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ADVANCED CASTES AT THE OUTSET OF EUSOCIALITY IN WASPS (VESPIDAE)

by

PATRICK KONRAD PIEKARSKI

B.S. University of Calgary, 2014

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science
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Major Professor: Barbara J. Sharanowski

ABSTRACT

A dominating and widespread view is that evolutionary change is gradual and waits upon mutation. Likewise, it is thought that workers and queens of eusocial insects diverged gradually and stepwise. That is, rudimentary castes preceded advanced castes. This paradigm hinges on eusociality having evolved once in Vespidae, and primitively eusocial paper wasps lacking ontogenetic caste biasing (differentiation of castes during larval development). Using a phylogenomic approach this study shows strong evidence for two origins of eusociality in vespid wasps, wherein one origin is likely characterized by the sudden appearance of castes with physiological, and perhaps even morphological, differences established before adulthood (i.e. advanced castes). Ontogenetic caste biasing was likely present at the outset of eusociality in the most recent common ancestor of paper wasps, yellowjackets and hornets. This suggests that the definitive, non-temporal castes of eusocial wasps evolved from interactions between mothers and daughters, rather than same generation females. These results challenge the idea that castes began with only rudimentary differences. A model of stepwise caste divergence, which assumes an independent mutation event for each phenotypic caste difference, is unwarranted. It is hypothesized that phenotypic plasticity and cryptic genetic variation may explain how some eusocial societies emerged, and began with advanced castes. The results suggest that evolution can produce alternative phenotypes with many aspects of the phenotype being discrete at the outset. Thus, the emergence and divergence of castes in eusocial vespids was not necessarily a gradual process.

I dedicate this thesis to my brother, and best friend, Daniel Piekarski, and my loving, supporting parents Renata and Boguslaw Piekarski.

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INTRODUCTION

Current Paradigm and Its Roots

Eusocial animals, such as ants, bees, and wasps, exhibit organized societies characterized by nest-sharing between mothers and daughters, cooperative brood care, and reproductive division of labor (i.e. presence of a discrete worker and queen caste)¹. The extent of divergence between workers and queens varies across eusocial species, with some showing primarily behavioral differences between castes, and others showing elaborate physiological and/or morphological differences^{1,2}. Varying degrees of eusociality are found in Vespidae³, making these wasps exceptional for studying the emergence and divergence of castes, and polyphenism in general. Polyphenism involves a switch-like mechanism, whereby environment, not genetic make-up, determines which phenotype is expressed by an individual⁴. In caste polyphenism, environmental cues experienced during larval development and/or adulthood determine whether a female becomes a worker or queen^{2,4,5}. Although caste determination is not strictly controlled by environmental factors and can involve genetic effects in some eusocial insects, it appears that genetic caste determination is negligible in vespid wasps^{5,6}.

Espoused by most researchers⁷⁻¹⁵ is the idea that castes of eusocial vespids, and Hymenoptera in general^{1,2,16}, have advanced in a stepwise fashion, with rudimentary castes that have only differences in behavior and ovarian status, gradually giving rise to advanced castes with physiological and morphological differences that are established during ontogeny. Specifically, it is thought that lack of ontogenetic (preimaginal) caste biasing (i.e. biasing during larval development, hereafter referred to as PCB), whereby all females are born with relatively

equal reproductive physiology, was the starting point of vespid eusociality⁷⁻¹⁵, rather than physiologically discrete, sub-fertile workers that are largely determined preimaginally. This framework has been popular because: (1) it is generally envisioned that the evolution of definitive, non-temporal castes stems from interactions among same generation females and began in a casteless nest-sharing (i.e. polygynous) context⁸; (2) the well-ascribed phylogeny supporting a single origin of eusociality implies the ancestral state of all eusocial wasps to have been rudimentary, temporal castes without preimaginal physiological differences^{10,13,17}; and (3) the observed caste totipotency of adult females in *Polistes*⁷, which has made the idea that primitively eusocial wasps lack PCB “influential”¹⁸. However, the absence of PCB as the groundplan for primitively eusocial wasps has been challenged before^{11,18-21}. These challenges have gone largely unheeded, because the dominating phylogeny implies a lack of PCB (i.e. castes without any distinct traits established before adulthood) as the groundplan condition for all eusocial vespids (Figure 1). If the well-ascribed single origin of eusociality hypothesis is demonstrably false, then the current framework becomes dubious. Currently, a single origin of eusociality in vespids is the dominating view^{13,17,22}, but a small minority of researchers are convinced of two independent origins^{23,24}.

Single Origin of Eusociality Hypothesis

The first phylogenetic study of vespids consisted of a parsimony analysis of 50 phenotypic characters coded for over 500 species²⁵. The study formalized the six-subfamily classification, and found that Stenogastrinae, Polistinae and Vespinae shared a recent common ancestor (Figure 1). At that time, it was thought that eusociality characterized only some

stenogastrines. This led to the conclusion that eusociality had evolved at least twice, with an independent origin(s) occurring within Stenogastrinae²⁵. After the accumulation of detailed accounts on stenogastrine behavior during the 1980's, it was clear that eusociality was the groundplan condition of the subfamily. It then followed that eusociality evolved once in Vespidae¹⁰.

More recently, two molecular studies found support for an opposing phylogeny of Vespidae^{23,26} wherein Stenogastrinae was distantly related from the other eusocial wasps, and thus argued eusociality arose twice. However, the first study²⁶ has been dismissed due to small taxon sampling (only 13 vespids) and an absurd result, namely inclusion of honey bees (Apidae) within Vespidae. More importantly, parsimonious realignment of their dataset supported a single origin of eusociality²⁷. The second study retrieved a molecular phylogeny based on sequences from four genetic loci (28S, 18S, abdominal-A, and RNA polymerase II) across 30 species (three outgroup, 27 ingroup) supporting two origins of eusociality²³. This work has had limited traction, due to its small taxon and site sampling, suboptimal alignments, and neglect of morphological and behavioral data¹⁷. Furthermore, parsimonious realignment of that dataset returned a phylogeny that rejected a paraphyletic Eumeninae¹⁷ – a crucial aspect of those authors interpretation of how eusociality evolved^{3,23}.

After the most comprehensive analysis to date, wherein four loci (CO1, 28S, 12S, 16S) and 333 phenotypic characters for 130 in-group species were simultaneously analyzed, the single origin of eusociality hypothesis was reinforced¹⁷. However, a recent study reanalyzed this dataset and confirmed that simultaneous analysis of all available evidence does support a single origin of eusociality, but that analysis of the molecular data alone supports a dual origin²². It was

concluded that molecular and phenotypic evidence were at odds, and convergent evolution of behavioral and morphological traits was a possible explanation²². A recent study that focused on reconstructing the evolutionary history of Hymenoptera, and thus had very limited sampling of vespids, recovered two origins of eusociality within Vespidae²⁸.

Levels of Eusociality

The term caste-biasing applies to factors that affect the probability of a female becoming a worker or queen, which means that pre-imaginal caste-biasing is universal in eusocial insects given that nutritional and other differences during ontogeny affect the reproductive potential of adults even in solitary insects. Since minor PCB is ubiquitous and unavoidable due to natural variation in the inputs that larvae receive during development, I reserve the term PCB for cases of exaggerated biasing, wherein females exhibit alternative phenotypes with at least behavioral and physiological differences that were established before adulthood. One phenotype greatly increases the likelihood of becoming a queen (i.e. a gyne phenotype), and the other decreases the likelihood of becoming a queen (i.e. a non-gyne, putative worker, phenotype). I use the term gyne to refer to females that have been sent down a developmental trajectory during larvalhood that results in a distinct phenotype (typically, prolonged behavioral quiescence and inactive reproductive physiology after eclosion) that heavily *biases* the individual to become a queen. More simply, they are putative queens who may or may not attain queen status (i.e. they can become a subordinate cofoundress that exhibits a worker behavioral phenotype). Typically, the term gyne refers to late season non-working, hibernating females that become foundresses²⁹. However, it may be that overwintering only serves to sharpen the distinctiveness of the gyne

phenotype, but does not necessarily sharpen the bias of a gyne becoming a queen. Nonetheless, physiological priming for diapause is generally a caste-biasing factor that increases the likelihood of becoming a queen.

Vespidae includes facultatively, primitively, and highly eusocial species³. Facultative eusociality exclusively describes hover wasp (Stenogastrinae) social behavior. In hover wasps, there is likely an absence of PCB, and reproductives (queens) and non-reproductives (workers/helpers) differ primarily in their behavior and stage of ovarian development¹³. The helper phenotype is temporary, and helpers eventually leave or take over the natal nest and become a reproductive¹³. In other words, all females are capable of laying eggs, and usually do so after helping rear their mothers brood. Thus, stenogastrine caste determination is almost exclusively regulated by environmental and social factors experienced during adulthood.

The paper wasps (Polistinae) are subdivided into independent-founders and swarm-founders (Epiponini, *Polybioides*, and some *Ropalidia*). In the latter, a swarm of workers accompanied by a small number of idle queens found nests rather than a single, or small group of foundresses³⁰. Independent-founding paper wasps (*Belonogaster*, *Mischocyttarus*, *Parapolybia*, *Polistes*, and some *Ropalidia*) are considered primitively eusocial. Primitive eusociality is characterized by a lack of morphological differences between workers and queens, but worker status is typically lifelong despite workers having the potential for direct reproduction^{24,31}. As in Stenogastrinae, ovary development is influenced by age, social status and task performance during adulthood³²⁻³⁴. However, it has been demonstrated in some primitively eusocial species that most workers are born permanently incapable of direct reproduction^{35,36} (but see³³), and some females refuse to copulate³⁷. This suggests that although workers of many primitively

eusocial species can become reproductive (replacement queens), there may be some degree of preimaginal caste biasing^{18,37}. In contrast to hover wasps, many paper wasps, whether swarm or independent-founding, exhibit PCB, whereby females undergo differentiation during larval development that biases them to become either a queen or worker^{11,12,18,21,35-43}. PCB is evident in temperate and subtropical independent-founding species, where future foundresses (i.e. gynes) are born with physiological differences that prime them for diapause^{21,40,44}. Diapausing gynes are future foundresses, and thus are more likely to become queens than non-diapausing offspring (i.e. non-gynes). Hence, factors that fate offspring to express either a non-diapause or diapause phenotype are usually preimaginal caste biasing factors. Nutritional and vibrational signals received by larvae have been implicated in caste-biasing in primitively eusocial paper wasps^{5,19,40,45}. Thus, caste determination in some, maybe most^{18,19}, primitively eusocial polistines is regulated by environmental and social factors experienced during adulthood *and* early development.

Highly eusocial vespids have the most advanced caste polyphenism, whereby workers are permanently sterile and morphologically distinct. Morphological decoupling of workers and queens characterizes yellowjackets and hornets (Vespinae)³, most swarm-founding paper wasps^{12,43,46}, and some independent-founding *Belonogaster*, *Polistes* and *Ropalidia*^{18,36,37,42,47}. I consider castes with physiological differences that have an ontogenetic basis as advanced, while morphologically discrete castes represent an elaboration of ontogenetic caste biasing. Ontogenetic differentiation that results in preimaginal physiological, and sometimes morphological, queen/worker dimorphism is thought to be a derived condition of more advanced societies.

This study uses a phylogenomics approach to test the well ascribed single origin of eusociality hypothesis, and investigates whether simple, monomorphic castes characterized the early stages of eusocial evolution. If eusocial wasps with the simplest of castes are distantly related from those with more advanced castes, then it follows that castes at one origin of eusociality were more pronounced than previously suspected. Current models for vespid eusocial evolution suppose that eusociality arose in a polygynous (multiple egg-layers on a shared nest) context in the absence of meaningful PCB, such that eusocial lineages descended from a casteless nest-sharing ancestor^{8,11} or began with rudimentary reproductive division of labor¹⁰. To test this supposition, and understand how castes originated and diverge, we require a robust phylogeny. The sudden appearance of eusocial societies with advanced castes would conflict with the current conception of stepwise caste divergence, and would require a mechanistic explanation of how castes began with numerous differences – one that does not rely on independent mutation events for each phenotypic difference. Instead, phenotypic plasticity and standing/cryptic genetic variation may explain how an advanced caste polyphenism (i.e. castes with at discrete, bimodal physiological differences that have an ontogenetic basis) suddenly appeared.

METHODS

Taxon Sampling

Sequence data for 137 species (136 ingroup, 1 outgroup) was successfully captured. Publicly available hymenopteran genomes, and genomic sequences from 11 ichneumonoid wasps that were collected for probe design (see probe design), were also included for analysis, which added 2 ingroup and 24 outgroup representatives (Table 1). Thus, in total 163 taxa are incorporated into phylogenetic analyses, 138 of which are ingroup taxa. This serves as the first molecular study to sample all recognized subfamilies and tribes of Vespidae. The ingroup samples come from 26 countries (Table 2). More taxa are sampled here than any other previously published phylogenetic study of Vespidae. For a complete list of the 142 taxa that were processed through the anchored hybrid enrichment workflow, see Table 2.

Data Collection

Extraction

Genomic DNA was extracted from specimens following Qiagen protocols in conjunction with the DNeasy™ Tissue Kit (Qiagen, Valencia, CA, U.S.A.). Voucher specimens were deposited in a variety of institutions (list available upon request). DNA from dry and ethanol preserved specimens that had collection dates ranging from 1987 to 2015 were successfully extracted. Downstream Illumina sequencing was successful for 137 of 142 species.

Locus selection and probe design

Loci were selected following the general criteria outlined in ref.⁴⁸. Probes were designed from available hymenopteran genomes (*Apis mellifera*, *Nasonia vitripennis*, *Microplitis demolitor*, *Diachasma alleoleum*, and seven ant species), and genomic sequences from 11 ichneumonoid wasps (4 braconids, 7 ichneumonids) that were sequenced across two rapid run HiSeq Illumina lanes. From these reference sequences, collaborator A. Lemmon utilized his probe design pipeline to select 57,066 probes tiled across 200 KB of sequence at 2.15X tiling density. These probes cover 541 loci for ~400 bp nt each belonging to ~450 genes.

Library preparation, anchored enrichment and sequencing

Genetic data was obtained through the Center for Anchored Phylogenomics at Florida State University (<http://www.anchoredphylogeny.com>) using the general methods outlined in Lemmon and Lemmon, 2012 (ref.⁴⁸). First, each genomic DNA sample was sonicated to a fragment size of 150-350bp using a Covaris E220 focused-ultrasonicator. Subsequently, library preparation and indexing were done following a protocol outlined in Meyer and Kircher, 2010 (ref.⁴⁹). Indexed samples were then pooled at equal quantities determined using a Qubit® fluorometer (16 samples per pool), and enrichments were done on each pool using an Agilent Custom SureSelect kit (Agilent Technologies) with custom designed probes (details above). The general enrichment process uses streptavidin-coated magnetic beads to isolate targeted genomic fragments that hybridize with the oligonucleotide probes⁵⁰. Enriched fragments were pooled in groups of three (three pools total with 48 samples in each) before sequencing on the PE150

Illumina HiSeq2000 (three lanes, 48 samples per lane). Sequencing was performed in the Translational Science Laboratory in the College of Medicine at Florida State University.

Data processing

Data were processed following methods described in Prum et al. 2015 (ref.⁵¹). Briefly, a bioinformatics pipeline completes a workflow that: merges overlapping paired-end reads (Merge.java), assembles reads into contigs and generates a consensus sequence for each locus per sample (Assembler.java), filters out consensus sequences derived from assemblies with low coverage (IdentifyGoodSeqsViaReadsMapped.r, GatherALLConSeqsWithOKCoverage.java), determines orthologues (GetPairwiseDistanceMeasures.java, plotMDS5.r), aligns sequences within each orthologous set using MAFFT v7.023b⁵², and trims/masks the alignment for each locus (TrimAndMaskRawAlignments3).

Coalescent-based Species Tree Estimation

Gene tree incongruence caused by incomplete lineage sorting presents a challenge to species tree estimation, and is especially problematic when time between speciation events is short⁵³⁻⁵⁶. In the presence of gene tree heterogeneity, concatenation-based species tree estimations may result in incorrect trees with high support^{53,54}. On the other hand, coalescence-based approaches are statistically consistently in the presence of gene tree incongruence⁵⁴⁻⁵⁶.

Gene tree generation

The best evolutionary model for each locus was determined using jModelTest 2.1.7⁵⁷. The loci were not partitioned by codon position, and the single model with the highest BIC score was assigned to each locus. Gene trees were then inferred using MrBayes3.2.6⁵⁸. Analyses were submitted in batch to the computing system at the UCF Advanced Research Computing Center. Branch lengths were given an unconstrained, exponential prior of 100 to prevent the MCMC chain from being trapped in parameter space characterized by unrealistically long tree lengths⁵⁹. All gene tree analyses were comprised of four runs with four chains each, being sampled a total of 2000 times with a 0.25 burn-in. In general, loci with more sites require more generations to reach stationarity. Loci that were <250bp were run for two million generations; >250bp but <600bp for five million generations; >600bp for 10 million generations. A total of 378 gene trees were estimated.

Locus exclusion

Loci that did not reach stationarity, perhaps due to model inadequacy or homoplasy, were excluded in the second coalescent-based analysis. A locus was considered to have failed the stationarity test if at least one criterion was true: 1) Average standard deviation of split frequencies above 0.05; 2) Potential scale reduction factor for any one estimated parameter above 1.025; 3) Average estimated sample size for any one estimated parameter below 100. Only 12 of 378 loci failed to reach stationarity (not shown). To ensure that loci with taxon incompleteness and ambiguously aligned regions were not impacting species tree estimations, species trees were estimated after processing the alignments through Gblocks 0.91b⁶⁰. The

following Gblocks 0.91b parameters make up the trimming scheme: the minimum number of sequences for a conserved position was set to 82 (~50% of taxa), the minimum number of sequences for a flank position was 122 (~75% of taxa), the maximum number of contiguous nonconserved positions was 8, the minimum length of a block was 10, and allowed gap positions was set to 'with half'. After gblocks trimming, 60 of 378 loci were entirely trimmed and had a sequence length of zero. For the remaining 318 loci a base composition heterogeneity test was performed within PAUP4.0b10⁶¹. Loci with base composition heterogeneity introduce systematic error^{62,63}, and so 83 loci that failed the heterogeneity test were excluded.

Coalescent-based analyses

Coalescent-based species tree estimations were done using ASTRAL4.10.6^{55,56,64}. Two ASTRAL trees were estimated. The first is based on all 378 gene trees, and the second on 228 gene trees (83 had base heterogeneity; 60 dropped post gblocks; 7 failed the stationarity test). Both analyses used gene trees that were generated at the start, prior to trimming with Gblocks (see Gene tree generation). Local posterior probabilities calculated within ASTRAL were favored over multi-locus bootstrapping (MLBS) support values⁶⁴. These posterior probabilities have higher precision (percentage of supported branches that are correct) and recall (the percentage of all true branches that are supported) compared to MLBS, and are not prone to false positives even with high levels of gene tree estimation errors⁶⁴.

Concatenation-based Species Tree Estimation

Poorly resolved gene trees caused by low phylogenetic information within individual genes may make concatenation-based estimations outperform coalescence-based estimations⁶⁵. Thus, concatenation-based species tree estimations were also performed. Analyses were done on two primary datasets consisting of 378 loci (all loci) and 235 loci. The second dataset contains gblock trimmed loci, and excludes loci that failed the base composition heterogeneity test. The concatenations are of the following length: 378 loci = 162919 bp; 235 loci = 63712 bp (Table 3).

Parsimony

Parsimony analyses were performed within TNT v1.5⁶⁶. Uninformative characters were left active for two reasons. First, these bootstrap values will be most comparable to RAxML bootstrap values. Secondly, uninformative characters deflate bootstrap support values⁶⁷, and so by leaving them active the potential for false positives is reduced (high support for a clade that is not true). *Nasonia vitripennis* was set as the outgroup and gaps were treated as missing data. All characters were weighted equally, which is especially justifiable with molecular datasets⁶⁸. The tree search was comprised of a driven search with initial addition sequence =5, find min. length =15, and sectorial search (defaults), tree fusing (defaults), drift (20 reps) and ratchet (200 reps, 8 upfactor) activated. This was followed by branch breaking, and an xmult search. The best tree length scores recovered were 364354 for the 235 gene tree, and 963153 for the 378 gene tree. Next, 1000 standard bootstraps were performed using traditional searches (defaults), with support values output as frequency differences⁶⁹.

Maximum likelihood

PartitionFinderV2.0.0⁷⁰ was used to find the best-fit models and partitioning scheme for the two concatenations. Each locus was treated as a subset, for a total of either 378 or 235 subsets. PartitionFinder was run with and without a user-specified fixed topology (the TNT parsimony tree inferred from 378 genes). The kmeans algorithm⁷¹ was implemented, with the entire concatenation treated as one subset, and the 378 gene parsimony tree used as the starting tree. Subsequently, PartitionFinder was run using the subsets retrieved by the kmeans algorithm without a starting tree specified. For each treatment an rcluster search⁷² was performed, and branch lengths were set as linked and model selection as BIC. The treatment with the lowest BIC score is favored (Table 4). Two ML analyses were performed using RAxML v8.2.4⁷³, each using the best partitioning scheme found for the individual concatenations (Table 4). The PartitionFinder results favored the GTR Γ model for most partitions, but the GTR or GTR+I+ Γ for a few others. A GTR Γ model was assigned to each partition following the recommendations of the creator of RAxML⁷³. Each RAxML analyses consisted of three stages: 1) executing 100 ML inferences (50 GTR Γ , 50 GTR Γ X) using 100 distinct randomized MP trees; 2) executing 1000 rapid bootstrap replicates (GTR Γ); and 3) mapping bootstrap values onto the best ML tree.

Ancestral State Reconstruction

Ancestral state reconstruction was performed on two traits (presence/absence of morphologically differentiated castes; presence/absence of preimaginal caste biasing), using the 235 loci Maximum Likelihood (ML) phylogeny. A parsimony unordered model and both ML models for ancestral state reconstruction (Markov k-state one parameter, Mk1, and asymmetrical

two-parameter Markov k-state, Assym.2) were implemented within Mesquite version 3.2⁷⁴. The Mk1 model assumes a similar rate of evolution for gains (0 → 1) and reversals (1 → 0). The Assym.2 model estimates a separate rate for gains and reversals. For both traits, the Assym.2 model favored reversals as more likely, especially when more solitary vespid taxa were included. This is because as more solitary vespids are included, the more observed instances of no gains. The models high estimate of a loss of morphological castes seems to contrast with the idea that morphologically distinct castes mark the “point of no return”, whereby eusocial life becomes irreversible^{1,16}. However, both maximum likelihood models for ancestral state reconstruction of morphological castes show that a loss most likely occurred within the swarm-founding Epiponini (Table 5). This is not to say that morphologically distinct castes do not mark the “point of no return” for eusociality (all Epiponini are eusocial), but that morphological caste differences can be lost.

The robustness of the reconstructions to taxon sampling was tested by performing them while including: all vespids; only Zethinae, Polistinae and Vespinae; only Polistinae + Vespinae. All vespines were coded as having morphological castes, and thus PCB^{3,18}. The only independent-founding polistines with documented evidence of morphological castes sampled in this study are *Polistes olivaceous*³ and *Polistes dominulus*⁷⁵. Coding of morphological castes for Epiponini follows ref.¹², and swarm-founding *Ropalidia* and *Polybioides* were coded as having morphological castes based on refs.^{3,43,46}. Coding species for state of PCB is a difficult task with much ambiguity – one of the arguments being made in this study is that there are limited studies directly testing for PCB in independent-founding polistines (especially tropical ones), and PCB may be more widespread than currently thought. Morphological castes are clear evidence of PCB. If ontogenetic determination of a diapause phenotype is a caste biasing factor, then

temperate or subtropical independent-founding polistines with seasonal nesting cycles (i.e. presence of gynes) have PCB present also. However, coding is complicated by the fact that many paper wasps have distributions that span tropical and subtropical/temperate regions, resulting in some populations with seasonal nesting cycles and others with aseasonal nesting cycles¹¹. *Polistes canadensis* have both seasonal and aseasonal populations⁷⁶, and *Polistes carnifex* likely does to. Other complications involve a lack of biological information on individual species and taxon sampling. For example, *Belonogaster* is primarily distributed in sub-Saharan Africa and most species may have aseasonal nesting cycles. However, the exemplars of *Belonogaster* (and *Ropalidia flavoviridis*) in this study are from Madagascar, which has distinct dry/wet seasons. A seasonal nesting cycle and queen/worker dimorphism has been shown in *Ropalidia* from Madagascar⁴⁷, as well as *Belonogaster* in South Africa³⁶, which suggests *Belonogaster* and *Ropalidia* in Madagascar likely have seasonal nesting cycles. These ambiguous cases were coded as having PCB absent (state = 0) following the prevailing idea that tropical independent-founding polistines lack PCB, which ultimately biases the ancestral state reconstruction to support the current paradigm that PCB was absent at the shared ancestor of Polistinae + Vespinae. Another analysis was done with these ambiguous cases coded as unknown (state = ?) for comparative purposes. Swarm-founding epiponines that lack morphological castes were coded as having an absence of PCB^{77,78}. However, *Polistes nimpha* and *Parapolybia indica* are known to have seasonal nesting cycles with hibernating gynes¹¹, and thus PCB.

RESULTS

Taxonomic Revisions

Overview of major changes

The molecular phylogeny (Figure 2) conflicts with the current accepted phylogeny (Figure 1) in several respects. Masarinae is currently divided into two tribes, Gayellini and Masarini²⁵. *Gayella* is recovered as sister to *Euparagia* (Euparagiinae), which renders Masarinae paraphyletic (Figure 2). Thus, Gayellini is raised to Gayellinae (also, Masarini = Masarinae). Eumeninae is currently divided into three tribes, and the previous relationships between them are: Zethini + (Eumenini + Odynerini)⁷⁹. Zethines are recovered as sister to Polistinae + Vespinae, while the remaining eumenines form a separate clade (Figure 2). Thus, Zethini is raised to Zethinae, and Eumeninae is restricted to Odynerini and Eumenini.

Detailed taxonomic discussion

Provespa and *Vespa* are referred to as hornets, while *Vespula* and *Dolichovespula* are known as yellowjackets. *Vespula* species typically build enveloped nests underground, while *Dolichovespula* nest aerially⁸⁰. Originally, cladistic analysis of phenotypic characters suggested the following relationships: *Vespa* + (*Provespa* + (*Dolichovespula* + *Vespula*))⁸¹. A recent study using data from nine loci ambiguously supported monophyly of yellowjackets, and argued that a close relationship between *Dolichovespula* and the hornets was possible⁸². The results of this

study match the most recent reported phylogeny⁸³, wherein yellowjackets are not monophyletic, *Vespula* is the basal vespine, and hornets are monophyletic (Figure 2).

Phylogenies based primarily on morphological and behavioral evidence suggest *Polistes* to be the basal polistine^{10,84,85}, while molecular phylogenies favor Ropalidiini as basal^{17,22,23}. The recent finding of Polistini and Epiponini as sisters¹⁷ receives support from this study, as does a basal Ropalidiini (Figure 2). *Polistes* has been a focal study organism in research addressing eusocial evolution, in part because it was suspected to be a basal polistine. Future research should be directed at ropalidiines if the purpose is to uncover the mode of eusociality in the ancestor of all Polistinae. Regarding *Polistes* subgeneric relationships, recovered with high support in all analyses is: (*Polistes s.str.* + (*Polistella* + (*Gyrostoma* + *Aphanilopterous*))). Previously, *Megapolistes*, *Nygmopolistes*, and *Gyrostoma* were synonymized, and subgeneric relationships were unresolved with the exception of a sister relationship between *Polistes s. str.* and *Aphanilopterous*⁸⁶. A more recent *Polistes* phylogeny supported this sister relationship, and recovered a paraphyletic *Gyrostoma* with *Megapolistes* sister to the rest of *Polistes*⁸⁷. However, other studies recover the sister of *Aphanilopterous* to be a *Polistella* + *Gyrostoma* clade⁸⁵, or *Megapolistes*⁸⁸. The most recent molecular study, which analyzed data from six genes across 58 *Polistes* species, found the relationships to be: (*Polistes s.str.* + ((*Polistella* + *Gyrostoma*) + *Aphanilopterous*))⁸⁹.

The molecular phylogeny for the Epiponini retrieved here conflicts with the current recognized phylogeny^{12,84}. Firstly, *Angiopolybia* are sister to the remaining Epiponines rather than *Apoica*. Secondly, *Apoica* and *Agelaia* are sister taxa (both have caste dimorphism where queens are smaller than workers in some aspects¹²). Thirdly, *Epipona* are sister to *Synoeca*,

rather than *Polybia*. It was previously thought that *Synoeca*, *Clypearia*, *Metapolybia* and *Asteloeca* formed a monophyletic grouping¹². These four genera lack morphological castes, but have sterile workers in the absence of PCB^{12,77,78}. Fourthly, *Brachygastra*, *Chartergus*, *Charterginus*, *Protonectarina*, and *Protopolybia* form a monophyletic group (Figure 2).

Eumeninae is currently divided into three tribes, and the proposed relationships between them are: Zethini + (Eumenini + Odynerini)⁷⁹. Monophyly of Eumeninae (*sensu* Carpenter) is supported by 11 phenotypic synapomorphies¹⁷. Thus, paraphyly of Eumeninae is the most surprising result in this study. Zethines are recovered as sister to Polistinae + Vespinae, while the remaining eumenines form a separate clade (Figure 2). Thus, Zethini is raised to Zethinae, and Eumeninae is restricted to Odynerini and Eumenini. These results are partly reminiscent of Richards (1962) classification for "Eumenidae", which contained Raphiglossinae, Discoeliinae (=Zethinae), and Eumeninae⁹⁰. Bohart and Stange (1965) considered *Raphiglossa*, *Raphiglossoides* and *Psiliglossa* to be included in Raphiglossinae⁹¹. Currently, Zethini contains both the raphiglossines and zethines⁷⁹. Unfortunately, raphiglossines were not obtainable. Thus, it can not be determined if the raphiglossines belong to Zethinae. The molecular phylogeny demonstrates non-monophyly of both Odynerini and Eumenini (Figure 2). Specifically, *Hypodynerus* renders Eumenini paraphyletic. Despite short, truncate mandibles being a main feature of zethines, *Zetheumenidion* is placed within Zethini due to the shared acute propodeal orifice and the propodeal valvula free posteriorly⁷⁹. Placement of *Zetheumenidion* within Zethini (now Zethinae) is incorrect. Strong support for the clade *Zetheumenidion* + (*Odynerus* + *Pterocheilus*) is recovered, with it placed amongst other odynerines (Figure 2). Until hundreds more taxa are sampled, a natural classification for Eumeninae cannot be proposed. The tribes

Odynerini and Eumenini are unnatural, but should be used for practical purposes until a more thorough phylogenetic reconstruction of Eumeninae is complete.

Zethus is currently comprised of four subgenera: *Zethus* (196 sp.), *Zethusculus* (27 sp.), *Zethoides* (42 sp.), and *Madecazethus* (2 sp.). Nest building behavior of this genus, and Zethines generally, is known for only ~10% of species⁹¹. The former two subgenera typically nest in twig, wood, or ground burrows, and use macerated vegetable matter to plug cells⁹¹. *Zethoides* are presumed to build nests *de novo* out of masticated plant material⁹¹. The molecular phylogeny is not compatible with this subgeneric concept. The two *Zethoides* (*Z. binodis* and *Z. toltecus*) are distantly related, and *Zethus* is paraphyletic with respect to *Ctenochilus* (Figure 2). If aerial nest construction out of plant material is the general state of *Zethoides*, then it would follow that this trait evolved more than once within *Zethus*. Use of plant material in nests may be ancestral for Zethinae: *Discoelius*, *Ischnocoelia*, *Protodiscoelius* and most *Zethus*, use vegetable matter for cell closure in nest burrows; *Psiliglossa* and *Raphiglossa* use pith; and *Calligaster*, and *Zethus* (*Zethoides*), build aerial nests using plant material^{91,92}. It has been argued that nest construction using plant material, of the kind seen in *Zethus* and *Calligaster*, is “a behavior that could precede the construction of nests from long-fiber wood pulp in the manner of Vespinae and Polistinae”²³. On the contrary, what is evident from the phylogeny is that these aerial paper nests resembling eusocial wasp nests must have evolved independently, and did not act as a precursor to polistine and vespine nests. Most likely, the shared ancestor of zethines, polistines and vespines lived in burrows (not necessarily underground) and used macerated vegetable matter to plug cells.

Masarinae is currently divided into two tribes, Gayellini and Masarini²⁵. Only two extant genera, *Paramasaris* and *Gayella*, belong to Gayellini⁹³. *Gayella* is recovered as sister to

Euparagia (Euparagiinae), which renders Masarinae paraphyletic (Figure 2). Thus, Gayellini is raised to Gayellinae (also, Masarinae = Masarini). Gayellinae and Masarinae (i.e. the pollen wasps) are unique among vespids in their provisioning of larvae with nectar and pollen. Either parallel evolution, or a reversal to provisioning larvae with arthropod prey in Euparagiinae, has occurred. Currently, Masarini is split into 3 subtribes, the relationships being *Priscomasarina* + (*Paragiina* + *Masarina*)⁹⁴⁻⁹⁷. This study lacks a *Priscomasarina* representative and only contains one Paragiina representative (i.e. *Paragia*). Nonetheless, a paraphyletic Masarina with Paragiina nested within it is recovered in all analyses (Figure 2). Furthermore, an unexpected sister relationship between *Celonites* and *Pseudomasaris* is highly supported across all analyses. All together, these results draw much uncertainty towards the evolutionary relationships among the Masarini.

The current proposed stenogastrine relationships, based on cladistic analysis of phenotypic characters, are^{9,98}: *Liostenogaster* + (*Anischnogaster* + (*Stenogaster* + *Eustenogaster*) + (*Parischnogaster* + (*Metischnogaster* + *Cochlischnogaster*))). The recovered phylogeny rejects *Liostenogaster* as basal, and show them most closely related to *Eustenogaster* with high support (Figure 2). There is discrepancy across analyses as to whether *Anischnogaster* or *Parischnogaster* is basal. Future phylogenetic studies focused on stenogastrine evolutionary relationships are needed to confidently ascertain how eusociality evolved in this lineage.

Phylogeny and Ancestral State Reconstruction

Two independent origins of eusociality in Vespidae are supported by a robust molecular phylogeny (Figure 2). Stenogastrinae is sister to all other vespids, and thus its eusocial habits are

independently evolved from those of Polistinae and Vespinae. This decreases the likelihood that rudimentary castes (i.e. phenotypic differences between castes lack an ontogenetic basis) were the groundplan condition for Polistinae + Vespinae (as shown in Figure 1), and suggests castes were more pronounced than expected at an origin of eusociality. Consequently, explanations of how caste divergence began and progressed over evolutionary time need to be reconsidered. Stenogastrinae should no longer serve as a phylogenetic intermediate between solitary and advanced eusocial life. It is possible that another group, now extinct, served as an intermediate instead. But, until such transitional fossils are found we cannot assume they exist. Thus, we must grapple with the idea that castes with ontogenetically determined physiological and morphological differences surfaced alongside eusociality in the ancestor of Polistinae + Vespinae.

Despite treating absence of PCB as the default for paper wasps, an ancestral state of no PCB at the most recent common ancestor of Polistinae + Vespinae was not supported (proportional likelihood for presence: Mk1 = 0.6134; Assym.2 = 0.9760; Figure 3). A likelihood ratio test identifies the Assym.2 model as superior for this trait ($\chi^2 = 29.73$, $df = 1$, $p = 0.001$). Furthermore, when coding ambiguous cases as unknown, presence of PCB was the most likely ancestral state (proportional likelihood for presence: Mk1 = 0.6916; Assym.2 = 0.9783; Figure 4). The Assym.2 model is superior ($\chi^2 = 22.21$, $df = 1$, $p = 0.001$). Maximum likelihood ancestral state reconstructions failed to significantly support the hypothesis that morphological castes were absent in the ancestor of Polistinae + Vespinae (proportional likelihood for presence: Mk1 = 0.1939; Assym.2 = 0.9655; Figure 5). A likelihood ratio test identifies the Assym.2 model as superior for this trait also ($\chi^2 = 24.67$, $df = 1$, $p = 0.001$). All taxon sampling schemes retrieved presence of morphological castes as the most likely ancestral state for Polistinae + Vespinae

(Table 5). The result of PCB and morphologically discrete castes at the ancestor of Polistinae + Vespinae may be an artefact of incomplete taxon sampling. But, whether it is appropriate to extrapolate PCB, or lack of it, to paper wasps more broadly is precisely the issue. The main reason why researchers have not expected or sought out PCB in independent-founding paper wasps is because workers can become reproductive depending on social contexts (i.e. caste totipotency)²⁰. However, that some workers may become replacement queens does not mean that caste biasing during larval development is completely absent. The results of this study suggest it is unwarranted to presume that simple castes without an ontogenetic basis necessarily characterize the early stages of eusocial evolution.

Some swarm-founding Epiponini (*Synoeca*, *Clypearia*, *Metapolybia* and *Asteloeca*) lack morphological castes, but have an absence of workers with intermediate ovarian development (presumably in the absence of PCB)^{12,77,78}. Ancestral state reconstruction suggests these genera likely descended from an ancestor with morphologically discrete castes (proportional likelihood for presence: Mk1 = 0.6325, Assym.2 = 1.0; Figure 5), indicating a loss of morphological differences between castes. It was previously considered that the syndrome of worker sterility seen in *Synoeca*, *Clypearia*, *Metapolybia* and *Asteloeca*, surfaced in an ancestor without morphological castes¹². Instead, it is more likely that a preceding ancestor had both morphological castes and worker sterility, and morphological caste differences were lost. A loss of morphologically distinct castes suggests that the degree of PCB is labile, and selection can cause the worker and queen phenotype to converge.

DISCUSSION

The case for sudden advanced castes

An independent origin of eusociality in the ancestor of Polistinae + Vespinae suggests that rudimentary castes may not be a required prerequisite to advanced castes. Current conceptions of Vespidae eusociality posit that PCB is characteristic of "more-derived species" and that imaginal determination is "less-derived"^{12,15}. Whether the most ancient or "less-derived" eusocial societies had only imaginal caste determination is *not* clear. Ancestral state reconstruction favors PCB as the ancestral condition of paper wasps, yellowjackets and hornets (Table 5; Figure 3 and 4). In the presence of PCB, physiological and incipient morphological differences among adults are determined before adulthood^{18,21}. This can include differences in number of Malpighian tubules, Van der Vecht organ shape, levels of hexameric storage proteins, maturation time, body size, fat content, ovary status, and responsiveness to juvenile hormone^{18,21,39,40,42,44,99,100}. The latter four differences are known to effect reproductive potential^{34,37,100,101}. Even differences in facial color patterns between workers and queens of *Polistes dominula*, which correlate with social dominance, are determined before adulthood⁷⁵. Given these demonstrated effects of PCB on reproductive potential, if PCB was the ancestral condition of paper wasps, yellowjackets and hornets, then a polyphenism with a distinct sub-fertile phenotype (i.e. non-gynes) was likely present at this origin of eusociality.

Most swarm-founding, and many independent-founding, paper wasps exhibit PCB, including species of *Polistes*, *Ropalidia* and *Belonogaster*^{11,12,18,21,34-40,42,45-47,75,99,102,103}. The evidence of PCB in *Mischocyttarus* is mixed^{41,104}, but larvae in pre-emergence colonies of *M.*

immarginatus give up saliva more readily to soliciting adults than larvae in post-emergence colonies¹⁰⁵, and in *M. flavitarsis* the first females to emerge reliably become workers and are often smaller than queens and cofoundresses¹⁰⁶. Furthermore, vibrational signaling by adult females characterizes all independent-founding paper wasps⁵, and has been shown to play an important role in PCB in *Polistes fuscatus*⁴⁵. It remains to be tested whether vibrational signalling plays a role in caste biasing in other independent-founding paper wasps⁵. Nonetheless, prevalence of vibrational signaling in independent-founding paper wasps⁵, and the accumulated discoveries of PCB in primitively eusocial paper wasps^{11,18,21,34-40,42,45,47,75,99,102,103}, both suggest PCB was the ancestral state of Polistinae. Lastly, Vespinae is comprised of species with morphologically discrete castes, although *Vespa analis* and *V. tropica* are well known exceptions wherein size dimorphism between castes is lacking. However, *V. tropica* has been demonstrated to have caste forewing dimorphism¹⁰⁷. Regardless, presence of PCB is certainly the ancestral state of Vespinae. Given that hover wasps are distantly related from the remaining eusocial wasps (Figure 2), it is likely that the ancestor of Polistinae + Vespinae had PCB and, perhaps even morphologically discrete castes (Table 5).

It has previously been argued that preimaginal differentiation of putative workers and putative queens (i.e. PCB) is an underlying, unifying feature of polistines and vespines^{3,11,18-20}. The molecular phylogeny (Figure 2) supports this previously proposed framework of eusocial evolution³, whereby two distinct developmental trajectories exist for polistine and vespine female offspring during larval development: the putative worker and gyne (i.e. future foundresses/putative queens) trajectory. These putative workers are biased to become ontogenetic workers (terminology from ref.^{19,20}) but may become replacement queens¹⁰⁸. The outcome of these alternative trajectories (putative worker vs. gyne) is most apparent in vespines,

and temperate, seasonal paper wasps with overwintering gynes^{21,40}. Like temperate primitively eusocial paper wasps, subtropical species show a markedly seasonal nesting cycle, wherein hibernating gynes are produced late in the colony cycle following the ‘worker production substage’. Subtropical species without hibernating individuals can still exhibit a seasonal nesting cycle (e.g. *Parapolybia varia*) with a distinct ‘nonworker production substage’¹¹. In such species, the putative worker and gyne phenotype appear to be determined before adulthood, with traits of these alternative phenotypes being apparent at eclosion⁴⁴. This framework predicts that signatures of the developmental bifurcation between putative workers and gynes should also be present in primitively eusocial polistines living in nonseasonal tropics⁴⁴. That is, PCB is the groundplan condition of paper wasps (i.e. Polistinae) and is incipient in tropical polistines.

There are a few reasons to expect there to be a physiologically distinct putative queen (i.e. gyne) phenotype among eclosing females in non-seasonal tropical primitively eusocial polistines: a) they typically follow an ‘aseasonal determinant nesting cycle’, wherein there is a clear ‘worker production substage’ and a ‘reproductive production substage’¹¹ (e.g. *Belonogaster juncea juncea*¹⁰⁹); b) foundress-reared brood in tropical species also tend to be smaller and develop faster than worker-reared brood, due to a low worker-to-larva ratio early in the colony cycle¹⁰⁸; and c) vibrational communication between adults and larvae is thought to be universal in vespines and independent-founding paper wasps, and has been shown to play an important role in preimaginal caste biasing in *Polistes fuscatus*⁵. Presence of alternative ontogenetic developmental trajectories is an inherent consequence of the underlying colony cycle shared by seasonal and aseasonal independent-founding paper wasps.

Recent work is showing ‘incipient’ morphological caste differences in tropical *Polistes*⁴², which necessarily signifies PCB. Secondly, in *R. marginata*, a species with an aseasonal indeterminate nesting cycle, half of the females isolated at eclosion do not build nests or lay eggs^{35,110}, and ~40% of females are born with lifelong sterility³⁴. Also, the tropical *Belonogaster grisea* shows worker-queen dimorphism, with morphological and physiological differences of castes being a result of preimaginal factors³⁷. The example of *B. grisea* is decisive. The apparent morphophysiological caste dimorphism in *B. grisea* led authors to believe that the genus did not exhibit a primitive (*sensu* ancestral) form of social organization that represents a transitional stage from solitary to social life³⁷. On the contrary, the phylogenetic position of *Belonogaster* does make it representative of the transitional stage from solitary to social life (Figure 2) and suggests that the advanced castes seen in *B. grisea* are the primitive (ancestral) eusocial condition (Table 5).

PCB at the outset of eusociality implies that castes of paper wasps, yellowjackets and hornets first evolved as a result of interactions between adults and larvae in a context of solitary nesting^{3,44}. This contrasts with the hypothesis that castes first evolved among nest-sharing casteless females of the same generation, wherein losers of social contests would become subordinate workers⁸. Although the evolution of a worker caste in a polygynous context is more applicable to hover wasps, it is still unlikely. The predominant mode of nest initiation is by a single foundress (i.e. haplometrosis), and most eggs in a shared nest are produced by a single female (i.e. monogyny) despite the presence of other fertilized females¹³. A possible explanation for the lack of PCB and morphophysiological worker/queen dimorphism in hover wasps may be the longer developmental time of brood and reduced number of brood that are simultaneously reared by a single foundress compared to paper wasps, yellowjackets and hornets¹³. These

aspects of hover wasp biology raise the worker-to-larva ratio in the early stages of the colony cycle, meaning brood raised by a single foundress will generally not be malnourished.

Although workers likely did not first evolve in a polygynous context, dominance interactions among group-living adults are significant to polistine and vespine eusociality^{7,32,108}. Putative queens (i.e. gynes) become foundresses, and a subset of gynes will be losers of social interactions and become subordinate cofoundresses that exhibit a worker phenotype. Queens are always *determined* after they win a dominance interaction and become egg layers. Like females in presocial wasps, variation in genetic and developmental backgrounds among gynes biases certain individuals to lay more eggs (i.e. win social interactions and attain queen status). Variation in reproductive potential among polistine gynes was likely a driving force behind the evolution of multiple foundress associations, dominance hierarchies, and mechanisms of imaginal caste determination (physical and pheromonal control over workers), but likely not the emergence of allomaternal care (altruism) and worker/queen dimorphism in eusocial vespids. Size differences among inseminated and uninseminated foundresses in pre-emergence colonies and gynes in overwintering clusters has been reported for *Polistes satan*¹⁰², suggesting that social competition among same generation females can give rise to worker/queen dimorphism. But this relates to how a dimorphism between subordinate workers and queens evolved (a relatively rare phenomenon), and not how a dimorphism between ontogenetic workers and queens evolved. In *Zethus miniatus* and other presocial wasps, allomaternal care did evolve out of a polygynous (casteless, nest-sharing) context^{8,111}. A lack of true castes (i.e. incomplete reproductive division of labor) in presocial wasps may be due to the absence of simultaneous progressive provisioning, wherein a mother provisions multiple brood at once. Although Zethinae is sister to Polistinae + Vespinae (Figure 2), the presocial *Zethus miniatus* is buried deep within Zethinae. Thus, the

evolution of casteless nest sharing in this species is an independent phenomenon from the evolution of eusociality. The molecular phylogeny (Figure 2) is incompatible with the idea that the rudiments of castes in a polygynous society gave rise to a definitive, non-temporal worker caste (c.f.⁸).

What further challenges the notion of advanced castes rising out of rudimentary castes is the fact that hover wasp lineages generally do not progress from temporal castes (i.e. working daughters will eventually become queens; all females are putative queens) to permanent castes with physiological and morphological differences established during ontogeny. Although permanent sterility has been reported in *Liostenogaster flavolineata*, this is exceptional and may be due to ecological/social cues experienced during adulthood having a pronounced effect in this species¹³.

The reason PCB has many losses in the ancestral state reconstruction (Figure 3) is because it was assumed to be lacking in most independent-founding paper wasps. If PCB is in fact widespread among paper wasps, then it would follow that losses are uncommon. What seems plausible is that the ontogenetic switch producing discrete alternative phenotypes of offspring (putative workers and putative queens) is rarely gained or lost, but the degree of ontogenetic caste-biasing is evolutionary labile. If this is true, we would expect many gains and losses of morphological differences between workers and queens. This is generally the case, given that various *Polistes* lineages show incipient morphological castes, as do *Polybioides*, various *Ropalidia*, some Epiponini, yellowjackets and hornets. Furthermore, some hornets lack a size difference between workers and queens, and this study presents a discovery that certain lineages of Epiponini lost morphological caste differences (Table 5). These likely represent cases

of caste convergence. Presence of PCB as the groundplan condition of paper wasps, hornets and yellowjackets would serve as an explanation for why many disparate lineages have gained and lost morphologically discrete castes. The commonness of gains and losses of morphological differences in castes, and the factors causing selection to favor caste convergence rather than divergence, needs to be investigated in future studies.

Although putative workers in primitively eusocial paper wasps emerge with active reproductive physiology, meaning their ovaries are on track to develop during early adulthood^{7,24,40,112}, it is not contradictory to argue that they bear a sub-fertile phenotype at birth. For example, if a wasp is born with active reproductive physiology but is too small to usurp a nest, or establish dominance on a nest it founded, then it's potential for direct reproduction is relatively low (i.e. that small wasp with active reproductive physiology is sub-fertile). Two distinct temporal phenotypes are seen in solitary, progressive provisioning wasps: a 'cell building, aggressive and egg-laying' phase followed by a 'brood-guarding, foraging, and ovary depleted' phase¹¹¹. The ovarian groundplan hypothesis posits that these temporal phenotypes are the substrate from which queens and workers, respectively, emerged¹¹². An absence of mature eggs (ovary depleted phase) correlates with foraging and brood care in solitary progressive provisioning wasps, and suppressed ovarian development correlates with the same behaviors in eusocial wasps¹¹². With respect to the ovarian groundplan hypothesis, it would be suppression of active reproductive physiology (reminiscent of the ovary-depleted phase) that makes brood care inducible in putative workers, and inactive reproductive physiology that prevents gynes from engaging in brood care. An ontogenetically determined difference of active and inactive reproductive physiology between putative workers and gynes, respectively, as the groundplan of Polistinae + Vespinae is compatible with the ovarian groundplan hypothesis.

The mechanism for sudden advanced castes

If castes started off advanced at the outset of eusociality, meaning castes had discrete morphophysiological differences that were a product of PCB, we require an evolutionary explanation for this anomaly. Cooption of a developmental switch regulating diapause in a bivoltine ancestor^{20,21,38,44} is one means by which castes would necessarily start off preimaginally differentiated and physiologically decoupled. The bivoltine model of eusocial evolution posits that the diapause and non-diapause phenotype of a bivoltine solitary ancestor corresponds to the gyne and putative worker phenotype of an eusocial descendent. If a seasonal polyphenism (e.g. bivoltinism) was coopted for the evolution of castes⁴⁴, then the underlying physiological differences between the diapause (gyne) and non-diapause (putative worker) phenotype would have preceded any behavioral specialization of castes. Alternatively, the elaboration of castes is not a gradual process that waits upon mutation to create differences between castes. Phenotypic plasticity and standing (cryptic) genetic variation may have carried a solitary, monophenic ancestor across the threshold of eusociality, and promptly produced an advanced caste polyphenism.

Many behavioral traits shared by all eusocial vespids – simultaneous progressive provisioning, prey mastication, extended parental care, construction of nests that hang free from the substrate, cell reuse, nest sharing among adults, adult-adult trophallaxis, cooperative brood care, allomaternal care and reproductive division of labor¹⁰ – appear simultaneously in the ancestor of *both* eusocial lineages. This hints at large-scale and rapid phenotypic change in both ancestors of eusocial wasps, which further suggests a pivotal role of phenotypic plasticity and

standing genetic variation in the origins of eusociality (i.e. phenotypic accommodation and genetic accommodation).

Among evolutionary biologists, the ‘plasticity-first’ hypothesis posits that phenotypic plasticity precedes and facilitates most adaptation, rather than mutation¹¹³. That is, novel traits may first appear as developmental variants in response to a changing environment (environment includes developmental contexts caused by other traits which may or may not have originated directly from a mutation). Phenotypic accommodation, adaptive adjustment without genetic change, is potentially a significant source of phenotypic innovation¹¹³⁻¹¹⁶, and has been postulated to be of chief importance in the emergence of vespid eusociality^{24,111}. Through phenotypic plasticity, a novel environment or trait (i.e. a contextual shift) can trigger the expression of a new (previously cryptic) phenotypic variant. This process (i.e. phenotypic accommodation) could lead to the simultaneous incipience of many novel traits in the absence of a mutation event specific to each newly emerged trait¹¹⁵. It is conceivable that a single innovation, perhaps simultaneous progressive provisioning, resulted in phenotypic accommodations that carried an ancestor across the threshold of eusociality. Adaptive morphological plasticity could have even produced morphological differences between castes at the outset of eusociality¹¹⁵ (Figure 5). Genetic accommodation follows phenotypic accommodation, whereby selection refines the novel phenotypic variants (i.e. traits) that were a consequence of plasticity by acting on previously cryptic alleles¹¹³.

That genetic accommodation can produce an ontogenetic color polyphenism (in just 13 generations) has already been demonstrated in the tobacco hornworm moth¹¹⁷. Therefore, the idea that genetic accommodation could promptly produce an ontogenetic physiological

polyphenism, whereby some daughters express a sub-fertile phenotype, is plausible. By selecting the greenest variants following heat shock for 13 generations, researchers produced a color polyphenism controlled by an environmental cue (temperature) in the monophenic tobacco hornworm moth¹¹⁷. Therefore, natural variation in the timing and extent of ovarian development in daughters³⁴ represents variation in the degree of subfertility (analogous to greenness), and selection would simply need to favor mothers that raise daughters that are highly sub-fertile early in the colony cycle for a polyphenism to evolve. Low nutrition for daughters born early in the colony cycle is an intrinsic consequence of simultaneous progressive provisioning²⁴. If standing genetic variation for altruism was hidden in a solitary ancestor (i.e. if altruism was an environmentally inducible phenotype), this would explain how mothers that raise sub-fertile daughters could have a selective advantage. Since the cost of altruism is minimized for sub-fertile females¹¹⁸, kin selection would favor previously cryptic genetic variants (i.e. allelic combinations) that produce both a sub-fertile and altruistic phenotype. In other words, if rearing sisters maximizes inclusive fitness of sub-fertile daughters, kin selection would favor previously cryptic/latent allomaternal care alleles. However, even if altruism is disadvantageous to the sub-fertile daughter, selection could still theoretically favor altruism by selecting for mothers that rear altruistic daughters (i.e. parental manipulation hypothesis)¹¹⁸. Regardless, if daughters are sub-fertile, it reduces the amount of antagonistic selection between daughters (workers) and mothers (queens) because mothers benefit from submissive altruistic daughters, and sub-fertile females gain inclusive fitness by becoming submissive altruistic daughters.

A plasticity-first model of eusocial evolution places variation in the reproductive potential of daughters as the foundation from which eusociality originated, emphasizing the cost component of Hamilton's equation in explaining the evolution of altruism. Because altruism

explains how a sub-fertile phenotype could have high fitness, the sudden appearance of a physiologically discrete (sub-fertile) could be explained if altruism was latent and inducible in a solitary ancestor. But is there any evidence that allomaternal care (altruism) was derived from hidden genetic variation in a solitary ancestor? In the ancestral solitary condition, maternal care genes are expressed after mating and egg laying (post-reproductively). The maternal heterochrony hypothesis posits that the altruistic phenotype involves a change in the timing of expression of maternal care genes to before mating (pre-reproductively)¹¹⁹. This hypothesis, that allomaternal care (i.e. worker behavior) involves expression of maternal care genes pre-reproductively, was supported in a gene expression study of *Polistes metricus*¹²⁰. If altruistic behavior is heterochronic maternal care, only the timing of expression of pre-existent maternal care genes needs to change. Thus, allomaternal care may have been latent in a solitary ancestor, was induced by environmental change (including change in developmental contexts caused by other traits) and is ultimately derived from standing genetic variation.

Conclusions

The most robust phylogeny produced to date shows eusociality emerged twice within Vespidae (Figure 2). Eusocial vespids with the simplest of castes (hover wasps) are distantly related from wasps with more advanced castes (paper wasps, yellowjackets and hornets). Thus, the rudimentary temporal castes seen in hover wasps were not a prerequisite to more advanced castes (i.e. morphophysiological queen/worker dimorphism due to preimaginal caste biasing). Yellowjackets, hornets, and many primitively eusocial paper wasps exhibit preimaginal caste biasing, suggesting that presence was the condition of their most recent common ancestor. The

results suggest that preimaginal caste biasing, wherein a category of sub-fertile females (i.e. putative workers) are produced early in the colony cycle, may have been established at the outset of eusociality in the ancestor of Polistinae + Vespinae (Figures 3-5). If castes were this advanced at the origin of eusociality, caste evolution was neither stepwise or gradual. Phenotypic accommodation and genetic accommodation may explain the sudden appearance of discrete castes with morphophysiological differences that have an ontogenetic basis. A plasticity-first model of eusocial evolution places variation in the reproductive potential of daughters as the foundation from which eusociality originated, and emphasizes the cost component of Hamilton's equation in explaining the evolution of altruism. Because altruism explains how a sub-fertile phenotype could have high fitness, the appearance of a discrete category of sub-fertile daughters could be explained if altruism was latent and inducible in a solitary ancestor. It is hypothesized that the standing genetic variation required for altruism and advanced castes was present in a solitary, monophenic ancestor, and thus, eusociality emerged *via* phenotypic plasticity.

The idea that the rudiments of castes in a polygynous society (multiple egg layers on a shared nest with variation among same generation females in direct reproductive output) was the starting point of caste divergence in eusocial vespids⁸ needs to be revisited. Given the molecular phylogeny (Figure 2), there is no hint of pleometrosis and polygyny being the condition at either origin of eusociality. The origins of the ontogenetic worker caste can be found in interactions between mothers and daughters, while the origin of subordinate workers can be found in interactions between same generation females.

The frequently written opinion that Vespidae are especially suitable for studying the early stages of eusocial evolution implies that the earliest stages of eusociality were necessarily

characterized by the least sophisticated of conditions (i.e. a lack of PCB, and only temporal castes or partial division of labor). This study highlights how in some instances, it might be advanced eusociality that is the primitive (i.e. ancestral) condition. Therefore, despite ants and termites only bearing highly eusocial species, they are perhaps equally well-suited for addressing how eusociality originated. By invoking phenotypic plasticity and standing genetic variation it is possible to explain how phenotypic revolutions, such as the sudden emergence of eusociality with advanced castes, can occur. Castes with discrete morphophysiological differences that were established before adulthood at the origin of eusociality (Figures 3-5) showcase how caste divergence, and divergence of alternative phenotypes in general, is not always stepwise and gradual.

APPENDIX A: FIGURES

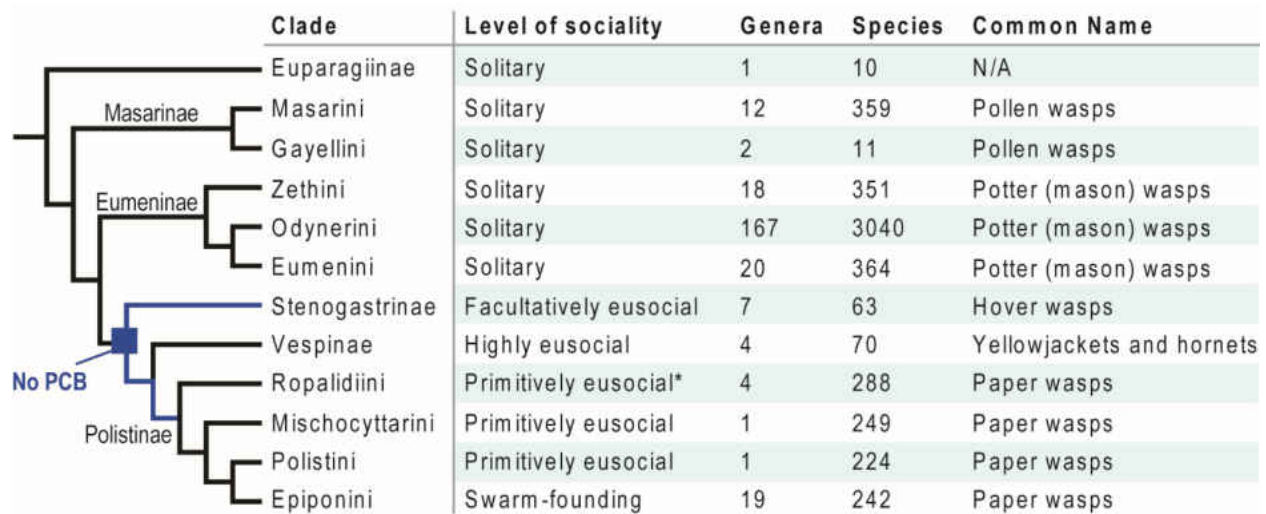


Figure 1 – Current accepted phylogeny of Vespidae. The groundplan condition of rudimentary castes with no preimaginal caste biasing (PCB) is shown in blue; castes lack preimaginal differences, and behavioral and physiological differences between workers and queens are assumed to have been established during adulthood. Definitions for facultatively, primitively, and highly eusocial are described in-text. (*) Ropalidiini also includes swarm-founding species.

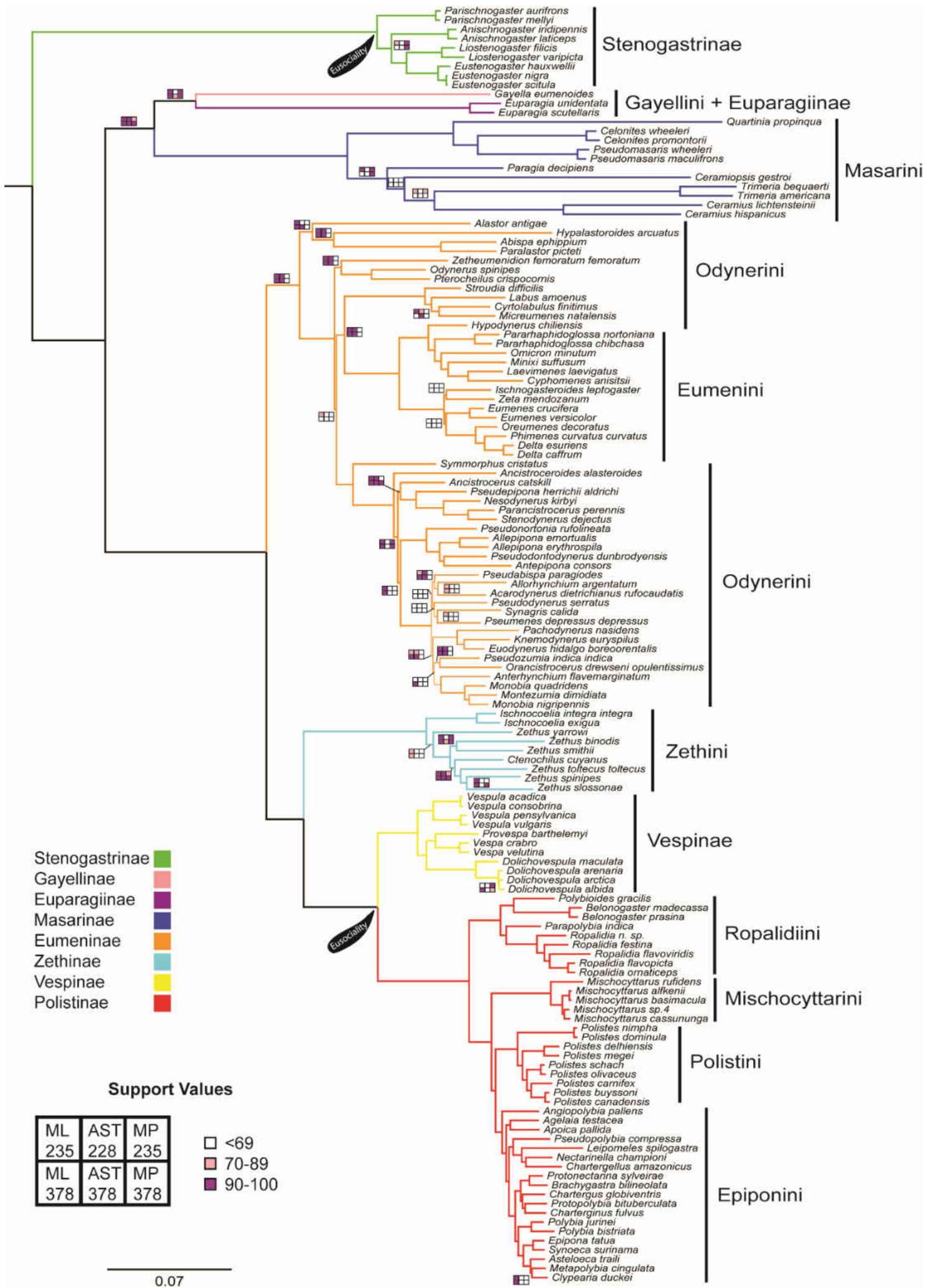


Figure 2 – Maximum-Likelihood tree of Vespidae inferred from 235 selected loci sequenced across 163 taxa (138 ingroup). A six-celled box summarizes branch support values (bootstrap or posterior probabilities) where each cell corresponds to a separate phylogenetic analysis (ML = Maximum Likelihood; AST = ASTRAL; MP = Maximum Parsimony; 228, 235 and 378 = # of loci used), and its color represents support obtained in that analysis (white = 0-69; pink = 70-89; purple = 90- 100). Branches that had >90 support in all 6 analyses are unmarked.

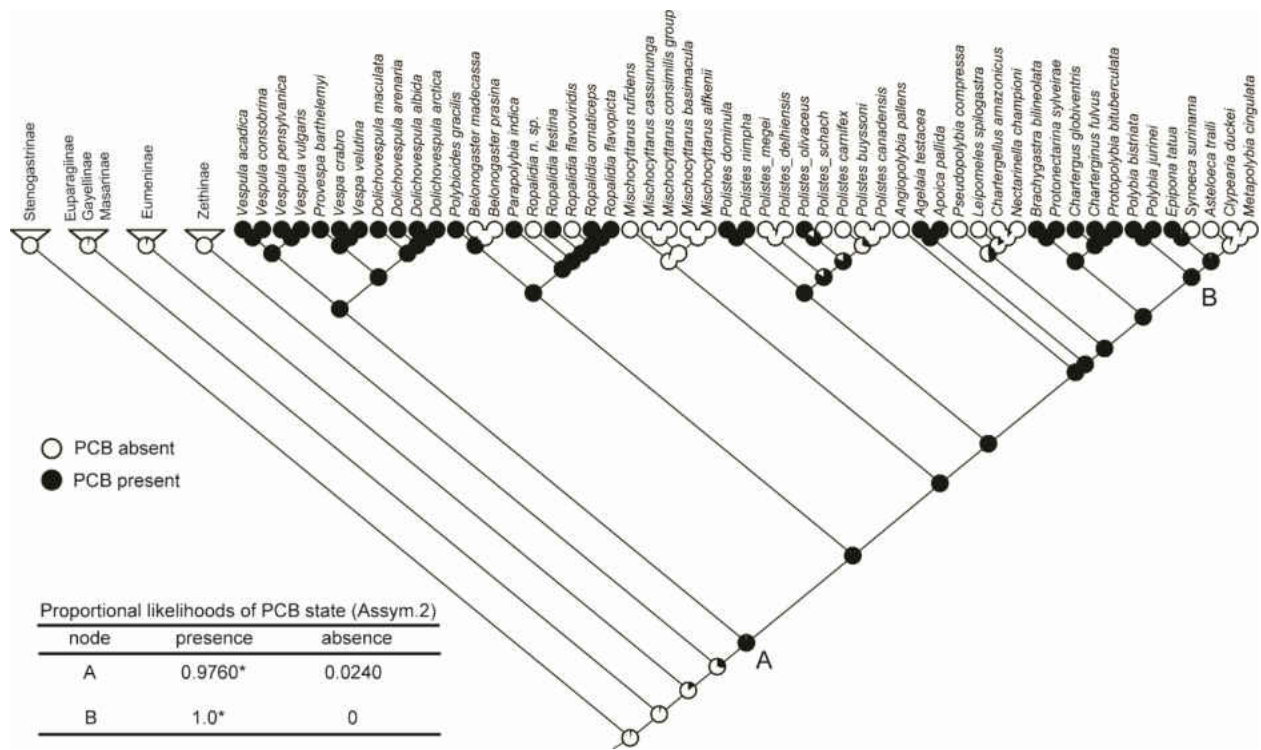


Figure 3 – Best maximum likelihood ancestral state reconstruction (Assym.2 model) of preimaginal caste biasing (PCB) on the maximum likelihood phylogeny obtained from 235 loci, while assuming independent-founding paper wasps lack PCB. All vespid samples were coded as having PCB absent or present. Swarm-founding *Ropalidia* and *Polybioides*, and *Polistes dominula* and *P. olivaceous* have documented PCB. Some clades collapsed for visualization purposes. Node A represents an origin of eusociality at the shared ancestor of paper wasps, hornets and yellowjackets.

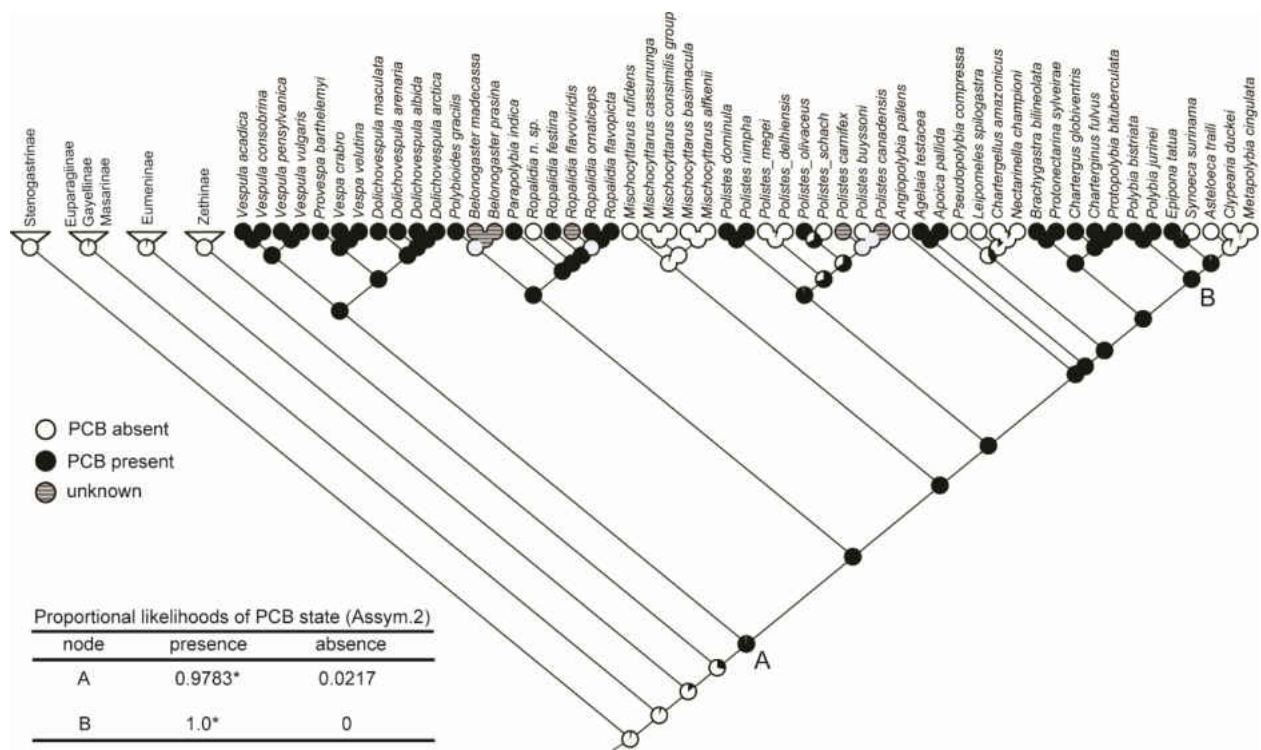


Figure 4 – Best maximum likelihood ancestral state reconstruction (Assym.2 model) of preimaginal caste biasing (PCB) on the maximum likelihood phylogeny obtained from 235 loci, while not assuming independent-founding paper wasps lack PCB. All vespid samples were coded as having PCB absent, present or unknown. Swarm-founding *Ropalidia* and *Polybioides*, and *Polistes dominula* and *P. olivaceus* have documented PCB. Some clades collapsed for visualization purposes. Node A represents an origin of eusociality at the shared ancestor of paper wasps, hornets and yellowjackets.

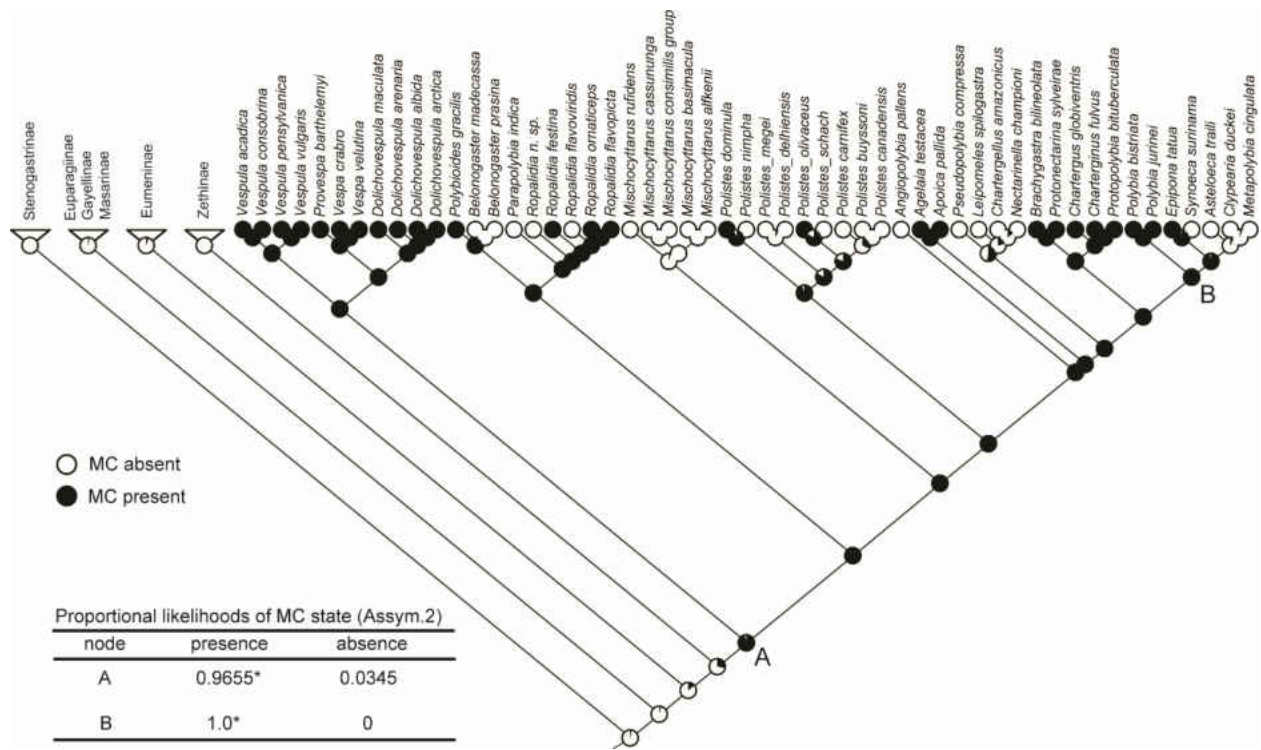


Figure 5 – Best maximum likelihood ancestral state reconstruction (Assym.2 model) of morphological castes (MC) on the maximum likelihood phylogeny obtained from 235 loci. All vespid samples were coded as having MC absent or present. Some clades collapsed for visualization purposes. Node A represents an origin of eusociality at the shared ancestor of paper wasps, hornets and yellowjackets.

APPENDIX B: TABLES

Table 1. Taxon sampling overview for this study. In total, 163 species were included for phylogenetic analyses. There are 138 ingroup taxa (2 Genbank, 136 new), and 25 outgroup taxa (13 Genbank; 11 from probe design; 1 new). * denotes data found on Genbank; ^ denotes unpublished genomic sequences gathered prior to probe design. Classifications following Carpenter (1982) and Hermes et al. (2013).

Families	Subfamilies/Tribes	# in tribe	# in subfamily	# in family		
Pteromalidae	\			1*		
Ichneumonidae				7^		
Braconidae				4^,2*		
Formicidae				7*		
Scoliidae				1		
Megachilidae				1*		
Apidae				2*		
Vespidae	\					
				Euparagiinae		2
				Masarinae		12
				Gayellini	1	
				Masarini Masarina	10	
				Masarini Paragiina	1	
				Eumeninae		62
				Zethini	10	
				Eumenini	14	
				Odynerini	38	
				Stenogastrinae		9
				Polistinae		42
				Ropalidiini	9	
				Mischocyttarini	5	
				Polistini	7,2*	
				Epiponini	19	
				Vespinae		11

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Table 2. Taxa selected for processing through the anchored hybrid enrichment workflow. Of 142 species total, 137 were captured successfully. Taxa with insufficient sequence captures are marked with an asterisk (*). The date and locality of specimen collection, and the collaborators from which specimens were loaned are listed. AMNH = American Museum of Natural History; SAM = Iziko South Africa Museum; and NMNH = National Museum of Natural History (France).

Genus	species	Author	Locality	Loaner	Date collected
<i>Abispa</i>	<i>ephippium</i>	(Fabricius)	Australia	B. Sharanowski	6.Jan.2014
<i>Acarodynerus</i>	<i>dietrichianus rufocaudatis</i>	(de Saussure)	Australia	AMNH	24.Jan.2002
<i>Agelaia</i>	<i>testacea</i>	(Fabricius)	Brazil	A. Somavilla	
<i>Alastor</i>	<i>antigae</i>	Buysson	Spain	AMNH	29.May.2011
<i>Allepipona</i>	<i>erythrospila</i>	(Cameron)	South Africa	SAM	8.Sep.2014
<i>Allepipona</i>	<i>emortualis</i>	(de Saussure)	South Africa	SAM	30.x.2009-22.ii.2010
<i>Allorhynchium</i>	<i>argentatum</i>	(Fabricius)	Malaysia	AMNH	1.Jul.2009
<i>Ancistroceroides</i>	<i>alasteroides</i>	(de Saussure)	Paraguay	AMNH	3.Feb.2007
<i>Ancistrocerus</i>	<i>catskill</i>	(de Saussure)	Agassiz	B. Sharanowski	21.Jun.2013
<i>Angiopolybia</i>	<i>pallens</i>	(Lepeletier)	Brazil	A. Somavilla	10-16.Aug.2010
<i>Anischnogaster</i>	<i>laticeps</i>	van der Vecht	Papa New Guinea	AMNH	24.Sep.2000
<i>Anischnogaster</i>	<i>iridipennis</i>	(Smith)	Papa New Guinea	AMNH	7.Jul.2001
<i>Antepipona</i>	<i>nr. consors</i>		Vietnam	T.Mita	6.Mar.2013
<i>Anterhynchium</i>	<i>flavomarginatum</i>	(Smith)	Japan	AMNH	12.Aug.2007
<i>Apoica</i>	<i>pallida</i>	(Olivier)	Brazil	A. Somavilla	
<i>Asteloeca</i>	<i>traili</i>	(Cameron)	Peru	AMNH	23.Jan.2011
<i>Belonogaster</i>	<i>prasina</i>	de Saussure	Madagascar	S. Yamane	12.Dec.2014
<i>Belonogaster</i>	<i>madecassa</i>	de Saussure	Madagascar	S. Yamane	9.Dec.2014
<i>Brachygastra</i>	<i>bilineolata</i>	(Spinosa)	Brazil	A. Somavilla	10-16.Aug.2010
<i>Celonites</i>	<i>promontorii</i>	Brauns	South Africa	SAM	16.Apr.2008
<i>Celonites</i>	<i>wheeleri</i>	Brauns	South Africa	SAM	14.Apr.2008
<i>Ceramiopsis</i>	<i>gestroi</i>	Zavattari	Paraguay	AMNH	2.Feb.2007
<i>Ceramius</i>	<i>hispanicus</i>	Dusmet	Spain	AMNH	22.May.2011
<i>Ceramius</i>	<i>lichtensteinii</i>	(Klug)	South Africa	S. Gess	Dec.2014
<i>Chartergellus</i>	<i>amazonicus</i>	Richards	Brazil	A. Somavilla	10-16.Aug.2010
<i>Charterginus</i>	<i>fulvus</i>	Fox	Brazil	A. Somavilla	10-16.Aug.2010
<i>Chartergus</i>	<i>globiventris</i>	de Saussure	Brazil	AMNH	12.Jul.2010
<i>Clypearia</i>	<i>duckei</i>	Richards	Brazil	AMNH	12.Jul.2010
<i>Ctenochilus</i>	<i>cuyanus</i>	Soika	Argentina	AMNH	11.Jan.2006
<i>Cyphomenes</i>	<i>anisitsii</i>	Brèthes	Brazil	AMNH	8.Jul.2010
<i>Cyrtolabulus</i>	<i>finitimus</i>	(Kohl)	South Africa	SAM	30.x.2009-22.ii.2010

<i>Delta</i>	<i>caffrum</i>	(Linnaeus)	South Africa	SAM	8.Sep.2014
<i>Delta</i>	<i>esuriens</i>	(Fabricius)	USA	AMNH	8.May.2010
* <i>Discoelius</i>	<i>japonicus</i>	Pérez	Japan	AMNH	12.Aug.2007
<i>Dolichovespula</i>	<i>maculata</i>	(Linnaeus)	Canada	R. Longair	6-15.Aug.2014
<i>Dolichovespula</i>	<i>arenaria</i>	(Fabricius)	Canada	R. Longair	6-15.Aug.2014
<i>Dolichovespula</i>	<i>albida</i>	Sladen	Canada	R. Longair	6-15.Aug.2014
<i>Dolichovespula</i>	<i>arctica</i>	Rohwer	Canada	P. Piekarski	10-15.Jul.2014
<i>Epipona</i>	<i>tatua</i>	(Cuvier)	Brazil	A. Perrard	10-16.Aug.2010
<i>Eumenes</i>	<i>crucifera</i>	Provancher	Canada	B. Sharanowski	9-23.Aug.2012
<i>Eumenes</i>	<i>versicolor</i>	(de Saussure)	Brazil	AMNH	10.Jul.2010
<i>Euodynerus</i>	<i>hidalgo boreoorientalis</i>	(de Saussure)	USA	AMNH	13.Jul.2006
<i>Euparagia</i>	<i>unidentata</i>	Carpenter & Kimsey	USA	AMNH	9.Jul.2008
<i>Euparagia</i>	<i>scutellaris</i>	Cresson	USA	AMNH	2.Jun.2001
<i>Eustenogaster</i>	<i>hauxwellii</i>	(Bingham)	Singapore	AMNH	1.Oct.2006
<i>Eustenogaster</i>	<i>scitula</i>	(Bingham)	Vietnam	AMNH	13.Mar.2013
<i>Eustenogaster</i>	<i>nigra</i>	Saito & Nguyen	Vietnam	P.L. Nguyen	3.Jun.2014
<i>Gayella</i>	<i>eumenoides</i>	Spinosa	Chile	AMNH	Jan.2008
<i>Hypalastoroides</i>	<i>arcuatus</i>	(Brèthes)	Paraguay	AMNH	2.Feb.2007
<i>Hypodynerus</i>	<i>chiliensis</i>	(Lepeletier)	Chile	AMNH	11.Jan.2015
<i>Ischnocoelia</i>	<i>integra integra</i>	(von Schulthess)	Australia	Queensland Museum	4-7.Dec.2008
<i>Ischnocoelia</i>	<i>exigua</i>	Borsato	Australia	AMNH	20.Jan.1999
<i>Ischnogasteroides</i>	<i>leptogaster</i>	(Walker, 1871)	Oman	A. Polazek	21.Nov.2014
* <i>Jugurtia</i>	<i>braunsi</i>	(von Schulthess)	South Africa	SAM	8.Sep.2014
<i>Knemodynerus</i>	<i>euryspilus</i>	(Cameron)	South Africa	SAM	30.x.2009- 22.ii.2010
<i>Labus</i>	<i>amoenus</i>	van der Vecht	Malaysia	AMNH	30.Jun.2009
<i>Laevimenes</i>	<i>laevigatus</i>	(Brèthes)	Paraguay	AMNH	12.Feb.2007
<i>Leipomeles</i>	<i>spilogastra</i>	(Cameron)	Brazil	A. Somavilla	22.Jun.2012
<i>Liostenogaster</i>	<i>varipicta</i>	(Rohwer)	Singapore	AMNH	23.Jun.2009
<i>Liostenogaster</i>	<i>filicis</i>	Turillazzi	Vietnam	P.L. Nguyen	8-10.Jun.2007
* <i>Metaparagia</i>	<i>maculata</i>	(Meade-Waldo)	Australia	AMNH	19.Jan.1987
<i>Metapolybia</i>	<i>cingulata</i>	(de Saussure)	Peru	K. Taisuke	30.Jul.2014
<i>Micreumenes</i>	<i>nr. natalensis</i>		Ghana	R. Longair	23-25.Dec.2008
<i>Minixi</i>	<i>suffusum</i>	(Fox)	Brazil	AMNH	6.Jul.2010
<i>Mischocyttarus</i>	<i>cassununga</i>	(von Ihering, 1903)	Brazil	A. Somavilla	July.2011
* <i>Mischocyttarus</i>	<i>drewseni</i>	de Saussure	Brazil	A. Somavilla	26-30.Jul.2009
<i>Mischocyttarus</i>	<i>basimacula</i>	(Cameron)	Belize	R. Longair	May.5.2014
<i>Mischocyttarus</i>	<i>rufidens</i>	(de Saussure)	Belize	R. Longair	May.5.2014

<i>Mischocyttarus</i>	<i>sp. 4</i>	(<i>consimilis</i> group)	Peru	B. Sharanowski	27.Jul.2014
<i>Mischocyttarus</i>	<i>alfkenii</i>	(Ducke)	Peru	K. Taisuke	25-30.Jul.2014
<i>Monobia</i>	<i>nigripennis</i>	de Saussure	Belize	R. Longair	Apr.30 May.5 2014
<i>Monobia</i>	<i>quadridens</i>	(Linnaeus)	USA	AMNH	14.Jul.2007
<i>Montezumia</i>	<i>dimidiata</i>	de Saussure	Peru	K. Taisuke	30.Jul.2014
<i>Nectarinella</i>	<i>championi</i>	(Dover)	Panama	AMNH	18.Dec.2012
<i>Nesodynerus</i>	<i>kirbyi</i>	(Dalla Torre)	USA	AMNH	18.May.2010
<i>Odynerus</i>	<i>spinipes</i>	(Linnaeus)	France	AMNH	13.Jun.2007
<i>Omicron</i>	<i>minutum</i>	(Fabricius)	Brazil	AMNH	12.Jul.2010
<i>Orancistrocerus</i>	<i>drewseni opulentissimus</i>	(Giordani Soika)	China	NMNH	2.Aug.2010
<i>Oreumenes</i>	<i>decoratus</i>	(Smith)	China	NMNH	2.Aug.2010
<i>Pachodynerus</i>	<i>nasidens</i>	(Latreille)	USA	AMNH	18.May.2010
<i>Paragia</i>	<i>decipiens</i>	Shuckard	Australia	AMNH	16.Nov.2013
<i>Paralastor</i>	<i>picteti</i>	(de Saussure)	Australia	B. Sharanowski	26.Dec.2013
<i>Parancistrocerus</i>	<i>perennis</i>	(de Saussure)	USA	AMNH	14.Jul.2007
<i>Parapolybia</i>	<i>indica</i>	(de Saussure)	China	A. Perrard	24.Jul.2010
<i>Pararhaphidoglossa</i>	<i>chibchasa</i>	Giordani Soika	Peru	K. Taisuke	30.Jul.2014
<i>Pararhaphidoglossa</i>	<i>nortoniana</i>	(de Saussure)	Belize	AMNH	27.Apr.2008
<i>Parischnogaster</i>	<i>nr. aurifrons</i>		Vietnam	P.L. Nguyen	2.Oct.2014
<i>Parischnogaster</i>	<i>mellyi</i>	(de Saussure)	Thailand	S. Yamane	29.Jan.2015
<i>Phimenes</i>	<i>curvatus curvatus</i>	(de Saussure)	USA	AMNH	17.May.2010
<i>Polistes</i>	<i>olivaceus</i>	(DeGeer)	Madagascar	S. Yamane	9.Dec.2014
<i>Polistes (Aphanilopterus)</i>	<i>buyssoni</i>	Brethes	Chile	B. Santos	11.Jan.2015
<i>Polistes (Aphanilopterus)</i>	<i>carnifex</i>	Fabricius	Belize	R. Longair	5.May.2014
<i>Polistes (Gyrostoma)</i>	<i>schach</i>	(Fabricius)	Australia	B. Sharanowski	30.Dec.2013
<i>Polistes (Polistella) sp.</i>	<i>megei</i>	Pérez	China	NMNH	2.Aug.2010
<i>Polistes (Polistella) sp.</i>	<i>delhiensis</i>	Das & Gupta	Vietnam	T. Mita	15.Mar.2013
<i>Polistes (s.str)</i>	<i>nimpha</i>	(Christ)	Hungary	AMNH	28.Jun.2010
<i>Polybia</i>	<i>bistriata</i>	(Fabricius)	Brazil	A. Somavilla	10-16.Aug.2010
<i>Polybia</i>	<i>jurinei</i>	de Saussure	Brazil	A. Somavilla	10-16.Aug.2010
<i>*Polybia</i>	<i>sericea</i>	(Olivier)	Brazil	A. Somavilla	26-30.Dec.2009
<i>Polybioides</i>	<i>gracilis</i>	van der Vecht	Thailand	S. Yamane	25.Jan.2015
<i>Protonectarina</i>	<i>sylveirae</i>	(de Saussure)	Brazil	AMNH	8.Aug.1992
<i>Protopolybia</i>	<i>bituberculata</i>	Silveira & Carpenter	Brazil	A. Somavilla	10-16.Aug.2010
<i>Provespa</i>	<i>barthelemyi</i>	(du Buysson)	Vietnam	T. Mita	13.Mar.2013
<i>Pseudabispa</i>	<i>paragiodes</i>	(Meade-Waldo)	Australia	AMNH	24.Nov.2007
<i>Pseudepipona</i>	<i>herrichii aldrichi</i>	(Fox)	Canada	P. Piekarski	10-15.Jul.2014

<i>Pseudodontodynerus</i>	<i>dunbrodyensis</i>	(Cameron)	South Africa	SAM	30.x.2009- 22.ii.2010
<i>Pseudodynerus</i>	<i>serratus</i>	(Fox)	Paraguay	AMNH	12.Feb.2007
<i>Pseudomasaris</i>	<i>maculifrons</i>	(Fox)	USA	AMNH	28.Mar.2005
<i>Pseudomasaris</i>	<i>wheeleri</i>	Bequaert	USA	AMNH	30.Mar.2005
<i>Pseudonortonia</i>	<i>rufolineata</i>	(Cameron)	South Africa	SAM	30.x.2009- 22.ii.2010
<i>Pseudopolybia</i>	<i>compressa</i>	de Saussure	Brazil	A. Somavilla	10-16.Aug.2010
<i>Pseudozumia</i>	<i>indica indica</i>	(de Saussure)	Vietnam	T. Mita	24.Jul.2010
<i>Pseumenes</i>	<i>depressus depressus</i>	(de Saussure)	Vietnam	T. Mita	24.Jul.2010
<i>Pterocheilus</i>	<i>crispocornis</i>	Bohart	USA	AMNH	30.Mar.2005
<i>Quartinia</i>	<i>propinqua</i>	von Schulthess	South Africa	AMNH	5.Feb.2006
<i>Ropalidia</i>	<i>flavoviridis</i>	Kojima	Madagascar	S. Yamane	12.Dec.2014
<i>Ropalidia</i>	<i>flavopicta</i>	(Smith, 1857)	Vietnam	T. Mita	12.Mar.2013
<i>Ropalidia</i>	<i>n. sp.</i>		Vietnam	T. Mita	15.Mar.2013
<i>Ropalidia</i>	<i>festina</i>	(Smith)	Papa New Guinea	A. Perrard	Oct.2012
<i>Ropalidia</i>	<i>ornaticeps</i>	(Cameron)	Vietnam	S. Yamane	13.Mar.2013
<i>Scolia</i>	<i>verticalis</i>			AMNH	2004
<i>Stenodynerus</i>	<i>dejectus</i>	(Cresson)	Puerto Rico	AMNH	23.Nov.2008
<i>Stroudia</i>	<i>nr. difficilis</i>		South Africa	SAM	30.x.2009- 22.ii.2010
<i>Symmorphus</i>	<i>cristatus</i>	de Saussure	Canada	B. Sharanowski	28.Jun.2013
<i>Synagris</i>	<i>calida</i>	(Linne)	?	R. Longair	?
<i>Synoeca</i>	<i>surinama</i>	(Linnaeus)	Brazil	A. Somavilla	10-16.Aug.2010
<i>Trimeria</i>	<i>americana</i>	(de Saussure)	Paraguay	AMNH	12.Feb.2007
<i>Trimeria</i>	<i>bequaerti</i>	Willink	Paraguay	AMNH	3.Feb.2008
<i>Vespa</i>	<i>crabro</i>	Linnaeus	Poland	P. Piekarski	3.Jul.2015
<i>Vespa</i>	<i>velutina</i>	Lepeletier	France	NMNH	1.Oct.2014
<i>Vespula</i>	<i>acadica</i>	(Sladen)	Winnipeg	B. Sharanowski	Jun.6-21.2013
<i>Vespula</i>	<i>consobrina</i>	(de Saussure, 1854)	DeWinton	P. Piekarski	15.Jul.2014
<i>Vespula</i>	<i>vulgaris</i>	Linnaeus	Chile	B. Santos	20.Jan.2015
<i>Vespula</i>	<i>pensylvanica</i>	de Saussure	Canada	P. Piekarski	18.Aug.2014
<i>Zeta</i>	<i>mendozanum</i>	(Schrottky)	Argentina	AMNH	19.Dec.2004
<i>Zetheumenidion</i>	<i>femoratum femoratum</i>	(von Schulthess)	South Africa	SAM	30.x.2009- 22.ii.2010
<i>Zethus</i>	<i>binodis</i>	(Fabricius)	Brazil	AMNH	8.Jul.2010
<i>Zethus</i>	<i>slossonae</i>	(Fox)	USA	AMNH	23.Jan.2009
<i>Zethus</i>	<i>spinipes</i>	Say	USA	AMNH	25.Aug.2009
<i>Zethus</i>	<i>yarrowi</i>	Giordani Soika	South Africa	AMNH	25.Jan.2006
<i>Zethus</i>	<i>toltecus toltecus</i>	de Saussure	Belize	AMNH	22.Apr.2008
<i>Zethus</i>	<i>nr. smithii</i>		Paraguay	AMNH	4.Feb.2007

Table 3. Summary statistics of concatenated datasets (378 loci and 235 loci) used for maximum likelihood and maximum parsimony phylogenetic analyses. The smaller 235 loci dataset was produced after trimming all 378 loci using gblocks (parameters in text), and excluding loci that showed base heterogeneity.

Properties	Dataset 1	Dataset 2
# of loci	378	235
# of taxa	163	163
# of sites	162919	63712
# of informative sites	82659	29151
# of characters	26555797	10385056
% missing characters	18.35	9.73

Table 4. PartitionFinderV2.0.0 BIC scores for various treatments of the data. The partitioning scheme with the best BIC score was implemented (in bold).

PartitionFinder treatment	378 gene BIC's/# of partitions	235 gene BIC's/# of partitions
By gene, MP topology specified	8527369.9316/57	3225716.93673/33
By gene, no specified topology	8525832.50372/51	3224763.56618/30
Kmeans, no specified topology	8117756.29444/28	3214269.07331/36

Table 5. Summary of ancestral state reconstructions for morphological castes (MC) and preimaginal caste biasing (PCB) using the 235 loci RAxML phylogeny. Three separate sampling schemes were used: all vespids; just Zethinae, Polistinae, and Vespinae (Z+P+V); or just Polistinae and Vespinae (P+V). Two maximum likelihood models (Mk1 and Assym2), and a parsimony (unordered) model were implemented. Two separate reconstructions, each with a different coding scheme, were performed for PCB. In the first reconstruction (PCB1), ambiguous cases were coded as absent, and in the second (PCB2) ambiguous cases were coded as unknown. Asterisk (*) indicates strong support as the ancestral state by a likelihood decision threshold of 2.0. Models in bold were favored by a likelihood ratio test ($p < 0.05$).

Trait	Sampling	Model	Ancestral State (absence:presence)	Most likely	Ancestral State (absence:presence)	Most likely	
			Polistinae + Vespinae		<i>Polybia-</i> <i>Metapolybia</i>		
PCB1	All vespids	Mk1	0.3866:0.6134	present	0.2898:0.7102	present	
		Assym2	0.0240:0.9760*	present*	0:1*	present*	
		Parsimony	ambiguous	?	ambiguous	?	
	Z+P+V	Mk1	0.2493:0.7507	present	0.2889:0.7111	present	
		Assym2	0.1981:0.8019	present	0.0242:0.9758*	present*	
		Parsimony	ambiguous	?	ambiguous	?	
	P+V	Mk1	0.2988:0.7012	present	0.2832:0.7168	present	
		Assym2	0.2586:0.7414	present	0.5036:0.4964	absent	
		Parsimony	present	present	ambiguous	?	
	PCB2	All vespids	Mk1	0.3084:0.6916	present	0.2734:0.7266	present
			Assym2	0.0217:0.9783*	present*	0:1*	present*
			Parsimony	ambiguous	?	ambiguous	?
Z+P+V		Mk1	0.2109:0.7891	present	0.2749:0.7251	present	
		Assym2	0.1783:0.8217	present	0.0552:0.9448*	present*	
		Parsimony	ambiguous	?	ambiguous	?	
P+V		Mk1	0.2623:0.7377	present	0.2758:0.7242	present	
		Assym2	0.1720:0.8280	present	0.7978:0.2022	absent	
		Parsimony	ambiguous	?	ambiguous	?	
MC		All vespids	Mk1	0.8061:0.1939	absent	0.3675:0.6325	present
			Assym2	0.0345:0.9655*	present	0:1*	present*
			Parsimony	absent	absent	ambiguous	?
	Z+P+V	Mk1	0.3223:0.6777	present	0.3542:0.6458	present	
		Assym2	0.3510:0.6490	present	0.0808:0.9192*	present*	
		Parsimony	absent	absent	ambiguous	?	
	P+V	Mk1	0.3392:0.6608	present	0.3208:0.6792	present	
		Assym2	0.3176:0.6824	present	0.3984:0.6016	present	
		Parsimony	present	present	ambiguous	?	

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