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Bilton, David

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Frequent discordance between morphology and mitochondrial DNA in a species group of European water beetles (Coleoptera: Dytiscidae)

David T. Bilton[1], Lucy Turner[1] and Garth N. Foster[2]

[1] Marine Biology & Ecology Research Centre, University of Plymouth, Plymouth, United Kingdom
[2] Aquatic Coleoptera Conservation Trust, Ayr, Scotland

ABSTRACT

The *Hydroporus memnonius* species group includes both widespread and range restricted diving beetle taxa in the western Palaearctic, some of which have been divided into a number of geographical subspecies. Of these, *Hydroporus necopinatus* is distributed in the far west of Europe, from central Spain to southern Britain, and has been split into three subspecies, occurring in Iberia (*necopinatus* sst.), France (*robertorum*) and England (*roni*) respectively—the last of these being a rare example of an insect taxon apparently endemic to northern Europe. Here we explore interrelationships between populations and subspecies of *H. necopinatus* and related members of the *Hydroporus melanarius* subgroup, using mitochondrial COI sequence data. We reveal widespread discordance between mitochondrial DNA sequence variation and morphology in areas where *H. necopinatus* and *H. melanarius* come into contact, consistent with historical introgressive hybridization between these taxa. In light of this discordance, the lack of clear genetic divergence between *H. necopinatus* subspecies, and the fact that both *robertorum* and *roni* are morphologically intermediate between *H. necopinatus* sstr. and *H. melanarius*, we suggest that these taxa may be of hybridogenic origin, rather than representing discrete evolutionary lineages.

INTRODUCTION

Hydroporus Clairville, 1806, with 188 described species (*Nilsson, 2016*), is one of the largest genera of diving beetles, most species occurring in the Holarctic realm. The genus is currently arranged into a number of 'species groups' (sensu *Nilsson, 2001*), these initially being defined on external morphology alone. Recent molecular analyses have suggested that many of these groups are monophyletic, albeit with some modifications to their original membership and scope (e.g., *Ribera et al., 2003*; *Hernando et al., 2012*). Most *Hydroporus* are relatively small dytiscids, with total body lengths in the order of 2–5 mm (*Nilsson & Holmen, 1995*), and the genus includes a number of taxonomically challenging species groups and complexes, particularly in the southern and eastern Palaearctic.

The *Hydroporus memnonius* species group is one such example, being comprised of a number of Palaearctic taxa, most of which are distributed around the Mediterranean Basin and in western Asia. As defined by *Fery (1999)*, the group comprised 14 species, distributed across the *memnonius*, *melanarius* and *ferrugineus* subgroups. The *memnonius* group was later redefined by *Hernando et al. (2012)*, in light of data from four mitochondrial genes, to include only the 11 species of the *memnonius* and *melanarius* subgroups, both of which were recovered as monophyletic; *H. ferrugineus* Stephens, 1829 and its relatives as well as *H. obsoletus* Aubé, 1838, apparently representing additional, distinct, lineages within the genus. Both the *memnonius* and *melanarius* subgroups are composed of a mixture of well-characterised species (e.g., *Hydroporus memnonius* Nicolai, 1822 and *Hydroporus longicornis* Sharp, 1871) and complexes of closely related taxa, including, in some cases, a number of geographical subspecies (*Fery, 1999*). In addition to the widespread *H. melanarius* Sturm, 1835 which ranges across the western Palaearctic from Ireland to West Siberia (*Nilsson & Hájek, 2015*), the *melanarius* subgroup includes three morphologically similar species: *H. hebaueri* Hendrich, 1990, distributed from Hungary, through the Balkans to Anatolia, *H. lenkoranensis* (*Fery, 1999*) known only from Azerbaijan, and *H. necopinatus* (*Fery, 1999*), whose range stretches from Iberia to southern England. The last of these species was further split into three subspecies by *Fery (1999)*: *H. necopinatus necopinatus Fery, 1999* (Iberia), *H. necopinatus robertorum Fery, 1999*, (France, including the UK Channel Islands) and *H. necopinatus roni Fery, 1999* (southern England), these taxa being diagnosed on the basis of subtle, but apparently consistent, differences in both external and aedeagal morphology (*Fery, 1999*). In addition to morphology, species of the *melanarius* subgroup differ consistently in their ecology. Whilst *H. melanarius* is characteristic of highly acidic, oligotrophic waters, very often with *Sphagnum* mosses and in partial shade (*Balfour-Browne, 1940*; *Nilsson & Holmen, 1995*), both *H. hebaueri* and *H. necopinatus* are species of temporary pools, typically more productive than those occupied by *H. melanarius* (*Fery, 1999*; *Foster, Bilton & Nelson, 2016*).

Given its restriction to heathlands around the Poole Basin in southern England, *H. necopinatus roni* is a rare example of an insect taxon apparently endemic to northern Europe. Such narrow-range endemics are generally absent at such high latitudes in the western Palaearctic, where repeated cycles of glaciation and recolonization have led to a relatively depauperate biota (e.g., *Dynesius & Jansson, 2000*). Despite being situated south of the ice sheet during the Last Glaciation, areas currently occupied by *H. necopinatus roni*, for example, would have experienced severe cold, with permafrost conditions, until ca. 15,000 bp (e.g., *Yalden, 2002*), and have been unsuitable for occupancy by this temperate Atlantic taxon until relatively recently. In terms of their evolutionary origins, such northern endemics have either experienced range shifts to areas which are entirely outside those in which they originated—e.g., by moving north on deglaciation with glacial refugial populations going extinct (see *Calosi et al., 2010*)—or these taxa are very recent entities which have evolved following the Postglacial recolonization of high latitudes (e.g., *Piertney, Summers & Marquiss, 2001*; *Robertson, Newton & Ennos, 2004*; *Ennos, French & Hollingsworth, 2005*). Here we explore mitochondrial DNA sequence variation in members of the *H. melanarius* subgroup, with a focus on the *necopinatus* complex, in an
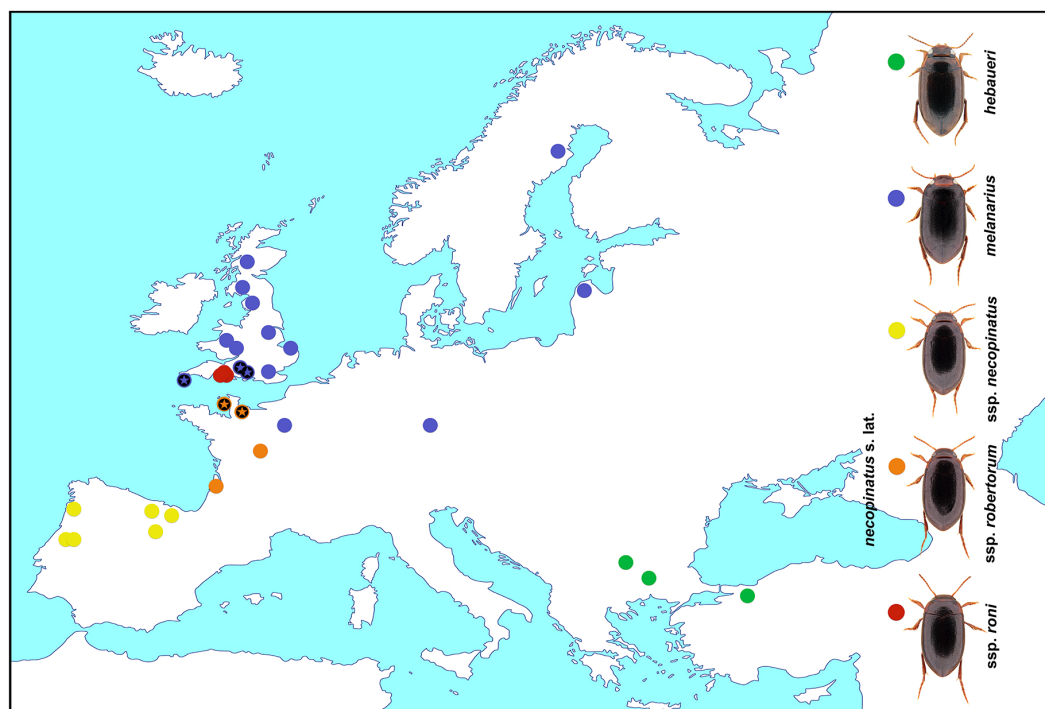
Figure 1 *Hydroporus melanarius* **subgroup taxa, together with localities sampled in this study.** Asterisks indicate populations where mismatches between mitochondrial DNA and morphological assignment occurred in *H. melanarius* and *H. necopinatus.*

attempt to better understand the evolutionary history of this group of beetles, including the status and origin of *H. necopinatus roni.*

MATERIALS AND METHODS

Specimens of the *Hydroporus* were collected using a D-framed pond net with 1 mm mesh at known localities across their geographical ranges (Fig. 1 and Table 1). Beetles were sorted in the field, and immediately killed and preserved in 100% ethanol, changed after 3–12 h to avoid dilution from body fluids. All specimens were identified following the morphological criteria of *Fery (1999)*, and stored at −20 °C prior to DNA extraction.

Genomic DNA extraction was performed using Wizard SV 96-well plates (Promega, UK). A ca. 800 bp fragment of the mitochondrial COI gene was amplified using primers Pat and Jerry (*Simon et al., 1994*). Sequencing was performed in both directions using a BigDye v. 1.1 terminator reaction and the same primers as in PCR. Sequences were analysed on an ABI3730 automated sequencer. Sequence chromatograms were scored and paired reads were assembled using PHRED/PHRAP as implemented in STARS (http://www.phrap.org/phredphrapconsed.html) on a dedicated bioinformatics unix server.

Sequences were aligned using the CLUSTALW program (*Thompson, Higgins & Gibson, 1994*). All alignments and base substitutions were confirmed visually. In addition to the COI data generated in this study, sequences for members of the *memnonius* group,

Table 1 Material used in this study, with GenBank Accession Numbers. Sequences obtained from GenBank shown in bold; all other sequences new from this study.

Taxon	Locality	Code	*cox1*
Hydroporus melanarius	Kentmere Tarn, Cumbria UK	Cumbria UK a	JN790719
Hydroporus melanarius	Kentmere Tarn, Cumbria UK	Cumbria UK b	JN790720
Hydroporus melanarius	Kentmere Tarn, Cumbria UK	Cumbria UK c	JN790721
Hydroporus melanarius	Kentmere Tarn, Cumbria UK	Cumbria UK d	JN790722
Hydroporus melanarius	Gaujas, Latvia	Gaujas, Latvia a	JN790736
Hydroporus melanarius	Gaujas, Latvia	Gaujas, Latvia b	JN790731
Hydroporus melanarius	Gaujas, Latvia	Gaujas, Latvia c	JN790732
Hydroporus melanarius	Gaujas, Latvia	Gaujas, Latvia d	JN790737
Hydroporus melanarius	Mains of Auchenfranco, Scotland, UK	Mains of Auchenfranco, UK	JN790733
Hydroporus melanarius	Manordeilo, Wales UK	Manordeilo, UK	JN790730
Hydroporus melanarius	Mrs Myhill's Marsh, Norfolk UK	Norfolk, UK	JN790734
Hydroporus melanarius	Guan Garthenor, Wales UK	Guan Garthenor, Wales UK	JN790735
Hydroporus melanarius	Crockford Bottom, New Forest UK	New Forest 1, UK a	JN790776
Hydroporus melanarius	Crockford Bottom, New Forest UK	New Forest 1, UK b	JN790777
Hydroporus melanarius	Crockford Bottom, New Forest UK	New Forest 1, UK c	JN790778
Hydroporus melanarius	Crockford Bottom, New Forest UK	New Forest 1, UK d	JN790779
Hydroporus melanarius	Burley Rocks, New Forest UK	New Forest 2, UK a	JN790780
Hydroporus melanarius	Burley Rocks, New Forest UK	New Forest 2, UK b	JN790781
Hydroporus melanarius	Burley Rocks, New Forest UK	New Forest 2, UK c	JN790783
Hydroporus melanarius	Burley Rocks, New Forest UK	New Forest 2, UK d	JN790782
Hydroporus melanarius	Bavaria, Germany	Bavaria, Germany a	JN790752
Hydroporus melanarius	Bavaria, Germany	Bavaria, Germany b	JN790755
Hydroporus melanarius	Bavaria, Germany	Bavaria, Germany c	JN790759
Hydroporus melanarius	Bavaria, Germany	Bavaria, Germany d	JN790753
Hydroporus melanarius	Bavaria, Germany	Bavaria, Germany e	JN790754
Hydroporus melanarius	Lizard Peninsula, Cornwall, UK	Lizard, UK a	JN790758
Hydroporus melanarius	Lizard Peninsula, Cornwall, UK	Lizard, UK b	JN790756
Hydroporus melanarius	Lizard Peninsula, Cornwall, UK	Lizard, UK c	JN790757
Hydroporus melanarius	Strath of Orchy, Scotland, UK	Strath of Orchy, UK a	JN790738
Hydroporus melanarius	Strath of Orchy, Scotland, UK	Strath of Orchy, UK b	JN790739
Hydroporus melanarius	Crowle Waste, Lincolnshire, UK	Lincolnshire, UK a	JN790740
Hydroporus melanarius	Crowle Waste, Lincolnshire, UK	Lincolnshire, UK b	JN790741
Hydroporus melanarius	Crowle Waste, Lincolnshire, UK	Lincolnshire, UK c	JN790742
Hydroporus melanarius	Crowle Waste, Lincolnshire, UK	Lincolnshire, UK d	JN790743
Hydroporus melanarius	Chobham Common, Surrery, UK	Surrey, UK a	JN790747
Hydroporus melanarius	Chobham Common, Surrery, UK	Surrey, UK b	JN790748
Hydroporus melanarius	Forêt de Fontainbleau, France	Forêt de Fontainbleau, France a	JN790749
Hydroporus melanarius	Forêt de Fontainbleau, France	Forêt de Fontainbleau, France b	JN790750
Hydroporus melanarius	Forêt de Fontainbleau, France	Forêt de Fontainbleau, France c	JN790751
Hydroporus melanarius	Vindeln, Västerbotten, Sweden	Västerbotten, Sweden	JN790803
Hydroporus necopinatus roni	Studland pool 2, Dorset, UK	Studland Pool 2, UK a	JN790760

Table 1 (*continued*)

Taxon	Locality	Code	*cox1*
Hydroporus necopinatus roni	Studland pool 2, Dorset, UK	Studland Pool 2, UK b	JN790761
Hydroporus necopinatus roni	Studland pool 2, Dorset, UK	Studland Pool 2, UK c	JN790762
Hydroporus necopinatus roni	Studland pool 2, Dorset, UK	Studland Pool 2, UK d	JN790763
Hydroporus necopinatus roni	Hartland Moor, Dorset, UK	Hartland Moor, UK a	JN790764
Hydroporus necopinatus roni	Hartland Moor, Dorset, UK	Hartland Moor, UK b	JN790765
Hydroporus necopinatus roni	Hartland Moor, Dorset, UK	Hartland Moor, UK c	JN790766
Hydroporus necopinatus roni	Hartland Moor, Dorset, UK	Hartland Moor, UK d	JN790767
Hydroporus necopinatus roni	Studland pool 1, Dorset, UK	Studland Pool 1, UK a	JN790768
Hydroporus necopinatus roni	Studland pool 1, Dorset, UK	Studland Pool 1, UK b	JN790769
Hydroporus necopinatus roni	Studland pool 1, Dorset, UK	Studland Pool 1, UK c	JN790770
Hydroporus necopinatus roni	Studland pool 1, Dorset, UK	Studland Pool 1, UK d	JN790771
Hydroporus necopinatus roni	Godlingstone Heath, Dorset, UK	Godlingstone Heath, UK a	JN790772
Hydroporus necopinatus roni	Godlingstone Heath, Dorset, UK	Godlingstone Heath, UK b	JN790773
Hydroporus necopinatus roni	Godlingstone Heath, Dorset, UK	Godlingstone Heath, UK c	JN790774
Hydroporus necopinatus roni	Godlingstone Heath, Dorset, UK	Godlingstone Heath, UK d	JN790775
Hydroporus necopinatus robertorum	Canne de Squez, Jersey, CI	Jersey, UK a	JN790723
Hydroporus necopinatus robertorum	Canne de Squez, Jersey, CI	Jersey, UK b	JN790724
Hydroporus necopinatus robertorum	Canne de Squez, Jersey, CI	Jersey, UK c	JN790725
Hydroporus necopinatus robertorum	Canne de Squez, Jersey, CI	Jersey, UK d	JN790726
Hydroporus necopinatus robertorum	La Teste-de-Buch, France	La Teste-de-Buch, France a	JN790784
Hydroporus necopinatus robertorum	La Teste-de-Buch, France	La Teste-de-Buch, France b	JN790785
Hydroporus necopinatus robertorum	La Teste-de-Buch, France	La Teste-de-Buch, France c	JN790793
Hydroporus necopinatus robertorum	La Teste-de-Buch, France	La Teste-de-Buch, France d	JN790786
Hydroporus necopinatus robertorum	Rosnay, Indre, France	Indre, France	JN790798
Hydroporus necopinatus robertorum	Forêt de Cerisy, Manche, France	Manche, France a	JN790727
Hydroporus necopinatus robertorum	Forêt de Cerisy, Manche, France	Manche, France b	JN790728
Hydroporus necopinatus robertorum	Forêt de Cerisy, Manche, France	Manche, France c	JN790729
Hydroporus necopinatus necopinatus	Serra Estrela 1, Beira Alta, Portugal	Beira Alta Estrela 1, Portugal a	JN790788
Hydroporus necopinatus necopinatus	Serra Estrela 1, Beira Alta, Portugal	Beira Alta Estrela 1, Portugal b	JN790789
Hydroporus necopinatus necopinatus	Serra Estrela 1, Beira Alta, Portugal	Beira Alta Estrela 1, Portugal c	JN790790
Hydroporus necopinatus necopinatus	Serra Estrela 1, Beira Alta, Portugal	Beira Alta Estrela 1, Portugal d	JN790792
Hydroporus necopinatus necopinatus	Serra Estrela 1, Beira Alta, Portugal	Beira Alta Estrela 1, Portugal e	JN790794
Hydroporus necopinatus necopinatus	Serra Estrela 2, Beira Alta, Portugal	Beira Alta Estrela 2, Portugal a	JN790795
Hydroporus necopinatus necopinatus	Serra Estrela 2, Beira Alta, Portugal	Beira Alta Estrela 2, Portugal b	JN790787
Hydroporus necopinatus necopinatus	Serra Estrela 2, Beira Alta, Portugal	Beira Alta Estrela 2, Portugal c	JN790797
Hydroporus necopinatus necopinatus	Serra Estrela 2, Beira Alta, Portugal	Beira Alta Estrela 2, Portugal d	JN790796
Hydroporus necopinatus necopinatus	Serra de Arga, Minho, Portugal	Minho, Serra de Arga, Portugal a	JN790799
Hydroporus necopinatus necopinatus	Serra de Arga, Minho, Portugal	Minho, Serra de Arga, Portugal b	JN790801
Hydroporus necopinatus necopinatus	Serra de Arga, Minho, Portugal	Minho, Serra de Arga, Portugal c	JN790802
Hydroporus necopinatus necopinatus	Serra de Arga, Minho, Portugal	Minho, Serra de Arga, Portugal d	JN790791
Hydroporus necopinatus necopinatus	Serra de Arga, Minho, Portugal	Minho, Serra de Arga, Portugal e	JN790800
Hydroporus necopinatus necopinatus	Reinosa, Cantabria, Spain	Cantabria, Spain a	JN790744
Hydroporus necopinatus necopinatus	Reinosa, Cantabria, Spain	Cantabria, Spain b	JN790745

Table 1 (*continued*)

Taxon	Locality	Code	*cox1*
Hydroporus necopinatus necopinatus	Reinosa, Cantabria, Spain	Cantabria, Spain c	JN790746
Hydroporus necopinatus necopinatus	Sierra de Arbasa, Vitoria, Spain	Sierra de Arbasa, Spain	JN790804
Hydroporus necopinatus necopinatus	Sierra de la Demanda, Burgos, Spain	Burgos, Spain a	JN790805
Hydroporus necopinatus necopinatus	Sierra de la Demanda, Burgos, Spain	Burgos, Spain b	JN790806
Hydroporus necopinatus necopinatus	Sierra de la Demanda, Burgos, Spain	Burgos, Spain c	JN790807
Hydroporus necopinatus necopinatus	Sierra de la Demanda, Burgos, Spain	Burgos, Spain d	JN790808
Hydroporus hebaeuri	E Rhodopes, Bulgaria	Rhodopes, Bulgaria a	JN790809
Hydroporus hebaeuri	E Rhodopes, Bulgaria	Rhodopes, Bulgaria b	JN790810
Hydroporus hebaeuri	Samokov, Bulgaria	Samokov, Bulgaria	JN790811
Hydroporus hebaeuri	Road to Samandere from Kaynasli, Turkey	Düzce, Turkey	JN790812
Hydroporus cantabricus	Reinosa, Cantabria, Spain	a	JN790813
Hydroporus cantabricus	Reinosa, Cantabria, Spain	b	JN790814
Hydroporus cantabricus	Reinosa, Cantabria, Spain	c	JN790815
Hydroporus cantabricus	Reinosa, Cantabria, Spain	d	JN790816
Hydroporus cantabricus	**Reinosa, Cantabria, Spain**	**e**	HE599653
Hydroporus brancoi gredensis	Serra Estrela, Beira Alta, Portugal		JN790817
Hydroporus brancoi brancoi	**Serra de Arga, Minho, Portugal**		HE599652
Hydroporus memnonius	**New Forest, UK**	**a**	AF518300
Hydroporus memnonius	**St Gottardo Pass, Switzerland**	**b**	HE599667
Hydroporus lluci	**Mallorca, Lluc**		AY365307
Hydroporus longicornis	**Vindeln, Vasterbotten, Sweden**		HE599663
Hydroporus normandi	**Santed, Zaragoza, Spain**		AY365312
Hydroporus longulus	**Loch Einich, Scotland**		AY365326
Hydroporus sanfilippoi	**Berceto, Emilia Romagna, Italy**		HE599672
Hydroporus cuprescens	**Paphos Forest, Ayia, Cyprus**		HE599655

and selected representatives of the *longulus* and *ferrugineus* groups were obtained from GenBank. Phylogenetic reconstruction was carried out with Bayesian inference (BI) and maximum likelihood (ML) and approaches. MRMODELTEST version 2.3 (*Nylander, 2004*—conducted in PAUP* (*Swofford, 2002*)) was used to identify the most appropriate model of sequence evolution based on the Alkaike Information Criterion (AIC). MRBAYES version 3.1.2 (*Huelsenbeck & Ronquist, 2001*) was then employed for BI analysis using a Metropolis-coupled Markov chain Monte Carlo (MCMC) search with four chains (one cold, three heated). Five million generations were produced from each set, sampling every 1,000 generations (i.e., 5,000 sampled trees). Convergence was assessed by visual examination of log-likelihood scores from both runs in MrBayes at 10,000 generations. The first 25% of samples were discarded as burn-in Prior to ML analysis, MODELTEST version 3.07 (*Posada & Crandall, 1998*) was used to select the most appropriate model of sequence evolution using AIC. ML searches were conducted using Garli version 0.951 (http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html) and the model selected by MODELTEST. All other settings were left as defaults. Support was measured with 1,000 bootstrap replicates. Only clades with significant support values (defined here as >0.90 posterior probabilities or >70 bootstrap) are shown.

RESULTS

COI sequences were compiled for 97 individuals of the *Hydroporus melanarius* subgroup, together with 15 sequences from 10 taxa of the *memnonius*, *longulus* and *ferrugineus* groups (Table 1). All described taxa of the *melanarius* subgroup were included, with the exception of *H. lenkoranensis,* known only from the holotype (*Fery, 1999*). New sequences from this study were deposited in GenBank (accession numbers: JN790719–JN790817—see Table 1). The total analysed alignment length used for phylogenetic analyses was 781 bp.

The *melanarius* subgroup was recovered as monophyletic, with high support, in both analyses (asterisks in Figs. 2A–2B). *H. longicornis* was not recovered in this clade, however, in accordance with *Hernando et al. (2012)*. Topologies obtained from both BI and ML analyses were broadly similar (Figs. 2A–2B) and did not reflect the boundaries of the morphologically defined species or subspecies within the *melanarius* subgroup. In both analyses there was a well-supported clade (*a* in Figs. 2A–2B) mainly composed of *H. necopinatus*, and containing all *H. necopinatus roni* and *H. necopinatus necopinatus* specimens, together with *H. necopinatus robertorum* from southern France and some southern English *H. melanarius*. In almost all cases, beetles from individual localities were either entirely inside or outside this clade; only those from the Lizard Peninsula, in the far southwest of England, grouping in both parts of the trees. Both analyses revealed a second clade (*b* in Figs. 2A–2B), well-supported in BI, and a bootstrap of 65 in ML, dominated by the remaining *H. melanarius*, as well as *H. necopinatus robertorum* from northern France and Jersey, and *H. hebaeuri*, which was nested within the second clade, a position well supported in BI analyses. Whilst the position of some of the taxa outside the *melanarius* subgroup differed between analyses, all of these were consistently recovered outside the *melanarius* subgroup itself.

DISCUSSION

Mitochondrial COI sequence variation is clearly at odds with previously recognized taxonomic boundaries in this group of diving beetles. This is true not only of the different subspecies of *Hydroporus necopinatus*, but also *H. hebaeuri* and *H. melanarius*. Whilst *H. hebaeuri* is morphologically similar to *H. necopinatus*, being distinguished primarily on the detailed structure of the male median lobe, *H. melanarius* differs from these two taxa on a suite of external and genitalic characters, including habitus (see Fig. 1) and the setation of the metacoxal process (*Fery, 1999*). Our results confirm the monophyletic status of the *melanarius* subgroup (see *Hernando et al., 2012*), but show that within it taxa are not reciprocally monophyletic on COI sequence, despite differing in ecology and morphology.

Mismatches between taxonomic boundaries and mitochondrial DNA phylogenies can arise for a number of reasons, including incomplete lineage sorting, introgression resulting from historical or ongoing hybridization, infection with *Wolbachia* and differential selection on nuclear and mitochondrial genomes (*Wirtz, 1999*; *Funk & Omland, 2003*; *Chan & Levin, 2005*; *Werren, Baldo & Clark, 2008*; *Toews & Brelsford, 2012*). Distinguishing between these potential drivers of discordance can be difficult in practice (*Hawlitschek et al., 2012*; *Toews & Brelsford, 2012*), although when there is strong geographical inconsistency between
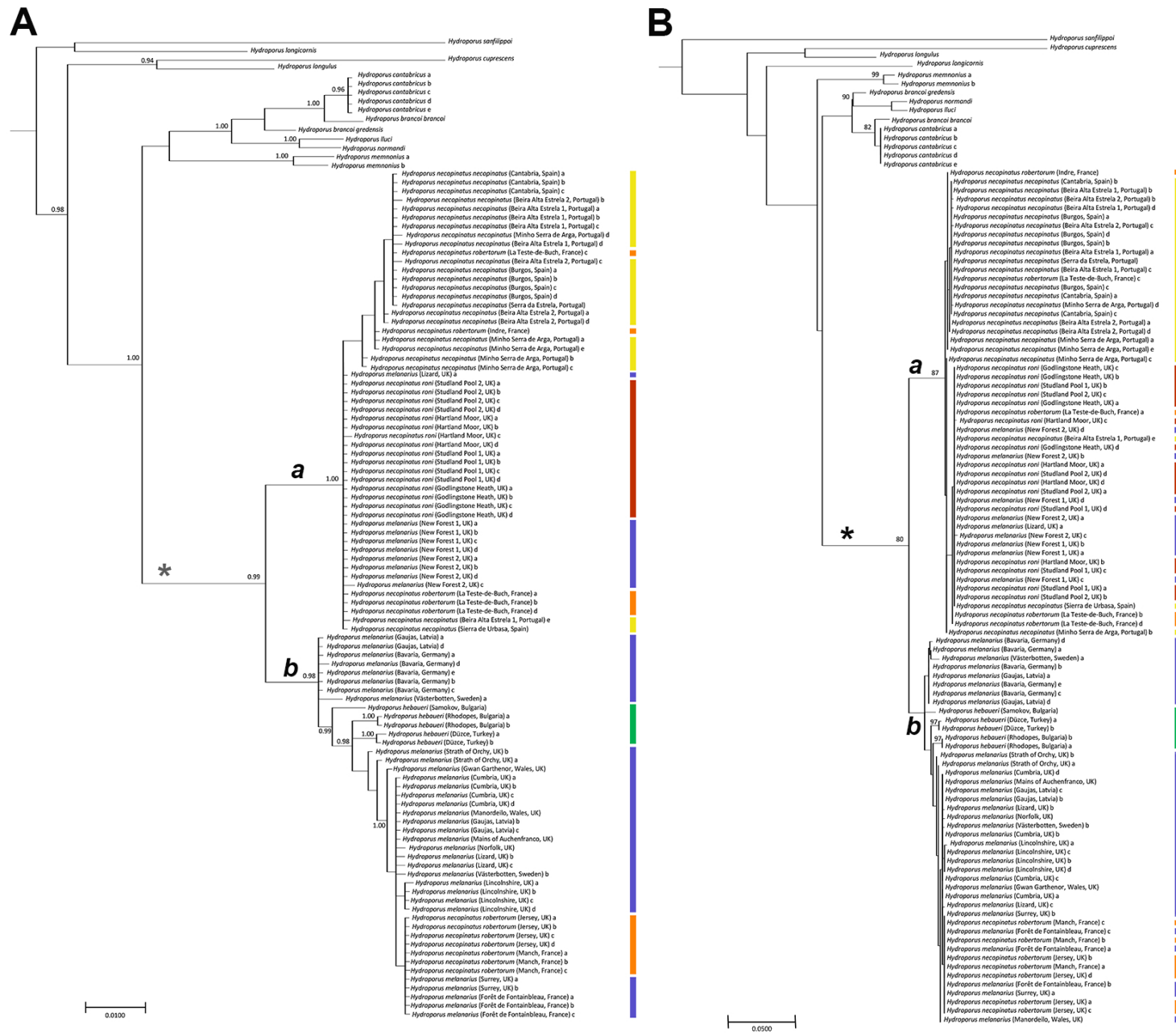
Figure 2 Phylograms of *Hydroporus memnonius* groups taxa. (A) bayesian majority-rule consensus phylogram of COI dataset obtained using MRBAYES. Numbers are posterior probabilities from Bayesian analysis; only values equal or greater to 0.90 are shown. Coloured bars correspond to taxa illustrated in Fig. 1; (B) maximum likelihood consensus phylogram of COI dataset obtained using Garli. Numbers represent bootstrap values (1,000 replicates); only values equal or greater to 70 are shown. Asterisks indicate the *melanarius* subgroup (excluding *H. longicornis*); coloured bars correspond to taxa illustrated in Fig. 1.

patterns in mitochondrial and nuclear DNA, incomplete lineage sorting can generally be ruled out. In these *Hydroporus*, whilst we lack sequence data from the nuclear genome, mitochondrial-morphological mismatches between species are concentrated in areas of range overlap between taxa, a pattern more consistent with introgressive hybridization than incomplete lineage sorting (*Toews & Brelsford, 2012*), a process which has been suggested to be responsible for similar mismatches in other western Palaeartic diving beetle clades (*Ribera, Bilton & Vogler, 2003*; *García-Vázquez & Ribera, 2016*).

Clade *a* in our analyses is composed largely of *H. necopinatus* individuals, of all three described subspecies, but also includes some specimens bearing all the diagnostic morphological features (parallel-sided habitus, setose metacoxal process, less elongate apex to the male median lobe) of *H. melanarius*. All of these mitochondrially misplaced individuals originate from wet heathland habitats in southern England, which are located either side of the restricted region of the UK (Dorset), occupied by *H. necopinatus*. Some southern English *H. melanarius* have more elongate apices to their median lobes than those from populations elsewhere in the UK (RB Angus, pers. comm.; DT Bilton & GN Foster, pers. obs., 1970), making them somewhat intermediate between *H. melanarius* and *H. necopinatus* on this character—this also being the case for the specimens discussed here. Such an observation, coupled with our genetic data, suggests that introgressive hybridization may have occurred between *H. melanarius* and *H. necopinatus* in this region, resulting in both the intermediate morphologies and mitochondrial mismatch. In the case of one southern English locality, the Lizard Peninsula in the extreme southwest of the country, such individuals of *H. melanarius* are distributed across clades *a* and *b* (Fig. 2), indicating that *necopinatus* and *melanarius* mitochondrial DNAs can occur in the same population.

Whilst only strongly supported in BI, clade *b* was recovered in both analyses, and included the majority of studied individuals identified as *H. melanarius* (Fig. 2). In addition, this group included a number of specimens of *H. necopinatus robertorum*. These all originate from the UK Channel Islands and northern France, the latter in areas where the ranges of *H. necopinatus* and *H. melanarius* still broadly overlap (*Bameul & Queney, 2014*). Again, such a distribution of individuals with discordant morphology and mitochondrial DNA is consistent with this resulting from introgressive hybridization on secondary contact (*Toews & Brelsford, 2012*), with this likely to have occurred before the species colonized the UK Channel Islands, in a manner analogous to that reported by *Prager et al. (1993)* in the case of Scandinavian house mice. Clade *b* also includes the eastern European-Anatolian *H. hebaueri*, which is consistently grouped with *H. melanarius* on the basis of COI sequence. A similar scenario was revealed by *Hernando et al. (2012)*, albeit on the basis of mitochondrial sequences from only three individuals. Such a placement of *H. hebaueri* may result from introgression or the retention of ancestral polymorphism, something which should be explored through the study of both mitochondrial and nuclear sequence variation across a wider range of populations in the future. On the basis of current data, the nesting of all *hebaueri* individuals within *H. melanarius* suggests that this may be due to incomplete lineage sorting.

Our COI data reveal very limited differentiation between the proposed subspecies of *H. necopinatus*. Whilst such a finding could be consistent with these representing distinct

taxa of very recent origin, we favour a somewhat different interpretation. Other than geography, the differences between *necopinatus* subspecies identified by *Fery (1999)*, involve characters of body shape, surface sculpture and the relative length of the apex of the male median lobe. In the case of *H. necopinatus robertorum* and *H. necopinatus roni*, these features are progressively more like those seen in *H. melanarius*, in that these two forms are progressively more parallel-sided, have progressively higher frequencies of strongly reticulated individuals and progressively shorter apices to the male median lobe than *H. necopinatus necopinatus*. In light of the morphological-mitochondrial mismatches discussed above, we suggest that the morphological variation seen across the geographical range of *H. necopinatus* may, at least in part, have resulted from bi-directional introgressive hybridization between it and *H. melanarius*, in areas where the two taxa came into secondary contact during Postglacial range expansion. This hypothesis could be tested in the future, with data from highly variable nuclear DNA markers in the species group. If correct, it suggests that both *H. necopinatus robertorum* and *H. necopinatus roni* have appeared through recent hybridization events, rather than representing discrete evolutionary lineages. The fact that *H. necopinatus* sensu lato and *H. melanarius* remain distinct over most of their ranges, however, suggests that such hybridization is limited, this perhaps being mediated by genotype x environment interactions in these beetles.

Competing Interests

The authors declare there are no competing interests.
   Garth Foster is not an employee of ACCT, which is a registered charity.

Author Contributions

- David T. Bilton conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Lucy Turner analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Garth N. Foster conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The new COI sequences from this study are accessible via GenBank (accession numbers: JN790719–JN790817).

Data Availability

The following information was supplied regarding data availability:

The only raw data are the COI sequences, deposited in GenBank.

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13/13