DNA BARCODING CONFIRMS BREEDING BY KING DIVING BEETLE *DYTISCUS DIMIDIATUS* (BERGSTRÄSSER 1778) AT THREE SITES IN THE SOMERSET PEAT MOORS

A.F. SERJEANT

Abstract

A molecular ecological solution to the problems of identification allowed the larval stages of Great Diving Beetle (*Dytiscus marginalis*) and King Diving Beetle (*D. dimidiatus*) to be separated, and provided conclusive evidence for the first time of the latter species breeding at three sites in the Brue valley. The DNA barcoding technique is described in detail, as is its application to elucidate the habitat preferences of *Dytiscus* larvae in the Somerset Levels and Moors. The implications of the findings for conservation practice are discussed.

INTRODUCTION – THE GREAT DIVING BEETLES

Beetles of the genus *Dytiscus* are important predators in still freshwater aquatic ecosystems. Experiments and observations in the wild have shown that the colonisation of a pond by a relatively small number of *Dytiscus* beetles can have a profound effect on the balance of invertebrate, vertebrate and plant species which occur (Cobbaert *et al*. 2010). Great Diving Beetles have proved themselves to be serious economic pests of commercial fisheries yet also highly beneficial to humans, through their control of the vectors of major diseases such as bilharzia and malaria (Bhimachar and Tripathi 1966; Reddy *et al*. 1967).

Like most other water beetles, Great Diving Beetles are carnivorous in both the adult and larval forms. The adults are streamlined and admirably adapted for active underwater pursuit of prey. The larvae are consummate ambush predators and can often be seen hanging motionless in the water column suspended just below the surface with only their abdominal breathing apparatus protruding into the air. The somewhat fearsome appearance of the larvae is enhanced by their possession of large, strongly curved mandibles which they use

Fig. 1 Female Dytiscus dimidiatus *(source:AfroBrazilian 2012)*

to pierce the bodies of their victims and through which they pump toxins and enzymes to subdue their prey and to begin the process of digestion.

Four out of six *Dytiscus* species that occur in the UK have been recorded in historic times in Somerset (Duff 1993) and the Somerset Levels and Moors is an important stronghold for the rarest and most threatened of the genus in the UK: the King Diving Beetle (*Dytiscus dimidiatus*) (Fig. 1). It is accorded RDB 3 status which means that it is considered to be rare but not in immediate danger of extinction. Ranging from 32 to 38 mm in length, the adult beetle is the largest of the UK *Dytiscus* species and, where water beetles are concerned, it is second in size only to another Levels and Moors denizen, the Great Silver Water Beetle (*Hydrophilus piceus*). The adult beetle may be distinguished readily from other *Dytiscus* species, such as the much commoner Great Diving Beetle (*D. marginalis*), by its size and also by the markings on its upper surface, reddish underside and the shape of the body armour at the points where the rear legs attach to the body (ie metacoxal processes) (Beebee 1991; Foster and Friday 2011).

PROBLEMS IN THE IDENTIFICATION OF *DYTISCUS* LARVAE

While adults of *D. dimidiatus* are easy to tell apart from other *Dytiscus* species, the larvae are very similar in appearance and present greater identification challenges. There are keys to the larvae of northern European species (eg Rozkošnỹ 1980; Nilsson 1982; Klausnitzer 1991) but these rely on features such as width of head in relation to neck, comparative length of certain body segments, the position of pores or hairs located on mouthparts or on legs and other features that are hard to measure or even to see in the field. The keys mentioned mostly depend on observations made, at best, on a handful of individual larvae that have been reared subsequently to adulthood, so they tend to be based on very small samples from populations of species that may vary in size and form in different geographical localities.

Several years ago I began researching the habitat requirements of *D. dimidiatus* in the Somerset Levels and Moors by examining the characteristics of the ditches in which the species is found. In parts of the Levels and Moors adults of *D. dimidiatus* occur in the same ditches as those of *D. marginalis* and, in many of these ditches *Dytiscus* larvae may also be found. Because of the difficulties outlined above in making positive identifications of larval material, at the beginning of my study I could not be sure whether the larvae that I caught alongside the adults belonged to one or other of these species, were a mixture of both or, indeed, whether other *Dytiscus* species were present in catches. To help clarify the situation, I tried both molecular techniques (specifically, 'DNA barcoding') and microscopic examination.

DNA BARCODING AS AN IDENTIFICATION AID

Traditional taxonomy relies upon morphology to identify and separate species but this can have obvious drawbacks when two or more species resemble each other very closely, as in the case of the larval stages of *Dytiscus* beetles. The rapid development in the last few decades of techniques to probe the genetic make-up of organisms has created opportunities for species identity to be established by molecular means. 'DNA barcoding' is one such technique. Essentially, it depends on finding sequences of bases in certain genes that are unique to a particular species and which occur in each individual of that species but not in other species. The principles underlying DNA barcoding are explained in Box 1.

To enable identification of larvae, firstly I extracted a sample of DNA from the adult beetles which, as we have seen, can be identified readily in the field to species level. For barcoding purposes, I found that it was possible to extract sufficient DNA from a single leg of a beetle. This is because there are so-called 'amplification' techniques available now which can be used to generate huge numbers of copies of particular fragments of a gene from relatively small amounts of source DNA. Using such methods I amplified parts of the CO1 genes in *D. dimidiatus* and *D. marginalis* adults from genetic material which was sent to a specialist laboratory for automated sequencing.

Using these initial sequencing results I identified four short sections of DNA in the CO1 gene that varied in each species by substitution of one base (see Box 2). Thus, for example, part of the gene that read 'GGTTT' on one of the strands of the DNA molecule in *D. marginalis* read

Box 1 DNA Barcoding

Every cell in a multicellular organism is compartmentalised with different parts of the cell performing different functions. The bulk of the genetic material, the DNA, is maintained in the cell nucleus, but small amounts can also be found in structures known as mitochondria which are the cell's energy generating apparatus. The mitochondrial DNA codes for proteins involved in energy production. There are genetic sequences in the mitochondria that are specific, for example, to the Cytochrome Oxidase enzyme which is vital in aerobic respiration. Different genes code for different sub units of the enzyme. The CO1 gene for example controls the building of the sub unit 1.

The proper functioning of the Oxidase enzyme depends crucially on its three dimensional shape which is determined by the order in which the enzyme's protein's building blocks – the amino acids – are added into the growing molecule as it is synthesised. This order is decided by the DNA code, so it can be readily appreciated that alterations to the code can have catastrophic consequences if the cell's entire stock of such a vital enzyme comes to be put together in such a way that it cannot function properly. This means there is great evolutionary pressure on the CO1 gene to stay highly conserved. Yet, despite this the CO1 sequence does vary from species to species. Crucially for taxonomists, it has proved possible to find sections of the CO1 gene that are the same for all members of a species and which can be used to identify individuals of that species and distinguish them from members of closely related species.

The sequencing of DNA fragments was once a time-consuming and expensive affair but the advent of gene sequencing machines has meant that the order of bases in a short length of DNA can be obtained in a matter of hours. The output from these machines often comes in the form of banding patterns indicating the presence or absence of particular DNA bases. These patterns, which are unique to a particular species, suggest analogies with supermarket barcodes which are unique patterns identifying particular products, hence the term 'DNA barcoding' was coined to describe the concept (Hebert *et al* 2004).

Box 2. Identifying sequences in 159 base fragment of CO1 gene from *Dytiscus* beetles from Somerset Levels

D. dimidiatus: ....GTTTA…..GGTTT…..GGATA…..GAGCAT….

D. marginalis: ....GTCTA…..GATTT…..GAATA…..GGGCAT….

G = Guanine T = Thymine A = Adenine C = Cytosine

'GATTT' in the corresponding place along the DNA in *D. dimidiatus.*

Dytiscus larvae were trapped at six separate sites within the Brue valley and a middle leg was removed from each, from which DNA was obtained.The relevant fragment of the CO1 gene was amplified and this amplified DNA was sequenced. Larvae were assigned to a species on the basis of at least two out of the four identifying sequences being found within the larval sequence as reported by the automated laboratory.

IDENTIFICATION THROUGH BIOMETRICS AND MORPHOLOGY

Over the course of several seasons' research I collected over one hundred specimens of *Dytiscus* larvae. I preserved these in 100% ethanol. Legs were taken from most of these preserved larvae so that DNA could be extracted for barcoding. At the same time as the legs were removed the larvae were examined microscopically. I used a Meiji Techno Binocular Zoom microscope with eyepiece graticule to make observations and to measure aspects of larval anatomy, such as head width and lengths of appendages, that are used in the larval keys previously mentioned. A total of 16 observations and measurements were made on each intact larva.

THE TWO APPROACHES COMPARED

Between 2006 and 2011, I caught a total of 223 *Dytiscus* larvae. I released 85 back into the wild after a middle leg was removed, which meant 138 specimens were available for biometric examination. The material examined included not only whole larvae but also specimens that were damaged, possibly due to attempted predation, as well as ones that consisted of partially digested and even fragmentary remains. 119 individuals were sufficiently intact that they could be keyed out using either the key in Klausnitzer (1991) or that in Rozkošnȳ (1980).The great majority of the larvae examined under the microscope (ie 102) were identified as *D. marginalis*, while nine were keyed out as possibly *D. dimidiatus* and eight as possibly *D. circumflexus* (a species with a distinctly coastal distribution in Somerset).

In all but nine instances DNA was extracted successfully for sequencing and in 90% of cases where sequences were obtained the specimens could be assigned unambiguously to a species on the basis of genetic evidence. The majority of specimens assigned to species were identified as *D. marginalis.* No individuals were *D. circumflexus* on the basis of genetic evidence but 17 larvae were identified as *D. dimidiatus* (Table 1). In terms of the latter species, there was no agreement between identifications based on genetics and tentative determinations obtained from keying out specimens.

The failure of the keys to distinguish larvae that were genetically *D. dimidiatus* from those which were *D. marginalis* prompted me to investigate whether there was a suite of morphological characters that might be reliably used to separate the species. I subjected the measurements obtained from intact larvae to Principle Components Analysis (PCA), a statistical technique that arranges a dataset so that similar individuals are

TABLE 1: DETAILS REGARDING LARVAL MATERIAL FROM SOURCES IDENTIFIED AS *DYTISCUS DIMIDIATUS* 2007-2008

Date caught	**Site**	**Number Trapped.**	**Taken (T) or Released (R)**	**Condition if taken**
8/4/2007	Shapwick Heath	1	R	-
5/5/2007	Shapwick Heath	1	T	Whole
20/5/2007	Shapwick Heath	3	3R	-
17/6/2007	Shapwick Heath	2	2R	-
10/5/2008	Westhay Heath	2	1R, 1T	Fragments
2/6/2008	Westhay Heath	1	T	Whole
8/6/2008	Shapwick Heath	1	T	Whole
27/6/2008	Tealham Moor	1	T	Whole
29/6/2008	Westhay Heath	3	2R, 1T	Whole
17/7/2008	Westhay Heath	2	2R	-

grouped together in a conventional 2D graph. PCA summarises many variables in a single plot and allows an objective evaluation of the main causes of variation in the data. In this case, the analysis demonstrated that the *Dytiscus* larvae varied from one another mainly on the basis of length of major body segments and secondly according to the size of appendages.

If gross morphology is a useful way to distinguish one species from another then, ideally, the individuals of one species would cluster together in the PCA plot and there would be clear separation between this cluster and the rest of the individuals in the dataset. Although most *D. dimidiatus* larvae did cluster together there was not sufficient separation from other larvae to enable a definite identification to be made. I concluded from this that only the results from the DNA barcoding could really be relied upon for larval identification.

HABITAT REQUIREMENTS OF *DYTISCUS DIMIDIATUS*

When there is a long run of records of a particular species of beetle from a site it may be legitimate to assume that there is an established breeding colony there, but this is by no means certain when it comes to large species that might disperse over relatively long distances. As with dragonflies, observations of pairs of beetles in copulation may be good evidence that breeding occurs at a particular site, but proven presence of the later stages of larvae is even better.

The results in Table 1 indicate breeding by *D. dimidiatus* at three sites in the Brue Valley. Duff (1993) cites several records from the 1930s and 1940s of this species at Shapwick Heath, where I confirmed larvae present in 2007 and 2008. There is a 1988 record due to A. J. Parsons of the King Diving Beetle at Westhay Heath (in Duff 1993) and I found larvae of the species there in relative abundance in old peat workings. I am not aware that the beetle has been recorded previously from Tealham Moor, so my record of a single larva from this site might be the first. Interestingly, I did not record adult *D. dimidiatus* from Tealham Moor, nor from any of the more open sites that I set traps in during 2008 (East Waste and Catcott Heath). Adults were recorded in numbers at Westhay Moor, but no larvae were caught.

The fieldwork conducted in 2008 included trapping with equal intensity on particular days at pairs of sites chosen to represent examples of shaded habitats (Shapwick Heath, Westhay Heath, Westhay Moor) and unshaded habitats (Catcott Heath, East Waste and Tealham Moor). All the *D. dimidiatus* larvae except for one were captured at sites with a high degree of tree cover shading the water-bodies from which specimens were collected. Adult *D. dimidiatus* too were caught in statistically significantly greater numbers at shaded sites than would be expected if they displayed an equal preference for unshaded ditches as for shaded ones. Although *D. marginalis* larvae also seemed to prefer shaded sites, the adults displayed no particular bias. These results seem to confirm the view that *D. marginalis* is something of a generalist species, whereas *D. dimidiatus* is more specialised.

A typical habitat where *D. dimidiatus* larvae were found is illustrated in Fig. 2. There is more than a passing similarity between this type of habitat – a shaded ditch with relatively low duckweed cover – and that reported by Boyce

Fig. 2 Typical shaded aquatic habitat favoured by Dytiscus dimidiatus *larvae at Westhay Heath, Somerset (Photo taken September 2008 © Pamela Serjeant)*

(2004) as favoured in the Levels and Moors for breeding by the rare Lesser Silver Water Beetle (*Hydrochara caraboides*). Boyce drew attention to a possible association in terms of habitat requirements between *D. dimidiatus* and *H. caraboides* and my study tends to support his hypothesis.

There is some evidence from my study that *D. dimidiatus* abundance may be negatively correlated with high duckweed cover and high electrical conductivity of waters. Since high duckweed cover and high conductivity may both be associated with agricultural pollution, it is possible that absence of *D. dimidiatus* from otherwise suitable habitats could be an indicator of water quality issues.

Other studies of the habitat preferences of *D. dimidiatus*, most notably of the species in Germany (Braasch 1989), concluded that larvae and adults have different requirements. So far as adult beetles are concerned, Braasch found adult beetles in a range of different still waters and he formed the view that *D. dimidiatus* adults were relatively flexible with regards to the water bodies they would frequent so long as these habitats catered for some aspect of adult requirements (in terms of breeding, food, hibernation, dispersal, etc), whether this was over the long term or in the short term (Braasch 1989). Nevertheless, according to Braasch, the adults displayed some preferences for particular types of macro- and micro-habitat, being found more often in fens than in peat bogs and tending to favour richly-vegetated still waters. In contrast to the adults, size of water body seemed important to the larvae identified as *D. dimidiatus* in Braasch's study and he reported that 'nymphs require temporary, semipermanent [sic] and permanent waters of mostly small size'. It was crucial that water should remain over the whole period of larval development (which in his study area, like mine, was generally between May and June). He also concluded that larval water-bodies must have 'little cover of Lemnaceae' (ie duckweed), provide an abundance of prey organisms and support a diversity of vegetation; water bodies with dense algal cover and/or eutrophic conditions were not favourable to *D. dimidiatus* larvae (Braasch 1989).

My findings generally agreed with many of Braasch's. In particular, I found the adults of *D. dimidiatus* in a variety of sites and could not detect a strong niche separation between adults of *D. dimidiatus* and *D. marginalis*, the latter being considered a good example of a generalist species with a wide ecological niche. The larvae of *D. dimidiatus* were far more restricted than the adults, however, and were found in much fewer numbers than *D. marginalis* both in overall terms and in proportion to the adults, which would support the hypothesis that the larval needs of *D. dimidiatus* are different from those of the adult beetles.

With regards to eutrophication, breeding at Tadham Moor (the most eutrophic of my study sites) appears to be not wholly consistent with Braasch's findings. However, it must be appreciated that the Moor is not grossly polluted and it does match some of the other criteria that Braasch identified as important, having well-vegetated waterbodies with an abundance of potential prey (including many aquatic snails).

RECOMMENDATIONS FOR CONSERVATION

So far as *D. dimidiatus* is concerned, the key to maintaining a viable population appears to be retaining landscapes across the Levels and Moors with a diversity of aquatic habitats. For the moment, blocks of wet woodland in close association with waterbodies (whether these are ditches, shallow ponds or lakes), appear to be crucial to breeding success, as the vast majority of larvae that I confirmed as *D. dimidiatus* occurred in heavily-shaded wetland sites. Ditches in more open areas with rich vegetation and diverse invertebrate communities may provide other breeding opportunities (as testified by the finding of a *D. dimidiatus* larva at Tealham Moor), and certainly they offer rich foraging areas for adult beetles. Generally, an improvement in water quality would probable favour *D. dimidiatus*, particularly if this resulted in a reduction in the prevalence of duckweed-choked ditches.

ACKNOWLEDGEMENTS

The research reported here was undertaken as part of a DPhil study supervised by Professor Trevor Beebee. I am greatly indebted to Trevor for his generous support.Thanks are due also to Dr Johannes Bergsten for providing some *Dytiscus marginalis* and *D. dimidiatus* CO1 sequences. The Aquatic Coleoptera Research Trust helped with financial support towards the CO1 gene

sequencing. I am fortunate to have received generous support from my employers, Somerset County Council, not only financially through the award of a training grant, but also in allowing me access to the County Council laboratory. Yanina Pelegri-Fairfax patiently supervised my use of the equipment and instruments in the laboratory and made many helpful suggestions regarding the DNA extraction. I am grateful to Natural England and to the Somerset Wildlife Trust for their kind permission to sample from ditches on their land. The late Dr Pat Hill-Cottingham encouraged me very greatly to take an interest in the water beetle fauna of the Levels and Moors. It was in no small part the example she set as a mature doctoral student working on the Shining Ramshorn Snail (*Segmentina nitida*) that persuaded me that I might also contribute something towards the conservation of the wetland's unique wildlife through a study focussing on a particular Levels and Moors rarity.

Author contact
Tony Serjeant, Somerset County Council, County Hall, Taunton, TA1 4DY
email: afserjeant@somerset.gov.uk

References

AfroBrazilian, 2012. *Dytiscus dimidiatus 01.JPG* [Photograph]. Wikimedia Commons [Online]. Available at http:/commons.wikemedia.org/wiki/File:Dytiscus_dimidiatus_01.JPG

Beebee, T.J.C., 1991. 'Britain's biggest water beetles', *British Wildlife*, 2, 295-300.

Bhimachar, B.S., and Tripathi, S.D., 1966. *A Review of Culture Fishery Activities in India*, Central Inland Fisheries Research Institute, FAO Fisheries Reports No 44, 2. http://www.fao.org/docrep/005/61036T/61036T00.htm#TOC

Boyce, D.G., 2004. *A study of the distribution and ecology of the lesser silver water beetle* Hydrochara caraboides *on the Somerset Levels*, English Nature Research Report No 591, English Nature, Peterborough.

Braasch, D., 1989. 'Zur Habitatwahl von *Dytiscus dimidiatus* BERGSTR.,177 (Insecta, Coleoptera: Dytiscidae)', *Faunistiche Museum für Tierkunde Dresden*, 17, 31-5.

Cobbaert, D., Bayley, S.E., and Greter, J-L., 2010. 'Effects of a top predator (*Dytiscus alaskanus*; Coleoptera: Dytiscidae) on fishless pond ecosystems', *Hydrobiologia*, 644, 103-14.

Duff, A., 1993. *Beetles of Somerset*, Somerset Archaeological and Natural History Society, Taunton.

Foster, G.N., and Friday, L.E., 2011. *Keys to the adults of the water beetles of Britain and Ireland (Part 1),* Handbooks for the Identification of British Insects, Vol. 4, Part 5 (2nd edn), Field Studies Council and Royal Entomological Society.

Hebert, P.D., Cywinska, A., Ball S.L., and deWaard J.R., 2003. 'Biological identifications through DNA barcodes', *Proceedings of the Royal Society B: Biological Sciences*, 270 (1512), 313-21.

Klausnitzer, B., 1991. *Die Larven der Käfer Mitteleuropas. L1 Larvae Adephaga*, Goecke and Evers, Krefeld.

Nilsson, A.N., 1982. A key to the larvae of the Fennoscandian Dytiscidae, *Fauna Norrlandica*, 5, 2, 1-45.

Reddy, K. R., Rao, D.R., and Emaduddin, M., 1967. 'Biological control of fresh water snails by aquatic beetles *Dytiscus marginalis* and *Hydrophilus triangularis* (a preliminary note)', *Indian Vet. J.*, 2, 748-51.

Rozkošnỹ, R. (ed.), 1980. *Klíč vodních larev hmyzu*, Československá Akademie Věd, Praha.