



## Phylogeny and DNA barcoding of inquiline oak gallwasps (Hymenoptera: Cynipidae) of the Western Palearctic

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### ABSTRACT

We examine phylogenetic relationships within the *Synergus* complex of herbivorous inquiline gallwasps (Hymenoptera; Cynipidae; Synergini) associated with cynipid host galls on oak, a biologically diverse group whose genus-level morphological taxonomy has long been considered stable but whose species level taxonomy is problematic. We incorporate data for over 70% of recognised Western Palearctic species in five morphology-based genera (*Ceroptres*, *Saphonecrus*, *Synergus*, *Synophrus*, *Ufo*), comprising sequence for two mitochondrial loci (*cox1*, *cytb*) and one nuclear locus (28S D2). In particular, we assess the evidence for monophyly of two long-established, morphology-defined sections within the genus *Synergus* that differ in a range of biological traits. To aid analyses of ecological interactions within oak cynipid communities, we also consider the utility of cytochrome oxidase I (*cox1*) DNA barcodes in the oak inquilines. In this assessment, we do not assume that species are delineated at a single threshold value of sequence divergence for a single gene, but examine concordance in the composition of molecular operational Taxonomic units (MOTUs) across a range of sequence divergences in each gene and across genes. We also assess the impact of sampling effort on MOTU stability.

Phylogenetic reconstructions for all three loci support monophyly for *Synergus* and *Synophrus*, but reject monophyly for *Saphonecrus* and for the two sections within *Synergus*. The suites of traits associated with the two sections of the genus *Synergus* are thus homoplasious. All three loci also reject monophyly for three *Synergus* species (*S. hayneanus*, *S. pallipes*, *S. umbraculus*). Sequences for each locus identify robust MOTUs that are largely concordant across loci for a range of cut-off values. Though many MOTUs correspond to recognised Linnean species, there is significant, multigene disagreement between groupings supported by morphology and sequence data, with both allocation of different morphospecies to the same MOTU and allocation of the same morphospecies to multiple MOTUs, regardless of cut-off value. Our results imply that while DNA barcoding has considerable utility within this group, morphology-based identification needs major revision at both genus and species levels. Further, life history traits currently attributed to single morphospecies probably confound attributes of multiple lineages. Revealing patterns of character state evolution in *Synergus* requires collection of new host association and life history data explicitly linked to DNA barcode data for the specimens concerned.

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### 1. Introduction

Accurate taxonomy and sample identification are crucial to analyses of the ecology and evolution of species, higher-level taxonomic groups and communities. Morphological taxonomy has long been used to define species using consistent differences in

external characters, but the scale of the challenge of identifying and classifying all species in this way is prohibitive (Tautz et al., 2003). While approximately 1.5 million, predominantly insect, species have been described to date (de Meeus and Renaud, 2002), these represent only a small proportion of estimated global diversity and molecular studies continue to reveal cryptic taxa inseparable on the basis of morphological taxonomy (e.g. Papakostas et al., 2005; Smith et al., 2006, 2007; Bergmann and Russell, 2007; Starrett and Hedin, 2007), increasing the magnitude of the challenge.

Approaches based on genetic markers, particularly DNA sequence data, are increasingly used to augment or replace morphological taxonomic analyses (Tautz et al., 2003). As discussed by Vogler and Monaghan (2006), at least 3 conceptually different but related approaches have been used. DNA taxonomy uses patterns of variation in DNA sequence data to define taxa *a priori*, without reference to morphological data, though it can enable the identification of diagnostic morphological characters. This approach uses no pre-defined level of difference (e.g. % sequence divergence) to define taxa, but attempts to identify independently evolving lineages. Because the topology of an individual gene tree can differ significantly from population and species trees, identification of such lineages is best approached using data for multiple loci (Meyer and Paulay, 2005; Hickerson et al., 2006; Vogler and Monaghan, 2006). A second approach captures the sequence diversity present in a group of samples by identifying molecular operational taxonomic units (or MOTUs; Floyd et al., 2002), defined as a group of sequences differing from one another by a specified maximum number of base pairs (Blaxter, 2004). MOTU richness is a useful summary measure of sequence diversity, particularly in describing samples of morphologically cryptic taxa. However, MOTU approaches have the drawback in comparison with DNA taxonomy that the link between MOTU membership and biological species status remains unclear (Vogler and Monaghan, 2006).

DNA barcoding is the third approach, and rather than defining taxa *a priori*, it uses sequence similarity at a single 'barcode' locus (in Metazoa, usually the mitochondrial locus cytochrome c oxidase subunit I, *coxI*) to allocate unknown specimens to morphologically determined voucher taxa (Floyd et al., 2002; Blaxter, 2004; Hebert et al., 2004a,b; Powers, 2004; Blaxter et al., 2005; Hajibabaei et al., 2005; Lambert et al., 2005; Ward et al., 2005). The underlying rationale for DNA barcoding is that sequence variation among species is greater than (and discrete from) variation within species: in other words, that sequence variation at the selected locus shows a 'barcoding gap' (Fig. 1). Given this assumption, unidentified specimens that differ by less than a threshold sequence divergence (operationally taken as 2% for *coxI* in Metazoa; Hebert et al., 2003 and see <http://www.barcodinglife.com>) from voucher sequence for a reference species can be allocated to that species, and sequences more divergent than the selected threshold from any reference taxon will

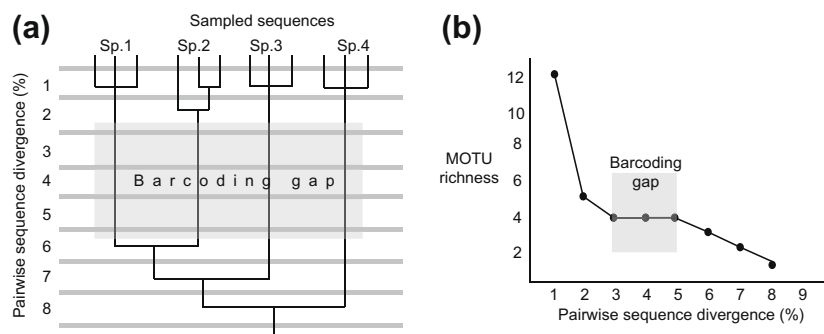
be allocated to a new species (Hebert et al., 2003). This approach can be effective in revealing morphologically cryptic taxa, and so inform revision of morphological taxonomy (e.g. Smith et al., 2006, 2007; Challis et al., 2007; Stone et al., 2008).

However, there are major potential drawbacks of using a single mitochondrial barcode marker in this way. Coalescent theory predicts that both intraspecific sequence diversity and the probability of shared barcode sequences among discrete biological species will be sensitive to population demography, particularly species age and past effective population size (Moritz and Cicero, 2004; Meyer and Paulay, 2005; Hickerson et al., 2006; Nielsen and Matz, 2006; Knowles and Carstens, 2007). Young species with large effective population sizes are predicted not to be monophyletic for their barcode sequence, and where past population sizes vary substantially within a group of taxa, a single threshold divergence is unlikely to separate intraspecific variation from variation among species – in other words, there may be no barcoding gap (Funk and Omland, 2003; De Salle et al., 2005; Meyer and Paulay, 2005; Cognato, 2006; Vogler and Monaghan, 2006; Rubinoff, 2006). Heteroplasmy, introgression (with or without selective sweeps imposed by *Wolbachia* and other symbionts), potential selection on nucleotides, and the existence of nuclear pseudogenes can all lead to conflicts between species membership and grouping by DNA barcode (Hebert et al., 2004a,b; Hurst and Jiggins, 2005). DNA barcoding is thus best used where (i) the presence of a barcoding gap for the barcoding locus can be demonstrated empirically, and (ii) concordance in gene tree topology can be demonstrated between the barcoding locus and at least one nuclear locus (Blaxter, 2004; Ahrens et al., 2007; Smith et al., 2007; Cardoso et al., 2009). More broadly, the utility of DNA barcoding is arguably greatest where existing morphological taxonomic expertise allows molecular and morphological approaches to be compared, revealing the shortfalls of traditional approaches but providing biological context to sequence-based taxa (e.g. Cardoso et al., 2009).

Here we examine the molecular taxonomy and potential for DNA barcoding of a group of insects whose morphological taxonomy is problematic – the inquiline gallwasps of the tribe Synergini (Hymenoptera: Cynipidae).

### 1.1. The inquiline gallwasps

The tribe Synergini contains *ca.* 170 species that inhabit plant galls induced by other insects – primarily other gallwasps (Cynipidae; reviewed in Csóka et al., 2005). The inquilines are highly specialised herbivores, able to modify the host plant tissues on which they feed, but dependent on true gall inducers to initiate gall formation (agastoparasites *sensu* Ronquist, 1994; Brooks and Shorthouse, 1998; Sanver and Hawkins, 2000; Stone et al., 2002). They attack the galls of hosts in the gallwasp tribes Cynipini (oak gallwasps, hosts to



**Fig. 1.** Diagrammatic representation of the barcoding gap. (a) Phylogenetic representation of sampled sequences, showing the separation of intraspecific and interspecific sequence variation assumed in single locus barcoding. (b) The relationship between the threshold used to divide MOTUs (here, % sequence divergence) and the number of MOTUs defined in a given sample (MOTU richness). In this idealised example, the barcoding gap is revealed as a plateau in MOTU richness over a threshold range.

the inquiline genera *Ceroptres*, *Saphonecrus*, *Synergus*, *Synophrus* and *Ufo*), Diplolepidini (rose gallwasps, hosts to the inquiline genus *Periclistus*) and Aylacini (herb gallwasps, hosts to the inquiline genus *Synophromorpha*) (Ronquist, 1994, 1999; Csóka et al., 2005). The major exception to attack of cynipid hosts is the inquiline genus *Rhoophilus*, whose single species inhabits galls induced by Cecidosid moths on *Rhus* species (sumacs; van Noort et al., 2006).

Gall communities have been the subjects of numerous studies of community structure and evolution (Stone et al., 2002, 2009; Stone and Schönrogge, 2003; Bailey et al., 2009), and cynipid inquilines have a major impact on food web structure and community species richness (Schönrogge et al., 1995, 1996a,b; Schönrogge and Crawley, 2000; Stone et al., 2002). The morphological taxonomy of the inquilines has been studied in depth (Mayr, 1872; Wiebes-Rijks, 1979; Nieves-Aldrey and Pujade-Villar, 1985, 1986; Pujade-Villar and Nieves-Aldrey, 1990, 1993; Liljeblad and Ronquist, 1998; Nieves-Aldrey, 2001; Pujade-Villar et al., 2003). On the basis of morphology, the most species-rich genus, *Synergus*, is divided into two sections (Sections I and II; Mayr, 1872) that also differ in a range of biological traits. Section I species are predominantly univoltine, and their development in a host gall is rarely lethal to the gall inducer. In contrast, Section II species are predominantly bivoltine, and their development in a host gall often kills the gall inducer (Csóka et al., 2005). Section II species are also characterised by high between-generation variation in some adult morphological attributes, particularly size and colour (Nieves-Aldrey and Pujade-Villar, 1986; Pujade-Villar, 1992; Wiebes-Rijks, 1979), which can make morphology-based identification difficult. As a result, morphology-based identification of adults is often only possible to complexes of morphologically similar species, and identification of most immature stages, desirable in food web analyses, is currently impossible. Demonstration of effective DNA barcoding in this group would thus greatly facilitate the generation of more comprehensive food web data.

Here we assess the utility of two mitochondrial loci (*coxI* and cytochrome *b*, *cytb*) and one nuclear locus (the D2 region of the 28S ribosomal RNA gene) in resolving the relationships among inquiline oak gallwasp lineages, incorporating data for over 80% of recognised Western Palaearctic species and all recognised genera. We examine the concordance between mitochondrial and nuclear gene trees, particularly important here because two processes known to influence mitochondrial sequence diversity – introgression and infection with *Wolbachia* symbionts – occur in inquiline and gall inducing Cynipini (Rokas et al., 2001, 2002a, 2003a, 2003b). We choose 28S D2 as the nuclear locus because it has proven valuable in resolving species level relationships in cynipids in the past, and in contrast to alternatives such as long wavelength opsin and elongation factor 1, it can be amplified with highly conserved primers (see Methods: problematic for long wavelength opsin in some cynipids: Stone et al., 2009) and does not require cloning (sometimes required for opsin and elongation factor 1; Rokas et al., 2002b; Stone et al., 2009). The need for further phylogenetic analysis of oak inquiline gallwasps is supported by the fact that although previous analyses support monophyly for the complex as a whole (Ronquist, 1994; Ronquist and Liljeblad, 2001; Nylander et al., 2004a, 2004b; Nieves-Aldrey et al., 2005), the genera and species within the complex are difficult to distinguish morphologically (Pujade-Villar et al., 2003). Though some species in this complex have been included in previous molecular phylogenetic analyses (e.g. Rokas et al., 2002b; Nylander et al., 2004a, 2004b; Péntzes et al., 2009), sampling of *Synergus* has been very limited. A recent molecular analysis of the genera *Synophrus* and *Saphonecrus* (Péntzes et al., 2009) revealed the possible diphyly of the genus *Saphonecrus*, though placement of these genera relative to *Synergus* was not investigated. We address the following questions: (a) Are the genera *Synophrus*, *Saphonecrus* and *Synergus* monophyletic groups, and what are the phylogenetic relationships between them? (b) are Sections

I and II of *Synergus* monophyletic groups? If yes, then diagnostic biological traits have been conserved within two divergent lineages. Rejection of section monophyly would imply that biological trait evolution in *Synergus* is more labile than currently accepted. (c) Is there evidence of morphologically cryptic sequence diversity, suggesting the need for revision of current estimates of taxon diversity?

We then explore the potential for DNA barcoding in the oak-associated Synergini. First, we assess the empirical support for a barcoding gap in *coxI* and *cytb*. Rather than assuming a single cut-off threshold for definition of MOTUs (e.g. 2% sequence divergence for *coxI*), for the two mitochondrial genes we examine the impact of variation in the cut-off threshold from 0% to 12% on MOTU membership. Because MOTU richness and composition can be sensitive to taxon sampling (Meyer and Paulay, 2005), we also explore the impact of sampled sequence diversity on MOTU richness. We address the following additional questions: (d) Do *coxI* MOTUs capture Linnean species? If not, are separate Linnean species represented by sequences in the same MOTU (Type I error *sensu* Quicke, 2004)? (e) Do *coxI* barcodes reveal cryptic taxa unrecognised by existing morphology-based taxonomy (Type II error *sensu* Quicke, 2004)? (f) To what extent are MOTUs identified using *coxI* sequence data concordant with those supported by *cytb* and 28S D2? (g) To what extent is MOTU membership sensitive to the number of sequences included in the analysis?

## 2. Materials and methods

### 2.1. Taxon sampling

Because tests of the efficacy of DNA barcoding are highly dependent on thorough taxon sampling (Meyer and Paulay, 2005), we sampled as many of the described species of Western Palaearctic Synergini associated with oak cynipid hosts as possible. We obtained data for 184 specimens (see Supplementary online Appendix S1) comprising 33 of the 45 described species (73%: 2/2 *Ceroptres*, 6/6 *Saphonecrus*, 23/30 *Synergus*, 2/7 *Synophrus*; Csóka et al., 2005; Sadeghi et al., 2006; Penzes et al., 2009). Because the ability of DNA barcodes to assign individuals to species requires adequate sampling of intraspecific as well as interspecific variation (Moritz and Cicero, 2004; Morando et al., 2003), where possible we incorporated samples from multiple refugial centres of intraspecific diversity (Rokas et al., 2003b) across the Western Palaearctic from Spain to Iran (Appendix S1). Sampling was strongest for *Synergus*, in which 11 species were represented by four or more *coxI* sequences, and four species (*S. hayneanus*, *S. pallicornis*, *S. pallipes* and *S. umbraculus*) were represented by between 10 and 15 sequences (Appendix S1). This variation in sampling effort approximately reflected the abundance of species in our rearings, and for the most extensively sampled species was also driven by some existing concern over the difficulty of defining morphological species limits. Examination of conflicts between Linnean species designation and *coxI* MOTU membership is thus restricted to *Synergus*.

Unsampled species are either extremely local in distribution, such that we were unable to obtain samples (*Synophrus olivieri*, *Synergus ibericus*, *S. ilicinus*, *S. ruficornis*, *S. subterraneus* Pujade-Villar et al., 2003) or are morphologically very similar to (and probable synonyms of) sampled species (*S. dacianus* of the sampled species *S. crassicornis*; *S. synophri* of the sampled species *S. hayneanus*; and *S. radiatus* of the sampled *S. pallipes*). All sampled inquilines were reared from oak galls, except for *Rhoophilus loewi*, which was reared from *Scyrotis* sp. (Lepidoptera, Cecidosidae) galls induced on *Rhus* in South Africa. Specimens were identified by Melika and Pujade-Villar, recognised experts in the field of morphological cynipid taxonomy and the authors of the existing keys (Pujade-Villar et al., 2003). Due to difficulties in morphological identification, some individual

specimens were assigned >1 morphospecies name (see Appendix S1). The Eastern Palaearctic (Abe et al., 2007) and Nearctic (Burks, 1979) inquiline faunas are far less known. To begin the process of assessing relationships between these regional faunas, and in particular to explore whether Western Palaearctic MOTUs span the palaearctic into Asia, our analysis incorporates two *Synergus* species from China (*Synergus chinensis* and *S. xiaolongmeni*; det. G. Melika) (Melika et al., 2004), and one from Japan (*Synergus japonicus*; det. Y. Abe).

## 2.2. DNA extraction and sequencing

DNA was extracted from a single hind leg of most specimens using a simple Chelex-based protocol (Lopez-Vaamonde et al., 2001). Based on prior experience (though not a hard and fast rule), for insects less than 2 mm long we extracted DNA from the whole wasp using the DNeasy Tissue Kit (QIAGEN cat. 69504).

For cytochrome *b* (*cytb*), we first used the following primers: forward primer, CB1 TATGTACTACCATGAGGACAAATATC, reverse primer, CB2 ATTACACCTCCTAATTTATTAGGAAT (Jermiin and Crozier, 1994). Where amplification using the CB1/CB2 primer pair failed, an overlapping fragment was amplified using the CP1/CP2 primer pair: CP1 GATGATGAAATTGGATC, CP2 CTAATGCAATAACTCTCC (Harry et al., 1998). For cytochrome *c* oxidase subunit I (*coxI*), we used forward primer, LCO1490 GGTCAACAATCATAAAGATATTGG, and reverse primer HCO2198 TAAACTTCAGGGTGACCAAAAAAT (Folmer et al., 1994). For the D2 region of the nuclear 28S ribosomal RNA gene, we used forward primer CGTGTGCTTGATAGTGCAGC, and reverse primer TCAAGACGGTCTGAAAGT (Heraty et al., 2004). We also amplified the D3-5 region of the 28S ribosomal RNA gene, using forward primer ACACACTCCTTAGCGGA, and reverse primer GACCCGTCTTGAACACGGA (Friedrich and Tautz, 1995).

For all loci, 25 µl polymerase chain reactions (PCRs) were carried out in a PTC-200 DNA Engine (MJ Research) using 1 U Taq polymerase (Invitrogen or Promega), 2.5 µl 10× Taq buffer, 1.5 µl MgCl<sub>2</sub> (25 mM), 0.5 µl dNTPs (10 mM), 0.35 µl primers (20 pmol), 1.0 µl template DNA and 18.85 µl dH<sub>2</sub>O. PCR products were purified using shrimp alkaline phosphatase and *Escherichia coli* exonuclease I (USB Corporation, USA) and sequenced directly on an automated ABI Prism 3730 Genetic Analyzer machine using ABI BigDye v3.1 Terminator Sequencing chemistry. All PCR products were sequenced in both directions to minimise PCR artefacts, ambiguities and base-calling errors. Chromatogram output was checked by eye using Sequencher 4.1 (Gene Codes) or ProSeq (Filatov, 2002). Direct sequencing of a small proportion of the *cytb* PCR products revealed mixtures of multiple *cytb*-like fragments, or sequences possessing reading frames containing stop codons or indels, suggesting the possible presence of nuclear pseudogenes (Bensasson et al., 2001; Rokas et al., 2003a). In these cases, individual PCR products were amplified by cloning (TA cloning, Invitrogen) and only specimens for which a single, correct open reading frame (ORF) bearing sequence was identified have been included in the following analyses. Though heterozygotes have been detected for the 28S D2 region in cynipids (e.g. Stone et al., 2007) and other taxa (e.g. Smith et al., 2008), none were detected in our surveys.

Our analysis incorporates 404 new sequences (*coxI*, 106; *cytb*, 143; 28S D2, 108; 28S D3-5, 47), with Genbank accession numbers in Appendix S1. Of the 184 Synergini specimens in the study, 70 had full sequences for all three genes (*cytb*, *coxI* and 28S D2). The 67 discrete haplotype sets in these specimens comprise the **max-data** dataset.

## 2.3. Phylogenetic analyses

### 2.3.1. Alignment and phylogeny reconstruction

All *coxI* (660 bp) and *cytb* (433 bp) sequences were the same length, and each gene set could be aligned unambiguously by

eye. The 28S fragments were of variable length (D2: 520–572 bp, D3-5: 511–513 bp) and were aligned using MUSCLE 3.6 (Edgar, 2004) using default settings. There was very little sequence variation in the D3-5 region, and we do not consider it further. Bayesian phylogenetic inference was performed in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Phylogenies for individual genes and for combined gene datasets were constructed under the GTR + I + G model of sequence evolution, partitioned by codon position for *coxI* and *cytb*. For each data matrix, two independent Markov chain Monte Carlo (MCMC) runs of four Metropolis-coupled chains were performed with the gamma shape parameter, the proportion of invariant sites, base frequencies and substitution rates unlinked across all partitions, and with default priors. MCMC runs comprised either 4 million (*coxI*, 28S D2) or 8 million (*cytb*) generations, sampled every 1000 generations with a burn-in time of 3 or 7 million generations, respectively. Models were considered to have converged when the average standard deviation of split frequencies between the two independent runs fell below 0.01, and chain parameters examined in Tracer v1.4 (Rambaut and Drummond, 2003) showed stable distributions. All trees were rooted using *Ceroptres clavicornis*, since previous studies spanning multiple tribes of Cynipinae (Nylander et al., 2004a) have shown this genus to represent a lineage distinct from the *Synergus* complex.

Because molecular clock assumptions can significantly influence clade support, we compared support for clock and non-clock models for each gene in MrBayes using ln Bayes factors (ln BF). These were estimated as twice the difference in the natural log of the harmonic mean of model likelihoods of each model (2ΔlnHML), interpreted following Table 2 of Kass and Raftery (1995). By their criteria, ln BF of 2–6, 6–10 and >10 represent, respectively, positive, strong and very strong support for the model with higher likelihood. All three molecular markers showed sequence variation consistent with strict clock assumptions (ln BF in favour of a clock model for *coxI* = 32; *cytb* = 227, 28S D2 = 221).

### 2.3.2. Tests of taxon monophyly

Support for the monophyly of specific taxa was tested by using MrBayes and Bayes factors as above to compare the harmonic mean likelihoods of models in which taxon monophyly was constrained with models in which there was no such constraint.

### 2.3.3. Likelihood mapping

Likelihood mapping (Strimmer and von Haeseler, 1997) provides an estimate of the phylogenetic utility of a set of sequences, and was performed in TreePuzzle 5.0 (Schmidt et al., 2002) using the HKY model of nucleotide substitution. Likelihood maps were constructed for each gene with parameters estimated from the data set and using all possible quartets of taxa.

## 2.4. *CoxI* MOTU analyses

MOTUs were defined for the complete set of unique *coxI* haplotypes at cut-off values ranging from 1 to 100 base pairs (ca. 0–12% sequence divergence) using MOTU\_define 2.04 (Floyd and Blaxter, 2006). MOTU\_define clusters input sequences into MOTUs by adding each sequence in turn to a local BLAST database and then taking the next sequence and performing a BLAST similarity search against the entries in the local database. If the sequence has less than the user-defined cut-off number of differences to an existing MOTU then it is added to that MOTU, otherwise it is assigned to a new MOTU. The grouping of sequences in this way is sensitive to the order in which they are added (Blaxter et al., 2005) so 100 replicates using different random resampling orders were performed for each MOTU cut-off.

To examine the impact of sampling depth on MOTU richness and membership, we compared the results obtained from analysis

of the **maxdata** supermatrix (matrix containing data for all 3 loci) with those obtained for a second supermatrix intended to maximise sequence diversity present in all three loci, while minimising the number of specimens with missing data. Starting from the **maxdata** supermatrix, this **maxtaxa** supermatrix was constructed by adding all specimens that either individually contributed a unique new haplotype for at least one gene, or represented a novel combination of existing haplotypes. Where two individuals shared a haplotype not present in the **maxdata** supermatrix, we added data for specimens sequenced for two genes in preference to those sequenced for one. This approach resulted in addition of data for 58 specimens, resulting in a supermatrix containing sequence for 125 specimens, almost doubling the specimen sample size. The **maxtaxa** matrix lacked data for 100 of the 375 gene sequences making up the alignment, representing 26.7% missing data.

### 3. Results

#### 3.1. Phylogenetic relationships within the *Synergus* complex

##### 3.1.1. Phylogenetic utility of *coxI*, *cytb* and 28S D2

All three molecular markers used in phylogeny reconstruction had high phylogenetic utility, all showing fewer than 10% of quartets in the unresolved central portion of the likelihood map (Fig. 2). *CoxI* had the highest phylogenetic utility, with 95.8% of quartets in the well-resolved regions towards the corners, followed by *cytb* (92.9%) and 28S D2 (86.4%).

##### 3.1.2. Relationships among major lineages of the *Synergus* complex

All three genes supported broadly concordant relationships among major lineages (Figs. 3–5 for *coxI*, *cytb* and 28S D2 respectively). All three genes supported monophyly of the large genus *Synergus*. Bayes factor comparisons strongly supported monophyly in the *coxI* data (Fig. 3), while the *cytb* and 28S D2 data were equivocal (Table 1). *Synergus* was nevertheless supported as monophyletic with a posterior probability of >0.95 in the 28S D2

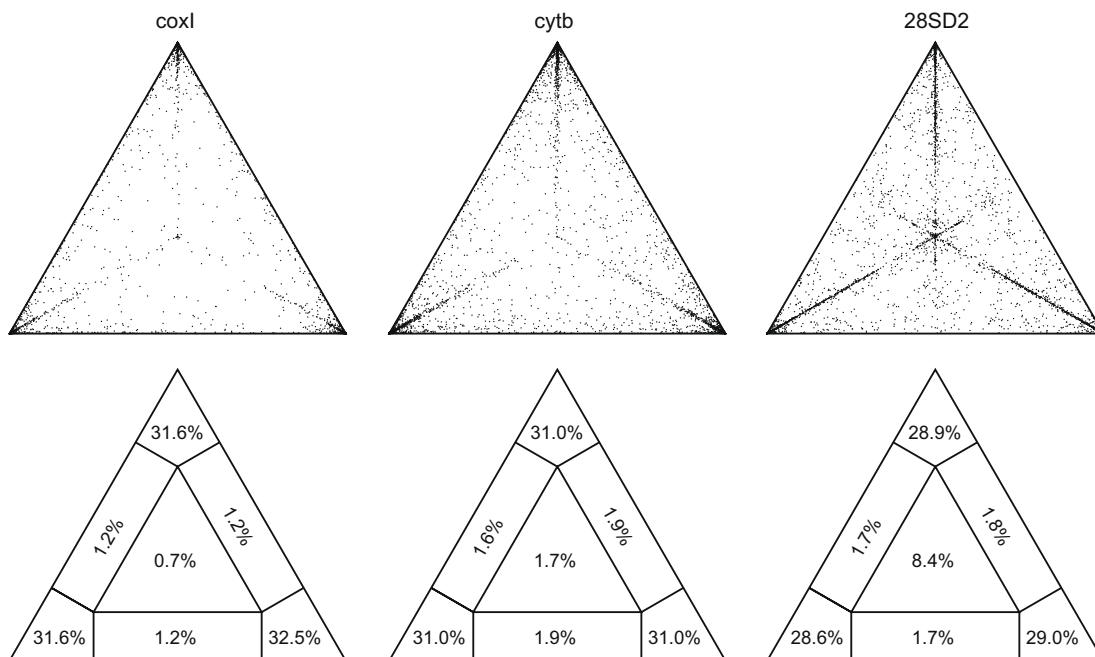
reconstruction (Fig. 5). All three genes supported monophyly for the small genus *Synophrus* (the posterior probability of monophyly is 1.0 in all analyses; Figs. 3–5), but while *Saphonecrus* monophyly was supported (albeit weakly) by the *cytb* data (Fig. 4), monophyly was rejected rather more strongly by *coxI* and 28S D2 (Table 1). Both *coxI* and 28S D2 divide *Saphonecrus* into two (Figs. 3 and 5): three species (*Saphonecrus barbotini*, *S. connatus* and *S. lusitanicus*) form part of a monophyletic clade including all sampled *Synophrus* species, while the remaining Western Palaearctic species (*Saphonecrus haimi*, *S. undulatus* and a recently described species *S. irani*) represent a separate lineage (the ‘haimi clade’ Figs. 3 and 5). Which of these two lineages is the sister group to *Synergus* remains poorly resolved in each of the single gene datasets, while the haimi clade of *Saphonecrus* is strongly supported (posterior probability >0.95) as the sister group in an analysis incorporating data for all three genes (Fig. S1).

##### 3.1.3. *Synergus* shows extensive non-monophyly of morphology-based taxa

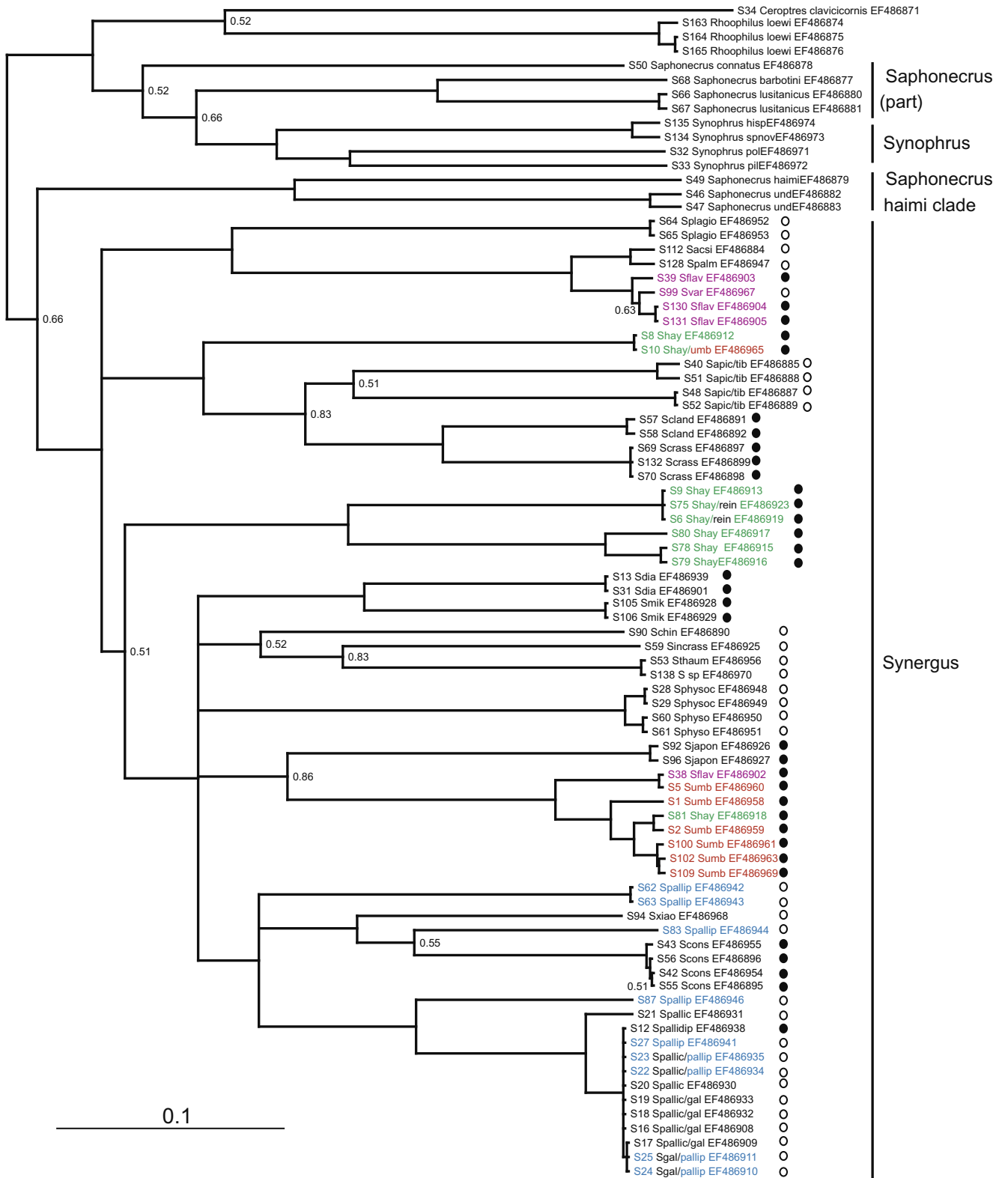
All three genes very strongly rejected monophyly of Mayr’s Sections I and II within *Synergus* (Table 1 and Figs. 3–5). This implies that the biological traits characteristic of species in these sections are evolutionarily labile within this genus. There are also conflicts in *Synergus* between morphological and molecular taxa at the level of Linnean species. Bayes factor comparisons for all three genes rejected monophyly of haplotypes attributed to *S. hayneanus*, *S. palipes* and *S. umbraculus* (highlighted in Figs. 3–5 and Table 1). Phylogenetically divergent haplotypes in all three genes were also attributed to *Synergus flavipes* (Figs. 3–5).

##### 3.1.4. Placement of Eastern Palaearctic taxa

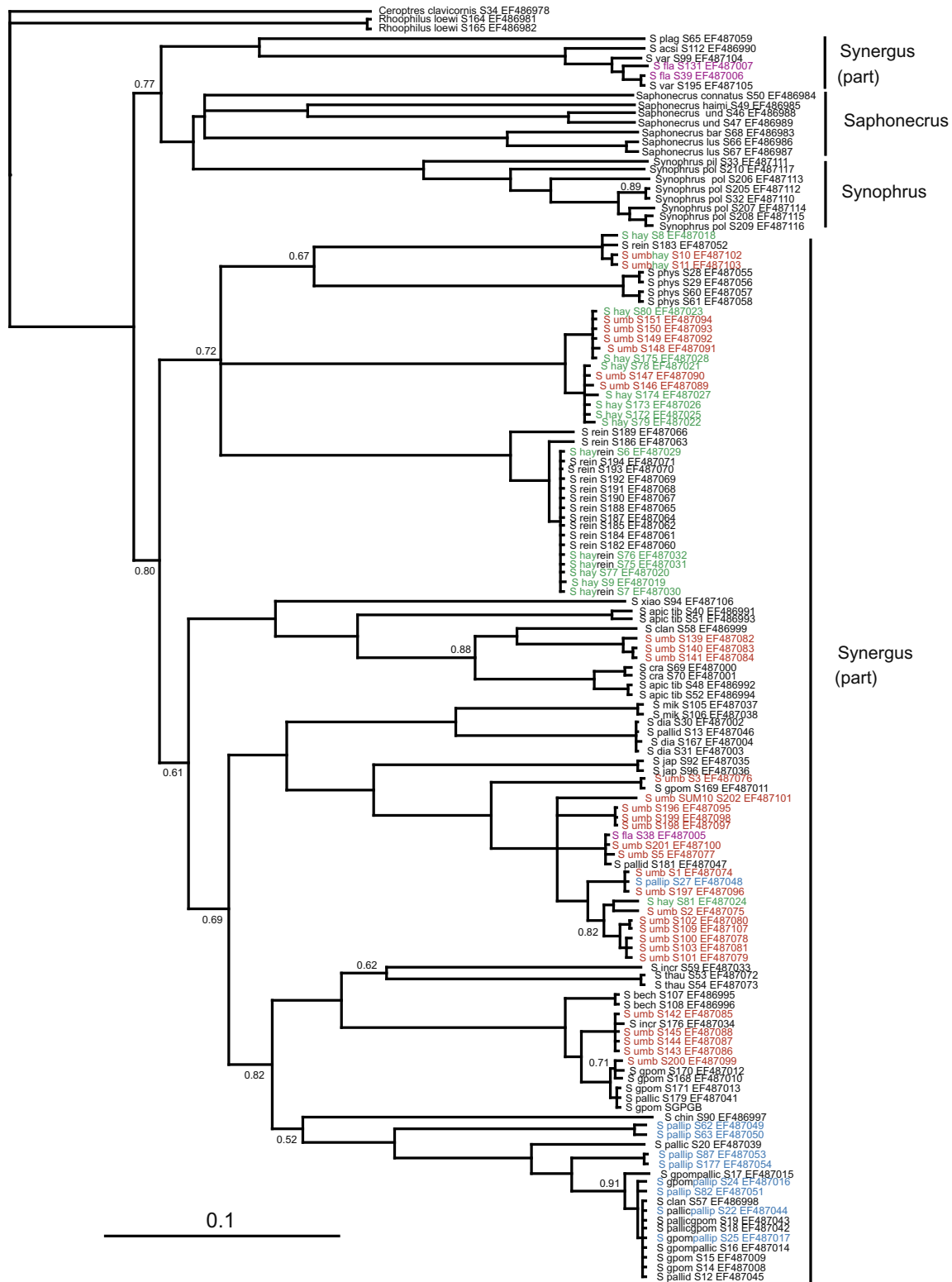
All single gene datasets show Chinese *Synergus chinensis* and *S. xiaolongmeni* and Japanese *Synergus japonicus* to represent distinct lineages nested among the Western Palaearctic lineages. There is no evidence to suggest that Eastern and Western Palaearctic *Synergus* represent discrete monophyletic radiations.



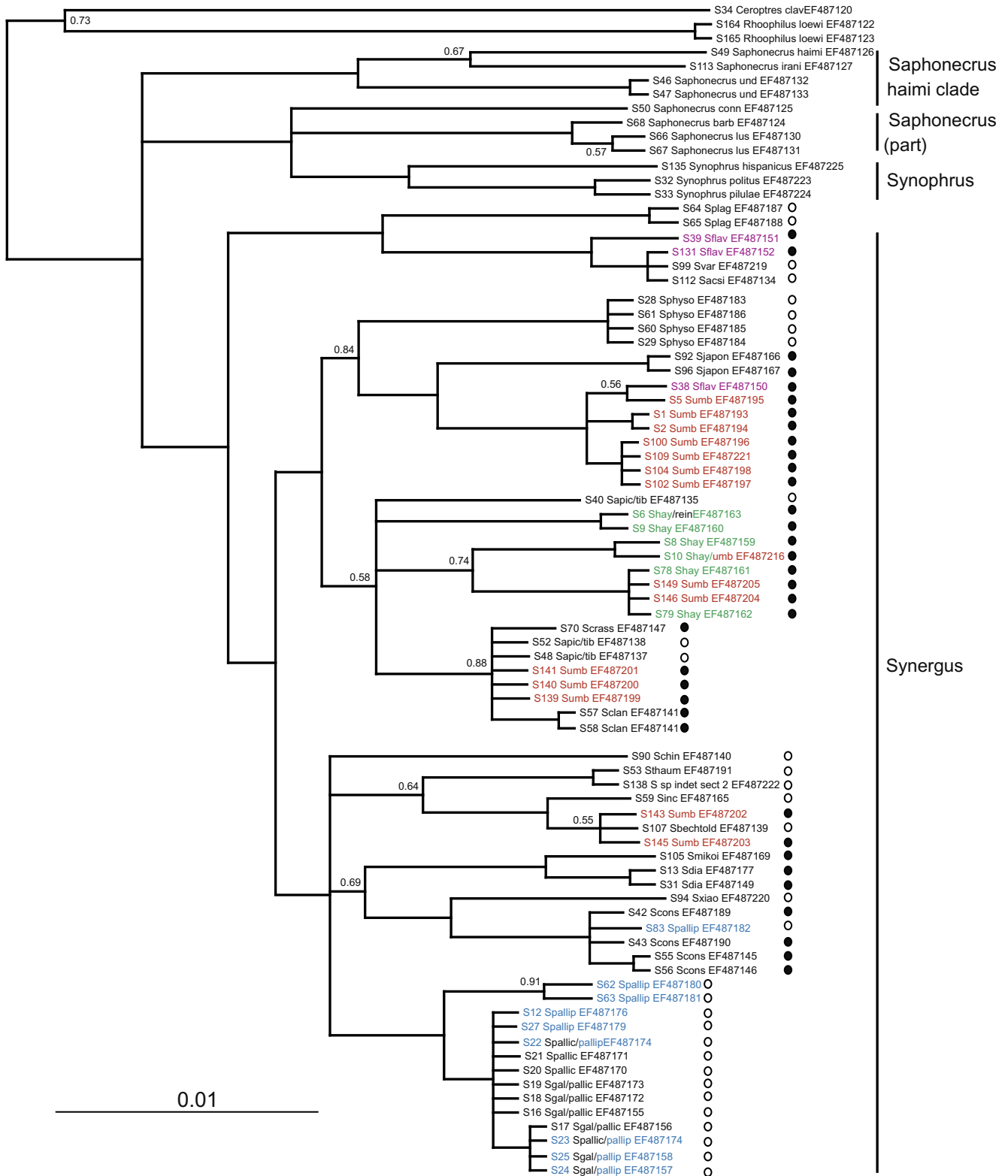
**Fig. 2.** Likelihood maps produced using TreePuzzle 5.0 (Schmidt et al., 2002) showing the phylogenetic utility of the three molecular markers used in this study. The upper row shows the distribution of likelihoods for each of the possible quartets of taxa for each gene. The lower row indicates the proportion of quartets that were poorly resolved (central portion) and well resolved (corners) for each gene. The number within the inner triangle indicates the percentage of quartets whose vectors place them less than halfway from wholly unresolved (the centre of the larger triangle) to fully resolved (any vertex of the larger triangle).



**Fig. 3.** Bayesian majority rule consensus phylogeny for *coxI*, assuming a GTR + I + G strict clock model of sequence evolution. Vertical bars at right indicate main clades within the *Synergus* complex of oak inquiline gallwasps. All unlabelled nodes have a posterior probability of  $\geq 95\%$ , and values for other nodes with support  $>50\%$  are shown. Membership of Mayr's *Synergus* sections is indicated by a filled circle after the sample name for Section I and by an open circle for Section II. Taxon labels of the form a/b refer to specimens showing morphological characters of species a and b within *Synergus*. Full morphology-based identifications are given for each specimen in Appendix S1. Coloured taxon labels illustrate the separation of four morphospecies (*Synergus hayneanus* in green, *S. pallipes* in blue, *S. umbraculus* in red, *S. flavipes* in purple) among multiple MOTUs. Scale bar indicates 0.1 substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Bayesian majority rule consensus phylogeny for *cytb*, assuming a GTR + I + G strict clock model of sequence evolution. Vertical bars at right indicate main clades within the *Synergus* complex of oak inquiline gallwasps. All unlabelled nodes have a posterior probability of  $\geq 95\%$ , and values for other nodes with support  $>50\%$  are shown. Taxon labels of the form a/b refer to specimens showing morphological characters of species a and b within *Synergus*. Coloured taxon labels illustrate the separation of four morphospecies (*Synergus hayneanus* in green, *S. pallipes* in blue, *S. umbraculatus* in red, *S. flavipes* in purple) among multiple MOTUs. Full morphology-based identifications are given for each specimen in [Appendix S1](#). Scale bar indicates 0.1 substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 5.** Bayesian majority rule consensus phylogeny for 28S D2, assuming a GTR + I + G strict clock model of sequence evolution. Vertical bars at right indicate main clades within the *Synergus* complex of oak inquiline gallwasps. All unlabelled nodes have a posterior probability of  $\geq 95\%$ , and values for other nodes with support  $>50\%$  are shown. Membership of Mayr's *Synergus* sections is indicated by a filled circle after the sample name for Section I and by an open circle for Section II. Taxon labels of the form a/b refer to specimens showing morphological characters of species a and b within *Synergus*. Coloured taxon labels illustrate the separation of four morphospecies (*Synergus hayneanus* in green, *S. pallipes* in blue, *S. umbraculus* in red, *S. flavipes* in purple) among multiple MOTUs. Full morphology-based identifications are given for each specimen in [Appendix S1](#). Scale bar indicates 0.01 substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**  
Summary of In Bayes Factor (BF) analyses of alternative models for the *cox1* ( $n = 83$  sequences), *cytb* ( $n = 136$ ) and 28S D2 ( $n = 81$ ) datasets. Ln Bayes Factors are calculated as twice the difference in lnHML ( $2\Delta\ln\text{HML}$ ) between alternative models, calculated from 2 runs for each model in MrBayes. For each single gene dataset, BF tests strongly supported a strict clock model, which is used as the reference for comparison with models in which taxon monophyly was enforced. Our inference follows Kass and Raftery (1995), with ln BF of 2–6, 6–10 and >10 taken to represent respectively positive, strong and very strong support for the model with higher likelihood. For all three loci we used a GTR + I + G model, partitioned by codon position in *cox1* and *cytb*.

Model	<i>Cox1</i>	<i>Cox1</i> inference	<i>Cytb</i>	<i>Cytb</i> inference	28S D2	28S D2 inference
HML for strict clock GTR + I + G	–7039.8		–5875.5		–2242.2	
<i>Synergus</i> monophyly	<b>20.9</b>	Very strongly supported	<b>–2.8</b>	Very weakly rejected	<b>–5.4</b>	Weakly rejected, but monophyletic with a posterior probability >0.95 in the MrBayes consensus tree.
<i>Synergus</i> Section 1 monophyly	–959.3	Rejected	–1457.5	Rejected	–273.9	Rejected
<i>Synergus</i> Section 2 monophyly	–961.5	Rejected	–1454.7	Rejected	–260.2	Rejected
<i>Saphonecrus</i> monophyly	–19.3	Rejected	<b>6.5</b>	Strongly supported	–63.5	Rejected
<i>Synergus hayneanus</i> monophyly	–267.1	Rejected	–753.8	Rejected	–245.3	Rejected
<i>Synergus pallipes</i> monophyly	–229.0	Rejected	–438.6	Rejected	–153.4	Rejected
<i>Synergus umbraculus</i> monophyly	–617.1	Rejected	–1343.6	Rejected	–242.0	Rejected

## 3.2. MOTU analysis

### 3.2.1. *Cox1* and *cytb* both show evidence of a barcoding gap

The relationship between numbers of MOTUs and the percentage sequence divergence used to define them is shown for all three genes in Fig. 6. Both *cox1* and *cytb* show a plateau of MOTU richness over a range of cut-off values compatible with the presence of a ‘barcoding gap’. Because there is no formal way to define the margins of a barcoding gap, we examined MOTU composition across a range of cut-off values spanning the barcoding gap for each gene (indicated on Fig. 6). For *cox1*, the selected sequence divergence values were 1.1% (most divisive, ‘splitter’), 3.8% (midpoint) and 6.4% (most inclusive, ‘lumper’), equivalent to divergences of 7, 25 and 42 base pairs (bp) and labelled A–C on Fig. 6. The equivalent values for *cytb* were 2.1, 4.4 and 6.2% (equivalent to 9, 19 and 27 bp, points D–F, Fig. 6). There is little evidence of a barcoding gap in the 28S D2 sequences, and to allow comparison of MOTU membership across the three genes, we selected a cut-off value of 2 bp (equivalent to 0.04% sequence divergence, point G, Fig. 6).

### 3.2.2. *Cox1* MOTUs and discordance with morphological taxonomy

The 98 *cox1* sequences assigned 31 *Synergus* complex species to 27 MOTUs at the inclusive 6.4% cut-off (Appendix A and Fig. S1), comprising 13 clusters and 14 singleton haplotypes (six of which were found in more than one individual). Comparison of morphology-based species identification with MOTU designations at this cut-off revealed that though many MOTU’s corresponded to recognised Linnean species, there was also substantial discordance: six

MOTUs (Table 2) contained samples representing more than one morphospecies (Type I error), while six species are represented by samples in more than one MOTU (Type II error) (Table 3).

The divisive 1.1% cut-off assigned the same haplotypes to 40 MOTUs, comprising 20 clusters and 20 singleton haplotypes (eight of which were found in more than one sample). At this cut-off, only 4 MOTUs contained more than one species (Table 2), while eight species were present in more than one MOTU (Table 3). Significantly for our inference of Type 1 and Type 2 errors, the specimens contributing to conflicts between morphological taxonomy and MOTU allocations were the same regardless of cut-off level, and included the non-monophyletic species described in Section 3.1.3. The morphospecies most commonly grouped together in a single MOTU were (i) *Synergus gallaepomiformis*, *S. pallicornis* and *S. pallipes* (all in Mayr Section II), grouped with *S. pallidipennis* (Section I) in MOTU 20, and (ii) *Synergus flavipes*, *S. hayneanus* and *S. umbraculus* (all in Mayr Section I) in MOTU 19 (Table 2 and Appendix B). The species most frequently allocated to multiple MOTUs were *Synergus hayneanus* (Section I; 4 MOTUs at 42 bp, 5 MOTUs at 7 bp), *S. pallipes* (Section II; 3 and 4 MOTUs, respectively) and *S. umbraculus* (Section I; 2 and 5 MOTUs, respectively) (Table 3). Although Section II of *Synergus* is characterised by greater intraspecific morphological diversity (see Section 1.1, above), there is no evidence that either Type I or Type II errors are more common in this section.

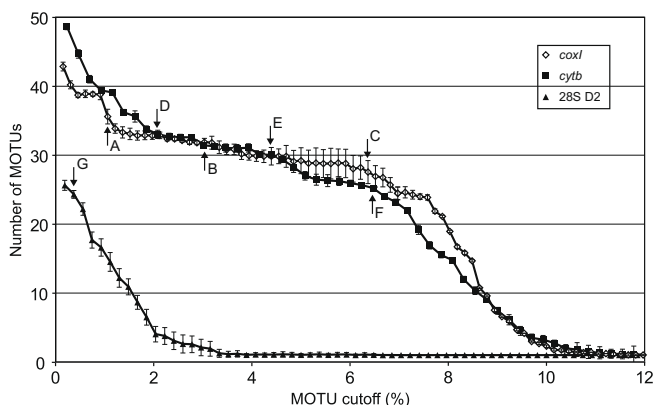
### 3.2.3. MOTU concordance across sampled genes

The three genes in our analysis identify highly concordant sets of MOTUs in the **maxdata** alignment (Appendix B). At the inclusive cut-off (6.4% for *cox1*, 6.2% for *cytb*), 8/14 clusters and all singletons inferred with the 67 distinct *cox1* sequences were also inferred with *cytb* (Appendix B). MOTUs defined by *cox1* were more stable over a range of cut-off values than those defined by *cytb*, apparent in Fig. 6 as the flatter plateau between points A–C for *cox1* than between points D–F for *cytb*.

Comparison between *cox1* (6.4%) and 28S D2 (2 bp, 0.04%) gave the strongest agreement of all between-gene comparisons. The 28S D2 data support 10/14 of the *cox1* clusters and all of the *cox1* singletons (Appendix B). This agreement in MOTU definition between genes supports the conclusion that the disagreement revealed between morphology- and sequence-based taxa is genuine, and unlikely to be an artefact associated with the use of a particular sequence to define molecular taxa.

### 3.2.4. MOTU designation is robust to variation in taxon sampling and missing data

All MOTUs inferred with greater than 50% support for each of the genes using the **maxdata** set (67 specimens) were also inferred using the **maxtaxa** set (125 specimens; see Appendices A and B).



**Fig. 6.** Variation in the numbers of MOTUs defined at cut-offs between 0% and 12% for *cox1*, *cytb*, and 28S D2. Arrows indicate the selected divisive, intermediate and inclusive cut-offs for *cox1* (A–C), *cytb* (D–F) and the single cut-off for 28S D2 (G). Values are means  $\pm$  1 standard error for 100 sampling replicates.

Addition of the 58 extra **maxtaxa** sequences had little impact on levels of Type I (the number of MOTUs containing >1 species) and Type II (the number of species in >1 MOTU) errors; each increased by 1 relative to values at corresponding cut-offs for the **maxdata** matrix, and involved almost identical sets of Linnean species (one additional MOTU containing two Linnean species is inferred in the **maxtaxa** dataset; Tables 2 and 3).

## 4. Discussion

### 4.1. Phylogenetic relationships within the *Synergus* complex

Our analyses provide well-resolved phylogenetic hypotheses for relationships between major lineages within the *Synergus* complex, but reveal widespread conflict with existing groupings based on morphology. While the genera *Synergus* and *Synophrus* are probably monophyletic, *Saphonecrus* is probably diphyletic. Our results thus confirm earlier doubts over the monophyly of this genus (Pujade-Villar and Nieves-Aldrey, 1990; Péntzes et al., 2009). The three *Saphonecrus* species allied to *Synophrus* (*S. barbotini*, *S. connatus* and *S. lusitanicus*) could reasonably be transferred to the genus *Synophrus*, while the distinct lineage comprising *Saphonecrus haimi* and *S. undulatus* could retain the genus name *Saphonecrus*. Within *Synergus*, we find no separation between Western Palaearctic taxa and our very limited sampling of three Eastern Palaearctic species from China and Japan. We find that Mayr's long-accepted morphology-based sections within the genus *Synergus* do not represent natural groups, and should be abandoned. The character used by Mayr to separate the two sections of *Synergus* – the dorso-ventral distribution of sculpturing on tergites of the metasoma (the abdomen behind the petiole) – is clearly relatively labile evolutionarily, and of no taxonomic use.

### 4.2. The potential utility of *coxI* barcodes for the *Synergus* complex

The widely used Folmer barcode region of the mitochondrial *coxI* gene appears to show a clear barcoding gap in the *Synergus* complex. MOTUs defined with this *coxI* region are relatively stable across a range of cut-off values representing 1.1–6.4% sequence divergence, and in many cases MOTU membership reflects current membership of Linnean species. The resampling inherent in MOTU\_define and comparison of results for the **maxdata** and **maxtaxa** analyses also shows that the compositions of the *coxI* MOTUs are also relatively insensitive to sampling order and variation in haplotype sampling effort. There has rightly been widespread criticism of the assumption of a single-sequence divergence threshold in a single locus in discriminating between biological species (see Section 4.3). We sug-

**Table 3**

Morphological species allocated to multiple *coxI* MOTUs in the **maxdata** and **maxtaxa** alignments. MOTU numbers refer to text Appendices 1 and 2, and Fig. S1. Specimens with combined species names showed morphological characters indicative of each species in current morphology-based taxonomy. Numbers in each cell refer to the **maxdata** alignment, with numbers of MOTUs in the **maxtaxa** alignment in brackets if different. Numbers after species names refer to membership of Mayr's *Synergus* Sections I and II.

Species	Number of MOTUs at the 6.4% cut-off	Number of MOTUs at the 1.1% cut-off
<i>Saphonecrus undulatus</i>	1	2
<i>Synergus apicalis/tibialis</i> (II)	2	2
<i>Synergus flavipes</i> (I)	2	2
<i>Synergus hayneanus</i> (I)	4	5
<i>Synergus pallicornis</i> (II)	1	2
<i>Synergus pallipes</i> (II)	2(3)	2(4)
<i>Synergus reinhardi</i> (I)	2	2
<i>Synergus umbraculus</i> (I)	2	5
Total number of species in >1 MOTU	6	8

gest that the stability of *coxI* MOTU membership and the congruence in MOTU membership across mitochondrial and nuclear genes supports the use of *coxI* barcodes in the *Synergus* complex.

### 4.3. Mismatches between morphological taxonomy and MOTU membership in *Synergus*

While some recognised *Synergus* species correspond to *coxI* MOTUs over the full range of cut-offs investigated here, others clearly do not. MOTU-based groupings suggest that both Type I errors (separation into discrete taxa where none exists) and Type II errors (cryptic lineages within single morphological species) (Quicke, 2004) exist in the *Synergus* complex. There are two general hypotheses for such mismatches between morphological taxonomy and MOTU membership: (i) that MOTU-based identification is correct, while identification based on morphological traits is flawed, and (ii) that MOTU-based identification is flawed, while identification based on morphological traits is correct. The latter hypothesis predicts a mismatch between specimen groupings based on mitochondrial sequence data and those based on nuclear sequence data. There are many reasons to expect such a mismatch (Hudson and Turelli, 2003; Machado and Hey, 2003; Moritz and Cicero, 2004; Hurst and Jiggins, 2005). In particular, coalescent theory predicts that species can share *coxI* barcodes through incomplete sorting of ancestral polymorphism (Hickerson et al., 2006; Knowles and Carstens, 2007), and many species are polyphyletic rather than monophyletic for mitochondrial genes (Johnson and Cicero, 2002;

**Table 2**

*CoxI* MOTUs identified from the **maxdata** and **maxtaxa** alignments containing specimens of more than one morphological species. MOTU numbers refer to text Appendices 1 and 2, and Fig. S1. The first two columns refer to results incorporating the inclusive 6.4% (42 bp) cut-off, while the righthand column summarises changes when the divisive 1.1% (7 bp) cut-off is used. Specimens with combined species names showed morphological characters indicative of each species in current morphology-based taxonomy. Numbers in brackets after species names refer to membership of Mayr's *Synergus* Sections I and II.

MOTU number	Species combined at the 6.4% cut-off	Differences observed at the 1.1% cut-off
3	<i>Saphonecrus barbotini</i> + <i>Saphonecrus lusitanicus</i>	– 2 single-species MOTUs
6	<i>Synergus hayneanus</i> (I) + <i>S. hayneanus/reinhardi</i> (I) + <i>S. hayneanus/umbraculus</i> (I)	No change
19	<i>Synergus flavipes</i> (I) + <i>S. hayneanus</i> (I) + <i>S. umbraculus</i> (I)	– 4 MOTUs: 2 multispecies ( <i>S. flavipes</i> + <i>S. umbraculus</i> ; <i>S. hayneanus</i> + <i>S. umbraculus</i> ), and 2 single-species (each <i>S. umbraculus</i> ).
20	<i>Synergus gallaepomiformis</i> (II) + <i>S. pallicornis</i> (II) + <i>S. pallipes</i> (II) + <i>S. pallidipennis</i> (I)	– 3 MOTUs: 1 multispecies containing all 4 species, and 2 single-species, single-sequence ( <i>S. pallicornis</i> ; <i>S. pallipes</i> ).
21	<i>Synergus clandestinus</i> (I) + <i>S. crassicornis</i> (I)	– 2 single-species MOTUs
24	<i>Synergus acsi</i> (II) + <i>S. flavipes</i> (I) + <i>S. palmirae</i> (II) + <i>S. variabilis</i> (II)	– 4 MOTUs: 3 single-species ( <i>S. acsi</i> ; <i>S. flavipes</i> ; <i>S. palmirae</i> ) and 1 multispecies ( <i>S. flavipes</i> + <i>S. variabilis</i> ).
Added only in the <b>maxtaxa</b> alignment		
26	<i>Synergus consobrinus</i> (I) + <i>S. pallipes</i> (II)	– 2 single-species MOTUs

Funk and Omland, 2003; Meyer and Paulay, 2005). Where sorting of ancestral polymorphism is complete, species can still share barcodes through introgression (Machado and Hey, 2003; Hurst and Jiggins, 2005). Exchange of mitochondrial genes by introgression has been demonstrated in gall inducing cynipids (Rokas et al., 2003b), and might thus be expected in inquiline gallwasps. These pitfalls of relying on a single mitochondrial locus in molecular barcoding are the same as those associated with its use in phylogeography, and can be avoided by using multiple unlinked nuclear loci to determine affinities between specimens (e.g. Knowles and Carstens, 2007; Starrett and Hedin, 2007). Although we have only used one nuclear locus here, the agreement in MOTU memberships between *cox1* and 28S D2 datasets despite the much lower levels of sequence variation in the nuclear locus (see also Rokas et al., 2002b; Blaxter, 2004; Ahrens et al., 2007) gives greater confidence that the MOTUs capture biologically meaningful entities.

If the molecular taxa identified here do represent discrete biological entities, then the morphological taxonomy of the Synergini must be flawed – either because the characters used do not adequately define taxa, or because the taxonomists identifying the specimens made mistakes in character recognition. The authors have considerable accumulated experience of working with inquiline cynipids (Nieves-Aldrey and Pujade-Villar, 1985, 1986; Pujade-Villar and Nieves-Aldrey, 1990; Pujade-Villar, 1992; Pujade-Villar et al., 2003; Sadeghi et al., 2006), so mistaken interpretation of specimen morphology should be rare. While it is possible that occasional placements of specimens in phylogenetically disparate MOTUs could be the result of identification error (e.g. the placement of *Synergus flavipes* Figs. 3–5), we regard taxonomist error as an unlikely explanation for two major persistent mismatches between morphological and sequence-based groupings: (i) The inability of sequence data to discriminate between *Synergus gallaeopomiformis*, *S. pallicornis*, *S. pallidipennis* and *S. pallipes*; and (ii) the widespread phylogenetic placement and the multiple MOTU allocation of specimens in the morphospecies *Synergus hayneanus* and *S. umbraculus* (Figs. 3–5 and Fig. S1). We note that mismatches between morphological and molecular taxa are clearest in the morphotaxa with strongest sampling, raising the possibility that further sampling of other morphotaxa would reveal further cryptic lineages.

#### 4.3.1. Failure of barcodes to discriminate among recognised morphological species

Several sets of *Synergus* species shared identical or very similar *cox1*, *cytb* and 28S D2 sequences (Figs. 3–5 and Fig. S1). Examples include *Synergus gallaeopomiformis*, *S. pallicornis*, *S. pallidipennis* and *S. pallipes* combined in *cox1* MOTU 20, *Synergus acsi*, *S. flavipes* and *S. variabilis* combined in *cox1* MOTU 24, and *Synergus hayneanus* and *S. umbraculus* (Fig. S1). Failure to discriminate these species using sequence barcodes implies either (i) that these morphospecies genuinely grade into each other (such that neither existing morphological characters nor barcode sequence can meaningfully discriminate among them) or (ii) that the morphological traits discriminating the species are real, but that the species have diverged so recently that sorting of both mitochondrial (*cox1*) and nuclear (28S D2) lineages between them is far from complete (Johnson and Cicero, 2002; Funk and Omland, 2003; Meyer and Paulay, 2005; Hickerson et al., 2006). We suspect that the first explanation applies. *Synergus* species are known to show substantial phenotypic variation within and between generations each year (Wiebes-Rijks, 1979; Pujade-Villar, 1992; Nieves-Aldrey and Pujade-Villar, 1986), and the characters currently used to discriminate species may simply represent redescrptions of phenotypic plasticity in a single taxon. We recommend that the morphological basis of the groups of species above should be thoroughly reviewed. If reanalysis supports their maintenance as separate taxa, then species relationships should be reinvestigated using multiple nuclear locus approaches

that provide greater statistical power when lineage sorting is likely to be incomplete (Jennings and Edwards, 2005; Knowles and Carstens, 2007; Starrett and Hedin, 2007).

#### 4.3.2. Placement of morphological species in phylogenetically diverse MOTUs

*Synergus hayneanus* and *S. umbraculus* are both placed in several phylogenetically divergent lineages for both mitochondrial and nuclear genes (Figs. 3–5 and Fig. S1). This pattern was reconfirmed when the morphology of a subset of specimens of both species was rechecked without knowledge of their phylogenetic placement. Our results suggest the need for careful revision of the morphological traits associated with separation of *S. hayneanus* and *S. umbraculus* (and *S. reinhardi*, which was often hard to separate morphologically from *S. hayneanus*). Further, the characters used to identify *S. hayneanus* and *S. umbraculus* are either homoplasious or conserved ancestral traits, and conceal phylogenetically divergent but morphologically cryptic lineages. While such cryptic lineages have been observed in other arthropod groups (e.g. Hebert et al., 2004a,b; Smith et al., 2006, 2007; Starrett and Hedin, 2007), the *Synergus* complex is unusual in that its member species have a long history of taxonomic and ecological study (Ross, 1951; Eady, 1952; Wiebes-Rijks, 1979; Nieves-Aldrey and Pujade-Villar, 1985, 1986; Pujade-Villar and Nieves-Aldrey, 1990; Pujade-Villar, 1992; Ronquist, 1994; Schönrogge et al., 1995, 1996a,b; Liljeblad and Ronquist, 1998; Schönrogge and Crawley, 2000; Pujade-Villar et al., 2003).

#### 4.4. Consequences of the need to revise oak inquiline gallwasp taxonomy

A striking feature of the original *Synergus* sections established by Mayr (1872) is the correlation between section membership and biological differences. Most Section I *Synergus* species are univoltine, and where there are two generations per year (in *S. crassicornis* and *S. umbraculus* in the Iberian Peninsula), adult morphology does not differ markedly between generations (Nieves-Aldrey and Pujade-Villar, 1985; Pujade-Villar, 1992). In contrast, most Section II *Synergus* species are bivoltine and show generational adult dimorphism (Ross, 1951; Eady, 1952; Wiebes-Rijks, 1979; Nieves-Aldrey and Pujade-Villar, 1986; Pujade-Villar, 1992). The distribution of section membership through the *Synergus* tree (Figs. 3–5 and Fig. S1) implies that these character state combinations have evolved repeatedly in the genus. It is then of interest to examine how strictly correlated evolution of these traits has been through diversification of *Synergus*. However, such an analysis may be impossible using existing published data, because for several Linnean species specimens attributed to a single morphological species are placed in multiple distinct phylogenetic lineages (discussed in Section 4.3 above). Unless DNA sequence data can be generated for the specimens examined in past work, it will be impossible to know which of alternative possible lineages should receive the character states attributed to current Linnean species (see also Knowlton and Jackson, 1994). Revealing patterns of character state evolution in *Synergus* requires collection of new host association and life history data explicitly linked to accessions from which DNA sequence data can be generated and lineage membership determined.

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## Appendix A

*Coxl* **maxtaxa** MOTUs showing% support for MOTU boundaries at inclusive (6.4%/42 bp), intermediate (3.8%/25 bp) and divisive (1.1%/7 bp) cut-offs. An 'x' indicates specimens that are also included in the **maxdata** dataset.

MOTU	Sample		Species	coxl				
				6.4%	3.8%	1.1%		
Outgroup 1	34	x	C. cerri	70	30	100	100	
Outgroup 1	35	x	C. cerri					
Outgroup 2	36	x	C. clavicornis	70		100	100	
Outgroup 2	37	x	C. clavicornis					
Outgroup 3	120		C. cornigera	100		100	100	
Outgroup 4	164	x	Rhoophilus loewi	100		100	100	
Outgroup 4	165	x	Rhoophilus loewi	100		100	100	
1	78	x	S. hayneanus	100		100	100	
1	79	x	S. hayneanus					
1	80		S. hayneanus					
2	6	x	S. hayneanus/reinhardi	100		100	100	
2	7		S. hayneanus/reinhardi					
2	9	x	S. hayneanus					
2	73		S. hayneanus/reinhardi					
2	75		S. hayneanus/reinhardi					
2	76		S. hayneanus/reinhardi					
2	77		S. hayneanus					
3	66	x	Saph. lusitanicus	100		100	100	
3	67	x	Saph. lusitanicus					
3	68	x	Saph. barbotini					
4	62	x	S. pallipes	100		100	100	
4	63	x	S. pallipes					
5	65	x	S. plagiotrochi	100		100	100	
6	8	x	S. hayneanus	100		100	100	
6	10	x	S. hayneanus/umbraculus					
6	11		S. hayneanus/umbraculus					
6	74		S. hayneanus/reinhardi					
7	92	x	S. japonicus	100		100	100	
7	96	x	S. japonicus					
8	59	x	S. incrassatus	100		100	100	
9	40	x	S. apicalis/tibialis	100		100	100	
9	51		S. apicalis/tibialis					
10	13	x	S. diaphanus	100		100	100	
10	30	x	S. diaphanus					
10	31	x	S. diaphanus					
11	32	x	Synophrus politus	100		100	100	
12	94	x	S. xiaolongmeni	100		100	100	
13	105	x	S. mikoi	100		100	100	
13	106		S. mikoi					
14	46	x	Saph. undulatus	100		100	100	
14	47	x	Saph. undulatus					
15	53	x	S. thaumacerus	100		100	100	
15	54		S. thaumacerus					
16	90	x	S. chinensis	100		100	100	
17	50	x	Saph. connatus	100		100	100	
18	49	x	Saph. haimi	100		100	100	
19	5	x	S. umbraculus	100	55	45	100	
19	38	x	S. flavipes					
19	1	x	S. umbraculus					
19	2	x	S. umbraculus			45		100
19	81		S. hayneanus					
19	100	x	S. umbraculus					
19	102	x	S. umbraculus					
19	109	x	S. umbraculus					

(continued on next page)

20	27	x	<i>S. pallipes</i>	100	100	100	
20	20	x	<i>S. pallicornis</i>				
20	17	x	<i>S. gallaepomiformis/pallicornis</i>				
20	12	x	<i>S. pallidipennis</i>				
20	14	x	<i>S. gallaepomiformis</i>				
20	15	x	<i>S. gallaepomiformis</i>				
20	16	x	<i>S. gallaepomiformis/pallicornis</i>				
20	18	x	<i>S. pallicornis/gallaepomiformis</i>				
20	19	x	<i>S. pallicornis/gallaepomiformis</i>				
20	23		<i>S. pallicornis/pallipes</i>				
20	22	x	<i>S. pallicornis/pallipes</i>				
20	24	x	<i>S. gallaepomiformis/pallipes</i>				
20	25	x	<i>S. gallaepomiformis/pallipes</i>				
20	26		<i>S. gallaepomiformis/pallipes</i>				
20	86		<i>S. pallipes</i>				
20	88		<i>S. pallicornis/pallipes</i>				
20	89		<i>S. pallicornis/pallipes</i>				
20	21		<i>S. pallicornis</i>				100
20	87		<i>S. pallipes</i>		100		100
21	57	x	<i>S. clandestinus</i>	100	100	100	
21	58	x	<i>S. clandestinus</i>				
21	69	x	<i>S. crassicornis</i>		100		100
21	70		<i>S. crassicornis</i>				
21	132		<i>S. crassicornis</i>				
22	48	x	<i>S. apicalis/tibialis</i>	100	100	100	
22	52	x	<i>S. apicalis/tibialis</i>				
23	28	x	<i>S. physocerus</i>	100	100	60	40
23	29	x	<i>S. physocerus</i>				
23	60	x	<i>S. physocerus</i>				40
23	61	x	<i>S. physocerus</i>				
24	112	x	<i>S. acsi</i>				100
24	39	x	<i>S. flavipes</i>			79	21
24	99	x	<i>S. variabilis</i>				
24	131	x	<i>S. flavipes</i>				21
24	130		<i>S. flavipes</i>				
24	128		<i>S. palmirae</i>				100
25	33	x	<i>Synophrus politus/pilulae</i>	100	100	100	
26	42		<i>S. consobrinus</i>	100	100	100	
26	43		<i>S. consobrinus</i>				
26	44		<i>S. consobrinus</i>				
26	45		<i>S. consobrinus</i>				
26	56		<i>S. consobrinus</i>				
26	83		<i>S. pallipes</i>		100		100
27	134		<i>Synophrus sp.nova</i>	100	100	100	
27	135		<i>Synophrus sp. nova</i>				100

**Appendix B**

**Maxdata** Comparison of MOTU boundaries between the three genes at various cut-offs. M = MOTU number, S = sample identification number in online Supplementary Appendix S1.

M	S	Species	cox1			cytb			28S D2
			42bp	25bp	7bp	27bp	19bp	9bp	2bp
OG1	34	<i>C. cerri</i>	69	31	100	100	100	100	100
OG1	35	<i>C. cerri</i>							
OG2	36	<i>C. clavicornis</i>	69		100	100	100	100	100
OG2	37	<i>C. clavicornis</i>							
OG4	164	<i>Rhoophilus loewi</i>	100	100	100	100	100	100	100
OG4	165	<i>Rhoophilus loewi</i>	100	100	100	100	100	100	100
1	78	<i>S. hayneanus</i>	100	100	100	87	7	100	100
1	79	<i>S. hayneanus</i>							
2	6	<i>S. hayneanus/reinhardi</i>	100	100	100			100	100
2	9	<i>S. hayneanus</i>							
3	66	<i>Saph. lusitanicus</i>	100	100	100	93		100	100
3	67	<i>Saph. lusitanicus</i>							
3	68	<i>Saph. barbotini</i>		100	100			100	100
4	62	<i>S. pallipes</i>	100	100	100	90	6	100	100
4	63	<i>S. pallipes</i>							68 (+M20)*
5	65	<i>S. plagiotrochi</i>	100	100	100	92		100	100
6	8	<i>S. hayneanus</i>	100	100	100	100	100	100	38*
6	10	<i>S. hayneanus/umbraculus</i>							
7	92	<i>S. japonicus</i>	100	100	100	97		100	100
7	96	<i>S. japonicus</i>							
8	59	<i>S. incrassatus</i>	100	100	100	97		100	100
9	40	<i>S. apicalis/tibialis</i>	100	100	100	98		100	100
10	13	<i>S. diaphanus</i>	100	100	100	100		100	100
10	30	<i>S. diaphanus</i>							
10	31	<i>S. diaphanus</i>							
11	32	<i>Synophrus politus</i>	100	100	100	100	100	100	100
12	94	<i>S. xiaolongmeni</i>	100	100	100	100	100	100	100
13	105	<i>S. miko</i>	100	100	100	100	100	100	100
14	46	<i>Saph. undulatus</i>	100	100	100	100	100	100	100
14	47	<i>Saph. undulatus</i>			100			100	
15	53	<i>S. thauaerucus</i>	100	100	100	100	100	100	100
16	90	<i>S. chinensis</i>	100	100	100	100	100	100	100
17	50	<i>Saph. connatus</i>	100	100	100	100	100	100	100
18	49	<i>Saph. haimi</i>	100	100	100	100	100	100	100
19	5	<i>S. umbraculus</i>	100	68	32	100	82	15	100
19	38	<i>S. flavipes</i>					15	100	63
19	1	<i>S. umbraculus</i>			32	100			23
19	2	<i>S. umbraculus</i>				100			20
19	100	<i>S. umbraculus</i>				100			
19	102	<i>S. umbraculus</i>							
19	109	<i>S. umbraculus</i>							
20	27	<i>S. pallipes</i>	100	100	100				68 (+M4)*
20	20	<i>S. pallicornis</i>				92	8	100	100
20	17	<i>S. gallaepomiformis/pallicornis</i>				8	100	51	49
20	12	<i>S. pallidipennis</i>							46
20	14	<i>S. gallaepomiformis</i>							
20	15	<i>S. gallaepomiformis</i>							
20	16	<i>S. gallaepomiformis/pallicornis</i>							
20	18	<i>S. pallicornis/gallaepomiformis</i>							
20	19	<i>S. pallicornis/gallaepomiformis</i>							
20	22	<i>S. pallicornis/pallipes</i>							
20	24	<i>S. gallaepomiformis/pallipes</i>							
20	25	<i>S. gallaepomiformis/pallipes</i>							
21	57	<i>S. clandestinus</i>	100	100	100	93		100	100
21	58	<i>S. clandestinus</i>							
21	70	<i>S. crassicornis</i>		100	100	67	15	93	7
22	48	<i>S. apicalis/tibialis</i>	100	100	100			7	88
22	52	<i>S. apicalis/tibialis</i>							12
23	28	<i>S. physocerus</i>	100	100	61	39	81	100	100
23	29	<i>S. physocerus</i>							
23	60	<i>S. physocerus</i>							39
23	61	<i>S. physocerus</i>							
24	39	<i>S. flavipes</i>			100		97	100	100
24	99	<i>S. variabilis</i>			61	39			100
24	131	<i>S. flavipes</i>				39			
24	112	<i>S. acsi</i>							
25	33	<i>Synophrus politus/pilulae</i>	100	100	100	100	100	100	100

\*Additional MOTU boundaries inferred that cannot be displayed in this representation.

## Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.12.004.

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