Estimating ancestral geographical distributions: a Gondwanan origin for aphid parasitoids?

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We tested the published hypothesis of a Gondwanan origin for the overwhelmingly northern hemisphere aphid parasitoids (Aphidiinae) as follows: (i) finding their sister group by a phylogenetic analysis of the entire Braconidae (Insecta: Hymenoptera) using sequence data from approximately 500 bp fragments of both the nuclear 28S (D2 region) and mitochondrial 16S rDNA genes, (ii) using this sister-group relationship and the more informative 28S D2 gene to estimate the phylogeny of the Aphidiinae and (iii) estimating the ancestral distribution for the Aphidiinae using maximum-likelihood and maximum-parsimony methods. Both methods indicated a Gondwanan origin.

Keywords: maximum likelihood; historical biogeography; Aphidiinae; phylogeny; Gondwanaland; character estimation

1. INTRODUCTION

The parasitoid wasp subfamily Aphidiinae presents a biogeographical puzzle. Today, along with their aphid hosts, the Aphidiinae have a Northern Hemisphere distribution, with only *ca.* 3% of species found outside this region (Mackauer 1968). However, a Gondwanan origin for the subfamily was proposed by Schlinger (1974) based on the apparently plesiomorphic host relationships and morphology of two Southern Hemisphere genera: *Pseudephedrus* (South and Central America) and *Parephedrus* (Australia). Any such tendency for Southern Hemisphere aphidiine genera to attack older aphid taxa would be a poor indicator of biogeographical history because putatively older aphid genera are better represented in the Southern Hemisphere where ancient gymnosperms persist (Gärdénfors 1986). To exclude the possibility of later range expansion into the Southern Hemisphere, an independently derived phylogeny is necessary. In this paper we estimate the ancestral geographical distribution of the Aphidiinae presenting, to our knowledge, the first use of maximum-likelihood (ML) methods for this purpose. ML has been used to estimate ancestral ecological, life history and biochemical characters (Schluter *et al.* 1997; Pagel 1999a), but the analysis of geographical distribution remains dominated by maximum-parsimony (MP) methods (e.g. Bremer 1992; Ronquist 1997).

The importance of the Aphidiinae as biological control agents has led to considerable interest in their phylogeny (Belshaw & Quicke 1997; Smith *et al.* 1999; Kambhampati *et al.* 2000; Sanchis *et al.* 2000), but this research did not include the Southern Hemisphere genera and has been hampered by the inability to identify a suitable outgroup. Although attempts have been made to develop a robust phylogeny for the Braconidae based on morphological (Quicke & Van Achterberg 1990) and molecular data sets (Belshaw *et al.* 1998; Dowton *et al.* 1998), the position of the Aphidiinae was uncertain. In this paper we combine and

expand the above molecular data sets in order to test the Gondwanan origin hypothesis. Extensive convergent evolution appears to have occurred among the morphological characters of this group (Quicke & Belshaw 1999) rendering them unreliable markers, so we do not include them.

2. MATERIAL AND METHODS

(a) Phylogenetic analyses

We performed two sequential phylogenetic analyses.

First, to find the sister group to the Aphidiinae (the most suitable outgroup) we inferred a phylogeny using representatives of most braconid subfamilies, including six aphidiine species representing all the tribes. We sequenced approximately 500 bp fragments from both the nuclear 28S ribosomal gene (D2 region) and the mitochondrial 16S ribosomal gene. Novel sequences are available in the EMBL/GenBank/DDBJ databases, accession numbers AF173217–AF173224, AF176048–176068, AJ231532, AJ231535, AJ245682–AJ245698 and AJ245958. To make robust inferences about relationships we employed the following three independent alignment and compatible tree-building protocols.

- (i) Protocol 1. Sequences aligned by eye (by D.L.J.Q.) with definable motifs among the variable length regions coded as additional binary characters (Barriel 1994). Most parsimonious tree(s) (MPTs) were found from 100 random additions, tree bisection and reconnection (TBR) branch swapping on a maximum of ten trees. A successive approximations analysis was then performed (Farris 1969): characters were reweighted using the maximum value of the rescaled consistency index and the search repeated until no further changes occurred.
- (ii) Protocol 2. Sequences aligned by eye (by M.D.) with variable length regions excised. MPTs were then found using equal weights or the six-parameter weighting scheme of Stanger-Hall & Cunningham (1998), with 100 and 50 random additions, respectively.
- (iii) Protocol 3. Sequences aligned using CLUSTAL W v. 1.5 (Thompson *et al.* 1994) with gap open penalty of 10, and

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gap extension penalty of 0.1 and 0.05 in pairwise and multiple alignments, respectively. We then constructed a neighbour-joining tree using the F81 model (selected because there were large differences in base composition).

Second, we performed a more thorough phylogenetic analysis of a smaller clade consisting of the Aphidiinae and its sister group. In addition to a wide range of Northern Hemisphere aphidiine genera, our analysis includes one of the two southern genera discussed by Schlinger (1974) (*Pseudephedrus*) plus a second more recently described genus from New Zealand (*Choreopraon* which attacks *Neophyllaphis totarae*) (M. Mackauer, personal communication). For this analysis we used the 28S D2 gene only, which was the more phylogenetically informative of the two (see below). Sequences were aligned using MALIGN v. 1.99 (Wheeler & Gladstein 1994) with a range of gap to substitution costs: 1:1, 3:2, 2:1 and 5:2 (a gap cost of 3:1 aligned obviously non-homologous regions and was rejected). In each case a single most parsimonious alignment was found from ten random additions (randorders=10) with single sequence swapping on alignments (alignswap). For each alignment we built trees using ML (ten random additions) with the following parameters estimated from unweighted MPTs (100 random additions): base composition, substitution rate (general time-reversible (GTR) model) and rate heterogeneity (gamma distribution).

Tree building was carried out using PAUP* (Swofford 1998) with gaps treated as missing data. Relative branch support was assessed by bootstrapping (Felsenstein 1985) with 100 replicates, each consisting of five random additions with branch swapping using TBR on a maximum of ten trees. The details of the DNA extraction, amplification and sequencing protocols are in Belshaw *et al.* (1998) and Downton *et al.* (1998). An additional 16S primer (5'-TACGCTGTTATCCCTAAGG) approximately 150 bases upstream of the main reverse primer was necessary to amplify some aphidiines.

The relative strength of the phylogenetic signal in the two genes was estimated using data decisiveness (DD) (Goloboff 1991): we calculated the unweighted MPT length, minimum length and mean length of 10 000 random trees for both genes in the alignment with variable length regions excised (protocol 2). We tested for incongruence between the genes using the incongruence length difference (ILD) test (Farris *et al.* 1994) implemented in PAUP*; 100 replicates each consisting of five random additions with branch swapping using TBR on a maximum of ten trees. We also used RASA 2.0 (Lyons-Weiler *et al.* 1996) to test for long branch attraction.

Details of the taxa included in this study and all alignments are available in electronic Appendix A on The Royal Society Web site.

(b) *Estimation of the ancestral distribution*

We estimated the ancestral distribution of the clade consisting of the Aphidiinae and its sister group using ML and MP. In both methods distribution was treated as a single character with two states: either Northern or Southern Hemisphere. Therefore, the ancestral distributions are estimated as being either Laurasian or Gondwanan, respectively, which is an appropriate categorization assuming a Cretaceous origin for the Aphidiinae (note that Australia remained linked to South America via Antarctica throughout this period; Smith *et al.* 1980). The fossil record of the Aphidiinae is poor; they are known from the Palaeocene period (Rasnitsyn 1980), but both their host group (Aphidoidea) and the family Braconidae appear before the middle of the Cretaceous period (Rasnitsyn 1983; Heie 1987). Subdividing ancestral states

to represent smaller continental units was not appropriate with a relatively small data set. All estimations of the ancestral distribution were made without restrictions or weightings and using trees from all DNA alignments.

We consider the ancestral distribution of the Aphidiinae to be the node representing the speciation event separating the Aphidiinae from its sister group; however, we also estimate the distribution at the first node separating lineages within the Aphidiinae.

The ML estimate of an ancestral state is the state with the highest likelihood, and the ratio of the likelihoods for the two possible states is a measure of support for this estimate (how much more likely is one character state at the node compared to the alternative). We used Discrete v. 1.0.1a (Pagel 1994, 1997) to calculate overall tree likelihoods with a single node successively fixed at one of the two states (Pagel 1999b). We then calculated the likelihood ratio (LR), which is the natural logarithm of the ratio of the two likelihoods. Deciding between competing hypotheses using an LR is a complex problem. In our case, because the two estimates are not nested, we cannot use the χ^2 -distribution to obtain a significance value; however, LR values of 2 and above may be treated as roughly analogous to 95% confidence limits (Schluter *et al.* 1997; Pagel 1999b). We found some variation in the likelihood values with repeated analyses, so we performed every analysis 30 times and used the highest likelihood value obtained. In our analysis, likelihood is proportional to the probability of obtaining the observed character states from a particular model of character change, and the program searches for the model which maximizes this value. For further explanation of likelihood and its relation to probability (see Page & Holmes 1998).

The MP estimate of an ancestral state is the one which minimizes the number of subsequent changes necessary to give rise to the present distribution of character states. This was made by mapping the character onto trees using MacClade v. 3.04. (Maddison & Maddison 1992). We repeated this estimation on MPTs built using gaps weighted the same as in the alignment protocol; this may be a more logical approach to tree building (Giribet & Wheeler 1999) but we argue that it can group unrelated taxa which share a similar sequence length.

3. RESULTS

(a) *The genes*

The amplified DNA fragments were between 421 and 492 bases for the 28S D2 gene (excluding *Ephedrus* and *Megalohelcon* which have large indels) and between 457 and 522 bases for the 16S gene. Both genes were highly variable: 76 and 53% of the positions were variable in the 28S D2 and 16S genes, respectively (these and the following figures are from protocol 3). However, the 28S D2 gene provides a stronger phylogenetic signal as indicated by its higher DD value: 0.537 compared to 0.357 (correspondingly, trees from simultaneous analysis were far more similar to those from separate analyses of the 28S D2 gene than the 16S gene). This does not appear to be attributable to differences in the between-site rate variation. Low values for the gamma shape parameter may make sequences less phylogenetically informative (Yang 1996). However, the values of the parsimony-based approximations of this parameter in the Yang-Kumar model (implemented in PAUP*) were similar among MPTs: 0.571–0.575 for the 28S D2 gene compared to 0.561–0.563 for the 16S gene. We suspect that the greater

Figure 1. Phylogenetic relationships between braconid subfamilies inferred from simultaneous analysis of 28S and 16S rDNA sequence data. The phylogram shown is from protocol 1. Branches are thickened if they are recovered in all other protocols with the range of bootstrap values for the between-subfamily branches shown (for clarity bootstrap support for within-subfamily branches is not shown). Tree statistics: length = 5319, consistency index = 0.257 and retention index = 0.548. For outgroups we have used two ichneumonids and an aculeate (the Braconidae and Ichneumonidae are sister taxa which together form the sister group of the Aculeata).

AT bias of the 16S gene is responsible for it being the less informative of the two. Whilst the base composition of the 28S D2 gene was slightly AT biased (mean = 57%, excluding outgroups and the aberrant *Megalohelcon*), the 16S gene was extremely AT rich (mean = 82%). A greater bias will make more of the positions effectively binary and, hence, more likely to have reverse mutations (two rather than four states available).

The ILD test found the genes to be incongruent ($p = 0.01$) and the correct treatment of such data sets is the subject of debate (Huelsenbeck *et al.* 1996). In addition to the emerging consensus that such data should be combined regardless (Davis *et al.* (1998) and references therein), we were confident doing so because we believe that the incongruence in our study is an artefact caused by noise rather than differing phyletic histories. Noise can

Figure 2. Estimation of the ancestral geographical distributions in the clade Aphidiinae plus *Mesostoa* plus *Aspilodemon* by ML and MP methods. The nodes show both the MP and ML estimations; for the ML method the LRs of 3.46 (basal node) and 4.60 have been converted to proportions in a pie diagram. The phylogram shown is from ML analysis of the 28S D2 sequence data aligned with MALIGN gap to a substitution cost of 2:1; branches are shown thickened if they are recovered in all other alignments. The branch represented by a dashed line shows a shift in base composition and is reduced to zero length for character estimation (the AT content is shown in parentheses after taxon names). Asterisks show cosmopolitan species, which we believe were introduced into the Southern Hemisphere. The phylogram was rooted using *Histeromerus* as an outgroup.

itself generate incongruence in ILD tests (K. Dolphin, R. Belshaw, D. Orme and D. Quicke, unpublished data) and excluding the ‘noisier’ elements from the alignment greatly reduces the apparent incongruence. The difference in length between the original and mean partition replicate is 2.87% for the complete sequences aligned by Clustal (protocol 3), but only 0.89% when the length variable regions are removed (protocol 2).

(b) *Phylogenetic relationships*

All simultaneous analyses recovered a clade within the Braconidae containing the Aphidiinae, *Mesostoa* and *Aspilodemon* with bootstrap support of above 80% (figure 1). This clade was also recovered in all analyses of individual genes. We are unable to estimate the geographical distribution basal to this clade because (i) the basal clade (Rhyssalinae plus Histeromerinae) of the sister group (the cyclostomes) is poorly resolved with both Northern and Southern Hemisphere genera present and (ii) basal to this sister group relationship the tree is unreliable for estimating character changes (the branches are both poorly supported and sensitive to the analysis protocol).

The result of the more thorough analysis of the above clade is shown in figure 2. This analysis was rooted using

one of the species (*Histeromerus*) in the basal clade of their sister group (cyclostomes) to calculate the two basal branch lengths. The topologies recovered under alternate alignment parameters are summarized in figure 3. The pairing of *Aspilodemon* with *Mesostoa* is not an outlier on relative apparent synapomorphy analysis (RASA) regression plots, which means the sister-group relationship recovered in most alignments is unlikely to result from long branch attraction (Lyons-Weiler & Hoelzer 1997).

(c) *Estimation of the ancestral distribution*

The sister group of the Aphidiinae is entirely from the Southern Hemisphere: *Mesostoa* together with all members of the Mesostoinae are Australian and *Aspilodemon* is a South and Central American genus currently grouped with other Southern Hemisphere genera, the Chilean *Hydrangeocola* and the Australian *Opiopterus* (note, these are therefore misplaced within the Hormiinae) (Wharton 1993).

All trees contain a long branch (dashed line in figure 2) caused by a shift in base composition (the minimum percentage AT in the derived clade is 5% higher than the maximum value elsewhere in this data set). Because this violates the ML assumption that branch length is proportional to time, we set the length of this

Figure 3. Reconstruction of the ancestral geographical distributions using other alignment parameters to those in figure 2 (the gap to substitution cost is shown under each phylogram). Other features are identical to the legend to figure 2. The LR_s (basal first) are 5.08 and 8.09 (1:1 alignment), 9.15 and -0.21 (3:2 alignment) and 3.03 and 3.54 (5:2 alignment).

branch to zero. ML estimation of the ancestral distribution of the Aphidiinae finds a southern origin of the aphidiines to be 'significantly' more likely under all alignment protocols (LR = 3.03–9.15) (figures 2 and 3).

MP estimation also supports a southern origin, but more weakly: in two alignments the MP estimation is southern, but in the other two it is ambiguous. All MP estimations are southern on the less preferred trees (MPTs built with gaps weighted as in the alignment protocol).

4. DISCUSSION AND CONCLUSIONS

ML may be an inappropriate method of ancestral character state estimation if its assumptions do not hold (Cunningham *et al.* 1998). (i) The assumption of rate constancy may be biologically unrealistic, with migration likely to have become more difficult after the continents broke away and (ii) multiple substitutions may reduce the internal branch lengths, favouring changes occurring on terminal branches. However, neither of these effects would bias the result in favour of a southern origin. We believe a more important concern here may be the applicability of ML to rare events as we cannot test how well the model for their change fits the data.

Nevertheless, in two alignments both MP and ML methods prefer a southern origin. In the remaining two, although MP is ambiguous, ML prefers a southern origin because the basal branches of the northern but relatively basal Ephedrini and Praini are longer than those of the Southern Hemisphere taxa (figure 2 and the 5:2 alignment in figure 3). Given the assumption that branch length is proportional to time, this means a longer period when the ancestors of the Ephedrini and Praini could have migrated from the Southern to the Northern Hemisphere. The ability to take into account the increased probability of change (here distributional) occurring on longer branches is a major advantage of ML over MP. A further caveat is necessary here. We did not include all species within the clade in our analysis; therefore, long branches could be caused by under-sampling. However, we consider this unlikely because the Ephedrini and Praini are represented in our analysis by more species than the total of the Southern Hemisphere species. Hence, the inclusion of additional taxa is more likely to reduce the branch lengths of southern taxa. Although we cannot predict how the inclusion of

all taxa would affect the results, we suggest they are as likely to strengthen as weaken the support for a southern origin: the omitted species in the putative sister group are all southern and one of the two omitted southern aphidiine genera (*Parephedrus*) has many plesiomorphic morphological characters (Gärdenfors 1986) (the other genus, *Vanhartenia*, is from sub-Saharan Africa; Starý & Van Harten 1974). Also consistent with the hypothesis of a Gondwanan origin for the Aphidiinae are the observations that (i) the basal braconid in our analysis, *Megalohelcon*, is Australian and (ii) the most recent simultaneous molecular and morphological analysis places the southern Labeninae as basal within its sister group the Ichneumonidae (Quicke *et al.* 2000).

On balance, therefore, we consider our data to support Schlinger's (1974) hypothesis of a Gondwanan origin for aphid parasitoids.

The origin of the Aphidiinae might alternatively be considered to be the first node that separates lineages within the subfamily. As shown in figures 2 and 3, this reduces the support for a southern origin: ML estimations remain southern in three alignments (LR = 3.54–8.09), but ambiguous in the fourth (LR = -0.21) and all MP support is lost, with estimates ambiguous in two alignments, southern in one and northern in one.

Finally, we notice that Eastop (1998) suggested that gall forming is ancestral in aphids. Both genera recovered as forming a clade with the Aphidiinae are associated with galls: *Mesostoa* is phytophagous, producing galls on *Banksia* (Proteaceae) (Austin & Dangerfield 1998), whilst *Aspilodemon* is a parasitoid of gall-forming Cecidomyiidae (M. Macedo, personal communication). Unfortunately the historical biogeography of aphids has yet to be estimated, although in anecdotal considerations of aphid origins the Southern Hemisphere plays a role disproportionate to the size of its current aphid fauna.

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