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Short communication

Phylogenetics of the new world honey ants (genus *Myrmecocystus*) estimated from mitochondrial DNA sequences

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1. Introduction

Honey pot workers that store immense amounts of food in the crop and are nearly immobilized due to their highly expanded gasters are known from a variety of ant species in different parts of the world, presumably as adaptations to regular food scarcity in arid habitats (Hölldobler and Wilson, 1990). Although these so called repletes are taxonomically fairly widespread, the phenomenon is most commonly associated with species of the New World honey ant genus *Myrmecocystus*, whose repletes have a long lasting reputation as a delicacy with indigenous people. As repletism supposedly occurs within all species of *Myrmecocystus*, it seems plausible that this key innovation promoted the diversification of honey ants after formation of the North American deserts. Accordingly, the distribution of the genus is centered in arid parts of southwestern North America, including the Mohave, Sonoran, Great Basin, and Chihuahuan Deserts.

The genus *Myrmecocystus* seems to be most closely related to *Lasius* (Snelling, 1976), and Bolton (1994) placed *Myrmecocystus* and the genera *Acanthomyops*, *Euprenolepis*, *Lasius*, *Paratrechina*, *Prenolepis*, *Protrechina*, *Pseudolasius*, and *Teratomyrmex* in the same tribe Lasiini. *Myrmecocystus* can be distinguished from all Nearctic genera of Formicinae by the elongated maxillary palpi, of which the fourth segment is as long as, or longer than, the combined lengths of the two following segments.

Snelling (1976) subdivided the genus into three subgenera: *Myrmecocystus*, *Eremnocystus*, and *Endiodioctes*. The nominate subgenus contains all the light colored, strictly nocturnal species, the subgenus *Eremnocystus* contains small, uniformly dark colored species, and the subgenus *Endiodioctes* contains species with a ferruginous head and thorax and darker gaster. Snelling (1976) proposed that the earliest division within the genus was that between the line leading to the subgenus *Myrmecocystus* and that leading to *Eremnocystus* plus *Endiodioctes* (maybe based on diurnal versus nocturnal habits) and that the second schism occurred when the *Eremnocystus* line diverged from that of *Endiodioctes*.

Based on external morphology and data from both behavior and distribution, Snelling (1976) recognized 27 species and assembled most of them into species groups. In a supplement to his revision, Snelling (1982) described another two species. Johnson and Ward (2002) have recently listed four additional, hitherto undescribed species of *Myrmecocystus* in their treatment of the ants of Baja California, that appear to be endemic to that region (*M.* sp. *cf. flaviceps*; *M.* sp. *cf. mendax*; *M. BCA-1*; *M. BCA-2*).

Fifteen of the 29 described species, including all subgenera and seven of the eight species groups, are represented in this analysis as well as samples from the four undescribed species from Baja California. A sample of workers from Maricopa, Arizona, (referred to as *M.* sp. *n.* in this study) was determined to be *M. kennedyi* (Det. R.R. Snelling), but the alate queens are distinctively different (R.A. Johnson, pers. comm.). We also include this species in our phylogeny.

This study is aimed at employing DNA sequence information to elucidate the proper systematic placement of these undescribed *Myrmecocystus* species and to evaluate the systematic relationships within the genus that have been proposed by Snelling (1976), mainly based on morphological data. A well supported phylogenetic

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framework will also provide a necessary background for future evolutionary and behavioral studies of the genus, addressing issues like the evolution of slavery (Kronauer et al., 2003).

2. Material and methods

2.1. Specimens

We sequenced 26 *Myrmecocystus* specimens as well as one specimen each of *Prenolepis imparis* and *Lasius fuliginosus* as closely related outgroup taxa. All samples were collected by the authors and several collaborators. Voucher specimens are deposited at the Bohart Museum of Entomology, University of California at Davis (UCDC). Detailed sample information is given in Table 1.

2.2. Laboratory protocols

All specimens were stored in 95% ethanol and genomic DNA was extracted from whole ants following a standard phenol/chloroform protocol (Gadau et al., 1996). PCR amplification was performed on a T1 Thermocycler (Biometra) with the following cycle parameters: 3 min at

Table 1
List of specimens included in this study

Species	Subgenus	Species Group	Locality (Collector)	GenBank Accession No.			Voucher No.
				LCO	Jerry	VARf	
M. mexicanus 1	*Myr*	*Mexicanus*	Yavapai, AZ (Cover)	AY519368	AY519396	AY519424	SC6256
M. mexicanus 2	*Myr*	*Mexicanus*	Portal, AZ (Hölldobler)	AY519369	AY519397	AY519425	Jmyr
M. testaceus	*Myr*	*Testaceus*	Big Pine Flat, CA (Gadau)	AY519370	AY519398	AY519426	J142
M. creightoni	*Ere*	n.a.	San Diego, CA (Cover)	AY519371	AY519399	—	SC4795
M. perimeces	*Ere*	n.a.	near La Chocera, BCN (Kronauer)	AY519372	AY519400	AY519427	DK44
M. yuma	*Ere*	n.a.	La Paz, AZ (Johnson)	AY519373	AY519401	AY519428	RAJ2243
M. depilis	*End*	*Mimicus*	Portal, AZ (Hölldobler)	AY519375	AY519402	AY519429	D4.10
M. flaviceps	*End*	*Flaviceps*	Death Valley Natl.Pk., CA (Ward)	AY519374	AY519403	—	PSW13523
M. kennedyi	*End*	*Kennedyi*	near Parker AZ (Gadau)	AY519376	AY519404	AY519430	J126
M. melliger	*End*	*Melliger*	Chihuahua, MEX (Johnson)	AY519377	AY519405	AY519431	RAJ2770
M. mendax	*End*	*Melliger*	near Globe, AZ (Gadau)	AY519378	AY519406	AY519432	J120
M. mimicus 1	*End*	*Mimicus*	Portal, AZ (Hölldobler)	AY519379	AY519407	AY519433	7.9
M. mimicus 2	*End*	*Mimicus*	Bahia Falsa, BCN (Johnson)	AY519380	AY519408	—	RAJ2268
M. mimicus 3	*End*	*Mimicus*	Portal, AZ (Gadau)	AY519381	AY519409	AY519434	J80
M. mimicus 4	*End*	*Mimicus*	Portal, AZ (Hölldobler)	AY519382	AY519410	AY519435	1.8
M. mimicus 5	*End*	*Mimicus*	near Casa Grande, AZ (Kronauer)	AY519383	AY519411	AY519436	DK28
M. mimicus 6 (dark form)	*End*	*Mimicus*	San Diego, CA (Cover)	AY519384	AY519412	AY519437	SC4794
M. nequatzcatl	*End*	*Kennedyi*	near Punta Narragansett, MEX (Ward)	AY519385	AY519413	AY519438	PSW13493
M. placodops	*End*	*Melliger*	Mountain State Park Tucson, AZ (Prchal)	AY519386	AY519414	AY519439	Mplac1
M. romainei	*End*	*Romainei*	Graham, AZ (Cover)	AY519387	AY519415	AY519440	SC6239
M. wheeleri	*End*	*Kennedyi*	near Banner, CA (Ward)	AY519388	AY519416	AY519441	PSW14302
M. sp. *BCA-1*	*End*	n.a.	near La Chocera, BCN (Johnson)	AY519389	AY519417	AY519442	RAJ2271
M. sp. *BCA-2*	*End*	n.a.	near Guerrero Negro, BCN (Kronauer)	AY519390	AY519418	AY519443	DK52
M. sp. *cf. flaviceps*	*End*	n.a.	near Vizcaino, BCN (Kronauer)	AY519391	AY519419	AY519444	DK58
M. sp. *cf. mendax*	*End*	n.a.	Hwy3, BCN (Kronauer)	AY519392	AY519420	AY519445	DK82
M. sp. *n.*	*End*	n.a.	Maricopa Co., AZ (Johnson)	AY519393	AY519421	AY519446	RAJ2810
L. fuliginosus			Würzburg, D (Kronauer)	AY519394	AY519422	AY519447	H4N12
P. imparis			UC Davis campus, CA (Kronauer)	AY519395	AY519423	AY519448	DK84

Brief description of the species sorting proposed by Snelling (1976) (n.a.: not assigned). All undescribed forms are assigned to the subgenus *Endiodioctes*. Specimen localities, collector's identities, collection voucher numbers, and GenBank accession numbers (partial sequences are referred to by the respective forward primer) are provided. Locality codes: AZ (Arizona/USA), BCN (Baja California Norte/Mexico), CA (California / USA), D (Germany), and MEX (Mexico). Species identities for all specimens have been confirmed by Philip S. Ward and vouchers have been deposited in the Philip S. Ward collection at the Bohart Museum of Entomology, UCDC.

94 °C, followed by 30 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 1.5 min, and a final extension time of 5 min at 72 °C. The PCR cocktail was set to a total volume of 25μl containing approximately 10 ng of template DNA, 1× PCR-buffer (10 mM Tris–HCl, 50 mM KCl, and 0.08% Nonidet P40), 2 mM $MgCl_2$, 240 μM dNTPs, 800 μM of each primer, and 2.5 U of *Taq* DNA polymerase (MBI Fermentas). Amplified PCR products were precipitated in 1/10 volume 3 M NaAc and 2 volumes 100% ethanol, washed in 70% ethanol and resuspended in H_2O. Purified PCR products and sequencing primers were sent to a sequencing facility (Sequence Laboratories Göttingen GmbH) and directly sequenced by cycle sequencing with Big Dye. Partial *cytochrome c oxidase subunit I* (*COI*) sequences were amplified and sequenced using primers LCO/HCO (Folmer et al., 1994) and Jerry (Simon et al., 1994)/Ben (Ted Schultz, pers. comm.). Primer VARf (Kronauer et al., 2003) was used in combination with the reverse primers VARr (specifically designed for this study) and Barbara (Simon et al., 1994), respectively, to amplify and sequence partial *COI*, an intergenic spacer (IS) between genes *COI* and $tRNA^{Leu}$, $tRNA^{Leu}$, and partial *cytochrome c oxidase subunit II* (*COII*). The reference position for primer VARr (5′-ATT ATTGG(TA)GTAAT(AT)GTTC-3′) relative to the *Apis mellifera* mitochondrial genome (Crozier and Crozier, 1993) is 3813–3832.

2.3. Phylogenetic analyses

Protein coding DNA sequences were unambiguously aligned using ClustalX (Thompson et al., 1997) and translated into protein sequences by the program MEGA v2.1 (Kumar et al., 2001). Sequences encoding $tRNA^{Leu}$ were aligned manually based on published inferred secondary structures of ant (Chiotis et al., 2000) and honeybee (Crozier et al., 1989) $tRNA^{Leu}$. Unalignable regions were excluded from analyses. Alignment of the IS region was ambiguous for the group as a whole and it was thus discarded from the data set. A file with the final alignment is available from the authors upon request. Base frequencies were calculated with MEGA (Kumar et al., 2001).

Sequences from all three genes were combined in a total evidence approach in the final analyses. The GTR + I + G model was determined as the most appropriate maximum likelihood model of evolution for our data by the program MrModeltest v1.1b (Nylander, 2002) and the model was implemented in the program MrBayes v3.0B4 (Ronquist and Huelsenbeck, 2003). Base frequencies, proportion of invariable sites and γ-shape parameters were estimated by the program in the course of the analysis. One cold and three heated Markov chains were run simultaneously for a million generations and every 100th tree was sampled resulting in a total of 10001 trees. ''Burnin'' was examined graphically and the first 1001 trees were discarded from further analysis. Posterior probabilities for all nodes, which are equivalent to the percentage of trees retrieving the respective node, were estimated by computing a consensus tree in PAUP* 4.0b10 (Swofford, 2002). The analysis was repeated three times with random starting points to avoid convergence on local optima.

MrBayes (Ronquist and Huelsenbeck, 2003) was also employed to estimate branch lengths and posterior probabilities of alternative phylogenetic hypotheses.

An unweighted maximum parsimony (MP) analysis with heuristic searches and 1000 bootstrap replicates (Felsenstein, 1985), treating gaps as a 5th character state, was implemented in PAUP* (Swofford, 2002) for comparison (TBR branch swapping using steepest descent and ACCTRAN optimization).

3. Results

PCR amplification and sequencing of the complete target sequence was successful in all samples except *M. mimicus* 6 (therefore we used primer VARf in combination with primer VARr instead of Barbara, yielding 190 bp instead of 398 bp of *COII* sequence), *M. creightoni*, *M. flaviceps*, and *M. mimicus* 2 (in these latter species only 1011 bp of *COI* sequence were available for the analyses).

The absence of unexpected stop codons or indels in all protein coding sequences supports the idea that the targeted mitochondrial sequences have been successfully sequenced rather than putative pseudogenes. The complete $tRNA^{Leu}$ TψC—arm was discarded from the alignment and the D—arm was cut to 7 bp (including deletions in some species).

Table 2
Properties of DNA data partitions used for phylogenetic analyses

Sequences	Total sites	Variable sites	Parsimony informative sites	Nucleotide frequencies (%)			
				A	T	C	G
All	1534	500	356	32.8	41.3	15.0	11.0
COI	1074	352	264	30.9	40.8	15.8	12.5
$tRNA^{Leu}$	62	7	4	39.0	36.7	11.3	13.0
COII	398	141	88	37.4	43.6	13.2	5.9

Values are averaged over all sites and taxa.

Detailed information on nucleotide composition of data partitions is given in Table 2. Base composition of the *COI*/*COII* locus in the studied species is highly AT biased (73.9%), as is expected for mitochondrial DNA in general and particularly for hymenopterans (Brady et al., 2000; Chenuil and McKey, 1996; Whitfield and Cameron, 1998). AT bias at third codon positions is more distinctive (90.4%) than at first (66.6%) or second (64.9%) positions.

IS sequences were highly AT biased with AT contents ranging from 82.5% (*M. perimeces*) to 96.9% (*M. mendax*) in *Myrmecocystus* and 100% in *Lasius fuliginosus* (AT content mean ± SD: 93.81 ± 3.20%). IS lengths ranged from 40 bp (*M. perimeces*) to 142 bp (*M.* sp. *BCA-1*) with an average of 110 bp (29.46 bp SD). As is the case in Attini (Wetterer et al., 1998), the IS is generally shorter in the basal groups (40–103 bp in the outgroups, *M. perimeces* and the subgenus *Myrmecocystus*)

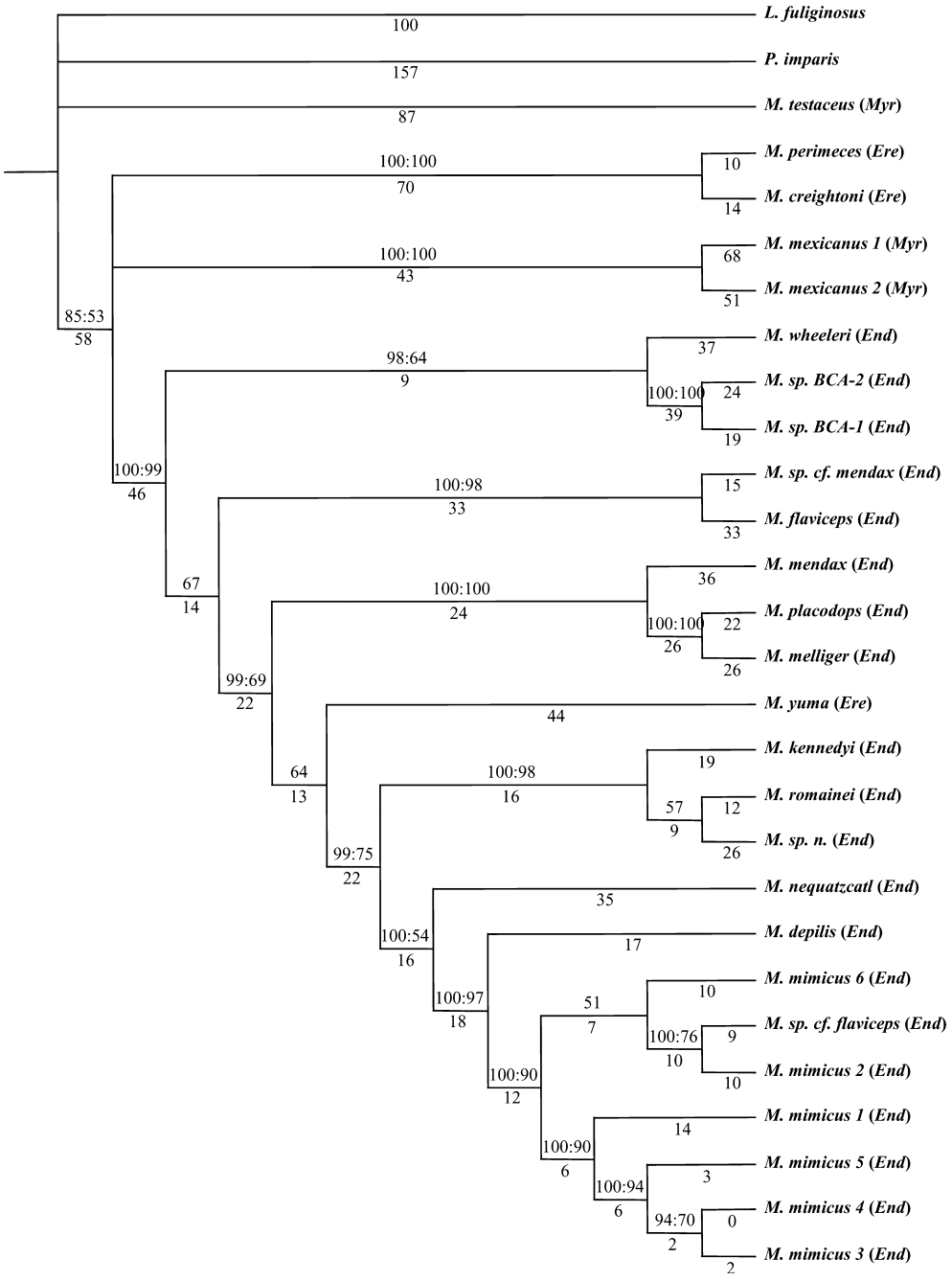

Fig. 1. Bayesian analysis of the combined *COI*, $tRNA^{Leu}$, and *COII* data sets comprising a total of 1534 bp. The first number above each branch gives the posterior probability for the respective node. Numbers below each branch indicate branch lengths. Where Maximum Parsimony bootstrap support was higher than 50, the value is given as the second number above each branch. See text for details of analyses. Subgenus affiliations of species are given in parentheses.

than in the more derived ones (118–142 bp in the subgenus *Endiodioctes*), which would be contradictory to a general trend to eliminate the IS from the mitochondrial genome altogether.

Both employed tree building algorithms agreed on the major topological features of the phylogeny (Fig. 1). Posterior probabilities gave consistently higher nodal support than the MP bootstrap, a phenomenon which has been repeatedly observed and critically assessed recently by Suzuki et al. (2002).

Major features of the phylogenetic estimates are:

- *M. yuma* (subgenus *Eremnocystus*) is placed within *Endiodioctes*, thereby rendering both *Eremnocystus* and *Endiodioctes* non-monophyletic. Not a single tree that would suggest monophyly of the three *Eremnocystus* species included in this study was visited by the Markov chain Monte Carlo samplers resulting in a posterior probability of zero for the alternative hypothesis of the subgenus being monophyletic.
- The *melliger* and *mimicus* species groups (Snelling, 1976) are confirmed (each supported by a posterior probability of 100), species belonging to the *kennedyi* group, however, are not grouped as monophyletic. Monophyly of the latter group has a posterior probability of zero.
- *M.* sp. *BCA-1* and *M.* sp. *BCA-2* are sister taxa. They appear basal within *Endiodioctes* with *M. wheeleri* being the most closely related species in the analysis. *M.* sp. *cf. mendax* is associated with *M. flaviceps* rather than *M. mendax*. The specimen designated *M.* sp. *cf. flaviceps* appears within the *M. mimicus* clade. *M. n.* sp. is indeed closely related to *M. kennedyi*, maybe even closer to *M. romainei*.
- The exact order in which the three subgenera branched off is not resolved. Monophyly of the subgenus *Myrmecocystus* is supported by a posterior probability of 2.3 (207 trees in the final analysis retrieved this group). A basal placement of the subgenus *Myrmecocystus* (whether monophyletic or not) with *Eremnocystus* plus *Endiodioctes* forming a clade has a posterior probability of 18.2 (displayed in 1639 trees).

4. Discussion

The fact that *M. yuma* is placed within *Endiodioctes* is surprising. Members of the subgenus *Eremnocystus* are rather homogeneous in morphology so that one would expect them to form a clade (Philip S. Ward, pers. comm.). Future work should therefore consider that at least one species of *Eremnocystus* should be classified as *Endiodioctes*.

Snelling's (1976) idea that the earliest division within the genus was that between the line leading to the subgenus *Myrmecocystus* and that leading to *Eremnocystus* plus *Endiodioctes* is not ultimately resolved. The basal node that makes *M. testaceus* an outgroup to the remaining *Myrmecocystus*, and that suggests that *Eremnocystus* and *Endiodioctes* have originated within a diverse ancestral group is only moderately supported (posterior probability and MP bootstrap of 85 and 53, respectively). Monophyly of both *Eremnocystus* and *Endiodioctes* is, however, strongly supported (considering the revised assignment of *M. yuma*).

The molecular analysis identifies the undescribed species *M.* sp. *cf. flaviceps* as *M. mimicus*, a phenomenon which is surprising, given the distinct phenotypes of the two forms. Both forms occur in sympatry at sites in Baja California and mixed samples were retrieved from single colonies. Future work, especially the use of nuclear markers, is needed to elucidate whether this represents two species that occasionally hybridize or whether the two morphological forms comprise a single species (*M. mimicus*).

The three species representing the *kennedyi* group (*M. kennedyi*, *M. wheeleri*, and *M. nequatzcatl*) do not form a monophyletic clade, which questions the legitimacy of this grouping. Although Snelling (1976) placed *M. nequatzcatl* in the *kennedyi* group, he noted that this species appears to be very closely related to *M. depilis*, an idea which is confirmed by our findings.

A sister species relationship between *M. mimicus* and *M. depilis* is strongly supported. Both species have been shown to enslave conspecifics, while *M. depilis* also enslaves *M. mimicus* in facultative interspecific slavery (dulosis) (Kronauer et al., 2003). The present results are therefore in accordance with the strict form of Emery's rule (Emery, 1909), which states that "the dulotic ants...generally originate from the closely related forms that serve them as hosts" (as cited in Wilson, 1971).

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