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Molecular systematics and phylogeography of the cryptic species complex *Baetis rhodani* (Ephemeroptera, Baetidae)

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Abstract

Genetic studies have highlighted cryptic diversity in many well-known taxa including aquatic insects, with the general implication that there are more species than are currently recognised. *Baetis rhodani* Pictet are among the most widespread, abundant and ecologically important of all European mayflies (Ephemeroptera), and used widely as biological indicators of stream quality. Traditional taxonomy and systematics have never fully resolved differences among suspected cryptic species in the *B. rhodani* complex because morphological characters alone do not allow reliable distinction. This is particularly true among larvae, the life-stage used most widely in monitoring. Here, we assess the molecular diversity of this complex in one of the largest such studies of cryptic species in the order Ephemeroptera to date. Phylogenies were constructed using data from the mitochondrial cytochrome oxidase subunit I (COI) gene. Two monophyletic groups were recovered consisting of one major haplogroup and a second clade of 6 smaller but distinct haplogroups. Haplogroup divergence ranged from 0.2–3% (within) to 8–19% (among) with the latter values surpassing maxima typically reported for other insects, and provides strong evidence for cryptic species in the *B. rhodani* complex. The taxonomic status of these seven haplogroups remains undefined. Their distributions across Western Europe reveal no obvious geographic pattern, suggesting widespread diffusion of genetic lineages since the last glacial maximum. The implications of these findings are far-reaching given the ecological and bioindicator significance of what now appears to be several taxa.

Keywords: *Baetis rhodani*; Ephemeroptera; Cryptic species; Molecular systematics; Phylogeography

1. Introduction

The abundance of morphologically unrecognised (or cryptic) species, even in well-known taxa, suggests that there are more species than are currently recognised or estimated (Barratt et al., 1997). While much of the effort to resolve such species has involved terrestrial organisms (e.g., for insects: Packer and Taylor, 1997; Schonrogge et al., 2002), cryptic diversity has also been highlighted in aquatic insects from the orders Odonata, Hemiptera, Trichoptera, Diptera, and Ephemeroptera. In the latter, four cryptic species of Ephemerellidae have been discovered, six species of Heptageniidae, three species of Isonychiidae, and one species of Behningiidae (Jackson and Resh, 1998). Even though well-developed conventional morphological taxonomy underpins much current ecological research, the taxonomic records of aquatic insects are generally considered to be incomplete (Hogg et al., 1998).

The taxonomy of Ephemeroptera has been documented for more than 100 years, with their identification and species status frequently revised. The use of morphological characters to separate imagines or immatures of different species is still expanding, such as in the Heptageniidae (Alba-Tercedor, 1998) and Siphlonuridae (Engblom et al., 1993). As a result, genera have been reorganised and new species described (Lugo-Ortiz et al., 1999) including within the family Baetidae (Alba-Tercedor, 2002; Alba-Tercedor and McCafferty, 2000). Nevertheless, some species have

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been consistently misidentified due to variation in putatively distinguishing morphological characters (Engblom et al., 1993). This is particularly true for larvae, which lack external genitalia, and hence cannot always be reliably linked to species recognised among adults. In addition to the need for accurate taxonomy in this well studied and ecologically important group, there are ramifications for the assessment and conservation of biodiversity, for example due to perceived endemicity in certain regions (Southwestern France) from the Baetidae, *Rhithrogena* and Habroleptoides (Thomas, 1996).

The ephemeropteran genus *Baetis* is cosmopolitan, and probably originated as a clade in the Northern Hemisphere (Lugo-Ortiz and McCafferty, 1998). Ogden and Whiting (2003) found that *Baetis* was frequently supported as the basal ephemeropteran lineage in their molecular study on Paleoptera. Among the genus, *Baetis rhodani* Pictet is particularly well studied because it constitutes a major part of the macroinvertebrate biomass in many European streams and rivers throughout the entire year. *B. rhodani* is not only important as a major grazer or prey species but its larvae are used widely as indicators of water quality (Elliott et al., 1988). Its commercial value includes its role as an indicator of organic pollution and as one of the most important model organisms in quantifying the recovery of streams from acidification (Bradley and Ormerod, 2002; Rutt et al., 1990).

Many taxonomists already consider *B. rhodani* a complex of several forms of unknown taxonomic status. Since the original description of *B. rhodani* by Pictet in 1843-45 there have been numerous records of species potentially part of the *B. rhodani* complex, some of which have only been found in specific countries or regions. In the early 20th Century Bengtsson proposed that *B. rhodani* consisted of three different species: *B. rhodani* Pictet, 1843-45, *B. wallengreni* Bengtsson, 1912, and *B. pusillus* Bengtsson, 1912 (Elliott et al., 1988). In 1969 Muller-Liebenau re-amalgamated these as the original species *B. rhodani* in a monograph now widely used for nomenclature in the genus *Baetis* (Elliott et al., 1988).

The most common morphological feature used to distinguish the larvae of *B. rhodani* from other baetids is the presence of marginal spines on the gills (Elliott et al., 1988). Among adults, however, there are no clear characters that separate forms of *B. rhodani*, particularly in females (Engblom, 1996; Harker, 1989). Among at least four forms of "*rhodani*" larvae are a *B. rhodani*-form, possibly the species originally described by Pictet, 1843-45; a *B. gemellus*-form that closely resembles the *B. pusillus* species described by Bengtsson in 1912; a *B. wallengreni*-form; and an un-named form that apart from adult male eye colour closely resembles the "Baetidae A1" species identified in Kashmir (Engblom, 1996; Engblom and Lingdell, 1999). At present, only body size supposedly distinguishes these forms, but overlaps exist. There may also be differences in mouthpart, terminal tail filament, mandibles, and gill shape. Taken together, we can infer that traditional morphological taxonomy has never clearly nor categorically resolved what may be a cryptic species complex. In part, this reflects a lack of definitive identification, and in part it reflects reliance on subtle morphological differences, particularly in larvae.

Molecular methods have been valuable in discriminating insect species that differ subtly morphologically (Jackson and Resh, 1998; Pilgrim et al., 2002) and mitochondrial (mt) DNA is a popular marker for studying systematics, population genetics, and phylogeny in both terrestrial (Kambhampati and Smith, 1995; Loxdale and Lushai, 1998; Lunt et al., 1996) and aquatic insects (Plague et al., 2001; Schultheis et al., 2002). The cytochrome oxidase subunit I (COI) region of mtDNA is particularly useful for determining intra- and inter-specific phylogenetic relationships at the genus and species level (Caterino and Sperling, 1999; Trewick, 2000) and within families (Logan, 1999). Several phylogenies have been constructed for aquatic insects, including studies on the order Trichoptera (e.g., Kjer et al., 2001; Kjer et al., 2002), the order Plecoptera (Terry and Whiting, 2005), the order Odonata (e.g., Chippindale et al., 1999; Rehn, 2003), and the order Ephemeroptera (e.g., McCafferty, 1998; Monaghan et al., 2005; Ogden and Whiting, 2003; Ogden and Whiting, 2005), although few of these have focused on cryptic species complexes.

Here, we address the systematics in the *B. rhodani* complex within Western Europe using mtDNA. To investigate localised patterns of diversity, we also include specimens collected within and among three adjacent catchments in Wales. This is the first large-scale study of the phylogeography of a European Ephemeropteran.

2. Materials and methods

2.1. Specimen collections

Larval specimens ($n = 350$) of "*B. rhodani*" were collected or provided by collaborators from 11 countries across Western Europe. The UK specimens originated from 31 different streams, some of which were adjacent (Fig. 1, Table 1). In England and Scotland (sites 1–15 and 20–5) specimens were taken from streams in different catchments. Within-catchment sampling was carried out in three adjacent Welsh catchments, the Tywi, Wye, and Usk in mid-south Wales (sites 17–19, Fig. 1). Two adjacent streams, and one non-adjacent stream were sampled in each catchment (insets in Fig. 1). In the Republic of Ireland and mainland Europe, 27 streams in different catchments were sampled from 24 sites in Ireland, Spain, Switzerland, France, Germany, Denmark, Sweden, and Norway (Fig. 1, Table 1).

In the Tywi, 10 specimens of *B. vernus* Curtis larvae were collected from stream LI5 (Fig. 1, site 17a) as reference samples for phylogenetic out-grouping. We attempted to gather information from existing phylogenetic analyses, based on morphological and/or molecular evidence, to identify optimal outgroup taxa for rooting phylogenetic trees (Smith, 1994). These data were extremely limited for Ephemeroptera. *B. vernus* was identified since it is currently

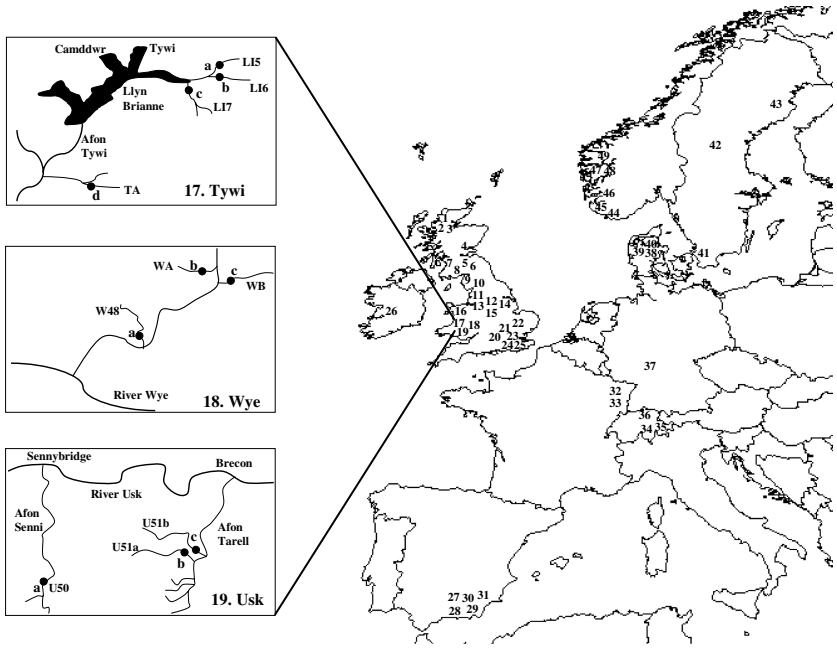

Fig. 1. Map of Western Europe showing the 49 different sites sampled for *B. rhodani* in England, Scotland, Wales, Republic of Ireland, Spain, Switzerland, France, Germany, Denmark, Sweden, and Norway. The insets illustrate the within-catchment sampling undertaken in the Tywi, Wye, and Usk catchments covering an area from mid to South Wales, as represented by the closed circles (Table 1).

the only morphologically similar species to *B. rhodani*. The topology of unrooted phylogenetic trees were studied to confirm the suitability of assigning this species to an outgroup.

Specimens were preserved first in 70–100% ethanol, and then transferred for long-term storage into 100% ethanol (Fisher Scientific) at 4 °C within 1–2 weeks of collection. The larvae of both *B. rhodani* and *B. vernus* were identified using Elliott et al. (1988). To distinguish *B. rhodani* from other baetid species, the gills of each individual were checked for the presence of marginal spines, clearly visible under a light microscope at 40× magnification.

2.2. DNA extraction and amplification

DNA was extracted from decapitated larvae using the PureGene™ DNA Isolation kit for Cell and Tissue (Gentra Systems cat #: D-5000A), and PCR amplified in 25 μl reaction volumes containing 40–100 ng template DNA, 0.625 U of *Taq* DNA Polymerase with 1× *Taq* buffer containing 20 mM Tris–HCl (pH 8.2) and 50 mM KCl, 4 mM $MgCl_2$, 200 μM total dNTP concentration (Gibco Life Technologies), and 0.5 μM of each primer (MWG Biotech) made up to 25 μl with tissue culture water (Sigma). 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 mtDNA. Initial denaturation (2 min at 94 °C) was followed by 35 cycles of 70 s at 94 °C, 70 s at 58 °C, 90 s at 72 °C, and a final extension of 72 °C for 5 min, using a Perkin-Elmer 9700 thermal cycler. PCR conditions were optimised following procedures outlined in Chippindale et al. (1998), Innis et al. (1990), and Logan (1999). PCR products were visualised under UV light on 1.3% ethidium–bromide stained agarose electrophoresis gels.

2.3. Sequencing

PCR products were purified using the GeneClean® Turbo for PCR kit, and sequenced in forward and reverse directions using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v2.0 (PE Biosystems). Cycle sequencing was carried out in 5 μl containing 2 μl of purified DNA, 1 μl of primer (MWG Biotech) at 0.8 μM concentration, 1 μl of the BigDye™ mix, and 1 μl of 2.5× buffer (PE Biosystems). Fragments were separated on an ABI 3100 Automated Sequencer, following the re-suspension of isopropanol purified products in Hi-Di™ formamide (PE Applied Biosystems).

DNA sequences were aligned and edited using the program SEQUENCHER 3.1.2 (Gene Codes Corporation, 1995). Ambiguous sequences were repeated. The *Drosophila mauritiana* Tsacas & David (Diptera) complete mtDNA sequence (GenBank Accession No. M57907) was used to align and position the reading frame, after BLAST analysis revealed that this sequence corresponded most closely with *B. rhodani*.

2.4. Nuclear copies

Nuclear copies of the COI region have been reported in insects (Loxdale and Lushai, 1998). To avoid inclusion of nuclear sequences into the data set, the guidelines outlined in Zhang and Hewitt (1996) were followed. PCR amplifications displaying >1 band under optimised conditions were

Table 1
Summary of the sample collections of *Baetis rhodani* analysed

Country	Map No.	Latitude	Longitude	No. of samples sequenced	Collector	Sample set ID
Scotland	1	N 58° 25′ 30″	W 04° 59′ 05″	6	R. Kowalik (Cardiff University)	NW35
	2	N 57° 32′ 42″	W 05° 28′ 38″	6	R. Kowalik (Cardiff University)	NW2
	3	N 57° 58′ 06″	W 04° 42′ 41″	6	R. Kowalik (Cardiff University)	NW21
	4	N 56° 19′ 57″	W 02° 49′ 58″	6	R. Milne (St. Andrews University)	Kinnessburn
	5	N 55° 29′ 48″	W 03° 16′ 32″	6	F. Edwards (University of Edinburgh)	Little Yarrow
	6	N 55° 26′ 29″	W 03° 13′ 52″	6	F. Edwards (University of Edinburgh)	Cranalt Burn
	7	N 55° 07′ 10″	W 04° 55′ 45″	6	R. Kowalik (Cardiff University)	GA22
	8	N 54° 58′ 28″	W 03° 53′ 59″	6	R. Kowalik (Cardiff University)	GA5
N. England	9	N 54° 34′ 32″	W 03° 25′ 45″	6	H.R. Wilcock (Cardiff University)	Crossgates
	10	N 54° 32′ 12″	W 03° 07′ 19″	6	H.R. Wilcock (Cardiff University)	Watendlath Tarn
	11	N 53° 49′ 01″	W 02° 43′ 55″	6	A. Dunstan (Environment Agency)	355
	12	N 53° 47′ 37″	W 02° 20′ 40″	6	A. Dunstan (Environment Agency)	169
	13	N 53° 33′ 02″	W 02° 46′ 32″	6	A. Dunstan (Environment Agency)	River Tawd
	14	N 53° 24′ 01″	W 00° 15′ 35″	6	C. Dodd (Cardiff University)	River Rase, Tealby
	15	N 53° 31′ 16″	W 01° 36′ 38″	6	S. Crofts (Pennine Guide Services)	River Don
N. Wales	16	N 52° 59′ 42″	W 03° 58′ 10″	10	H.C. Williams (Cardiff University)	HP1
Wales (Tywi)	17 a	N 52° 07′ 55″	W 03° 43′ 31″	10	H.C. Williams (Cardiff University)	LI5
	17 b	N 52° 07′ 55″	W 03° 43′ 15″	10	H.C. Williams (Cardiff University)	LI6
	17 c	N 52° 07′ 42″	W 03° 43′ 41″	10	H.C. Williams (Cardiff University)	LI7
	17 d	N 52° 06′ 05″	W 03° 43′ 36″	10	H.C. Williams (Cardiff University)	TA
Wales (Wye)	18 a	N 52° 07′ 49″	W 03° 18′ 11″	10	H.C. Williams (Cardiff University)	W48
	18 b	N 52° 08′ 44″	W 03° 17′ 20″	10	H.C. Williams (Cardiff University)	WA
	18 c	N 52° 08′ 38″	W 03° 16′ 37″	10	H.C. Williams (Cardiff University)	WB
Wales (Usk)	19 a	N 51° 52′ 28″	W 03° 33′ 46″	10	H.C. Williams (Cardiff University)	U50
	19 b	N 51° 53′ 23″	W 03° 29′ 42″	10	H.C. Williams (Cardiff University)	U51a
	19 c	N 51° 53′ 29″	W 03° 29′ 42″	10	H.C. Williams (Cardiff University)	U51b
S. England	20	N 51° 20′ 53″	W 01° 06′ 29″	6	B. McFarland (Environment Agency)	Silchester Brook
	21	N 51° 38′ 07″	W 00° 26′ 42″	6	Environment Agency (Herts)	Collve
	22	N 51° 46′ 21″	W 00° 07′ 02″	6	Environment Agency (Herts)	Lee @ Waterhall
	23	N 51° 48′ 05″	W 00° 00′ 15″	6	Environment Agency (Herts)	Ash @ Easneye
	24	N 51° 01′ 17″	E 00° 06′ 06″	6	H.R. Wilcock (Cardiff University)	Oldlands Hall
	25	N 51° 18′ 23″	E 00° 29′ 05″	6	Z. Masters (Cardiff University)	Tributary River Medway
Ireland	26	N 53° 03′ 50″	W 08° 25′ 05″	6	D. Lowson (Galway)	Woodford River
	26	N 53° 00′ 20″	W 08° 26′ 05″	6	D. Lowson (Galway)	Coos River
Spain	27	N 40° 01′ 16″	E 03° 28′ 15″	6	J. Alba-Tercedor (University of Granada)	Arroyo de Prada Negro, Granada
	28	N 39° 41′ 12″	E 03° 11′ 45″	6	J. Alba-Tercedor (University of Granada)	Guadalfeo, Granada
	29	N 39° 40′ 54″	E 03° 00′ 29″	6	J. Alba-Tercedor (University of Granada)	Junta Laroles-Bayarcal, Almeria
	30	N 39° 43′ 09″	E 03° 15′ 49″	6	J. Alba-Tercedor (University of Granada)	Rio Trevelez, Granada
	31	N 40° 24′ 30″	E 02° 47′ 33″	6	J. Alba-Tercedor (University of Granada)	Rio Castril, Los Laneros
France	32	N 48° 29′ 00″	E 07° 00′ 00″	3	J. Skriver (NERI, Denmark)	Halbach
	33	N 47° 48′ 00″	E 06° 55′ 00″	3	J. Skriver (NERI, Denmark)	Than/Thyr
Switzerland	34	N 46° 10′ 45″	E 08° 55′ 00″	6	F. Lepori (Cardiff University)	Valle di Cugnasco
	34	N 46° 02′ 47″	E 08° 55′ 25″	6	F. Lepori (Cardiff University)	Vedeggio
	34	N 46° 28′ 54″	E 08° 55′ 00″	6	F. Lepori (Cardiff University)	Val Marolta
	35	N 46° 38′ 15″	E 10° 11′ 05″	6	M. Monaghan (EAWAG)	Spoel
	36	N 47° 24′ 18″	E 08° 36′ 35″	10	M. Monaghan (EAWAG)	Chriesbach
Germany	37	N 50° 02′ 06″	E 09° 25′ 48″	6	S. Pauls (Frankfurt University)	Aubach A2 (A2 230401 B)
Denmark	38	N 55° 56′ 07″	E 10° 06′ 07″	6	J. Skriver (NERI, Denmark)	Bilsbaek
	39	N 56° 12′ 23″	E 09° 40′ 35″	6	J. Skriver (NERI, Denmark)	Voel Baek
	40	N 56° 21′ 07″	E 09° 55′ 38″	6	J. Skriver (NERI, Denmark)	Hadsten Lilleaa
Sweden	41	N 55° 53′ 11″	E 13° 04′ 45″	6	L. Sandin (University of Uppsala)	Svalovsbacken (S 05 0015)
	42	N 61° 56′ 42″	E 15° 15′ 49″	6	L. Sandin (University of Uppsala)	Angeran (S 02 0067)
	43	N 63° 57′ 53″	E 20° 08′ 52″	6	B. Malmqvist (University of Umea)	Lake Tavelsjon
Norway	44	N 58° 19′ 47″	E 7° 01′ 15″	2	A. Fjellheim (University of Bergen)	River Kvina
	45	N 59° 25′ 53″	E 5° 48′ 19″	2	A. Fjellheim (University of Bergen)	River Ogna
	46	N 59° 32′ 34″	E 5° 52′ 59″	2	A. Fjellheim (University of Bergen)	River Rodne
	47	N 60° 53′ 22″	E 5° 18′ 29″	2	G. Raddum (University of Bergen)	loc. 9
	48	N 61° 14′ 49″	E 5° 23′ 55″	2	G. Raddum (University of Bergen)	loc. 6
	49	N 62° 15′ 30″	E 5° 37′ 38″	2	G. Raddum (University of Bergen)	loc. 16

not sequenced. Nucleotide sequences displaying ambiguities, persistent background bands, frameshifts, stop codons, unexpected insertions/deletions, or showing excessive differences from the expected results, were repeated and/or omitted (Palsbøll and Arctander, 1998).

2.5. Data analysis

The translation of nucleotide sequences, identification of variable and parsimony informative sites, nucleotide base composition, pairwise genetic distances, and transition/transversion ratios, were all calculated using MEGA2 (Kumar et al., 2001). The uncorrected *p* distance was calculated when investigating amino acid and nucleotide substitutions prior to phylogenetic analysis. Amino acid substitutions were evaluated as in Graur and Li (2000). A minimum spanning network (MSN) was constructed using TCS (Clement et al., 2000), and the parsimonious connections at the 95% confidence level were established using ARLEQUIN version 2 (Schneider et al., 2000). A Chi-square test of homogeneity of base frequencies was calculated in PAUP* b10 (Swofford, 1998).

2.6. Phylogenetic analysis

Phylogenetic relationships among haplotypes were estimated using distance and maximum likelihood methods implemented in PAUP* b10 for Windows (Swofford, 1998), and viewed using TREEVIEW (Page, 1996). Models and parameters of DNA evolution were selected using the hierarchical Likelihood Ratio Tests (hLRTs) in MODELTEST version 3.06 (Posada and Crandall, 1998). Bootstrap resampling was used to test support for tree nodes (Felsenstein, 1985). The consensus method used was 50% majority-rule, and values of 70% bootstrap support were taken to correspond to a 95% confidence level.

Neighbour joining (NJ) phenograms were constructed using corrected distances and the NJ search algorithm, based on the HKY85 model of DNA evolution (Hasegawa et al., 1985) with a gamma distribution shape parameter of 2.4267, and a specified proportion of invariable sites of 0.6187. Bootstrap resampling was conducted on the complete data set, with 1000 replicates and repeated 10 times.

A maximum likelihood (ML) tree was constructed also based on the HKY85 model with a gamma distribution shape parameter of 2.4267, 0.6187 proportion of invariable sites, 4.5901 Ti/Tv ratio, and base frequencies of A = 0.2244, C = 0.1687, G = 0.1725, and T = 0.4344. The ML method was applied using the step-wise addition algorithm with the "as is" option, followed by a branch-swapping heuristic search using the methods of nearest neighbour interchange (NNI), subtree pruning regrafting (SPR), and tree bisection-reconnection (TBR). The difference between the likelihood values of these trees was evaluated using the Kishino and Hasegawa (1989) two-tailed χ^2 approximation test in PAUP* b10 (Swofford, 1998). The tree with the highest likelihood score was assessed by fast step-wise heuristic bootstrap for 1000 replicates. Data reduction taxon sampling was undertaken to reduce the computation time for bootstrapping. The most representative haplotypes were selected from each monophyletic haplogroup by omitting those with frequencies <3 (Hillis, 1998). In haplogroups with less than 3 haplotypes no omissions were made.

3. Results

The 472 base pair (bp) region of the COI gene was successfully sequenced for 360 specimens, and could be aligned unambiguously with no insertions/deletions or missing data. Seventy-eight haplotypes were identified (Genbank Accessions AY265230 through AY265307) which clustered into seven haplogroups not including *B. vernus* (see minimum spanning network, Fig. 2). The commonest haplogroup (I) comprised one dominant haplotype and 40 others that differed by 1-5 bp. Haplogroups II–VII comprised 34 different haplotypes, although these represented only 88 individuals as opposed to 265 in haplogroup I. These haplogroups differed from haplogroup I by between 39 and 150 substitutions, and several were divergent at least an equivalent level to the 87 differences found with *B. vernus*.

Haplogroups I, III, and VII were widespread across Britain and other European countries, whilst the remaining four haplogroups displayed a more restricted geographic range. A closer inspection of haplogroup frequencies revealed the widespread occurrence of haplogroup I in 10 of 11 countries studied. Haplogroup VII was the second most widespread, occurring in seven countries, followed by haplogroup III in five. Haplogroup VI only occurred in Denmark and France. The least variable samples were in Germany and Spain, where only one haplogroup occurred (I and VII, respectively). In the UK, haplogroup I occurred most frequently, followed by haplogroups VII and III and very similar frequencies were observed in the streams within the Welsh catchments. The most variable samples were from Denmark and Switzerland with four and five haplogroups, respectively. In Switzerland three haplogroups (II, IV, and V) were not found in any other sample.

The reading frame and translation of the nucleotide sequences was successfully carried out, with one sequence identified as a potential nuclear copy, displaying a total of eight stop codons, four insertions/deletions, and 68 amino acid substitutions of which 29 where classed as radical replacements. This sequence was removed from the data set prior to phylogenetic analysis. Approximately 70% of substitutions were at the 3rd codon position. Non-synonymous substitutions occurred in only seven of 75 *B. rhodani* haplotypes and also *B. vernus*, and at just four different sites. All 1st and 2nd codon substitutions were non-synonymous with only one observed in the 3rd codon position where an isoleucine was replaced by methionine at position 33 in haplotypes 50 and 51 (haplogroup I). All except one replacement were conservative. Non-synonymous substitutions within haplogroups were only found in haplotypes 63 and 72 in haplogroup VII. COI is the

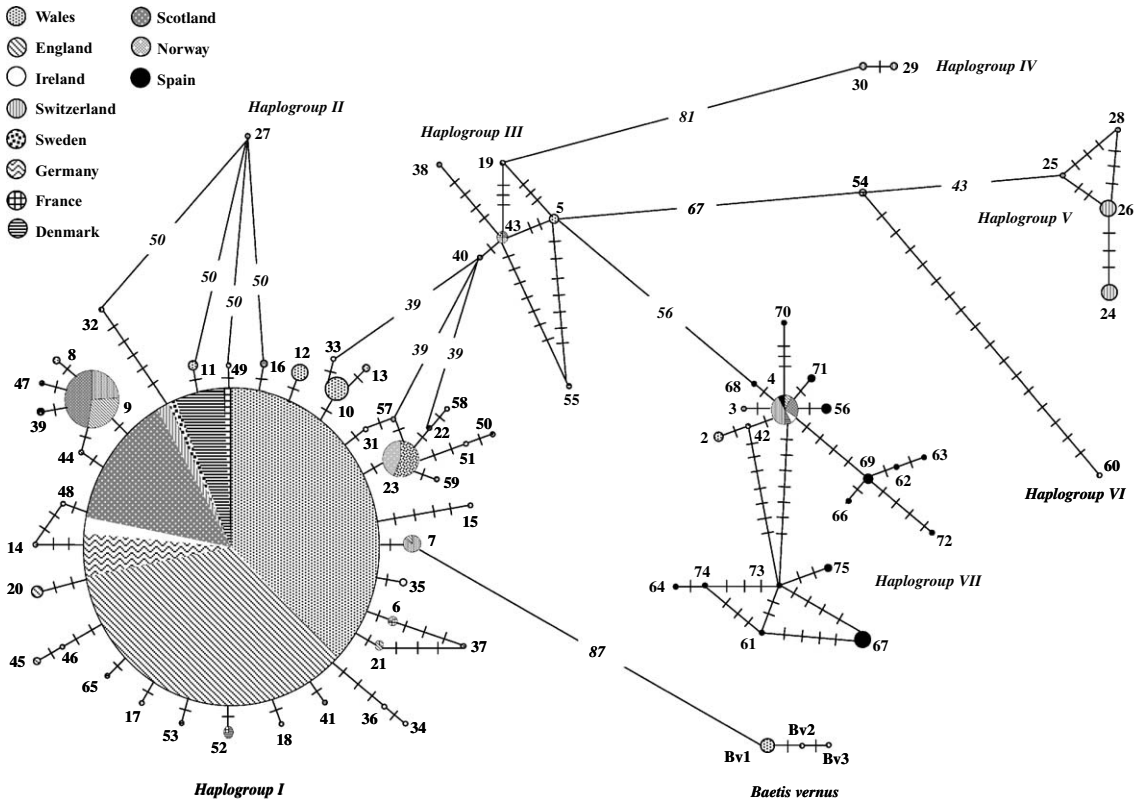

Fig. 2. Minimum spanning tree of the 78 haplotypes of which 75 were detected in *B. rhodani* haplogroups I–VII, and three in the species *B. vernus*. The size of the circles and lines are in proportion with the number of haplotypes and base substitutions, respectively. The number of base substitutions are represented by crossbars or italicised numbers, and the different shadings represent individual countries.

most conservative mitochondrial gene with respect to amino acid evolution (Simon et al., 1994) and non-zero values for uncorrected *p* distance were obtained only between *B. vernus* haplotypes Bv1-Bv3, and *B. rhodani* haplotypes 27, 50, 51, 54, 60, 63, and 72. Low amino acid divergence values were observed, ranging between 0.006 and 0.019, although higher values were observed for comparisons with *B. vernus*.

The 1st codon position had the most evenly distributed base composition, with a bias towards thymine at both 2nd and 3rd positions. The sequences for all haplogroups and *B. vernus* were A + T rich (60.3%), similar to the range (63% and 88.4%) reported in the COI gene of many insect orders (Brown et al., 1994; Litzenberger and Chapco, 2001). As reported in many of these studies, a higher A + T bias was observed in 3rd codon positions (73.8%) compared to the 1st and 2nd (51.5–54.8%). Base composition bias was not significantly different among haplotypes including *B. vernus*, regardless of codon position ($\chi^2 = 27.97$; df = 231; $p < 0.05$). 0.4–8.5% of variable sites (vs) and 0–3.2% of parsimony-informative sites (ps) were observed within haplogroups, similar to other insect species (Simon et al., 1994). Higher ranges of 8.3–35.6% vs and 0–34.3% ps were observed between haplogroups (Table 2), comparable to the 19.0–67.1% vs and 18.6–23.8% ps ranges reported between species in other insect orders, such as Hemiptera (Dohlen et al., 2002).

Uncorrected *p* distance values ranged between 0.002 and 0.191 for all pairwise comparisons of nucleotide sequences. Distances within haplogroups varied between 0.2–3% (Table 2), and were within the <1–7% range typically reported for other insects (Ross et al., 1997). However, ranges of 8–19% among haplogroups and with *B. vernus* surpassed these values, and such values have been interpreted as indicating cryptic species in other taxa (Buckley et al., 2001a). Divergences of 3–25% between species within a family or genus, and 23–28% between genera, have been published for insect orders (Chippindale et al., 1999; Landry et al., 1999; Simon et al., 1994).

The 1st and 3rd codon positions showed a linear relationship between the frequency of substitutions and *p*-distance (Fig. 3A). As in other insect orders (Morinaka et al., 2002), the 3rd codon position evolved most rapidly, followed by the 1st codon, with almost negligible substitutions at the 2nd. Transition substitutions accumulated faster than transversions, with no clear convergence between transition and tranversion accumulation rates at the highest genetic distance (Fig. 3B). This suggested that the gene sequences had not yet reached saturation (Rand et al., 2000), and were therefore useful for developing phylogenies (Logan, 1999; Weiblen, 2001). High transition rates are widely reported in related species (Davies and Bermingham, 2002; Nei and Kumar, 2000; Weiblen, 2001), and higher Ti/Tv ratios were observed at low sequence diver-

Table 2
Summary of the sequence divergence, transition/transversion ratios (Ti/Tv), the number of variable sites (Vs) and the number of parsimony-informative sites (Ps) for comparisons between individual haplogroups of *B. rhodani* and *B. vernus*, between multiple haplogroups, and within haplogroups with more then two haplotypes. The Ti/Tv ratios are presented for 1st, 2nd, and 3rd codon positions, and also summarised across all codon positions

Haplogroups (haplotypes)	Divergence (%)	Ti/Tv 1st	Ti/Tv 2nd	Ti/Tv 3rd	Ti/Tv all	Vs	Ps
Between individual haplogroups							
I (7) and *B. vernus*	18.40	∞	—	0.681	0.851	87	0
I (11) and II (27)	10.60	7.0	—	2.000	2.333	50	0
I (22) and III (40)	8.30	∞	—	2.182	2.545	39	0
I (22) and VI (54)	15.50	∞	—	1.952	2.429	72	0
I (22) and V (25)	16.90	∞	—	1.519	2.000	81	0
I (22) and VII (68)	14.20	∞	—	1.147	1.444	66	0
I (22) and IV (30)	19.10	∞	—	1.216	1.405	89	0
III (5) and VI (54)	14.20	∞	—	1.267	1.567	67	0
VI (54) and V (25)	9.10	∞	—	3.250	4.375	43	0
III (5) and VII (68)	11.90	∞	—	1.261	1.435	56	0
III (19) and IV (30)	17.20	∞	—	0.900	1.025	81	0
Between multiple haplogroups							
Haplogroups II–VII	0.20–19.7	∞	—	1.300	1.600	153	137
Haplogroups I–VII and *B. vernus*	0.20–22.72	∞	—	1.300	1.600	168	162
Within haplogroups							
Haplogroup I	0.20–2.12	∞	—	∞	∞	49	15
Haplogroup III	0.20–2.30	∞	—	5.400	6.900	15	1
Haplogroup V	0.40–1.30	—	—	∞	∞	7	1
Haplogroup VII	0.20–3.40	∞	—	3.700	4.300	30	15
B. vernus	0.20–0.42	—	—	∞	∞	2	0

gence levels (i.e., within haplogroups) compared with between haplogroups (Table 2; Fig. 3C). Overall, transversions occurred more frequently (Ti/Tv all $\leqslant 1$) in only one comparison involving *B. vernus* and haplogroup I (Table 2).

Four different bootstrap NJ phenograms were obtained and all reflected the seven haplogroups observed in the MSN. High bootstrap values of $\geqslant 95\%$ were observed for clustering within all haplogroups except II, which comprised only a single haplotype (data not shown). For ML trees, all search methods produced very similar likelihood scores (data not shown) and NNI was chosen based on time efficiency, since studies have suggested the SA + NNI search is usually as efficient as the more extensive TBR search (Nei and Kumar, 2000). The choice of search method has been shown to have little effect on bootstrap support (Buckley et al., 2001b). All seven haplogroups and *B. vernus* formed separate monophyletic groups in the ML tree, reflecting the same clustering observed in the MSN and NJ phenogram, and with only slight differences in topology (Fig. 4). In contrast to the NJ phenogram, haplogroup II did not diverge separately. A diphyletic structure was observed with haplogroups IV, V, VI, and *B. vernus* on one branch, and haplogroups II, III, and VII forming the other branch. The branching of these groups from haplogroup I was supported by 100% bootstraps. Values of >70% bootstrap support were observed for all other branches, except the divergence of haplogroups III and VII. The concordant ML and NJ results suggested that taxon sampling had not affected the main phylogenetic inference in the former. *B. vernus* was not separated from the other haplogroups by a single branch on unrooted trees, and so was not suitable as an out-group taxon. Preferred out-groups consist of sister taxa to the in-group, but the six haplogroups that were revealed are of unknown taxonomic status. Rooting trees is generally considered difficult (Smith, 1994), and so the probability of obtaining the correct tree was considered higher for unrooted trees.

4. Discussion

The *Baetis* sequence data displayed patterns of variation typical in the COI gene of other insects, including low amino acid divergences, A + T richness, expected frequencies of variable and parsimony-informative sites, and a transition substitution bias with unequal weighting across codon positions (Buckley et al., 2001b). The sequences do not yet appear to have reached a saturated divergence level and consequently the COI gene was considered useful for developing phylogenies (Logan, 1999; Weiblen, 2001).

Haplogroup I was identified as the main cluster of haplotypes in the MSN and in addition comprised the majority of specimens sequenced. A recent demographic expansion is suggested by the pattern of connections in haplogroup I although the demographic structure of this species is investigated elsewhere (Slatkin and Hudson, 1991; Williams et al., unpublished). A further six distinct haplogroups (II–VII) were identified and were separate to the *B. vernus* species group. Within haplogroups the sequence divergences of 0.2–3% are within the range reported in many other insect species (Simon et al., 1994). However, between haplogroups the divergences increased dramatically to 8–19%, with transition/transversion ratios approaching one. These values exceed those typically reported in other insect species and suggest that haplogroups II–VII are cryptic species (Buckley et al., 2001a; Ross et al., 1997).

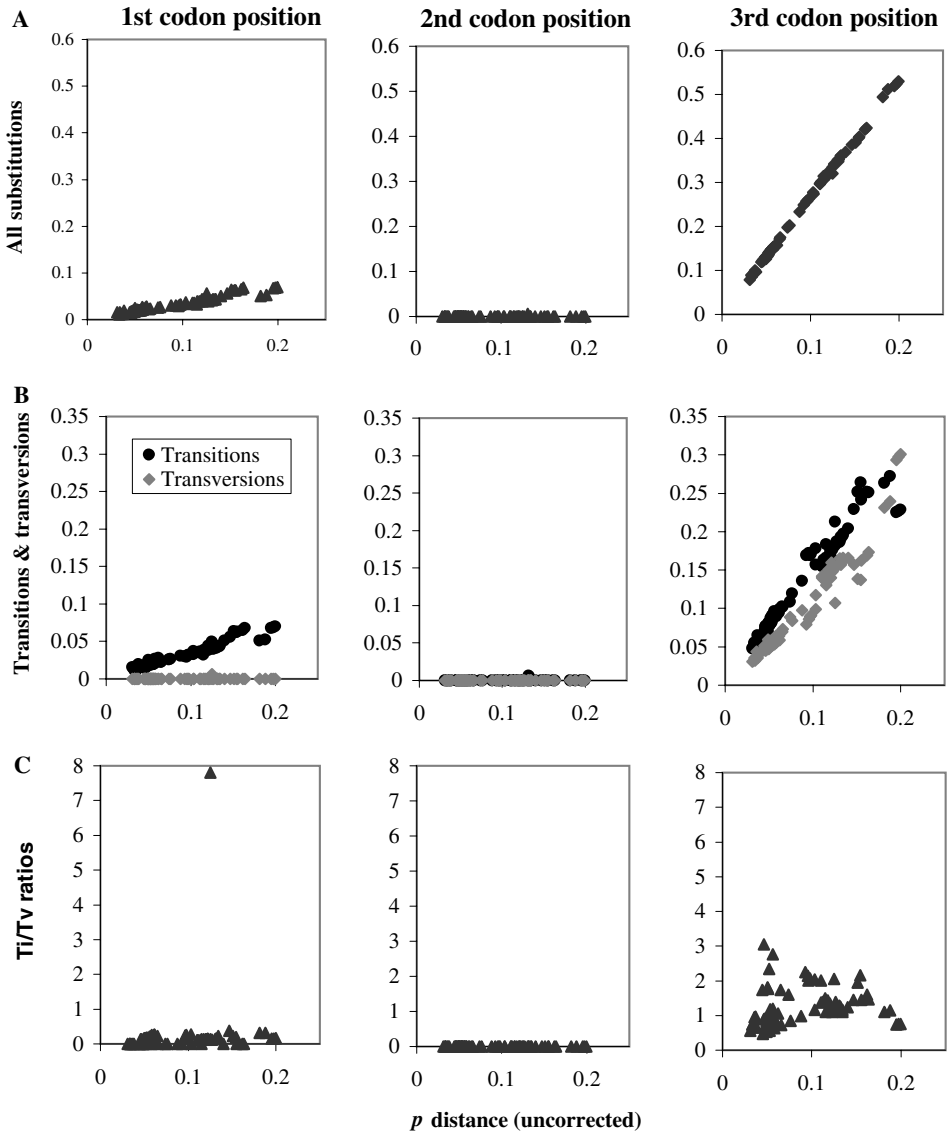

Fig. 3. Series of scatter graphs illustrating the relationships between substitution type and genetic distance (uncorrected *p*) for all *B. rhodani* and *B. vernus* haplotypes.

The clustering of sequences into haplogroups I–VII and *B. vernus* was strongly supported by the NJ and ML methods. Haplogroup I was separated by a single branch from the monophyletic group consisting of haplogroups II–VII and *B. vernus*, and this was supported by 100% bootstrap support values. High bootstrap support values were also obtained for branching within haplogroups II–VII and *B. vernus*, but not between haplogroups. There is therefore strong support for the presence of six distinct haplogroups that were clearly separated from haplogroup I, but their true branching order, including with respect to *B. vernus* remains unclear. Future phylogenetic studies within the order Ephemeroptera may reveal additional links within and between the distinct haplogroups identified in this study that will allow enhanced understanding of the phylogenetic relationships.

The distributions of the most frequently occurring haplogroups across Western Europe were not clustered by site. This suggests there has been widespread dispersal of multiple genetic lineages since the last ice age, and a recent range expansion was implicated in haplogroup I from the MSN. Many insects, including the meadow grasshopper *Chorthippus parallelus* Zetterstedt, have undergone south to north range expansion during inter-glacial periods from refugia in the south (Hewitt, 1996). The high divergence between several co-existing haplogroups suggest they may pre-date the time since the last glacial-interglacial cycles (Ross et al., 1997), although no molecular clock calibrations were attempted due to the lack of appropriate fossil records to conduct divergence time analyses. Putative refugia and/or hybrid zones could be present in Denmark, Switzerland, and Spain, since these regions are characterised by high haplotype and/or haplogroup diversity, and the presence of unique haplotypes. In many other organisms it has been discovered that during inter-glacial periods, mountain barriers such as the Pyrenees or Alps impeded northward

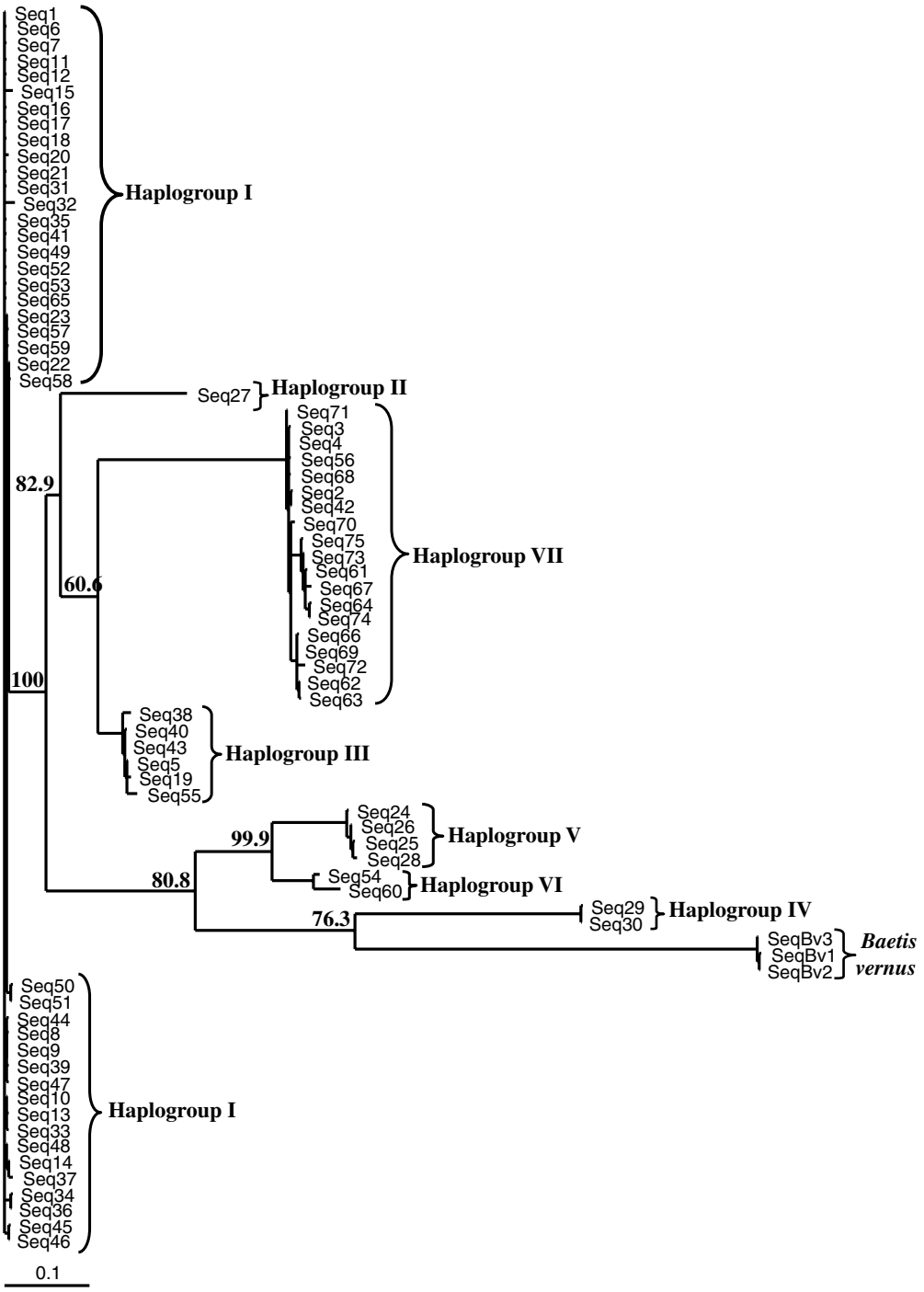

Fig. 4. The maximum likelihood tree with bootstrap values for all *B. rhodani* and *B. vernus* haplotypes based on the HKY85 model with the following parameters: gamma distribution shape parameter = 2.4267, proportion of invariable sites = 0.6187, Ti/Tv ratio = 4.5901, and base frequencies of A = 0.2244, C = 0.1687, G = 0.1725, and T = 0.4344. Bootstrap support values are displayed where haplogroups branch.

expansions of genomes from southern refugia. Hybrid zones formed in some of these areas due to subsequent mixing with expanding genomes from other regions (Hewitt, 2000). Although historical glacial events appear to have influenced the genetic structure of the *B. rhodani* species complex, different patterns of colonization events and refugia could exist between haplogroups. Further phylogeographic studies on individual haplogroups (I and VII) are currently in preparation (Williams et al., unpublished).

Four of seven haplogroups had restricted geographic distributions, including haplogroups II, VI, and V that were found only in Switzerland, and in particular haplogroup VI in Denmark and France. The low frequency of these haplogroups may reflect sampling. Careful taxonomic sampling designed to ensure coverage of all monophyletic groups for phylogenetic analysis (Hillis, 1998) was only partially achieved here. This is a difficult problem when studying morphologically undefined species complexes such as

B. rhodani. The molecular identification of the cryptic haplogroups II–VII provides a baseline for further work in the *B. rhodani* complex.

4.1. Delimiting the haplogroups as species

The difficulty in defining new species from haplogroups I to VII is in providing an accurate and consistent definition in the absence of detectable morphological distinction. Species are widely used as the fundamental recording units in areas of ecology and biogeography (Riddle and Hafner, 1999). The ability to identify species is essential for distribution and habitat studies (Pilgrim et al., 2002), in determining biological diversity, and providing a framework for conservation strategies (Zehr and Voytek, 1999). However, using molecular data singly and in combination with other approaches to defining species and other taxa can pose problems in some instances (e.g., Templeton, 1989; Kozak et al., 2006). Insect phylogenetic studies have indicated the need for a thorough taxonomic revision of many taxa at the family, genus and/or species level (Gentile et al., 2002), although only a few species descriptions are at least partly based on molecular data (Caterino et al., 2000; Kruse and Sperling, 2001). However this and other studies demonstrate that genetic data is a useful tool in revealing cryptic species complexes, and with more studies combining genetics and traditional taxonomy the classification of so called "cryptic" species may be possible.

A phylogenetic species is a basal cluster of organisms that are differentiated from other such clusters, and are monophyletic (Packer and Taylor, 1997). Based on this definition all of the haplogroups described here could be phylogenetic species, even those containing only a few sequences. The phylogenetic concept has been applied in the past for identifying conservation units of various species, including the tiger beetle *Cicindela puritana* Horn (Vogler et al., 1993). The genetically divergent haplogroups could also potentially be identified as evolutionary significant units (ESUs) regardless of their taxonomic status. ESUs are now being widely applied in conservation policies (Riddle and Hafner, 1999). With respect to conventional taxonomy they often correspond to species or subspecies boundaries, but in some circumstances can extend to isolated populations (Karl and Bowen, 1999).

Wiens and Penkrot (2002) discuss the advantages of using only mtDNA to delimit species based on exclusive haplotype monophyly. However, applying species definitions to haplogroups II–VII, including the ESU or phylogenetic concepts, could be considered inappropriate without combining evidence from other characters (Moritz, 1994; Crandall et al., 2000; Goldstein et al., 2000; Hewitt, 1996; Kelt and Brown, 2000). These haplogroups therefore represent several evolutionary lineages whose species status is currently undefined, and they are proposed here as distinct taxonomic units from *B. rhodani* and *B. vernus* worthy of further study and characterisation at the morphological and morphometric level. Extensive and rigorous morphological analysis has not been undertaken within this family, and there are no known diagnostic characters to support the proposed evolutionary taxa, although additional analysis using haplogroup-defined individuals is a direct recommendation of this study. Additional evidence from nuclear molecular markers, alternative mtDNA genes, morphology, physiology, and/or ecology, is necessary to corroborate or falsify the taxon limits that have been suggested by mtDNA (Hare, 2001; Hewitt, 1998; Leache and Reeder, 2002; Roderick, 1996).

4.2. Potential implications of cryptic species on ecological studies

B. rhodani larvae have been used in many ecological and/or toxicity studies dealing with the recovery of streams from disturbance or pollution, for example phenol and ammonium effluents (Khatami et al., 1998), inconsistent flows in regulated rivers (Malmqvist and Englund, 1996) and acidification (Fjellheim and Raddum, 1992; Fjellheim and Raddum, 2001; Guerold et al., 2000; Raddum and Fjellheim, 1987). Although there are no recognised morphological differences within the *B. rhodani* complex, they may vary biologically in life cycles and dispersal abilities, ecologically in habitat and/or food preferences, and physiologically. One or a combination of these factors could cause them to respond differently to disturbances within streams, and without appropriate taxonomic resolution, interpretations could be misleading.

The potential implications of cryptic species in the *B. rhodani* complex on current and future ecological studies are particularly far-reaching given the large number of studies carried out on what now appears to be several possible distinct taxa. These results have wider relevance since cryptic species have been detected in other aquatic insects (Jackson and Resh, 1998), and the presence and diversity of several taxa are widely used as biological indicators (Mason, 1996). The presence of cryptic species also has ramifications for the assessment of biodiversity in general, and the ability to account for them in future studies emphasises the need to correlate genetic differences from multi-locus data, with identifiable morphological characters and/or other factors including physiology. This will enable natural variations that occur within species to be separated from those indicating distinct species, including how this may potentially confound interpretations of population genetic variation and structure in this group (e.g., Rebora et al., 2005). Until the degree of adaptive differentiation between cryptic species can be established their effects on ecological studies remains questionable. This phylogenetic analysis should provide an appropriate reference for future studies within the *B. rhodani* complex and the group Ephemeroptera.

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References

Alba-Tercedor, J., 1998. Description of the imagines of *Rhithrogena goeldlini* Sartori and Sowa, 1988, and keys for the identification of imagines of the European species of the *R. diaphana* subgroup (Ephemeroptera: Heptageniidae). Aquat. Insect. 20, 125–130.

Alba-Tercedor, J., 2002. A new synonymy in Baetis: *Baetis nexus* Navas, 1918 is the valid name of *Baetis pentaphlebodes* Ujhelyi, 1966 (Ephemeroptera: Baetidae). Aquat. Insect. 24, 77–79.

Alba-Tercedor, J., McCafferty, W.P., 2000. *Acentrella feropagus*, new species (Ephemeroptera: Baetidae): formal new name for North American *A. lapponica* sensu Morihara and McCafferty. Entomol. News 111, 137–139.

Barratt, E.M., Deaville, R., Burland, T.M., Bruford, M.W., Jones, G., Racey, P.A, Wayne, R.K., 1997. DNA answers the call for pipistrelle bat species. Nature 387, 138–139.

Bradley, D.C., Ormerod, S.J., 2002. Long-term effects of catchment liming on invertebrates in upland streams. Freshwater Biol. 47, 161–171.

Brown, J.M., Pellmyr, O., Thompson, J.N., Harrison, R.G., 1994. Phylogeny of *Greya* (Lepidoptera: Prodoxidae), based on nucleotide sequence variation in mitochondrial cytochrome oxidase I and II: congruence with morphological data. Mol. Biol. Evol. 11, 128–141.

Buckley, T.R., Simon, C., Chambers, G.K., 2001a. Phylogeography of the New Zealand Cicada *Maoricicada campbelli* based on mitochondrial DNA sequences: ancient clades associated with cenozoic environmental change. Evolution 55, 1395–1407.

Buckley, T.R., Simon, C., Chambers, G.K., 2001b. Exploring among-site rate variation models in a maximum likelihood framework using empirical data: effects of model assumptions on estimates of topology, branch lengths, and bootstrap support. Syst. Biol. 50, 67–86.

Caterino, M.S., Sperling, F.A.H., 1999. *Papilio* phylogeny based on mitochondrial cytochrome oxidase I and II genes. Mol. Phylogenet. Evol. 11, 122–137.

Caterino, M.S., Cho, S., Sperling, F.A.H., 2000. The current state of insect molecular systematics: a thriving tower of babel. Annu. Rev. Entomol. 45, 1–54.

Chippindale, P.T., Whitmore, D.H., Dave, V.K., Valencia, T.G., Robinson, J.V., 1998. Effective procedures of the extraction, amplification and sequencing of odonate DNA. Odonatologica 27, 415–424.

Chippindale, P.T., Dave, V.K., Whitmore, D.H., Robinson, J.V., 1999. Phylogenetic relationships of North American Damselflies of the genus *Ischnura* (Odonata: Zygoptera: Coenagrionidae) based on sequences of three mitochondrial genes. Mol. Phylogenet. Evol. 11, 110–121.

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1660.

Crandall, K.A., Bininda-Emonds, O.R.P., Mace, G.M., Wayne, R.K., 2000. Considering evolutionary processes in conservation biology. TREE 15, 290–295.

Davies, N., Bermingham, E., 2002. The historical biogeography of two caribbean butterflies (Lepidoptera: Heliconiidae) as inferred from genetic variation at multiple loci. Evolution 56, 573–589.

Dohlen, C.D.V., Kurosu, U., Aoki, S., 2002. Phylogenetics and evolution of the eastern Asian-eastern North American disjunct aphid tribe, Hormaphidini (Hemiptera: Aphididae). Mol. Phylogenet. Evol. 23, 257–267.

Elliott, J.M., Humpesch, U.H., Macan, T.T., 1988. Larvae of the British Ephemeroptera: a key with ecological notes. Freshwater Biological Association Scientific Publication No. 49, Cumbria, England.

Engblom, E., 1996. Ephemeroptera, Mayflies. In: Nilsson, A.N. (Ed.), Aquatic Insects of North Europe—A Taxonomic Handbook. Apollo Books, Denmark, pp. 13–53.

Engblom, E., Lingdell, P.-E., 1999. Analyses of benthic invertebrates. In: Nyman, L. (Ed.), River Jhelum, Kashmir Valley Impacts on the Aquatic Environment. SWEDMAR, pp. 39–76.

Engblom, E., Longdell, P.E., Nilsson, A.N., Savolainen, E., 1993. The genus *Metretopus* (Ephemeroptera, Siphlonuridae) in Fennoscandia—identification, faunistics, and natural history. Entomol. Fennica 4, 213–222.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fjellheim, A., Raddum, G.G., 1992. Recovery of acid-sensitive species of Ephemeroptera, Plecoptera and Trichoptera in River Audna after liming. Environ. Pollut. 7, 173–178.

Fjellheim, A., Raddum, G.G., 2001. Acidification and liming of river Vikedal, Western Norway. A 20 year study of responses in the benthic invertebrate fauna. Water Air Soil Pollut. 130, 1379–1384.

Gentile, G., Torre della, A., Maegga, B., Powell, J.R., Caccone, A., 2002. Genetic differentiation in the African malaria vector, *Anopheles gambiae* s.s., and the problem of taxonomic status. Genetics 161, 1561–1578.

Goldstein, P.Z., DeSalle, R., Amato, G., Vogler, A.P., 2000. Conservation genetics at the species boundary. Conserv. Biol. 14, 120–131.

Graur, D., Li, W.-H., 2000. Fundamentals of Molecular Evolution, second ed. Sinauer Associates Inc., Massachusetts.

Guerold, F., Boudot, J.-P., Jacquemin, G., Vein, D., Merlet, D., Rouiller, J., 2000. Macroinvertebrate community loss as a result of headwater stream acidification in the Vosges mountains (N-E France). Biodivers. Conserv. 9, 767–783.

Hare, M.P., 2001. Prospects for nuclear gene phylogeography. TREE 16, 700–706.

Harker, J., 1989. Mayflies—Naturalists' Handbooks 13. Richmond Publishing Co. Ltd., Slough.

Hasegawa, M., Kishino, K., Yano, T., 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. 58, 247–276.

Hewitt, G., 1998. Diversity in insect species using DNA sequences. In: Karp, A., Isaac, P.G., Ingram, D.S. (Eds.), Molecular Tools for Screening Biodiversity. Chapman & Hall, London, pp. 418–425.

Hewitt, G., 2000. The genetic legacy of the quaternary ice ages. Nature 405, 907–913.

Hillis, D.M., 1998. Taxonomic sampling, phylogenetic accuracy, and investigator bias. Syst. Biol. 47, 3–8.

Hogg, I.D., Eadie, J.M., De Lafontaine, Y., 1998. Atmospheric change and the diversity of aquatic invertebrates: are we missing the boat? Environ. Monit. Assess. 49, 291–301.

Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), 1990. PCR Protocols—A Guide to Methods and Applications. Academic Press Inc., London.

Jackson, J.K., Resh, V.H., 1998. Morphologically cryptic species confound ecological studies of the caddisfly genus *Gumaga* (Trichoptera: Sericostomatidae) in Northern California. Aquat. Insect. 20, 69–84.

Kambhampati, S., Smith, P.T., 1995. PCR primers for the amplification of four insect mitochondrial gene fragments. Insect Mol. Biol. 4, 233–236.

Karl, S.A., Bowen, B.W., 1999. Evolutionary significant units versus geopolitical taxonomy: molecular systematics of an endangered sea turtle (genus *Chelonia*). Conserv. Biol. 13, 990–999.

Kelt, D.A., Brown, J.H., 2000. Species as units of analysis in ecology and biogeography: are the blind leading the blind? Global Ecol. Biogeogr. 9, 213–217.

Khatami, S.H., Pascoe, D., Learner, M.A., 1998. The acute toxicity of phenol and unionized ammonia, separately and together, to the ephemeropteran *Baetis rhodani* (Pictet). Environ. Pollut. 99, 379–387.

Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order on Hominoidea. J. Mol. Evol. 29, 170–179.

Kjer, K.M., Blahnik, R.J., Holzenthal, R.W., 2001. Phylogeny of Trichoptera (Caddisflies): characterization of signal and noise within multiple datasets. Syst. Biol. 50, 781–816.

Kjer, K.M., Blahnik, R.J., Holzenthal, R.W., 2002. Phylogeny of caddisflies (Insecta, Trichoptera). Zool. Scr. 31, 83–91.

Kozak, K.H., Weisrock, D.W., Larson, A., 2006. Rapid lineage accumulation in a non-adaptive radiation: phylogenetic analysis of diversification rates in eastern North American woodland salamanders (Plethodontidae: Plethodon). Proc. R. Soc. Lond. B 273, 539–546.

Kruse, J.J., Sperling, F.A.H., 2001. Molecular phylogeny within and between species of the Archips argyrospila complex (Lepidoptera: Tortricidae). Ann. Entomol. Soc. Am. 94, 166–173.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona, USA.

Landry, B., Powell, J.A., Sperling, F.A.H., 1999. Systematics of the *Argyrotaenia franciscana* (Lepidoptera: Tortricidae) species group: evidence from mitochondrial DNA. Ann. Entomol. Soc. 92, 40–46.

Leache, A.D., Reeder, T.W., 2002. Molecular systematics of the eastern fence lizard (Sceloporus undulates): a comparison of parsimony, likelihood, and Bayesian approaches. Syst. Biol. 51, 44–68.

Litzenberger, G., Chapco, W., 2001. A molecular phylogeographic perspective on a fifty-year-old taxonomic issue in grasshopper systematics. Heredity 86, 54–59.

Logan, J.A., 1999. Extraction, polymerase chain reaction, and sequencing of a 440 base pair region of the mitochondrial cytochrome oxidase I gene from two species of acetone-preserved Damselflies (Odonata: Coenagrionidae, Agrionidae). Environ. Entomol. 28, 143–147.

Loxdale, H.D., Lushai, G., 1998. Molecular markers in entomology. Bull. Entomol. Res. 88, 577–600.

Lugo-Ortiz, C.R., McCafferty, W.P., 1998. A new *Baetis*-complex genus (Ephemeroptera: Baetidae) from the Afrotropical region. Afr. Entomol. 6, 297–301.

Lugo-Ortiz, C.R., McCafferty, W.P., Waltz, R.D., 1999. Definition and reorganization of the genus *Pseudocloeon* (Ephemeroptera: Baetidae) with new species descriptions and combinations. Trans. Am. Entomol. Soc. 125, 1–37.

Lunt, D.H., Zhang, D.X., Szymura, J.M., Hewitt, G.M., 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol. Biol. 5, 153–165.

Malmqvist, B., Englund, G., 1996. Effects of hydropower-induced flow perturbations on mayfly (Ephemeroptera) richness and abundance in north Swedish river rapids. Hydrobiologia 341, 145–158.

Mason, C.F., 1996. Biology of Freshwater Pollution. Longman Group Limited, England.

McCafferty, W.P., 1998. Ephemeroptera and the great American interchange. J. N. Am. Benthol. Soc. 17, 1–20.

Monaghan, M.T., Gattolliat, J., Sartori, M., Elouard, J., James, H., Derleth, P., Glaizot, O., de Moor, F., Vogler, A.P., 2005. Trans-oceanic and endemic origins of the small minnow mayflies (Ephemeroptera, Baetidae) of Madagascar. Proc. R. Soc. Lond. B 272, 1829–1836.

Morinaka, S., Miyata, T., Tanaka, K., 2002. Molecular phylogeny of the *Eichhorni* group of *Delias* Hubner, 1819 (Lepidoptera: Pieridae). Mol. Phylogenet. Evol. 23, 276–287.

Moritz, C., 1994. Applications of mitochondrial DNA analysis in conservation: a critical review. Mol. Ecol. 3, 401–411.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Ogden, T.H., Whiting, M.F., 2003. The problem with "the Paleoptera Problem:" sense and sensitivity. Cladistics 19, 432–442.

Ogden, T.H., Whiting, M.F., 2005. Phylogeny of Ephemeroptera (mayflies) based on molecular evidence. Mol. Phylogenet. Evol. 37, 625–643.

Packer, L., Taylor, J.S., 1997. How many hidden species are there? an application of the phylogenetic species concept to genetic data for some comparatively well known bee "species". Can. Entomol. 129, 587–594.

Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Palsbøll, P.J., Arctander, P., 1998. Primers for animal mitochondrial DNA: the importance of species-specific primers. In: Karp, A., Isaac, P.G., Ingram, D.S. (Eds.), Molecular Tools for Screening Biodiversity. Chapman & Hall, London.

Pilgrim, E.M., Roush, S.A., Krane, D.E., 2002. Combining DNA sequences and morphology in systematics: testing the validity of the dragonfly species *Cordulegaster bilineata*. Heredity 89, 184–190.

Plague, G.R., Mulvey, M., Glenn, T.C., McArthur, J.V., 2001. Molecular genetic markers provide no evidence for reproductive isolation among retreat building phenotypes of the net-spinning caddisfly *Macrostemum carolina*. Mol. Ecol. 10, 243–248.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitutions. Bioinformatics 14, 817–818.

Raddum, G.G., Fjellheim, A., 1987. Effects of pH and aluminium on mortality, drift and molting of the mayfly *Baetis rhodani*. Ann. Soc. Roy. Zool. Bel. 117, 77–87.

Rand, D.B., Heath, A., Suderman, T., Pierce, N.E., 2000. Phylogeny and life history evolution of the genus *Chrysoritis* within the Aphnaeini (Lepidoptera: Lycaenidae), inferred from mitochondrial cytochrome oxidase I sequences. Mol. Phylogenet. Evol. 17, 85–96.

Rebora, M., Lucentini, L., Palomba, A., et al., 2005. Genetic differentiation among populations of *Baetis rhodani* (Ephemeroptera, Baetidae) in three Italian streams. Ital. J. Zool. 72, 121–126.

Rehn, A.C., 2003. Phylogenetic analysis of higher-level relationships of Odonata. Syst. Entomol. 28, 181–239.

Riddle, B.R., Hafner, D.J., 1999. Species as units of analysis in ecology and biogeography: time to take the blinders off. Global Ecol. Biogeogr. 8, 433–441.

Roderick, G.K., 1996. Geographic structure of insect populations: gene flow, phylogeography, and their uses. Annu. Rev. Entomol. 41, 325–352.

Ross, K.G., Krieger, M.J.B., Shoemaker, D.D., Vargo, E.L., Keller, L., 1997. Hierarchical analysis of genetic structure in native fire ant populations: results from three classes of molecular markers. Genetics 147, 643–655.

Rutt, G.P., Weatherley, N.S., Ormerod, S.J., 1990. Relationships between the physiochemistry and macroinvertebrates of British upland streams—the development of modeling and indicator systems for predicting fauna and detecting acidity. Freshwater Biol. 24, 463–480.

Schneider, S., Roessli, D., Excoffier, L., 2000. ARLEQUIN ver. 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schonrogge, K., Barr, B., Wardlaw, J.C., Napper, E., Gardner, M.G., Breen, J., Elmes, G.W., Thonas, J.A., 2002. When rare species become endangered: cryptic speciation in myrmecophilous hoverflies. Biol. J. Linn. Soc. 75, 291–300.

Schultheis, A., Hendricks, A.C., Weigt, L.A., 2002. Genetic evidence for 'leaky' cohorts in the semivoltine stonefly *Peltoperla tarteri* (Plecoptera: Peltoperlidae). Freshwater Biol. 47, 367–376.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.

Slatkin, M., Hudson, R.R., 1991. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129, 555–562.

Smith, A.B., 1994. Rooting molecular trees: problems and strategies. Biol. J. Linn. Soc. 51, 279–292.

Swofford, D.L., 1998. PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods) version 4 beta 10. Sinauer Associates, Sunderland, Massachusetts, USA.

Templeton, A.R., 1989. The meaning of species and speciation: a genetic perspective. In: Otte, D., Endler, J.A. (Eds.), Speciation and its Consequences. Sinauer Associates Inc.,, Massachusetts, pp. 3–27.

Terry, M.D., Whiting, M.F., 2005. Mantophasmatodea and phylogeny of the lower neopterous insects. Cladistics 21, 240–257.

Thomas, A., 1996. Mayflies of southwestern France 5. First list of recorded species since 1870 (Insecta, ephemeroptera). Int. J. Limnol. 32, 19–26.

Trewick, S.A., 2000. Molecular evidence for dispersal rather than vicariance as the origin of flightless insect species on the Chatham Islands, New Zealand. J. Biogeogr. 27, 1189–1200.

Vogler, A.P., Knisley, C.B., Glueck, S.B., Hill, J.M., DeSalle, R., 1993. Using molecular and ecological data to diagnose endangered populations of the puritan tiger beetle *Cicindela puritana*. Mol. Ecol. 2, 375–383.

Weiblen, G.D., 2001. Phylogenetic relationships of fig wasps pollinating functionally dioecious *Ficus* based on mitochondrial DNA sequences and morphology. Syst. Biol. 50, 243–267.

Wiens, J.J., Penkrot, T.A., 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Syst. Biol. 51, 69–91.

Zehr, J.P., Voytek, M.A., 1999. Molecular ecology of aquatic communities: reflections and future directions. Hydrobiologia 401, 1–8.

Zhang, D.-X., Hewitt, G.M., 1996. Nuclear integrations: challenges for mitochondrial DNA markers. TREE 11, 247–251.