Zootaxa 4394 (2): 295–300
http://www.mapress.com/j/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.4394.2.12
http://zoobank.org/urn:lsid:zoobank.org:pub:327D4316-98E7-42C1-B8AD-A480DE517BE2

Rediscovery of the critically endangered 'scarce yellow sally stonefly' *Isogenus nubecula* in United Kingdom after a 22 year period of absence

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Abstract

The critically endangered 'scarce yellow sally stonefly' *Isogenus nubecula* (Newman, 1833) (Plecoptera: Perlodidae) was rediscovered in the United Kingdom (UK) in 2017. This rediscovery comes after a 22-year period of absence despite numerous surveys since its last record in 1995. This species is one of the rarest stoneflies in the UK and Europe and its rediscovery is of international significance, being the westernmost point in Europe where the species is found, with the next nearest populations occurring in Austria and western Hungary, Slovakia, and central Sweden. The species is classed as pRDB2 (vulnerable), however is not listed in the British Red Data Book despite only being present (as far as records detail) in one river, the River Dee in North Wales, UK. Only fourteen individuals were caught and the need for conservation of this rare stonefly is therefore of paramount importance. We have made recommendations for the need to increase survey effort using environmental DNA (eDNA) techniques in order to fully understand the species range in this river and those in the surrounding area. The DNA sequence of *I. nubecula* has been uploaded on GenBank for further genetic studies. Captive rearing could also be explored with possible reintroductions to sites within its former UK range.

Key words: *Isogenus nubecula*, Scarce Yellow Sally Stonefly, Rediscovery, DNA

Introduction

Isogenus nubecula (Newman, 1833) is a rare stonefly species that has reportedly become extinct in many European countries from which it had historically been known (Davy-Bowker, 2003). Whilst Derka *et al.,* (2002) described *I. nubecula* as a species originally recorded from many localities in Western and Central Europe. Zwick (1992) noted its widespread disappearance in the second half of the 20th century. In central and eastern Europe, Claessens (1981) found no records since 1936 from Austria, Belgium, Switzerland and the Netherlands, and Landa *et al.,* (1997) considered that *I. nubecula* had probably also become extinct in the Czech Republic and Slovenia. Surviving European populations are few in number and occur mainly in eastern Europe in the Lafnitz and Rába rivers in South-Eastern Austria and western Hungary (Graf & Kovács, 2002; Kovács & Ambrus, 2001); the River Tisza in Hungary (Juhász *et al.,* 1998; Kovács & Ambrus, 2001); the River Jelesna, Slovakia where a single nymph was found in 1998 (Derka *et al.,* 2002); Northrhine-Westfalia in Germany (Graf *et al*. 2009); and in central Sweden (Hoffsten, 2003). *Isogenus nubecula* may potentially also occur in Finland, Estonia, Latvia and Lithuania.

Within the United Kingdom (UK), *I. nubecula* was first recorded in 1959 from the River Dee at Bangor-on-Dee in North Wales (Hynes, 1963). This site has a stone road bridge and a large within-channel island (Fig. 1a). The substratum consists of large gravel and cobbles, much of which is unstable due to high water velocity. A detailed account of the UK records of *I. nubecula* between its discovery in 1959 and 2003 is provided by Davy-Bowker (2003). In summary, its continued presence on the River Dee was confirmed in the surveys carried out in 1981 and 1982 by Mills and Andrew (1984) where its maximum range was recorded at nine of thirteen sites

Accepted by B. Kondratieff: 12 Jan. 2018; published: 14 Mar. 2018

surveyed over a 45km stretch of the river. In 1992, the range of *I. nubecula* had reduced significantly, to just two of its former sites (Bowker, 1993), however in 1993 it was found at five sites (Bowker, 1995), though this reduced again two years later and by 1995 only one nymph was found at one site (Tanner, 1997). Despite repeated surveys conducted in 1997 (Tanner, 1997); 1998 (Millband, 1998); 1999 (Millband, 1999); 2003 and 2004 (Davy-Bowker, 2003; Hammett & Wallace, 2005); 2007 (Davy-Bowker *et al.,* 2007) and unpublished surveys carried out by John Davy-Bowker in 2012 and 2015, no further specimens of *I. nubecula* were found on the River Dee. The September 1995 record was therefore the last record of *I. nubecula* in the UK. Its occurrence from a single river and none of its tributaries, its decline in range, and subsequent disappearance provided the basis for Macadam (2015) in his review of the stoneflies of Great Britain to assign *I. nubecula* the international threat category of 'Critically Endangered'.

Materials and methods

On the 12th March 2017, the River Dee at Bangor-on-Dee (Fig. 1a) was visited and sampled by John Davy-Bowker in an attempt to find *I. nubecula* nymphs. Sampling and bankside sorting was carried out for about two hours and all large Perlodidae nymphs found were transferred to sample containers with river water and oxygenation. Nymphs of different Perlodidae species, including *I. nubecula* (Fig. 1b) were kept in separate containers and aerated until adult rearing.

A follow up survey was also performed by Mike Hammett on the 15th March 2017, at Bangor-on-Dee and also at Erbistock a short distance upstream. The same procedure was followed, but over a 20 minute time period at both sites, with all large Perlodidae nymphs being kept in separate containers and aerated until adult rearing.

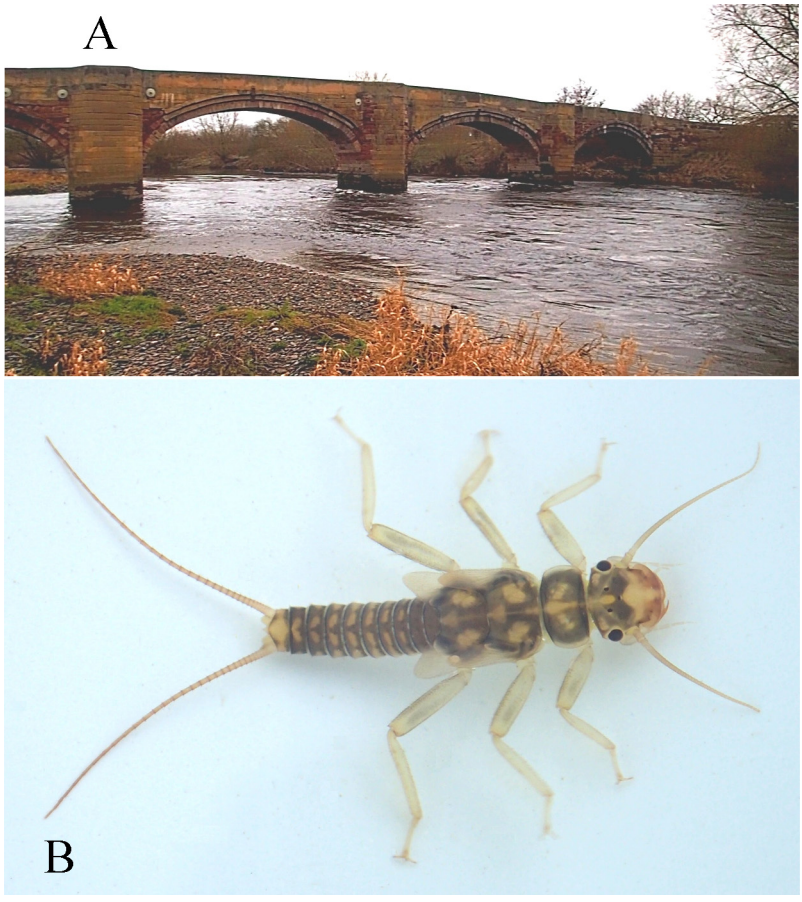

FIGURE 1. (a) The River Dee at Bangor-on-Dee, North Wales, UK and (b) *Isogenus nubecula* nymph (circa 20mm).

Following the completion of adult rearing, one of the prospective *I. nubecula* adults was preserved in >95% ethanol and sent to the University of Derby for DNA extraction and sequencing. All lab equipment was disinfected prior to doing any lab work with 10% bleach and 100% ethanol. Pipettes, tubes and tips were sterilised for 20 min

under UV light before use. Extraction was completed using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions and a polymerase chain reaction (PCR) was performed using primers (Forward) LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and (Reverse) HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al.,* 1994) which amplified the mitochondrial gene cytochrome oxidase 1.

The DNA extraction and sequencing procedure was as follows. PCR was performed in a 25 µL total volume with 12.5 µL of 2x PCRBIO Ultra Mix Red (PCRBIOSYSTEMS), 1 µL of each primer (10µM), 9.5 µL of ddH_2O and 1 µL of DNA template. The PCR programme included an initial 1 min denaturation at 95ºC, 35 cycles of denaturation at 95 ºC for 1 min, annealing at 40 ºC and elongation at 72 ºC for 1 min and 30 s. A final elongation step of 7 min at 72 ºC was added at the end of the PCR (Folmer *et al*. 1994). A negative control was added during the PCR to ensure the absence of contamination.

PCR products were confirmed by electrophoresis on 2% agarose gel stained with 3 µL of GelRed™ Nucleic Acid Gel Stain, Biotium. Product sizes were checked by comparing amplified DNA to 5 µL of PCRBio Ladder IV (PCRBIOSYSTEMS). 15 µL of PCR product added with 2 µL of Forward primer were then sent for sequencing by Eurofins Genomics UK.

Multiple sequences were created from the same individual to ensure no technical errors occurred during sequence amplification, these were aligned using Geneious Pro R6 and a representative sequence was submitted to GenBank under the accession number MF801622. A phylogenetic tree was developed following a global alignment with free end gap and using the Neighbor-Joining method within the Geneious Pro R6 software and using Tamura-Nei's Genetic Distance Model on the cytochrome oxidase subunit I (COI) fragments from stonefly species retrieved from the NCBI database. This phylogenetic tree (Fig. 2) illustrates where *I. nubecula* sits in relation to a range of other available COI sequences for stonefly species found or previously recorded from the UK (Appendix. 1).

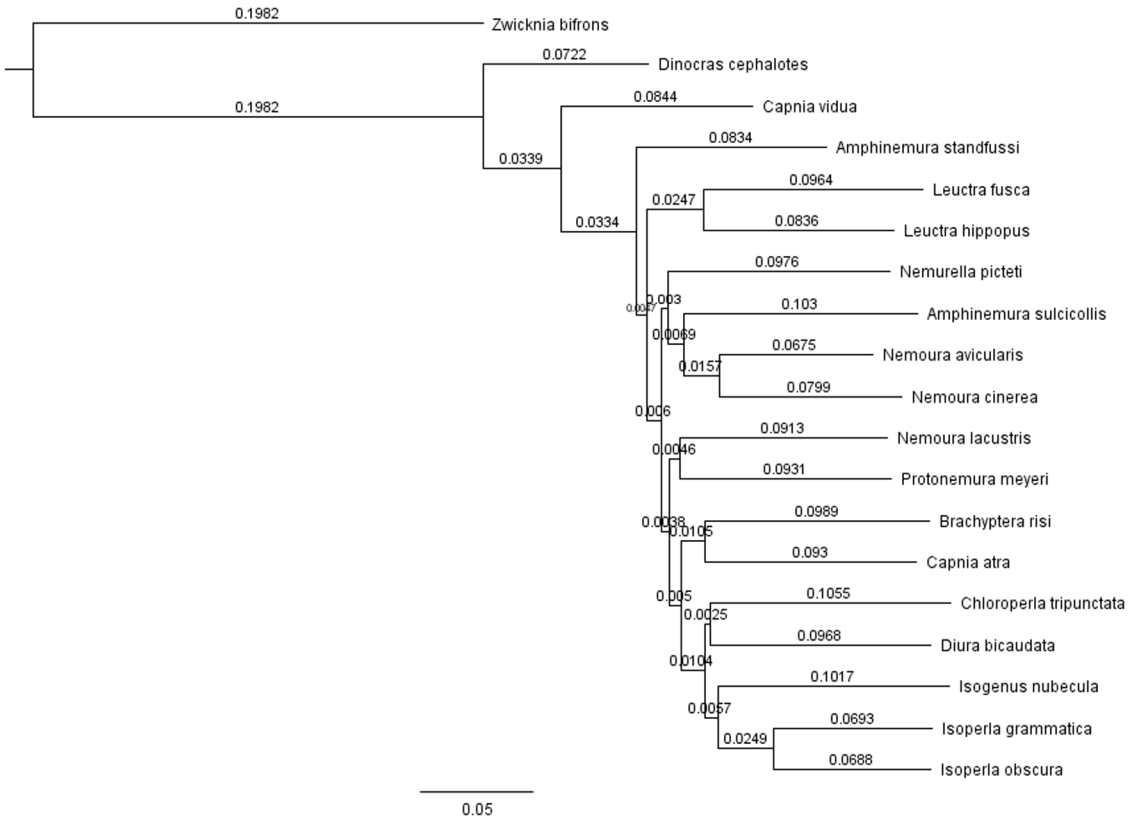

FIGURE 2. Phylogenetic tree obtained by global alignment with free end gap and using Neighbor-Joining method with Geneious Pro R6 software and using Tamura-Nei's Genetic Distance Model on cytochrome oxidase subunit I (COI) fragments from UK stonefly species retrieved on NCBI database. The tree was rooted with *Zwicknia bifrons.* See (Appendix. 1) for available sequences downloaded from GenBank and new sequence codes.

Results and discussion

Following subsequent adult rearing, definitive species identifications were then possible using the key of Hynes (1977). It was confirmed that *I. nubecula* has been rediscovered at Bangor-on-Dee. However, numbers of *I. nubecula* nymphs were low (Table 1). The second species was identified as *Perlodes mortoni* (Klapálek, 1906). (Table 1).

The rediscovery of *I. nubecula* makes this species one of the rarest stoneflies in the UK and Europe and its rediscovery is therefore of international significance. The River Dee population is important at a European level, as this record highlights the westernmost point in Europe where the species is found, with the next nearest populations occurring in Austria and western Hungary, Slovakia and central Sweden.

We draw readers attention to the importance of further monitoring of species such as *I. nubecula,* as stoneflies are an important food source for many species in river systems from fish to many birds (during different life stages). Moreover, they have an essential functional role inside river trophic webs as gatherer-collectors, scrappers, shredders or predators. The conservation of species such as *I. nubecula* is therefore important on many levels. The sequencing of the COI gene of *I. nubecula* (submitted to the NCBI database) can now allow for the development of novel tools for surveying this species.

In this context, further monitoring could be assisted with the development of eDNA techniques targeted for *I. nubecula*. eDNA is a relatively new tool available to conservationists, ecologists and environmental managers alike (Thomsen & Willerslev, 2014) and can significantly aid in understanding a species home range with reduced physical surveying needs and reduced costs (Mauvisseau *et al.,* 2017). Access to this DNA sequence and a greater comprehension of the phylogenetic relationships (Fig. 2) between close relative species are indeed crucial for developing species specific primers. Furthermore, public available repositories already contain COI sequences from various organisms. Supplementing the public database with DNA sequences from this endangered species will enhance our knowledge about phylogenetic relationships in the Perlodidae family. Our sequencing results place *I. nubecula* closest to the genera *Isoperla* and *Diura*. The close genetic relatedness we observe between *Isogenus*, *Isoperla*, and *Diura* is what we would expect within the same family.

Finally, we suggest that *I. nubecula* is also a potential candidate for a captive breeding program, whereby, if successful, individuals could be re-introduced into previously known and still suitable habitats through the River Dee catchment and surrounding area. This could make a significant contribution to safeguarding the future survival of this species in Europe.

TABLE 1. Perlodidae stonefly nymphs recorded from the River Dee at Bangor-on-Dee (national grid reference: SJ 38751 45500) and Erbistock (national grid reference: SJ 35421 41221) in spring 2017. Identifications of *I. nubecula* were confirmed by subsequent adult rearing.

Sampler	Date	Site	Species	Number found
John Davy-Bowker	12th March 2017	Bangor-on-Dee	*Isogenus nubecula*	8
			Perlodes mortoni	7
Mike Hammett	15th March 2017	Bangor-on-Dee	*Isogenus nubecula*	6
			Perlodes mortoni	2
Mike Hammett	15th March 2017	Erbistock	*Isogenus nubecula*	0
			Perlodes mortoni	0

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APPENDIX 1. List of stonefly species and GenBank accession number

Species	Accession number
Capnia atra	KF809156.1
Zwicknia bifrons	KF144842.1
Capnia vidua	JQ736348.1
Dinocras cephalotes	KF492802.1
Chloroperla tripunctata	HQ705654.1
Diura bicaudata	KJ675053.1
Isogenus nubecula	MF801622
Isoperla grammatica	KU955895.1
Isoperla obscura	KJ675043.1
Nemoura lacustris	MF801623
Protonemura meyeri	KF492803.1
Brachyptera risi	KF492801.1
Amphinemura standfussi	JX460920.1
Leuctra fusca	KT807840.1
Leuctra hippopus	KF809176.1
Amphinemura sulcicollis	JX495637.1
Nemoura cinerea	JX495661.1
Nemoura avicularis	JX905857.1
Nemurella pictetii	KF492804.1