	0.240	0.216	0.259	0.255								
Baetis rhodani	0.258	0.191	0.240	0.238	0.256	0.211	0.219	0.232	0.230							
Baetis vardarensis	0.247	0.201	0.227	0.269	0.269	0.225	0.230	0.263	0.259	0.240						
Baetis vernus	0.262	0.240	0.186	0.241	0.228	0.204	0.271	0.267	0.191	0.251	0.246					
Labiobaetis atrebatinus	0.221	0.201	0.219	0.248	0.233	0.191	0.259	0.241	0.226	0.200	0.246	0.196				
Labiobaetis tricolor	0.228	0.220	0.233	0.253	0.233	0.204	0.244	0.247	0.230	0.202	0.250	0.200	0.164			
Nigrobaetis digitatus	0.246	0.220	0.239	0.279	0.243	0.223	0.255	0.243	0.215	0.238	0.235	0.229	0.199	0.183		
Nigrobaetis gracilis	0.288	0.259	0.251	0.269	0.270	0.267	0.256	0.274	0.246	0.248	0.302	0.267	0.239	0.246	0.239	
Nigrobaetis niger	0.248	0.255	0.222	0.272	0.235	0.229	0.242	0.257	0.201	0.231	0.250	0.218	0.196	0.217	0.197	0.229

COI, cytochrome oxidase *c* subunit I gene.

species-groups were recovered as paraphyletic under at least one methodological approach (*Nigrobaetisi*, *B. vernus* species-group). However, branches containing these paraphyletic taxa gained very low support values and should be considered uncertain.

Parsimony analysis (Figures 18, 19)

Phylogenetic analyses recovered the 12 most-parsimonious topologies and a strict consensus tree of 1551 steps was constructed. The consistency and retention indices were 0.2979 and 0.7329, respectively.

The subfamily Baetinae was recovered as monophyletic, supported by four unique morphological apomorphies. Two large clades were recovered in the analysis. The first consisted of the genera *Labiobaetis* + *Alainites* + *Nigrobaetis*, the second of the genus *Baetis* s.str. Within the first clade, *Labiobaetis* was recovered as monophyletic and *Nigrobaetis* as paraphyletic (with *N. gracilis* nested close to *Alainites*); the monophyly of *Alainites* could not be tested, because only a single species was present in the sample. The clade consisting of *Alainites* + *Nigrobaetis* formed a sister lineage to *Labiobaetis*. Morphological characters uniting *Labiobaetis* + *Nigrobaetis* + *Alainites* lineage are mostly connected with the general body shape (distance between antennal bases and lateral compression of abdomen) and single common character for *Baetis* s.str. is absence of hairs between prostheca and incisors.

In the clade representing *Baetis* s.str., two major sister lineages were recognised (*B. vernus* + *B. fuscatus* + *B. buceratus* species-groups and *B. rhodani* + *B. lutheri* species-groups). From these five species-groups within *Baetis* s.str., two were recovered as monophyletic (*B. rhodani* and *B. lutheri*), one as paraphyletic (*B. vernus*) and two were represented by a single species, hence their monophyly could not be tested (*B. buceratus* and *B. fuscatus*). Within *B. rhodani* species-group, *B. baksan* was recovered as a sister lineage to *B. rhodani*. These two species formed a sister clade to the remaining representatives of the species-group.

The bootstrap support values of the resulting cladogram were relatively low in most basal nodes, sometimes not even reaching 50, therefore the branching scheme should be viewed with caution (see Figure 18). The highest bootstrap support values were obtained for the monophyly of all individual species, where more than a single specimen was analysed. The monophyly of the individual genera/species-groups gained much less support (only genus *Labiobaetis* and *B. lutheri* species-group exhibited higher bootstrap values than 50). The two major clades (*Labiobaetis* + *Alainites* + *Nigrobaetis* and *Baetis* s.str.) were supported by bootstrap support values only slightly overreaching 50. The bootstrap support values for the basal nodes within *Nigrobaetis* + *Alainites* lineage are also relatively low.

The PBS analysis revealed several inconsistencies between the phylogenetic signal provided by the molecular and morphological data in some deeper nodes (Figure 19, for details see Discussion). In the majority of subterminal branches, the data partitions were in accordance with, or at least did not contradict, each other.

Unique morphological apomorphies with consistency index 1.0 were mapped on the cladogram (Figure 18). In the set of 47 characters used in the analysis, 24 were recovered as unique apomorphies (~50%). Among eight genera/species-groups investigated, four were supported by one or more unique morphological apomorphies (Figure 18). However, several of these characters are shared by other taxa not included in the analysis (for details see Discussion).

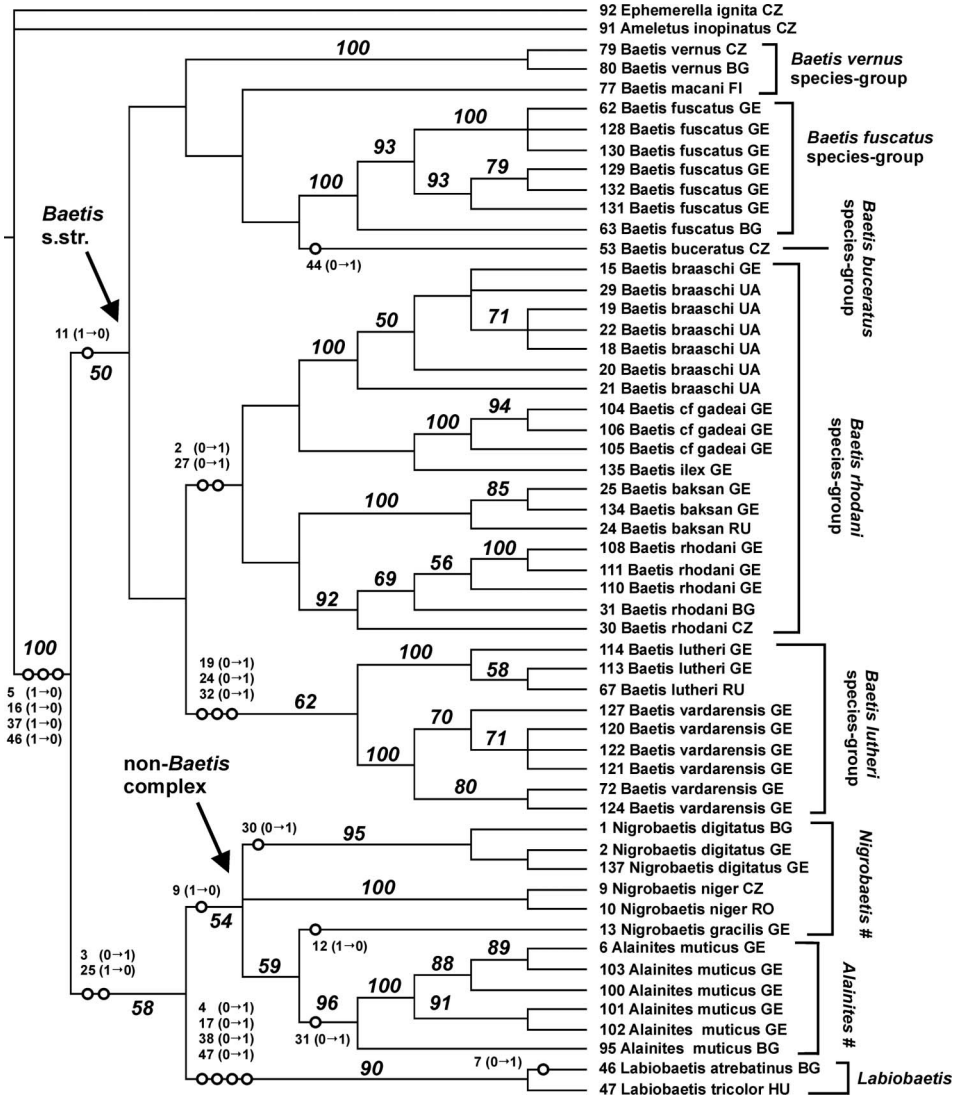


Figure 18. Strict consensus tree of the 12 most-parsimonious trees. The bold numbers near branches are bootstrap support values. Unique morphological apomorphies with consistency index = 1.0 are mapped on the topology. Non-*Baetis* complex genera are marked with "#".

Bayesian analysis (Figure 20)

At the end of 3,000,000 generations, the average standard deviation of split frequencies was ~0.008. Two large clades were recovered, one of which consisted of the genera *Labiobaetis* + *Alainites* + *Nigrobaetis* and another comprising the rest of the subfamily, grouped in the genus *Baetis* s.str. However, the values of the Bayesian posterior probability were low for these basal clades, being 0.52 and 0.68, respectively. The *Labiobaetis* + *Alainites* + *Nigrobaetis* lineage is further divided into two branches. One represents the monophyletic genus *Labiobaetis* (posterior probability 1.00), the other genera *Alainites* + *Nigrobaetis* (posterior probability

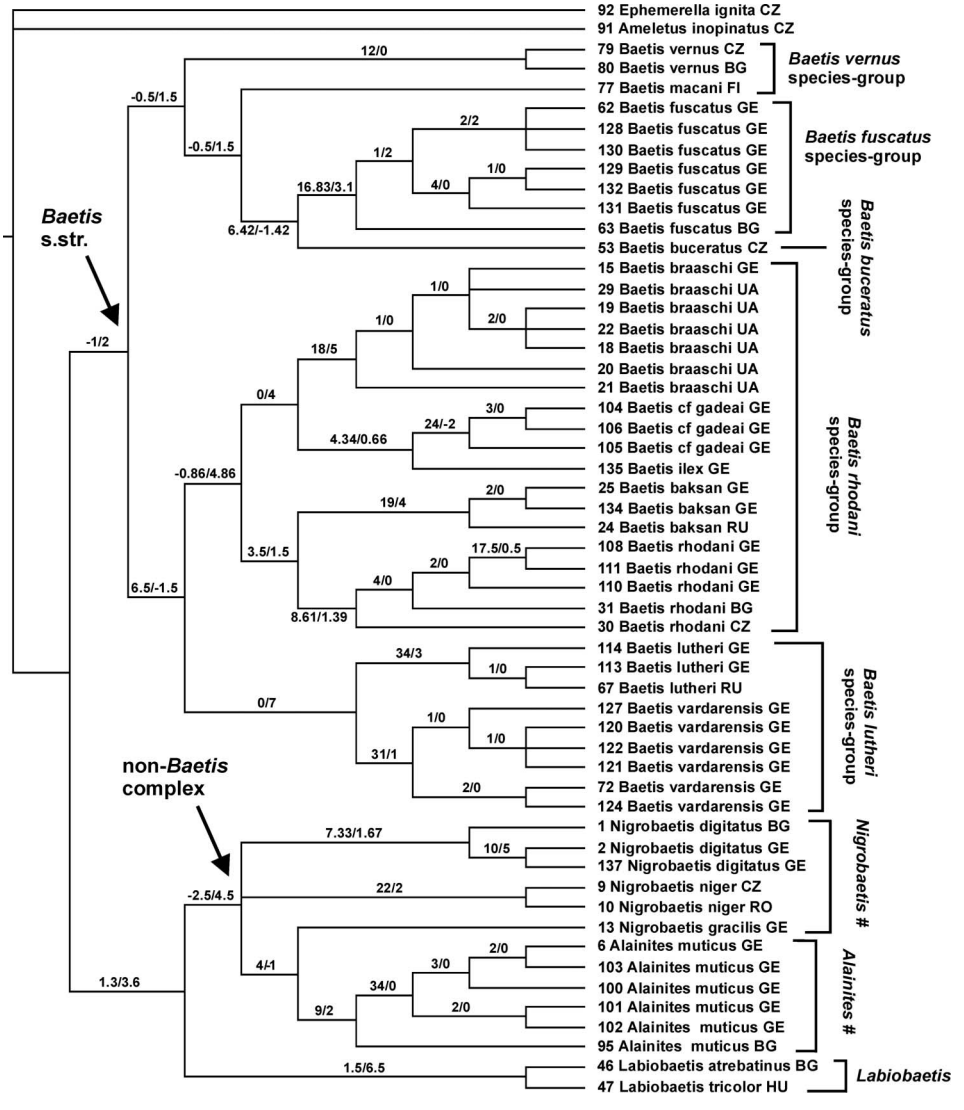


Figure 19. Strict consensus tree of the 12 most-parsimonious trees. The numbers near branches are partitioned Bremer support values (first value from the molecular data set, second value from the morphological data set). Non-*Baetis* complex genera are marked with "#".

0.75). The monophyly of *Alainites* could not be tested, because only a single species was present in the sample. The genus *Nigrobaetis* was recovered as paraphyletic, with *N. gracilis* nested close to *Alainites*; *N. gracilis* + *A. muticus* formed a common clade, supported by posterior probability of 0.98.

From the *Baetis* s.str. lineage, two of the established species-groups were recovered as monophyletic with high values of posterior probability. This applies to the *B. lutheri* species-group and *B. rhodani* species-group. The monophyly of the *B. buceratus* species-group and the *B. fuscatus* species-group could not be tested and the *B. vernus* species-group was recovered as paraphyletic.

The branching scheme within the clade comprising *Baetis* s.str. exhibited low values of posterior probability and should be viewed with caution. The close relationship of the *B. rhodani* species-group and the *B. lutheri* species-group is indicated, with a poorly supported position of *B. buceratus* as a sister lineage to the *B. lutheri* species-group.

Discussion

Intraspecific and interspecific divergences among Baetinae

The mean of interspecific divergences was counted as 0.256. In the study of Ball et al. (2005), the mean of congeneric interspecific divergences was 0.181 (in the sample of all 11 mayfly families studied). Webb et al. (2007) reported a mean value of congeneric interspecific divergences for North American *Heptagenia* of 0.1599 when excluding dubious species. So, the diversification of individual lineages within Baetinae can be considered as rather high and the recognition of several genera and species-groups within the subfamily is certainly justified.

The results do not support the presence of a distinct barcoding gap, with intraspecific and interspecific sequence divergences differing by at least an order of magnitude, as reported in mayflies by Ball et al. (2005) or Webb et al. (2007). On the contrary, intraspecific divergences gradually went over to interspecific divergences; the transitory zone being approximately 0.160–0.180 of K2P distance (slightly obscured by the probable existence of cryptic species). These results fully support the study of Wiemers and Fiedler (2007) who interpreted the barcoding gap as an artefact of insufficient sampling across taxa.

Nevertheless, most intraspecific sequence divergences lay far below the transitory zone and most Baetinae species were found to be clearly differentiated from their relatives.

The highest intraspecific divergences were recorded in *Alainites muticus*, where significant separation of the specimen from Bulgaria (DNA No. 95) from other haplotypes from Georgia occurred (K2P distances between five Georgian specimen reached at most 0.014, whereas distances between Bulgarian and Georgian specimens were 0.232–0.239). Such values usually occur interspecifically, so such results may indicate the presence of a cryptic species within *A. muticus*. This assumption remains to be confirmed in the future through the analysis of more samples and a detailed morphological study.

A similar situation occurred in *B. rhodani* (*B. rhodani* species-group, range of K2P distance 0.002–0.168, mean 0.122), which is consistent with the supposed existence of the cryptic species within this widespread and abundant species (see also Williams et al. 2006; Gattolliat and Sartori 2008). The definition of these cryptic species within *B. rhodani* is not clear, because all *Baetis* specimens with spines on the external gill margins were often uncritically attributed to the '*B. rhodani*' in the past and cryptic species revealed by molecular methods in the previous studies or herein remain without morphological characterisation so far. On the basis of subtle morphological differences, two subspecies different from *B. rhodani rhodani* have already been described in the past, namely *B. rhodani sinespinosus* Soldán and Thomas, 1983 (later raised to the species level by Soldán, Godunko and Thomas 2005) and *B. rhodani tauricus* Godunko and Prokopov, 2003. In order to fix the proper *Baetis rhodani rhodani*, the neotype was designated recently by Gattolliat and Sartori (2008). The *COI* sequences of *B. rhodani rhodani* specimens included in this

study cannot be compared with the population from the type locality, because only the *cob* sequence is known for the topotypes at present (Gattolliat and Sartori 2008). Subtle morphological differences within *B. rhodani rhodani* from the various regions and their relationship to the the pattern of the recorded *COI* differences will be the subject of a separate study (Sroka et al. in preparation).

A somewhat higher level of intraspecific divergences somewhere between known intraspecific and interspecific divergences was recorded in *Nigrobaetis digitatus*. The K2P distance between haplotypes from the two parts of its distributional range (Bulgaria and Georgia) reached 0.124, which is high compared with the usual intraspecific divergences in mayflies (Ball et al. 2005; Webb et al. 2007), indicating considerable variability of the species throughout its distributional range.

Intraspecific variability within other species where more than a single specimen was sequenced was usually well below 0.1 (see Table 3 and Figure 17), which is within the usual range of mayfly intraspecific variability (Ball et al. 2005; Webb et al. 2007). This applies either to the cases where all specimens originated from the closely situated localities (*Baetis baksan*, *Baetis* cf. *gadeai*, *B. lutheri*, *B. vardarensis*) or where relatively distant populations were analysed (*B. braaschi*, *B. vernus*, *B. fuscatus*).

On the other hand, considerably low interspecific K2P distances were found. The lowest interspecific divergence among the species studied was computed between *Labiobaetis tricolor* and *Labiobaetis atrebatinus*. Interspecific K2P distance between these two species was 0.165. Another close resemblance of *COI* sequences was identified between *B.* cf. *gadeai* and *B. baksan* from the *B. rhodani* species-group (mean K2P distance 0.172). However, these latter two species do not form sister groups in the phylogenetic analyses despite the high degree of *COI* similarity. That is probably because there are some important differences in morphology; *B.* cf. *gadeai* for example lack spines on the gill margins, a character typical for most other species from the *B. rhodani* species-group.

Nevertheless, these species pairs represent probably very closely related taxa, although individual species are morphologically well defined. Interspecific divergences among other species were always higher than 0.180; these species therefore constituting molecularly distinctly separated taxa.

Phylogenetic analyses

Basal nodes of Baetinae phylogeny

Combining molecular and morphological data in phylogenetic analyses is still a controversial issue (for a detailed methodological discussion see Huelsenbeck, Bull and Cunningham 1996; Wortley and Scotland 2006). Nevertheless, in many cases a combined approach led to more robust and reliable phylogeny reconstructions than using molecular data alone, as recorded by Wiens (2004). In the present study, incorporating morphological data into the analysis also considerably improved individual branch support values.

In both approaches, parsimony and Bayesian, the subfamily split into two lineages: a grouping of genera *Labiobaetis* + *Nigrobaetis* + *Alainites* and a second clade formed by the genus *Baetis* s.str. However, support values were relatively low in both types of analysis and the phylogenetic signal from the individual data partitions was different (according to the PBS analysis both major clades gained

more support from the morphological data partition, in addition the monophyly of *Baetis* s.str. group was not supported by the molecular data set, see Figure 19).

Nevertheless, in all analyses performed, *Baetis*-complex *sensu* Waltz and McCafferty (1997) was not supported as monophyletic. According to this concept, species from the genera *Nigrobaetis* and *Alainites* should form a monophyletic sister group to all remaining Baetinae taxa studied (Figure 1). However, both genera were always recovered as internal lineages, most probably as sisters to *Labiobaetis*. The presence of femoral villopore and antennal flat-tipped sensillae are therefore not supported as apomorphies of the *Baetis*-complex as considered by Waltz and McCafferty (1997) and Gaino and Rebora (1999). These characters can be regarded as evolutionary novelties at the base of Baetinae with secondary loss in the *Nigrobaetis* + *Alainites* lineage, or as the characters that independently evolved into two lineages of Baetinae (*Labiobaetis* and *Baetis* s.str.). From these two scenarios, reduction of this structure in one lineage seems to be more probable, as reduction is generally more likely to occur than independent evolution. Moreover, species with the villopore missing or at least poorly developed were recorded even within *Labiobaetis* (see Gattolliat 2001). Therefore a possibility of the secondary loss of the villopore seems likely. The lack of support for the monophyletic *Baetis* complex is in agreement with some previous studies where the *Baetis* complex was also not supported as monophyletic (e.g. Gattolliat et al. 2008).

Generally, in the framework of the present study the *COI* sequences worked well mainly in delimiting individual species. They were much less reliable in estimating phylogenetic relationships, mainly with regard to deeper nodes, where the support values were often very low (Figures 18, 19, 20). The consistency index in the MP analysis was also relatively low (0.2979), indicating a high percentage of homoplasies in the alignment and high diversification of the *COI* sequence of the individual genera and species-groups within Baetinae. Therefore, assumptions made on the basis of these data should be viewed with caution. Further studies are needed to resolve the basal branching scheme between *Baetis* s.str., *Nigrobaetis*, *Alainites* and *Labiobaetis*. The results proposed in the present study may alter when incorporating more conservative gene regions and also non-Palaearctic representatives of Baetinae (some of the taxa included in the analysis are probably more complicated when considering extralimital species. Afrotropical representatives of *Labiobaetis* were found to form a highly polyphyletic taxon (Gattolliat et al. 2008); the same probably applies to *Nigrobaetis* s.str. and *Alainites*, whose distributional ranges also considerably exceed the Palaearctic region).

According to the results of the present study, it is impossible to make any final statements about the possible polyphyly of Jacob's (2003) concept of *Baetis* s.l., because no Baetidae taxa of those that Jacob considered to be outside *Baetis* s.l. were included in the analysis. The concept of genera *Nigrobaetis* and *Alainites sensu* Waltz et al. 1994 is problematic, rendering *Nigrobaetis* paraphyletic. However, the branching scheme at the base of *Nigrobaetis* + *Alainites* lineage is ambiguous, with a contradictory signal from the morphological and molecular data partition. This also remains to be resolved in future studies incorporating more taxa and gene regions.

Individual taxa included in the analysis

From the taxa investigated, some genera/species-groups were supported by a single data partition; this applies to the *B. rhodani* species-group and *B. lutheri* species-

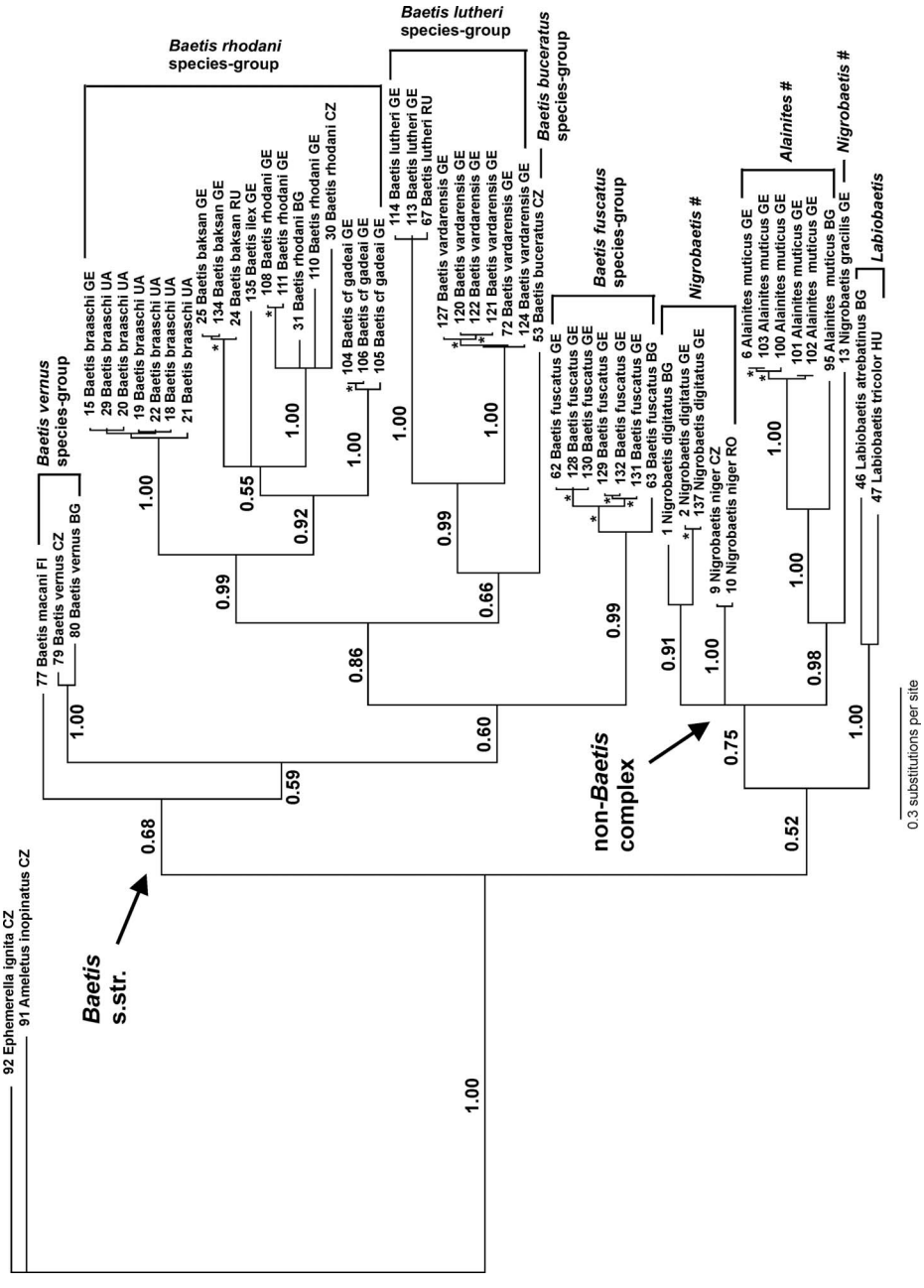


Figure 20. Topology of the Bayesian tree. The numbers near branches are values of Bayesian posterior probability. On some mostly terminal nodes, value of the posterior probability over 0.90 is replaced by an asterisk. Non-*Baetis* complex genera are marked with "#".

group, supported by the morphological data set only. Both of these groupings are characterised by several unique apomorphies. Apomorphy delimiting *B. rhodani* species-group is the presence of setae on basal antennal segments and terga; characters not recorded in other representatives of Baetinae. On the other hand, morphological characters supporting the *B. lutheri* species-group are present in other taxa, not included in the sampling (presence of sternal protuberances and similar arrangement of the paraproct teeth was recorded in genus *Acentrella*; subapical bristles are present in *B. alpinus* species-group, some species of *B. rhodani* species-group and genus *Acentrella*, see e.g. Jacob 2003; Sroka and Arnekleiv 2010).

In fact, many characters treated as the 'unique apomorphies' within the species sampled in the present study are known also in other more distant taxa of Baetidae which were not included in the analysis. Hence, they do not in reality always represent true unique apomorphies of the taxa studied here. The low number of the real unique morphological apomorphies supporting individual genera/species groups is not surprising because most of the higher taxa in Baetidae are known to be characterised rather by a combination of characters, which occur in different combinations in other Baetidae taxa. True unique apomorphies, present in only single genus/species-group are rare.

When considering a detailed scheme of relationships between genera, species-groups, and individual species, as recovered in the present study, they can be confronted with an already presumed hypothesis formulated in the past on the basis of morphological characters without using formal cladistic methodology (Müller-Liebenau 1969; Novikova and Kluge 1994; Jacob 2003; Soldán and Godunko 2009).

Within the *Nigrobaetis* + *Labiobaetis* + *Alainites* group, monophyletic clade *N. gracilis* + *A. muticus* was recognised in the present study. Therefore, Müller-Liebenau's (1969) and Jacob's (2003) original concept of the *B. gracilis* species-group (containing *N. gracilis* and *A. muticus*) was supported by the analysis, although the relationships between *N. digitatus* and *N. niger* to the *A. muticus* + *N. gracilis* clade were not resolved. The concept of Novikova and Kluge (1994), who established two different species-groups within the genera *Nigrobaetis* and *Alainites* (*niger-gracilis* species-group and *muticus-acinaciger-maxillaris* species-group), was not supported.

Within *Baetis* s.str., the branching scheme between the five species-groups investigated considerably differed between MP and Bayesian analysis; individual clades were also only poorly supported in both methodological approaches. Nevertheless, the *B. rhodani* species-group and the *B. lutheri* species-group may be seen as closely related, and these taxa nested within one clade in all analyses performed. In the MP analysis, the monophyly of the clade *B. rhodani* species-group + *B. lutheri* species-group was supported predominantly by molecular data partition. However, Soldán and Godunko (2009) have already claimed close relationships within the *B. rhodani* species-group + *B. lutheri* species-group on the basis of morphological similarities. However, no common unique apomorphy supporting these two species-groups was revealed in the present study. Within the *B. rhodani* species-group, *B. rhodani* was recovered as close to *B. baksan* in every type of analysis performed. This is fully in accordance with the high level of morphological similarity between these two species (see Soldán 1977).

The position of the *B. buceratus* species-group is uncertain; it was recovered as a sister clade to the *B. fuscatus* species-group (MP analysis), or to the *B. lutheri* species-group (Bayesian analysis). In each case, branch support was very low.

Conclusions

Previous studies investigating the *COI* sequence in mayflies were directed to the identification of species (Ball et al. 2005), to the investigation of species boundaries within a particular species or species-group (Williams et al. 2006; Ståhls and Savolainen 2007; Webb et al. 2007) or in the population genetic studies (e.g. Ogitani, Sekine and Tojo 2011; Baggiano, Schmidt, Sheldon and Hughes 2011). The *COI* sequence already proved to be a useful tool for estimating the extent of the species boundaries and its distance from the close relatives. These results were also confirmed by the present study. However, the presence of a distinct barcoding gap seems to be hardly possible in mayflies, and although *COI* sequences can help to identify and distinguish species it is necessary to use them in combination with morphological data to avoid misidentifications and confusion.

In the future, nevertheless, the construction of an extensive barcode library for mayflies will certainly help in disentangling relationships between individual populations of the more morphologically confusing species. Divergences in *COI* may also draw the attention of taxonomists to potentially cryptic species, which may subsequently be confirmed by detailed morphological study. Moreover, *COI* sequences can be helpful in associating larvae with adults, where rearing methods are unsuitable or difficult to perform (e.g. Gattolliat and Monaghan 2010).

For inferring phylogenetic relationships, *COI* sequences may also be useful to some degree on the lower taxonomic level, particularly in combination with the morphological data. However, its usefulness in disentangling basal nodes is limited because of the high number of homoplasies. As demonstrated in the present study, genera and species-groups within Baetinae are too diversified to gain reliable phylogeny using *COI* sequences. Therefore, incorporating more conservative gene regions will be necessary in future studies.

The results of this study have corroborated several partial phylogenetic hypotheses, already presumed on the basis of morphological data (the monophyly of some already established genera and species-groups, the close realationship of the *B. rhodani* species-group + *B. lutheri* species-group). However, the concept of the monophyletic *Baetis* complex was not supported. The genus *Nigrobaetis* (containing non-*Baetis* complex species) was recovered rather as a sister lineage to the genus *Labiobaetis*; the branch containing these two subgenera then formed a sister clade to the genus *Baetis* s.str. Nevertheless, as indicated above, rejection of the *Baetis* complex monophyly and clarification of the relationships between *Baetis* s.str., *Nigrobaetis, Alainites* and *Labiobaetis* remains as the subject of future studies using more taxa (covering also non-Palaearctic fauna) and more conservative gene regions.

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