A molecular phylogeny and social behaviour of Japanese
Ceratina (Hymenoptera, Apidae, Xylocopinae)

ADAM L. CRONIN


Primitively eusocial insect are often considered particularly useful for investigations into the processes underlying the origins of eusociality. Ceratinine bees have long been regarded as solitary, but sometimes exhibit odd social traits for solitary species, while other species are known to be social, and social behaviour has been artificially induced in others still (Maeta & Sakagami 1995; Sakagami & Maeta 1995). Recent studies have had some success in elucidating aspects of the evolution of social behaviour in some groups using phylogenetics to infer historical changes in social behaviour (eg: Danforth 2002; Schwarz et al. 2003; Bull et al. 2003). Phylogenetic treatments of Ceratina have only recently been attempted in earnest (Terzo 2000) and behavioural data is lacking for many Ceratina species. Nonetheless, the Japanese fauna represents some of the most comprehensively studied Ceratina, and this study uses nucleotide data from mitochondrial COI and CytB and nuclear EF-1α regions to infer a phylogeny of Japanese Ceratina species. Data give good resolution for the subgeneric groups, and provide some support for the recent morphological phylogeny of Terzo (2000), but little resolution for higher relationships. Behavioural data suggest that sociality is variable within subgenera. The diversity and primitive nature of social behaviour in this group make it one of the most promising to provide insights into the process of social evolution, but more data are clearly needed.

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Introduction
The subfamily Xylocopinae consists of four tribes; Manueliini, Xylocopini, Allodapini and Ceratinini. This group represents one of the most interesting for students of social evolution, as sociality is characterised by some traits unique among bees, is often primitive, and is expressed in a variety of different forms (Schwarz et al. 1997, 1998; Hogenhoorn & Velthuis 1992; Maeta & Sakagami 1995; Sakagami & Maeta 1995; Michener 1990). Such variability may exist in a single species or vary at different taxonomic levels, facilitating the investigation of mechanisms underlying the expression of sociality at a variety of levels.

Recent work on xylocopine and allodapine bees has provided a wealth of new data that has, coupled with phylogenetic analyses, permitted some inference of evolutionary history of social behaviour in these groups (Bull et al. 2003; Schwarz et al. 2003; Leyes et al. 2000). In contrast, there has been little or no concurrent expansion of our knowledge of the other tribes of Xylocopinae. Nonetheless, these groups offer opportunities for some revealing insights with further focus. Members of the tribe Ceratinini are usually considered solitary, though rudimentary social behaviour occurs in some species (review in Maeta & Sakagami 1995; Sakagami & Maeta 1995), and it remains unclear if this condition is vestigial or derived (West-Eberhard 1987; Sakagami & Maeta 1995). While the behavioural database for Ceratina remains somewhat scanty, there have been detailed experimental studies done on many of the Japanese fauna, which includes examples of primitively eusocial, semisocial, quasisocial, and strictly solitary species (Maeta & Sakagami 1995; Sa-
Table 1. Taxa used in this study. * dry specimens collected and donated by N. Sugiura and Y. Sugimoto, Kumamoto University, Kyushu, Japan. Previously unpublished outgroup specimen sequences D. acutigera and M. magengae donated by M. P. Schwarz and N. J. Bull (Flinders University of South Australia), sequence for A. micronota from Schwarz et al. (2003). Pithitis sp. specimen donated by N. J. Bull. All sequences submitted to Genbank by the author (except A. micronota).

<table>
<thead>
<tr>
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<th>Species</th>
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<th>Locality collected</th>
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<td>flavipes</td>
<td></td>
<td>Smith 1879</td>
<td>Kitami, Hokkaido, Japan 2001*</td>
<td>AY250210 – AY250190 - AY250200</td>
</tr>
<tr>
<td></td>
<td>japonica</td>
<td></td>
<td>Cockrell 1907</td>
<td>Nishioka, Sapporo, Hokkaido, Japan 2001</td>
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<td>okinawana</td>
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kagami & Maeta 1995). Eusociality, with overlapping generations, cooperative brood care and division of labour (Michener 1974), occurs frequently in only a few species, while species within the same subgenera may exhibit very limited capacity to form any multi-female associations or are strictly solitary.

Michener (2000) recognises 2 genera comprising some 17 subgenera within the tribe Ceratinini, though the status of some groups remains somewhat equivocal. Yasumatsu & Hirashima (1969) recognise 9 species from 3 subgenera in Japan; Ceratina sensu stricto, Neoceratina, and Ceratinidita. A recent phylogenetic analysis of Ceratinines by Terzo (2000) recognised 22 subgenera, including 8 from the oriental region. Terzo (2000) recognises 4 subgenera for the Japanese fauna, transferring C. iwataii and C. satoi from Ceratina sensu stricto to the new subgenera Yasumatina Terzo 2000. However, while the reclassification of Terzo (2000) is discussed, I retain the previous classification for reasons of consistency with previous works. The present study, while limited in generic scope, comprises the first molecular analyses of species relationships in this group.

Methods

Taxa

All known species of Japanese Ceratina excluding C. esakii Yasumatsu & Hirashima 1969 were included in analyses, totalling 9 species representing
three subgenera (Ceratina sensu stricto, Ceratinula and Neoeratina) of a total of 22 recognised subgenera (Terzo 2000). In addition, an Indian Ceratina (Pithita) species was included as part of the ingroup. Species and collection details are shown in Table 1. Most specimens were fresh, from sweeping or taken from nests and placed in 99% ethanol, though some specimens were dry or pinned (see table 1), and all ingroup sequences were obtained for this study. Outgroup taxa were chosen from the (probable) sister tribe Allostaphani (Schwarz et al. 2003), with sequences borrowed from a recent study by (Schwarz et al. 2003) or donated by Schwarz and co-workers representing 3 basal genera, Dalloaporella Michener 1975, Allo-
dape Lepeleter & Serville 1825 and Macrogala Cockerell 1930. Voucher specimens of all ingroup species used in this study are available in the Hokkaido University Museum.

DNA Extraction, Amplification and Sequencing

Bees were removed from ethanol and thoraxes or thoraces and abdomens (for C. satol and C. bon-
nersis) were pressed firmly between blotting paper. For DNA extraction, samples were homo-
genised in 0.7ml CTAB buffer with 45mg PVPP and 10μl of Proteinase-K (20mg/ml) and incubated for 2 hours at 55°C with occasional mixing.

One nuclear and two mitochondrial regions were amplified using the following primers: for the F1 copy of elongation factor-1α (EF-1α), primers designed by Danforth and Ji (1998); EF1-
For2: 5'- AAG GAG GC[CG] CAG GAG ATG GG – 3’; EF1-Rev2: 5’ – [T/C]TC [G/C]AC [T/C]TT CCA TCC GTA CC – 3' for; for Cytochrome b (Cytb) primers designed by Y. C. Crozier (James Cook University, Australia); clb1: 5’ – TAT GTA CTA CCA TGA GGA CAA ATA TC – 3’; clb2: 5’ – ATT ACA CCT CCT ATT GTA AAT GGA AT – 3’; and for Cytochrome Oxidase I (COI), primers designed by Lunt et al. (1996); UEAT7: 5’ – TAC AGT TGG AAT AGA CGT TGA TAC – 3’; UEAT10: 5’ – TCC AAT GCA CTA ATC TGC CAT ATT A – 3’. The following primers were designed when the above primers failed, modified from the Apis melliferat Linnaeus 1758 mitochondrial genome (Crozier & Crozier 1993), a Xylocopa col-
laris Lepeletier 1841 COI fragment (Tanaka et al. 2001) and/or sequences gained with the primers above, with the aid of the Primer3 program; for

Cyt b (internal primers); cytb NSF: 5’ – TTT TGA GGT GCA ACA GTT ATT – 3’, Cytb NSR: 5’ – GGT CAG ACT GTA AAA TTG AAT AAG CA – 3’, and for COI (external primers); COI NLF: 5’ – GTA GGG TTA GAT GTT GAT ACA CG – 3’, COI NLR: 5’ – TTC AAT GCA CCT ATT CTG CCA TA – 3’.

PCR amplifications were carried out in 50μl volumes containing 29μl MQH2O, 4μl dNTP’s, 5μl reaction buffer, 6μl MgCl2, 2μl each primer, 1 unit Taq gold Polymerase and 2μl 1:5 diluted DNA. PCR reactions were performed in a Takara TP400 thermal cycler, with the following program for EF-
1α and Cytb: 35 cycles (95°C, 45s; 52°C, 45s; 72°C, 60s) and a 6 minute extension phase at 72°C. The same program was used for COI but with an annealing temperature of 48°C. PCR products were purified using Qiagen-Quick spin columns following the manufacturers protocols. Cycle sequencing was performed in 20μl volumes using a Dye Terminator ready reaction kit, with 4μl Terminator mix, 5μl DNA, 1μl PCR primer. Products were purified by isopropanol precipitation, and sequenced on an ABI prism 310 sequencer.

Phylogenetic Techniques

Sequences were manually aligned using the BioEdit program (Hall 1999) and phylogenetic analyses were performed using PAUP*4beta10 (Swofford 1999). Recent phylogenetic studies of Xylocopineae have indicated a high A-T bias for mitochondrial regions with possibly saturation of 3rd positions, and have thus suggested down-weighting of transitions relative to transversions at mtDNA 3rd codon positions (Reyes et al. 1999; Leyes et al. 2000; Bull et al. 2003; Schwarz et al. 2003). Experimentation with various weighting schemes (transitions down-weighted 0.5, 0.3, 0.1, 0.05 and 0 for mitochondrial DNA 3rd positions) was thus explored for MP analyses here. In addition, the effect of removing 3rd positions entirely from the analysis was explored; for mtDNA genes and, subsequently for all regions.

Data were subject to MP and ML analyses. MP analyses employed heuristic searches with TBR branch swapping and 50 random sequence additions in PAUP*4beta10 (Swofford 1999), and were bootstrapped with 1000 pseudoreplicates with 50 random sequence additions per replicate. Cunningham (1997) recommended incongruence
length difference tests (Farris et al. 1994), as an indication of when combining data will generally improve phylogenetic accuracy (when $p > 0.01$). However, while ILD tests are included in this study to assess combinability of gene partitions, recent studies have questioned the validity of ILD tests in this role (Barker & Lutzoni 2002; Dowton & Austin 2002) and Dowton & Austin’s (2002) alternative recommendation of the exploration of a range of models during analyses is also followed here.

COI, CytB and EF-1α are known to show different substitution rates and/or base composition in Xylocopid bees (Reyes et al. 1999; Leyes et al. 2000; Schwarz et al. 2003), and ML analyses that attempt to combine gene partitions are problematic (Schwarz et al. 2003). Thus, ML analyses were carried out using the method described for similar gene fragments for Allophine bees in Schwarz et al. (2003); each gene partition was subjected to log likelihood tests applied to a series of 56 alternative substitution models using Posada and Crandall’s (1998) Modeltest 3.06 program, to obtain the optimal ML model for each gene fragment. The 659 most parsimonious trees were then obtained via an unmodified MP analysis, and log likelihood values were calculated for each tree for each gene partition using the relevant model. Log likelihood values were then summed for each tree to measure relative fit of trees to the partitioned likelihoods.

### Results

#### Base Pair Composition

Molecular analyses yielded 617 aligned nucleotides for COI, 417 for CytB, and 457 for EF1α, giving a total of 1491 aligned nucleotides. Of these, 409, 246 and 338 were invariant and 151, 114 and 82 (a total of 347) respectively were parsimony informative. Base pair compositions are summarised in Table 2, which includes percentage A-T richness and $\chi^2$ tests for nucleotide bias. Base pair composition was A-T rich for all mtDNA regions, but particularly so for 3rd positions, and differed significantly between taxa for COI and EF-1α 3rd positions.

#### Maximum Parsimony Analyses

The ILD test for unweighted MP analyses indicated that combining data would not likely result in a loss of resolution ($p = 0.01$). An unweighted heuristic search including all characters with 50 random sequence additions yielded a single most parsimonious tree. The result of bootstrap analysis utilising 1000 pseudoreplicates with 50 random sequence additions per replicate shown in Fig.1. This analysis indicates that although bootstrap support for sub-generic groups is high, there is little support for higher relationships, with an effective polytomy (the only resolved node has a bootstrap value of 59) at the subgeneric level. Recent
phylogenetic studies of Xylocopinae have suggested down-weighting of transitions to transversions at 3rd codon mtDNA positions due to probably saturation (Reyes et al. 1999; Leyes et al. 2000; Bull et al. 2003; Schwarz et al. 2003), and that weighting 3rd position mtDNA transitions 0 provided the highest resolution. Experimentation with various weighting schemes (transitions down-weighted 0.5, 0.3, 0.1, 0.05 and 0 for mitochondrial DNA 3rd positions) yielded trees of identical topology though bootstrap support did not improve over the initial analysis show in Fig 1. Once again, ILD tests indicated combining data was permissible (p = 0.01 in all cases). In addition, the effect of removing 3rd positions entirely from the analysis was explored; for mtDNA genes and, subsequently for all regions. Both analyses yielded fully resolved trees with identical topology which, though bootstrap support for basal nodes remained low, differed from other MP analyses in placing (Neoceratina + Ceratina sensu stricto) as the sister group to Ceratinidia, though bootstrap support remained low (62). However, the analysis with the highest bootstrap values (that with all 3rd positions weighted 0) suggests that Pithitis is the sister group to (Ceratinidia + (Neoceratina + Ceratina sensu stricto)), and is shown in Fig. 2 (ILD tests for these two latter treatments: p = 0.25 and p = 0.33 respectively).
Maximum Likelihood Analyses

The Modeltest 3.06 fit results indicated different models for each partition as follows: COI = GTR+I+G; CytB = TVM+G; EF-1α = K80+G. Fitting these models to the 659 most parsimonious trees resulted in combined log likelihood values ranging from 5944.418 to 6382.115. The tree with the lowest log likelihood, as shown in Fig. 3 maintains subgeneric groupings as per MP analyses. Whereas most inter-subgeneric relationships are similar to the MP tree with 3rd positions weighted 0, Pithitis now occupies a more distal position, with (Ceratina sensu stricto + (Pithitis + Neoceratina)) forming the sister group to Ceratinidia, in contrast to all MP analysis where Pithitis it is part of a basal polytomy or forms the basal group itself. However, in a strict consensus tree of the 10 trees with the lowest combined log likelihood scores the topology collapsed into a 4-way polytomy between recognised subgenera similar to the MP analysis in Fig. 1.

Discussion

Terzo (2000) provides a comprehensive phylogenetic analysis of Ceratina based on morphological characters. His reconstruction of oriental subgenera is shown in Fig. 4. Terzo reallocates C. iwatali and C. satoi to the novel subgenera Yasumatina, as distinct from previous classification within Ceratina sensu stricto. His morphologically based analysis suggests that Ceratina sensu stricto is a sister group to Yasumatina (Neoceratina (Ceratin-
Both MP and ML analyses presented here provide good support for the distinction of sub-genera Ceratina sensu stricto, Ceratinidia and Neoceratina, but little resolution for higher relationships between the subgenera. However, there is a suggestion that Pithitis forms the sister group to (Ceratina sensu stricto + Ceratinidia + Neoceratina; bootstrap = 81). Molecular data provide no indication of whether Terzo’s (2000) erection of Yasumatina is justified. C. megastigmata, C. iwataii and C. satoi are closely associated in all analyses, however, Yasumatina and Ceratina sensu stricto are sister groups in Terzo’s (2000) analysis if one takes into account the lack of molecular data for specimens from intervening subgenera in the present study. Higher relationships between subgenera lack bootstrap support in this analysis, and the suggestion that Ceratinidia forms the sister group to (Ceratina + Neoceratina) contrasts with morphological data (Terzo 2000).

Ceratina is considered to comprise mostly solitary species (Michener 1990; Maeta & Sakagami 1995), though eusociality has been confirmed in some species and social-like traits are found in other ‘solitary’ species (Sakagami & Maeta 1995; Maeta & Sakagami 1995). Social behaviour in Ceratina has been considered vestigial (West-Eberhard 1987) and rudimentary (Sakagami & Maeta 1995) but a lack of published behavioural data hinders a comprehensive treatment. One of the better-treated groups is the Japanese fauna, primarily from the life history and experimental stud-
ies of Sakagami and Maeta and coworkers (review in Maeta & Sakagami 1995), and some social traits of Japanese Ceratina are summarised in Table 3. Eusociality has been recorded in the subgenera Ceratina and Ceratinidia in Japan, and is known to vary within subgenera. Whereas C. japonica and C. flavipes rarely form social colonies in nature or in the lab, C. okinawana is primarily eusocial, and social degeneration during evolutionary change to temperate species is assumed by Maeta and Sakagami (1995). Furthermore, eusociality is known in C. iwataii; (Maeta 1993), and delayed eusociality in C. megastigmata (Katayama & Maeta 1979; Y. Maeta pers. com.), whereas the available data indicate that C. satoi is truly solitary (Maeta pers. com.). Behavioural data are lacking for the two species of Neoceratina. However, data from C. australiensis Perkins 1912 (Michener 1962; Cronin unpub.) suggest that this species is predominantly solitary, though at least one possibly social nest has been recorded. Other studies are scarce, but suggest solitary behaviour is predominant (review in Michener 1990; Maeta & Sakagami 1995; Sakagami & Maeta 1995).

In a recent phylogenetic study, Danforth (2002) concluded that there were three origins of eusociality in the Halictidae, with multiple (up to 12) reversions to solitary behaviour leading to a high degree of social variability within phylogenetic groupings (Danforth 2002). Australian allodapine bees, in contrast, exhibit generic conservatism with respect to social traits (Terney et al. 2000). Molecular data presented here provide good support for subgeneric groupings, and indicate a high level of intra-group variability with respect to social behaviour. Higher level relationships remain unclear, though there is a suggestion that Ceratinidia forms the sister group to (Ceratina + Neoceratina) (Figs 2,3) in contrast to morphological analyses (Terney 2000). This could indicate an origin of more advanced social characteristics in the ancestor to Ceratinidia or a loss of social traits in members of (Ceratina + Neoceratina). However, eusociality occurs in C. iwataii and scant published behavioural data are available for C. dentipes and C. boninensis. Furthermore, there is a suggestion that variation within Ceratinidia may be climate linked, with temperate species (C. japonica and C. flavipes) frequently limited to solitary behaviour while tropical species are eusocial (Maeta & Sakagami 1995). Further data may reveal cryptic behaviours in other species as have experimental studies of C. japonica and C. flavipes, and clearly more data are needed to reach any conclusions about social evolution in this group.
Table 3. Some social traits of Japanese Ceratina. # broods = number of broods per year; N females in MF: number of females in multifemale colonies; Sociality: E = eusocial, D = delayed eusocial, S = solitary, Q = quasisocial, Se = semi-social; Bo = eusocial, Re = reversed eusocial; Longevity: females live longer than 1 year vs/2 and (range in years); Natural-induced colonies: the percentage of multifemale colonies found to occur naturally or that could be induced in the laboratory; MF% new/reused: the percentage of naturally occurring new and reused nests that contained multifemale colonies.

<table>
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<tr>
<th>Subgenus</th>
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<th>Distribution</th>
<th>#Broods</th>
<th>N females in MF</th>
<th>Sociality</th>
<th>Longevity</th>
<th>Natural/Induced Colonies</th>
<th>MF% reused</th>
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<td>2-3</td>
<td>E S Re Q Eo</td>
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<td>1% or 31%</td>
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<td>Temperate</td>
<td>1</td>
<td>2-4</td>
<td>Re S De</td>
<td>Y (–4)</td>
<td>20%/easy</td>
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<tr>
<td>Ceratinia</td>
<td>flavipes</td>
<td>Temperate</td>
<td>1</td>
<td>2</td>
<td>Q</td>
<td>?</td>
<td>0.1%/3% easy</td>
<td></td>
<td>Sakagami &amp; Maeta 1987</td>
</tr>
<tr>
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<td>megastigma</td>
<td>Temperate</td>
<td>1</td>
<td>2+ (?7)</td>
<td>Q S De</td>
<td>Y (2-3)</td>
<td>5%/3%</td>
<td></td>
<td>Katayama &amp; Maeta 1979</td>
</tr>
<tr>
<td>Ceratina</td>
<td>iwatii</td>
<td>Both</td>
<td>2</td>
<td>2+ (?7)</td>
<td>E</td>
<td>some</td>
<td>55%? or 5%?</td>
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<td>Maeta 1993</td>
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</table>

Primitively social Hymenoptera are ideally suited to investigations of the evolutionary origin of sociality (Danforth 2002) and phylogenetic analyses provide an effective mechanism to reconstruct the historical pattern of social evolution in such groups (Danforth 2002; Schwarz et al. 2003). Ceratinae bees have the potential to yield numerous insights into processes underlying the evolution of sociality as social behaviour is varied and often exhibits primitive characters (see review in Sakagami & Maeta 1995). Further behavioural studies of these species, coupled with the collection of molecular data, should provide interesting counterparts to existing studies of other Hymenoptera.

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References


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