Genetic differentiation at species level in the Neotropical army ant *Labidus praedator*

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Abstract

The nomadic, swarm-raiding army ant *Labidus praedator* (Smith, 1858) is an important arthropod predator in the Neotropics with a strong ecological impact on invertebrate communities. However, despite its high abundance and ubiquity over a large distribution range, it received relatively little scientific attention. Moreover, the taxonomic status is confusing because some morphological descriptions point towards the co-occurrence of several distinct taxa which are lumped together as *L. praedator* in most classical keys. Yet, clarifying genetic studies are lacking. Here, we show strong genetic differentiation within an *L. praedator* population in Mexico. Both microsatellite genotype patterns and phylogenetic analyses (concatenated nuclear and mtDNA sequences, including the *coxI* genetic barcoding region), reveal the occurrence of two strongly isolated lineages. Colonies from the very same location, clearly identified as the same species (*L. praedator*) according to classical morphological keys, exhibit an extremely high average sequence divergence (9.7–12.8 %), which was well in the range of divergence among GenBank sequences from other *Labidus* species. Thus, our data very likely show genetic differentiation at species level or cryptic speciation within *L. praedator*, which should be recognized when investigating biodiversity and ecological importance of army ants (or other arthropods) in the Neotropics.

Keywords

Cryptic speciation, Genetic barcoding, Genetic diversity, Maximum likelihood, Microsatellites, Sequence divergence

Introduction

The nomadic, swarm-raiding army ants are important predators with a strong ecological impact in the Neotropics since they efficiently harvest a broad spectrum of invertebrates (Franks 1982; Franks and Bossert 1983; Gotwald 1995; Kronauer 2009). One of the most abundant and ubiquitous swarm-raiders is *Labidus praedator* (Smith, 1858), with extremely large colonies (probably over 1,000,000 individuals) and a wide distribution range spanning from Mexico to northern Argentina (Rettenmeyer 1963; Schneirla 1971; Watkins 1976; Longino 2005). Raids of *L. praedator* are frequent in the Neotropics (Kaspari and O'Donnell

2003; O'Donnell et al. 2007) and can deplete invertebrate biomass in the litter by up to 75 % (Kaspari et al. 2011). Despite this particularly strong ecological impact, most research on army ant biology has been based on swarm-raiding species of the genus *Eciton*, while *L. praedator* received much less attention. One reason might be its cryptic life history with unsteady and partially underground foraging activity (Rettenmeyer 1963; Schneirla 1971; Fowler 1979). Another problem is an unclear taxonomic status of *L. praedator* subspecies and local variants. For example, Borgmeier (1955) distinguishes two partially sympatric subspecies (*L. p.* s. str. and *L. p. sedulus*), but with weak morphological support for the worker caste (Rettenmeyer 1963; Longino 2005), and Longino (2005) suggests a morphologically distinct local variant (*L.* sp. JTL-001) from Costa Rico. Such ambiguities may in fact indicate cryptic speciation, which seems to be common in ants and needs to be addressed in biodiversity studies (Smith et al. 2005; Seifert 2009; Fournier et al. 2012). Given the ecological significance of *L. praedator,* it appears to be most timely to assess the intraspecific genetic divergence of these army ants using DNA tools. We do this in a case study for a Mexican *L. praedator* population, analyzing the genetic structure by microsatellite marker genotyping and sequencing fragments of the nuclear and mitochondrial genome, including parts of the genetic barcoding region of the cytochrome c oxidase subunit I (*coxI*) (Hebert et al. 2003a, b).

Materials and methods

Samples of 79 to 128 workers were taken from four *L. praedator* colonies between 2005 and 2008 in Chiapas, Mexico within a range of 10 km around the city of Tapachula (Table 1), preserved in 95 % ethanol and stored at −20 °C. Queens of army ants are highly polyandrous and mate with about 20 males from the surrounding colonies (Kronauer et al. 2007; Jaffé et al. 2009; Barth et al. 2014). By inferring the genotypes of the queen and the siring males from a large sample of workers of a colony, it is, therefore, possible to obtain information not only on the genotypic composition of the colony but also on the overall population structure. We used Chelex DNA extraction (Walsh et al. 1991) and standard polymerase chain reactions (PCR) to genotype all sampled workers (after removal of gasters) at nine microsatellite loci, four of which (Eb04, Eb25, Eb42, DmoD) were developed for other army ants (Denny et al. 2004; Kronauer et al. 2004), while five (Lp2, Lp4, Lp14a, Lp30, Lp38) were recently developed for *L. praedator* (Barth et al. 2014). Queen and male genotypes were reconstructed by Mendelian inferences and double checked with the software MATESOFT (Moilanen et al. 2004). By duplicating the queen genotypes and taking the males as homozygote diploids in the program FSTAT 2.9.3 (Goudet 1995), we achieved a diploid input file and estimated F_{ST} values for population differentiation (treating colonies as sub-populations).

Table 1: Sampling data, sample size of workers (n_w), reconstructed number of males (n_m), private allelic richness (A_{priv}) and GenBank accession numbers for the sequenced fragments of four *L. praedator* colonies in Chiapas, Mexico

Colony	Sampling date	Sampling location	GPS	n_w	n_m	A_{priv}	GenBank (*coxI*)	GenBank (28S)
Cac1	11/2005	Cacahoatán, coffee plantation	15°0′19″N, 92°10′17″W	128	21	3.85	KP455502	KP455506
Cac2	11/2005	Cacahoatán, coffee plantation	15°0′19″N, 92°10′17″W	106	26	1.34	KP455503	KP455507
Tap	12/2008	Tapachula, secondary forest	14°55′59″N, 92°17′3″W	109	18	0.80	KP455504	KP455508
TC	12/2008	Tuxtla Chico, agricultural research centre	14°58′25″N, 92°9′34″W	79	26	1.01	KP455505	KP455509

After standard phenol–chloroform DNA extraction of one additional *L. praedator* worker per colony, two mitochondrial (mtDNA) *coxI* fragments and one nuclear 28S fragment were amplified using the primer pairs CI13/CI14 and Jerry/Ben3R, and Bel28S/revBel28S, respectively, in PCR conditions as given in Brady (2003). Products were purified using the AMPure® XP kit (Beckman Coulter) and sequenced (MWG-Biotech) at the forward and reverse strands. The consensus sequences were compared in a phylogenetic analysis with the homologous GenBank sequences (Brady 2003) of the Neotropical army ant species *L. coecus*, *L. spininodis*, *Eciton burchellii*, *E. mexicanum*, *E. vagans*, *Nomamyrmex esenbeckii* and the African *Dorylus nigricans* as outgroup.

All 11 sequences were aligned using the MUSCLE algorithm (default settings) as implemented in the sequence analysis software MEGA5 (Tamura et al. 2011) resulting in a total length of 1387 bp of concatenated *coxI* (886 bp) and 28S (501 bp) fragments (GenBank accession numbers KP455502–KP455509, Table 1). MEGA5 was then also used to perform phylogenetic estimations (complete deletion of gaps and missing data). No gaps, stop-codons or discrepancies between forward and reverse strands were contained in the *coxI* alignment, so that the amplification of nuclear pseudogenes (Bensasson et al. 2001) could be excluded. Based on the lowest Bayesian Information Criterion score, a General Time Reversible (GTR) model + G (Gamma shape parameter = 0.23) was selected as best-fitting nucleotide substitution model for the data to construct a Maximum Likelihood (ML) phylogenetic tree using the nearest neighbor interchange algorithm (Nei and Kumar 2000; Tamura et al. 2011; Hall 2013). Likewise, a Neighbor Joining (NJ) tree was constructed, following the Tamura–Nei model estimated by the Maximum Composition Likelihood method (Tamura et al. 2004). As mtDNA and nuclear DNA sometimes represent different evolutionary signals (e.g., Rabeling et al. 2014), an ML tree was also estimated separately for the 28S alignment using a Tamura-3-parameter substitution model + G = 0.20. At last, pairwise nucleotide sequence divergence was estimated following the Kimura-2-parameter (K2P) model.

Additional sequencing of the *coxI* genetic barcoding region with the primers LepF/LepR (Hebert et al. 2004; Smith et al. 2014) yielded multiple peaks in the sequence chromatograms of colonies Cac2, Tap and TC indicating nuclear pseudogenes (Bensasson et al. 2001) of this fragment. We, therefore, used our CI13/CI14 fragments, which partially overlap with the barcoding region, to achieve a final alignment of 490 bp with eight barcoding fragments from GenBank (Smith et al. 2014) of *L. praedator* from Costa Rica, including variant JTL-001 and five more variants for which no morphological description is available yet. Phylogenetic analyses were performed as described above (GTR + G = 0.32, partial deletion with 90 % site coverage cutoff), using *L. spininodis* as outgroup (13 sequences in total). All alignments and trees were deposited in the internet data bank TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S16955).

Results

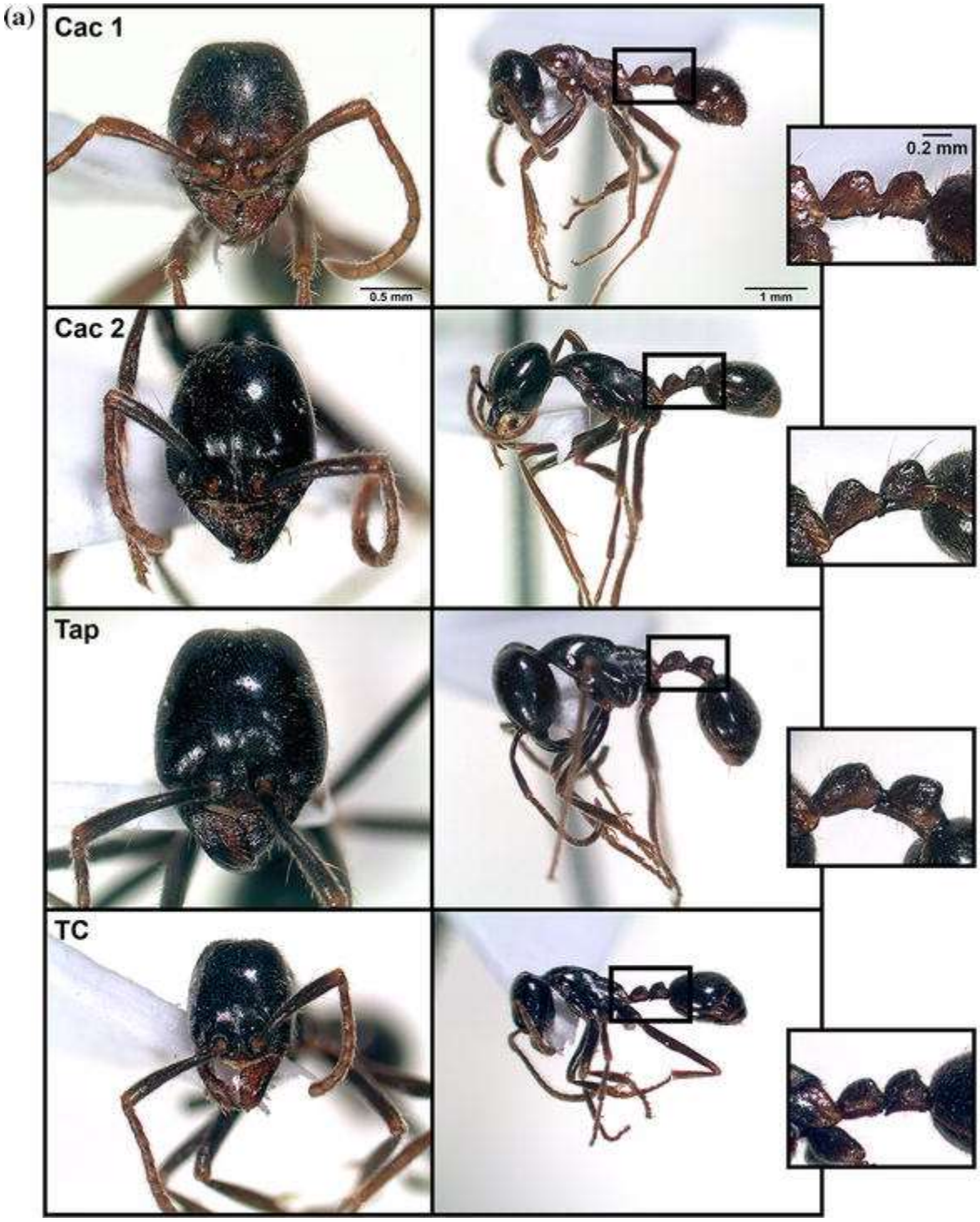
All four colonies were clearly identified as *L. praedator* for the absence of an anteroventral petiole tooth (Fig. 1), which is the character to discriminate *L. praedator* from other *Labidus* species following the classical morphological keys (Borgmeier 1955; Watkins 1976, 1982). However, those keys provide no worker characteristics to distinguish *L. p.* s. str. and *L. p. sedulus*. Moreover, only the online key of Longino (2005) separates the taxon *L.* sp. JTL-001, according to a 'smooth and shiny face' character, which may apply to our colonies Cac2, Tap and TC (Fig. 1).

In all colonies, a single queen genotype and 18–26 (22.75 ± 1.32 SD) male genotypes could be inferred from the workers (Table 1) and used to estimate population differentiation. Surprisingly, two of nine microsatellite loci were monomorphic in colony Cac1, but had more than three alleles in the other colonies, and the seven polymorphic loci differed considerably in allele size range, resulting in a high rate of private alleles for Cac1. While the allelic richness (6.39–7.53) did not differ between colonies (one-way ANOVA; $df = 24$, $F = 0.42$, $p = 0.74$), private allelic richness (Kalinowski 2005) was highest ($A_{priv} = 3.85$) in Cac1 ($F = 5.24$, $p = 0.006$; Table 1). Using all seven polymorphic loci resulted in considerable population differentiation of $F_{ST} = 0.102$ (95 % confidence from 15,000 bootstraps, CI 0.075/0.145), which dropped to $F_{ST} = 0.069$ (CI 0.035/0.118) when removing Cac1 from the analysis. Pairwise tests for Cac1 had twice as high F_{ST} values than between the other colonies (Table 2).

Table 2: Pairwise population differentiation (F_{ST}) values (above diagonal), and K2P values for sequence divergence (below diagonal) between four *L. praedator* colonies in Chiapas, Mexico

	Cac1	**Cac2**	**Tap**	**TC**
Cac1		0.173	0.128	0.153
Cac2	0.097		0.067	0.066
Tap	0.097	0.002		0.076
TC	0.098	0.001	0.002	

Both F_{ST} and K2P pairwise values are far higher between colonies Cac1 and Cac2, Tap or TC than between the latter three (more than 40-fold for K2P values)


(a)
Cac 1
0.5 mm
1 mm
0.2 mm
Cac 2
Tap
TC

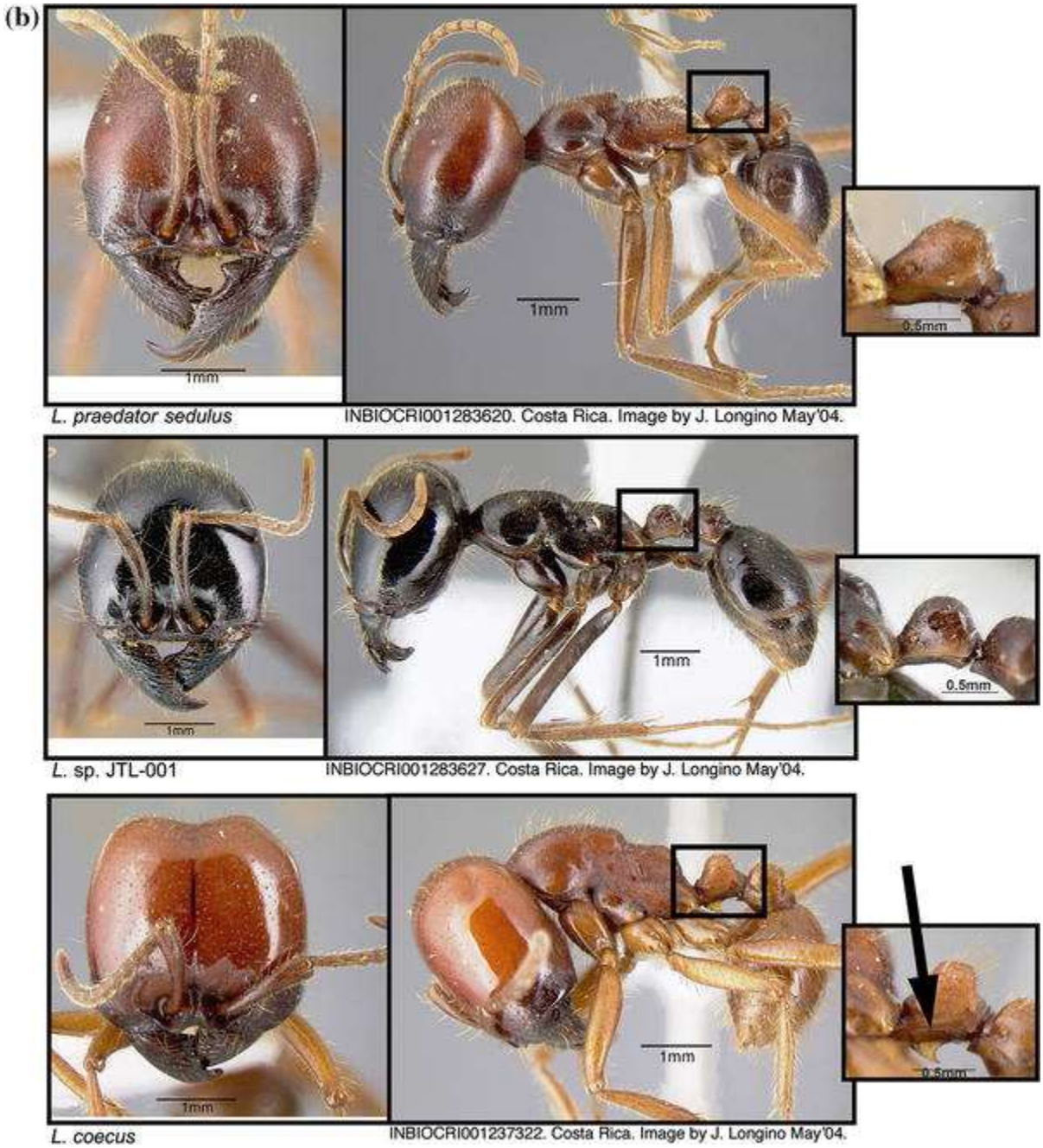


Fig. 1: Front (*left images*) and lateral (*right images*) view, and petiole (*small right images*) of **a** specimens of our four *L. praedator* colonies and **b** for comparison, images of Costa Rican specimens of *L. p. sedulus*, *L.* sp. JTL-001 and *L. coecus*, retrieved from Longino (2005, photo courtesy: J. Longino, with permission). *L. coecus* (*lowest images*) is the only *Labidus* species which co-occurs with *L. praedator* in Chiapas, Mexico, and can be distinguished from it by exhibiting an anteroventral petiole tooth (*arrow*), according to the classical morphological keys (Borgmeier 1955; Watkins 1976, 1982). Following these keys, all our specimens are clearly identified as *L. praedator*. However, following the 'shiny face' character in the online key of Longino (2005), the specimens of colonies Cac2, Tap and TC key out as *L.* sp. JTL-001

The whole 1387 bp alignment had 438 variable sites, of which 255 were parsimony informative, while the partial barcoding alignment had 151 variable sites (parsimony informative: 118), and the 28S alignment had 112 (parsimony informative: 29). For the whole and the barcoding alignment, ML trees with the highest log likelihoods (ln*L* = −4840.2 and ln*L* = −1978.8), based on GTR + G models, are given in Figs. 2 and 3. Both tree topologies

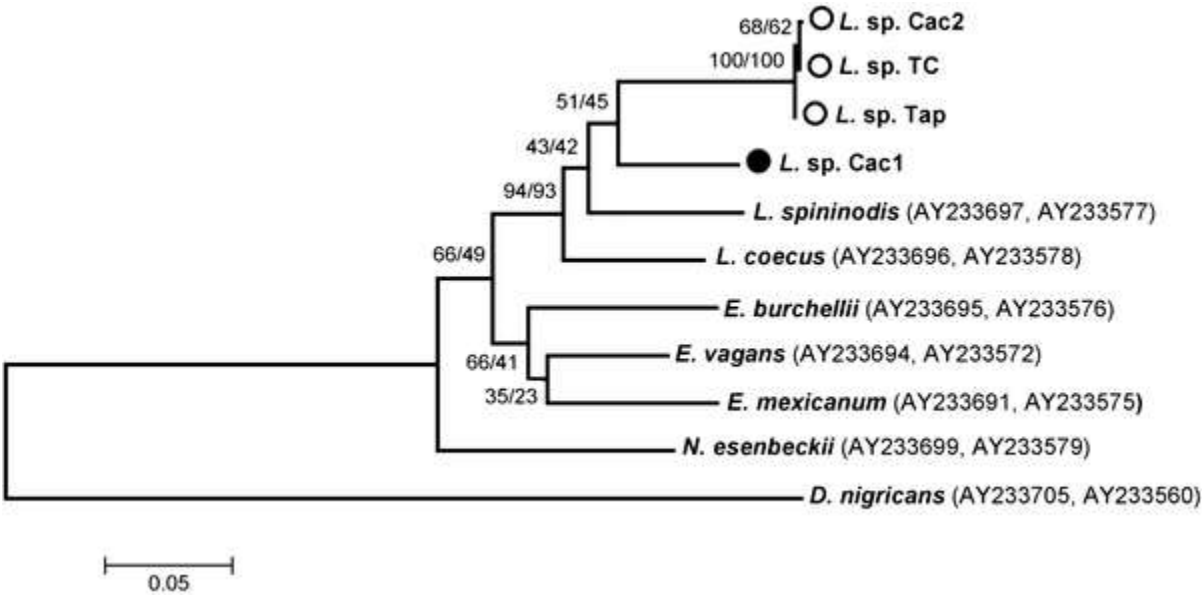


Fig. 2: Highest log likelihood ML phylogenetic tree of 11 sequences (1387 bp) of Mexican *L. praedator* and seven other army ant species (rooted with African *D. nigricans* as outgroup), based on a GTR + G model. Bootstrap values (500 replications) for the ML and NJ method are shown at the tree nodes. Branch lengths are drawn to scale (*small bar*) and measured in substitutions per site. Behind the taxa, colony labels are given for our samples and GenBank accession numbers (*coxI*, 28S) for the other species (Brady 2003). Colonies Cac2, Tap and TC (*open circles*) show an average sequence divergence of 0.2 %, but are separated from colony Cac1 (*black dot*) by 9.7 % divergence

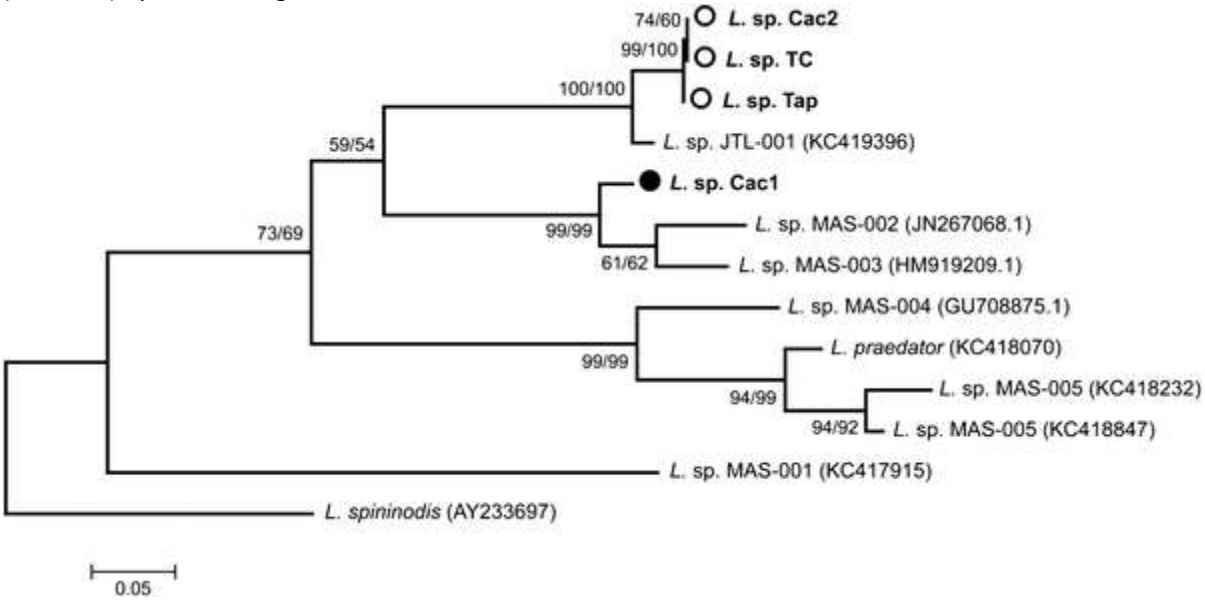


Fig. 3: Highest log likelihood ML phylogenetic tree of 13 *coxI* sequences (490 bp) of Mexican (*bold types*) and Costa Rican *L. praedator* lineages (rooted with *L. spininodis*), based on a GTR + G model. Bootstrap values (500 replications) for the ML and NJ method are shown at the tree nodes, and branch lengths in substitutions per site are drawn to scale (*small bar*). Colony labels or GenBank accession numbers (Smith et al. 2014) are given behind the taxa. The Mexican colonies Cac2, Tap and TC (*open circles*) and Cac1 (*black dot*) cluster in different clades with Costa Rican lineages, and show an average sequence divergence of 12.8 %, which is within the range (14.2 %) of overall divergence

were also obtained with NJ analyses (see TreeBase) with bootstrap values (500 replications) given at branches. The ML tree of 28S supports the topology of the concatenated sequence

tree with slightly longer branches of the latter (see TreeBase) which is expected considering the faster evolutionary rate of mtDNA (Avise 2000).

The concatenated sequence tree (Fig. 2) shows the phylogenetic relationship of our four *L. praedator* colonies and seven other army ant species. Colonies Cac2, Tap and TC cluster in one branch with very low K2P average sequence divergence (0.2 %), but are separated from Cac1 as sister group with an average pairwise divergence of 9.7 % (Table 2). Average divergence is also 9.7 % among all included *Labidus* species, and 11.7 % among all species (excluding the outgroup, *D. nigricans*). In the barcoding phylogeny of our sequences and those of several formally undescribed Costa Rican *Labidus* lineages, retrieved from Smith et al. (2014), colonies Cac2, Tap and TC cluster together as sister group of *L.* sp. JTL-001, while colony Cac1 is much closer related to the lineages *L.* sp. MAS-002 and 003 (Fig. 3). Within this *Labidus* group, the average pairwise divergence between colony Cac1 and the other three is 12.8 % (to 0.2 % among the latter), while average sequence divergence is 14.2 % among all lineages (excluding the outgroup).

Discussion

Our findings clearly show strong genetic differentiation among four Mexican *L. praedator* colonies, which may indicate distinction at species level between colony Cac1 and the other three. The lack of polymorphism of two otherwise highly diverse microsatellite loci and the particularly high rate of private alleles among all 21 males and the queen of Cac1 is highly unlikely a result of chance alone. The non-detection error of males (probability of genotypic identity by chance, Boomsma and Ratnieks 1996) is only 3.5 % for the two loci, and the non-sampling error is 0.53 on average, according to fitted Poisson distributions. Also population fragmentation can hardly explain this pattern because the well flying army ant males easily allow for gene flow within our sampling area (Berghoff et al. 2008; Jaffé et al. 2009; Barth et al. 2013), and colonies Cac1 and Cac2 were even sampled in the very same time and place (Table 1). Thus, our results suggest two sympatric, but strongly reproductively isolated *L. praedator* lineages within our samples, which are, however, hardly distinguishable with the classical morphological keys (Borgmeier 1955; Watkins 1976, 1982) (Fig. 1).

This is also confirmed by phylogenetic analyses. Even though some branches contain only weak bootstrap values (probably due to our restricted sampling), particularly the separate clustering of colonies Cac2, Tap and TC in the whole sequence tree (Fig. 2) and the clustering of those colonies and Cac1 within different clades in the *coxI* barcoding tree (Fig. 3) is well supported. Moreover, our whole sequence ML tree (Fig. 2) is well confirmed by the army ant phylogeny of Brady (2003). However, *L. praedator* was not included in this phylogeny, and shows strong intraspecific sequence divergence, especially among the 490 bp barcoding fragments of recently published lineages (Smith et al. 2014) from Costa Rica (Fig. 3). Interestingly, according to the latter data, our Mexican lineages seem to be more closely related to different Costa Rican lineages than to each other, suggesting the occurrence of several strongly isolated sympatric lineages across the very large distribution range of *L. praedator*.

Average pairwise sequence divergence of 9.7 % (whole alignment) between colony Cac1 and colonies Cac2, Tap and TC is over 40-fold higher than the average divergence of 0.2 %

among the latter (Table 2), which is clearly in the range of the more distant *Labidus* species from GenBank (also 9.7 %). This holds as well for the *coxI* barcoding fragments, while divergence becomes even more pronounced with 12.8 % on average between our Mexican lineages (14.2 % over all lineages). Though deep *coxI* divergence within some ant species seems not to be unusual (Smith et al. 2005; Fisher and Smith 2008), and particularly in army ants restricted maternal dispersal may foster mtDNA differentiation (Berghoff et al. 2008; Barth et al. 2013), the inclusion of nuclear 28S fragments shows that our results are not an artifact of mtDNA divergence only. Taking the 28S phylogeny alone not only confirms the whole sequence tree topology but still results in a divergence of 3.7 % between colony Cac1 and the other three, compared to 0.0 % among the latter (2.4 % among all *Labidus* species). This is also supported by the population genetic differentiation pattern of nuclear microsatellite data. Our results, therefore, easily meet the requirements of the genetic barcoding approach for species discrimination with thresholds of maximal 2 % intra- and a minimal tenfold higher interspecific divergence for the *coxI* region (Hebert et al. 2003a, b, 2004). Thus, the observed divergence very likely reflects differentiation at species level within *L. praedator*.

Although the subspecies *L. p. sedulus*, described on the basis of a male specimen from Colombia, has not been recorded for Mexico (Borgmeier 1955), we cannot exclude that we detected this type here because no prior genetic data exist. Morphologically, this subspecies can only be identified by the males or the largest soldier workers which we had not sampled, and which are difficult to obtain (Rettenmeyer 1963, p. 403). The variant *L.* sp. JTL-001, since it was first mentioned for Costa Rica, has been widely recorded throughout Central America including Mexico (Longino 2005; www.antweb.org). According to the 'shiny face' character, the three Mexican colonies Cac2, Tap and TC may belong to this or a closely related taxon (Fig. 1), which is also suggested by the *coxI* ML tree (Fig. 3). For the MAS variants from Smith et al. (2014), no morphological descriptions exist so far. However, the present study did not intend to cover the entire spectrum of morphological and genetic variance in *L. praedator*. Instead, our data show deep phylogenetic divergence over a very small geographic area, which may hold true for the whole *L. praedator* distribution range, while available morphological data insufficiently explain this diversity. Thus, we suggest the existence of several distinctive lineages or cryptic speciation in an '*L. praedator*-complex', and a phylogenetic and taxonomic revision seems timely.

Tropical forests may harbor over 20 sympatric army ant species (Rettenmeyer et al. 1983; Longino et al. 2002). It is this very diversity which facilitates their enormous impact at the ecosystem level (Kaspari and O'Donnell 2003; Kaspari et al. 2011; O'Donnell et al. 2007), and we show that this diversity, particularly of the swarm-raiders, may be still underestimated. Our study, therefore, demonstrates the importance of detecting cryptic speciation as an essential element of understanding biodiversity and community dynamics in these ants and in general.

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