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The species status of *Cathormiocerus britannicus*, an endemic, endangered British Weevil

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Abstract

This study uses DNA sequence and morphometric comparisons to investigate the systematic status of a broad-nosed weevil, *Cathormiocerus brittanicus* (Curculionidae; Entiminae), currently described as an endangered UK endemic. The nuclear marker ITS2 was sequenced from specimens of *C. brittanicus*, *C. myrmecophilus*, *C. curvipes* and *C. maritimus*. Little sequence differentiation was detected between *C. brittanicus* and *C. myrmecophilus*, although both species' sequences were different to those of the other two congeners under study. Multivariate analyses of eight morphometric characters also failed to distinguish *C. brittanicus* from *C. myrmecophilus*. The taxonomic status of the two species is therefore questionable. If they are the same species the endangered status of *C. brittanicus* shall need re-assessing and, because *C. myrmecophilus* also occurs in France, so will its status as a UK endemic. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Conservation; Endemic; ITS2; Parthenogenesis; Specific status

1. Introduction

The broad-nosed weevil genus *Cathormiocerus* (Curculionidae; Entiminae) is small, but zoogeographically interesting as it is restricted to the extreme west of the Palaearctic region (Morris, 1997). Of the 70 species in total only 10 are found outside the Iberian peninsula or Morocco (de la Escalera, 1918). Ten species have been reported from France (Tempére and Péricart, 1989) and five species are known from the British Isles (Morris, 1997).

The biology of the genus is very poorly known, but its species are believed to be similar in many respects to those in the related genus *Trachyphloeus*. *Trachyphloeus* species overwinter as adults (Hoffman, 1950; Hansen, 1965; Dieckmann, 1980; Borovec, 1989), and may be found around the roots of the host plants where they are believed to feed on fresh and partly decomposed plant material (Dieckmann, 1980). *Cathormiocerus* species are believed to be polyphagous and are to be found around the base of such plants as *Plantago* and *Thymus* spp. (Morris, 1997). The elytra are fused suggesting that

their dispersal ability is poor, as is the case in *Trachyphloeus* species (Jermin and Mahler, 1993).

All *Cathormiocerus* species exhibit a form of parthenogenesis termed ploidy stasis (Lamb and Wiley, 1986). The causative agent of parthenogenesis in *Cathormiocerus* weevils is unclear. In the *Aramigus tessellatus* complex (Curculionidae, Naupactini) parthenogenesis is probably caused by polyploidy (Normark, 1996). The bacteria *Wolbachia* has been found in parthenogenetic insects. In these insects it has been shown to be the cause of parthenogenesis and also involved in parasite resistance (Hsiao, 1985a,b; Stouthammer et al., 1990; Hsiao, 1996).

Cathormiocerus species are normally found in marginal habitats of relatively low diversity and it has been suggested that the superior colonising ability of parthenogenetic species enables them to monopolise appropriate niches in such habitats (Lanteri and Normark, 1995).

Cathormiocerus britannicus Blair, 1934 is currently listed as endemic to the UK (Morris, 1997), is described as endangered (Hyman and Parsons, 1992) and has its own Species Action Plan (UK Biodiversity Steering Group, 1999). *C. brittanicus* is very similar to *Cathormicenus myrmecophilus* Seidlitz and the species are presently separated on morphological grounds, namely, differences in the elytra, striae and the rostrum. In the UK both species are confined to coastal habitats

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in the Southern England where they are to be found in short, stony turf (Morris, 1997). *C. myrmecophilus* also occurs in continental Europe (in Normandy, Brittany and south into the Iberian peninsula; Hoffman, 1950).

Taxonomic rank is a very important criterion in assessing the conservation priority of an endangered organism. Because of the close similarity between *C. britannicus* and *C. myrmecophilus* and the complications generated by parthenogenesis and, potentially, by *Wolbachia* infection the taxonomic status of the putative UK endemic is unclear. This study is an investigation of the species status of *C. britannicus* using DNA sequencing and morphometric analysis.

2. Methods

2.1. Specimen collection and identification

Specimens of *Cathormiocerus myrmecophilus, C. britannicus* and *C. maritimus,* were collected with a suction sampler from short turf at known localities on England's south coast (Fig. 1). Very few sites are known for these species and they are all in England's southern counties. Collections were made between 21 and 28 May 1999. *Cathormiocenus curvipes* was collected by searching beneath stones and plants in the Oriental Pyrenees, southern France (Grid Ref: 113427) on 3rd February 2000. Individuals collected were transported live to laboratories where they were transferred to 100% ethanol and refrigerated or processed immediately.

All British specimens were identified using criteria described by Morris (1997). The genitalia of the specimens were dissected to confirm that only females were present. *C. curvipes* and *C. maritimus* can easily be distinguished from *C. myrmecophilus* and *C. britannicus* using morphological characters. Identification of *C. curvipes* was made by using Hoffman's key (1950).

2.2. DNA preparation PCR and sequencing

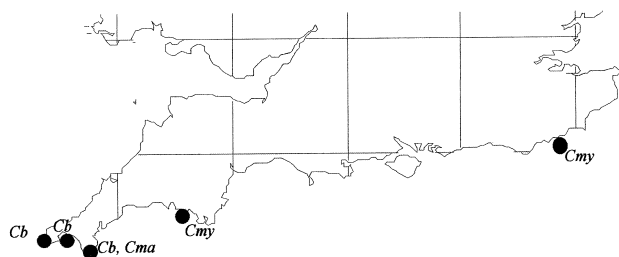The thorax, femurs and (to determine presence of *Wolbachia*) abdomen of specimens were ground to pro-

Fig. 1. Specimen collecting localities (Cb, Cathormiocerus britannicus; Cma, C. maritimus; Cmy, C. mymecophilus).

vide the DNA for the PCR amplification. The DNA was extracted using standard protocols (Juan et al., 1995)

Regions within the ITS nuclear ribosomal complex were amplified using PCR. The total volume of the reaction mixture was 25 µl. ITS2 amplification used the primers described by White et al. (1990): ITS2 (5.8s), 5'-TGTTGACTCTAAATATGACGTCGTCACCTCAC-CG-3' and ITS2 (28s), 5'-GACCCCGGGTAAAATG-TTACGAAAATAAAC-3'. The contents of this mixture were as follows: 17.5 µl H_2O, 2.5 µl Taq buffer, 3 µl $MgCl_2$ and *Taq* polymerase (buffer, salts and polymerase all Promega), 0.25µl dNTPs (20 mM), 0.175µl of each primer (20 mM) 0.25 µl of bovine serum albumen and 1 µl of DNA extract. Cycling involved an initial denaturation of 94°C for 30 s then 25 cycles of 92°C for 1 min, 50°C for 2 min, 72°C for 2 min and a final elongation of 72°C for 5 min. PCR products were concentrated and run through a 1% agarose gel. The stained bands containing the DNA were excised and the DNA was extracted in accordance with the QIAquick gel extraction protocol (Qiagen).

The DNA extracted from the gel was then applied to a sequence PCR reaction and sequenced following the manufacturers instructions (Perkin Elmer applied biosystems).

2.3. Wolbachia detection

The PCR approach used to detect *Wolbachia* used the primers desribed by O'Neill et al. (1992): 99f, 5'-GAA-TAGGTATGATTTTCATGT-3'; 994r, 5'-GAA-TAGGTATGATTTTCATGT-3'. Cycling involved an initial denaturation at 93°C for 5 mins then 34 cycles of 93°C for 1 min, 49°C for 1 min 30 s, 72°C for 1 min and a final elongation of 72°C for 5 min.

2.4. Morphometric analysis

Thirteen *C. myrmecophilus* and 36 *C. britannicus* were analysed using image analysis equipment and computer software (Micro measure, version 3). Eight measurements were made on each individual (Fig. 2) and these were used in a principal components analysis (PCA; SPSS). PCA reduces data from multidimensional distributions to newly derived axes that maximally explain variation in the dataset. PCA is a very useful tool for exploring the morphometric data which can then be analysed further using discriminant function analysis (DFA). DFA generates a set of vectors that best separate a priori determined groups. DFA compares the variation between groups to that within groups and assesses the significance of any differences observed. Thus, both analyses were performed, with the latter using only the characters with the highest factor loadings in the former.
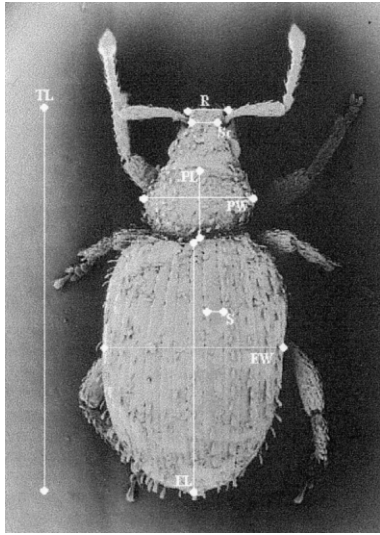
Fig. 2. Scanning electron micrograph of *Carthomiocerus britannicus* showing the morphological characters that were measured. EL, elytra length; EW, elytra width; S, striae convergence; PL, pronotum length; PW, pronotum width; R, rostrum convergence; Sc, distance between scrobes and TL, total length (total length = 3 mm approx.).

3. Results

Table 1 shows pair-wise Kimura two-parameter distances for all the pairs of the 13 ITS2 sequences. Approximately 670 bp of the ITS2 region were sequenced. One specimen for each of the *britannicus* sites was sequenced. Three specimens for the Sussex *C. myrmecophilus* and two specimens for the Devon *C. mymecophilus* were sequenced. The two sequenced specimens of *C. maritimus* came from the same Cornwall location. The two sequenced *C. curvipes* specimens came from the same location in the oriental Pyrenees. The degree of DNA differentiation between *C. myrmecophilus*

and *C. britannicus* is almost equal to the amount of differentiation that is observed within either species. However, when either of these species was compared to the two other congeners, the level of differentiation is much higher. There was no DNA differentiation observed between the *C. myrmecophilous* specimens (Table 1).

Results of the tests for *Wolbachia* showed that both *C. myrmecophilus* and *C. britannicus* are infected with the bacteria. The presence of the bacteria was observed in all of the populations that were sampled. No *Wolbachia* were found in *C. curvipes* or *C. maritimus*.

Generally, the morphometric characters measured were weakly correlated (Table 2) suggesting sufficient independence among variables for use in the subsequent multivariate analyses. The first two principal components of the PCA analysis explain 53.6% of the total variance in the dataset (Fig. 3, Table 3). Loadings of the first principal components are all positive. Consequently this component is related to overall size (Manly, 1986). The second component as well as any other component will relate to overall shape. Discriminant analysis reveals that, on the basis of the traits measured, the two weevil species are not readily distinguishable. Discriminant classification shows that 19% of the *C. myrmecophlilus* group were misclassified as *C. britannicus* and 23% of the *C. britannicus* were misclassified as *C. myrmecophlilus*.

4. Discussion

Neither the molecular or the morphometric analyses described here provide any evidence to support the species separation of *C. brittanicus* and *C. myrmecophilus*. On the basis of the multivariate analysis of the morphometrical data it is impossible to define *C. britannicus*

Table 1
Pair-wise Kimura two-parameter distances for rDNA ITS2 sequences (all gaps in the aligned sequences were removed for the purposes of the pair-wise analysis)[a]

	bri023	bri050	bri094	bri095	myr002	myr004	myr008	myr009	myr018	cur122	cur123	mar013	mar052
bri023	0.0000	0.0019	0.0059	0.0020	0.0019	0.0019	0.0019	0.0019	0.0019	0.1702	0.1684	0.2185	0.2179
bri050		0.0000	0.0039	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1683	0.1742	0.2167	0.2186
bri094			0.0000	0.0020	0.0039	0.0039	0.0019	0.0039	0.0039	0.1753	0.1746	0.2247	0.2167
bri095				0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1724	0.1682	0.2223	0.2157
myr002					0.0000	0.0000	0.0000	0.0000	0.0000	0.1676	0.1687	0.2157	0.2165
myr004						0.0000	0.0000	0.0000	0.0000	0.1647	0.1740	0.2147	0.2143
myr008							0.0000	0.0000	0.0000	0.1705	0.1735	0.2197	0.2163
myr009								0.0000	0.0000	0.1679	0.1745	0.2162	0.2176
myr018									0.0000	0.1647	0.1699	0.2147	0.2158
cur122										0.0000	0.0013	0.1290	0.1261
cur123											0.0000	0.1245	0.1279
mar013												0.0000	0.0015
mar052													0.0000

[a] bri, Caththormicerus brittannicus; myr, C. myrmecophilus; cur, C. curvipes; mar, C. maritimus; 023, Rinsey Head (Cornwall); 050, Cudden Point (Cornwall); 094, Lizard Penninsula (Cornwall); 095, Logan Rock (Cornwall); 002/004/008, Hastings Country Park (Sussex); 009/018, Stoke Point (Devon); 122/123, Tour Magdeloc (S. France); 013/052, Rinsey Head (Cornwall)].

Table 2
Correlation matrix of morphometric variables (* = correlation is significant at the 0.05 level. ** = correlation is significant at the 0.01 level)

	Elytra length	Elyrtra width	Rostrum convergence	Scrobe separation	Pronotum width	Pronotum length	Striae convergence	Total length
Elytra length	1							
Elyrtra width	0.60**	1						
Rostrum convergence	0.13	0.09	1					
Scrobe separation	0.22	0.37**	0.20	1				
Pronotum width	−0.21	0.09	0.03	0.32*	1			
Pronotum length	−0.04	0.15	0.03	0.37**	0.72**	1		
Striae converegnce	0.11	0.05	0.17	0.09	−0.10	−0.19	1	
Total length	0.52**	0.37**	0.06	0.24	−0.12	0.08	0.19	1

*$P < = 0.05$.
**$P = 0.01$.

as a species. The morphological variables that are used to separate *C. myrmecophilus* and *C. britannicus* are all of the continuous type therefore variation is expected to be apparent in these variables within, as well as between populations.

Although it may be argued that morphological features commonly used in identification, such as genitalia, were not compared in this study the use of genitalia in differentiating parthenogenetic species is best approached with caution (Jermin and Mahler, 1993). This is due to the fact that there will be disruption of the concurrent selection of male and female genitalia caused by the lack of males. Selective pressures on the genital characters will reduce or cease (Jermin and Mahler, 1993). This will result in the genital shapes varying more freely making them inappropriate for identification purposes.

The lack of genetic variation that was observed between *C. myrmecophilus* and *C. britannicus* leads to the conclusion that the two are very similar indeed. Although each species was different from *C. curvipes* and *C. maritimus*, no evidence was found for any significant degree of sequence divergence between *C. britannicus* and *C. myrmecophilus*. *C. britannicus* was originally described from 12 specimens (Blair, 1934) and

in this description the author mentions that it was very difficult to define which of the specimens were *C. myrmecophilus* or *C. britannicus*.

These findings have some important implications for conservation. The continued recognition of two separate species has no phylogenetic support. *C. britannicus* is currently listed as endemic with a status of endangered and its own species action plan. If it is to be viewed as the same as *C. myrmecophilus* then will the status of *C. myrmecophilus* (RDB3 rare) be modified? If the sites that are known for *C. britannicus* are added to the sites that are all ready known for *C. myrmecophilus* then the total of inhabited 10-km squares does not reach the threshold of 15 10-km squares. This is the cut off point for relegation into one of the lower priority categories (Soumalainen, 1950).

Concern has been expressed over the application of genetic techniques to populations of conservation importance. It is believed that these techniques will lead to the exaggeration of the number of 'species' in the absence of any other type of distinctiveness (Avise, 1989). The results of this study show that the opposite effect can be realised by addressing the genetic differentiation among morphs previously recognised as distinct species. Work on vertebrates has shown that the definition of a species based on colour are not reflective
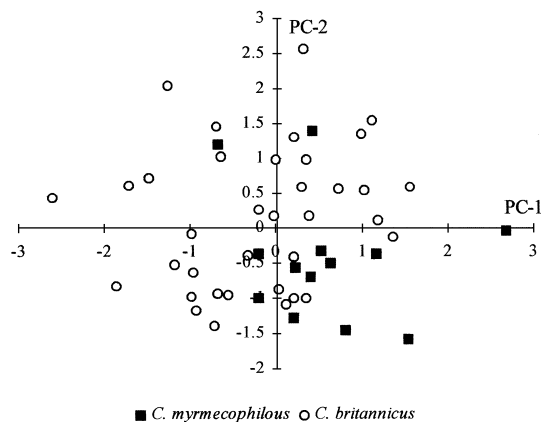

Fig. 3. First (PC-1) and second (PC-2) principal component axes for individuals in the two putative species.

Table 3
Factor loadings for the first two components extracted by the principal components analysis

Morphological characters	PC-1	PC-2
Elyrtra width	0.7789	−0.1605
Elytra length	0.7782	−0.4241
Total length	0.7328	−0.2793
Scrobe separation	0.5602	0.4507
Pronotum width	0.1336	0.8940
Pronotum length	0.2982	0.8344
Rostrum convergence	0.2490	0.1909
Striae converegnce	0.0423	−0.2005
% of total variance explained	27.9	25.7

of the underlying genetic differentiation (Avise and Nelson, 1989; Karl and Bowen, 1999).

The effect of environmental factors, such as temperature, have been shown to effect morphology. It has been shown that an array of developmental temperatures will produce variation in the morphology of the adult insects concerned (Hogue and Hawkins, 1991).

Very little is known of the biology of the *Cathormiocerus* species. Their conservation priority is based on their localised distributions. New sampling methods employed in this study have shown that in suitable habitat these beetles can be found in relatively high densities.

This study showed that *Wolbachia* is present in the species of interest. To examine if these bacteria are responsible for inducing parthenogenesis would require experiments with antibiotics. Some strains of this bacteria have been shown to induce parthenogenesis in the hymenopteran genus *Trichogramma* (Stouthammer et al. 1990). Experiments with tetracycline may aid examination of the effects of infection on morphology of the adult. In the weevil *Hypera postica* it has been shown that the bacteria are responsible not only for reproductive incompatibility (Hsiao and Hsia, 1985a, b) but also for conferring resistance to the hymenopteran parasite *Bathyplectes curculionis* (Hsiao, 1996). It would be most interesting to elucidate the exact relationship that *Wolbachia* has with *C. myrmecophilus*.

In conclusion it seems that the species status of *C. britannicus* is very questionable. It is therefore worth considering, on the basis of this evidence, if *C. britannicus* should be removed from the Biodiversity Action Plan and the resources better applied to another insect species of conservation concern where the systematic status is assured.

Acknowledgements

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