Myrmecological News	17	5-11	Vienna, August 2012

Molecular and morphological evidence for three sympatric species of *Leptanilla* (Hymenoptera: Formicidae) on the Greek island of Rhodes

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Abstract

Ants of the genus *Leptanilla* have a broad distribution in the Paleotropical and southern Palearctic regions. Workers are eyeless, strictly subterranean and rarely encountered, whereas the males possess eyes and fully developed wings, and are collected with greater frequency in pan traps, in Malaise traps, and at lights. As a result of these circumstances a parallel taxonomy has developed for workers and males, with little or no attempt at integration of the two systems. Molecular markers have the potential to link the two sexes and permit reconciliation of the two taxonomies. Here we analyze a sample of fourteen *Leptanilla* males from a single site on the island of Rhodes, Greece, using morphology and DNA sequence data (ten nuclear genes; 8.6 kb of aligned sequence data). The two sources of data are fully concordant, and indicate the occurrence of three sympatric species. Phylogenetic relationships of these taxa and two other *Leptanilla* species are estimated using Bayesian inference and maximum likelihood. Two of the species, *Leptanilla* GR01 and *Leptanilla* GR03 are sister taxa in these analyses, while *Leptanilla* GR02 is more distantly related. Within this sample we observe almost no intraspecific genetic or morphological variation, but there are substantial differences among the species. The divergent DNA sequences provide an opportunity to identify the corresponding workers of these *Leptanilla* species.

Key words: Formicidae, Leptanillinae, *Leptanilla*, systematics, taxonomy, species delimitation, molecular phylogenetics, male ants.

Myrmecol. News 17: 5-11 (online 22 February 2012)
ISSN 1994-4136 (print), ISSN 1997-3500 (online)

Received 10 October 2011; revision received 30 November 2011; accepted 2 December 2011
Subject Editor: Corrie S. Moreau

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Introduction

The ant subfamily Leptanillinae is an enigmatic group of pale, blind subterranean ants, distributed widely in tropical and warm temperate regions of the Old World (BARONI URBANI 1977, BOLTON 1990). Leptanillines appear to represent an ancient, early branching lineage of ants. In some molecular phylogenetic analyses they emerge as the sister group of all or most other extant ants (MOREAU & al. 2006, RABELING & al. 2008, KÜCK & al. 2011), although difficulties in correctly rooting the ant tree introduce uncertainty about the position of the group (BRADY & al. 2006, WARD 2007). The most widespread genus, *Leptanilla* EMERY, 1870, is found from southern Africa to the Mediterranean region of Europe, and east to China, Japan, southeast Asia and Australia (BARONI URBANI 1977). *Leptanilla* workers are small, eyeless, confined to subterranean abodes, and infrequently collected. The males, eyed and fully winged, are collected in light traps, pan traps or Malaise traps, and are often the only indication of the presence of *Leptanilla* in a locality. ROBERTSON (2000), for example, recorded males of nine *Leptanilla* species in this manner from the Brandburg Massif, Namibia, yet no workers were encountered. Because males are usually collected separately from the workers, a parallel taxonomy has developed for the two sexes (BOLTON 1990). DNA sequence data have the potential to link the two sets of names and thereby improve the taxonomy of the group. Here we provide molecular and morphological characterizations of three sympatric species of *Leptanilla* on the Greek island of Rhodes. Our sample consists of males only, but with the DNA sequence data it should be possible to identify the corresponding workers if they are collected.

Seventeen species of *Leptanilla* have been described from the Mediterranean region. Eleven of these are based on workers (or workers and queens), six are known from males only, and for no species have both sexes been associated (BARONI URBANI 1977, LÓPEZ & al. 1994, SCUPOLA & BALLARIN 2009, BOLTON & al. 2007). Thus, there is a strong likelihood of synonymy among some of these named taxa. In addition to the seventeen named species, four other male-based morphospecies have been described – but not formally named – from Tunisia and Spain (PETERSEN 1968, BARONI URBANI 1977) and Sicily (SCUPOLA & BALLARIN 2009).

Materials and methods

This study is based on a series of 14 *Leptanilla* males taken from a blue pan trap set in open woodland on the island of Rhodes. Collection data are as follows: Greece, Rhodes,

Corrigendum added between Online Earlier and print publication: see p. 11.

Tab. 1: Ant specimens sequenced and GenBank accession numbers. For *Leptanilla* GR01, *Leptanilla* GR02 and *Leptanilla* GR03 the specimen codes apply to the actual sequenced specimens. Specimen codes for the three outgroup species refer to voucher specimens from the same collection series as the sequenced specimen; full collection data for these are available on AntWeb (www.antweb.org).

Specimen	Specimen Code	18S	28S	Wg	AbdA	LW Rh	EF1aF1	EF1aF2	ArgK	TPI	Ubx
Leptanilla GR01a	CASENT0106505	EF012871	EF012999	EF013707	JN967846	JN967889	JN967837	JN967829	JN967880	JN967820	JN967809
Leptanilla GR01b	CASENT0106236	JN967870	JN967862	JN967854	JN967847	JN967890	JN967838	JN967830	JN967881	JN967821	JN967810
Leptanilla GR02a	CASENT0106062	JN967871	JN967863	JN967855	EF013127	EF013579	JN967839	EF013431	JN967882	JN967822	JN967811
Leptanilla GR02b	CASENT0106060	JN967872	JN967864	JN967856	JN967848	JN967891	JN967840	JN967831	JN967883	JN967823	JN967812
Leptanilla GR02c	CASENT0106061	JN967873	JN967865	JN967857	JN967849	JN967892	JN967841	JN967832	JN967884	JN967824	JN967813
Leptanilla GR03a	CASENT0106063	JN967874	JN967866	JN967858	JN967850	JN967893	JN967842	JN967833	JN967885	JN967825	JN967814
Leptanilla GR03b	CASENT0106058	JN967875	JN967867	JN967859	JN967851	JN967894	JN967843	JN967834	JN967886	JN967826	JN967815
Leptanilla GR03c	CASENT0106059	JN967876	JN967868	JN967860	JN967852	JN967895	JN967844	JN967835	JN967887	JN967827	JN967816
Leptanilla TH01	CASENT0119792	JN967869	JN967861	JN967853	JN967845	JN967888	JN967836	JN967828	JN967879	JN967819	JN967808
Leptanilla ZA01	CASENT0106085	AY867436	AY867452	AY867421	AY867468	AY867483	EF013270	EF013432	JN967878	JN967818	JN967807
Protanilla JP01	CASENT0007002	EF012925	EF013053	EF013761	EF013181	EF013633	EF013337	EF013499	JN967877	JN967817	JN967806

2.5 km N Psinthos, near summit of road to Maritsa, 7. - 9. VIII.2003, 36.33536° N 28.08847° E, 396m, leg. M. Ohl & C. Lüter. Voucher specimens are deposited in the California Academy of Sciences, San Francisco (CASC) and the Bohart Museum of Entomology, University of California at Davis (UCDC).

The series of males was scrutinized for external morphological differences. This led to the provisional recognition of three morphotypes, here termed *Leptanilla* GR01 (two specimens), *Leptanilla* GR02 (eight specimens) and *Leptanilla* GR03 (four specimens). These were characterized with the following metric measurements and indices:

ALI Antennal length index: (LA2 + LA3 + LA4) / HL
CI Cephalic index: HW / HL
FI Profemur index: FW / FL
FL Length of profemur
FW Width of profemur, measured orthogonal to FL
HL Midline length of head, from anterior clypeal margin to midpoint of a line drawn across posterior margin of head
HW Maximum width of head excluding eyes
LA2 Length of second antennal segment
LA3 Length of third antennal segment
LA4 Length of fourth antennal segment
SI Scape index: SL / HW
SI2 Scape index, using HL: SL / HL
SL Scape length: length of first antennal segment, excluding radicle
WL Weber's length: length of mesosoma in lateral view, from anterior pronotal margin, excluding cervical protrusion, to posteroventral extremity of mesosoma

Terms for wing venation and male genitalia follow YOSHIMURA & FISHER (2011).

To verify the distinctness of these forms we sequenced a subset of eight males: two of *Leptanilla* GR01, three of *Leptanilla* GR02 and three of *Leptanilla* GR03 (Tab. 1). DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia, California, U.S.A.). The extraction procedure followed the manufacturer's recommended protocol except that the final elution used water rather than AE buffer. One male (*Leptanilla* GR01a) was destructively extracted; two males (*Leptanilla* GR02a, *Leptanilla* GR03a) had their genitalia removed and saved prior to destructive extraction; and the remaining five males were non-destructively extracted, i.e., the entire specimen was salvaged after extraction. For non-destructive extraction the same protocol was followed except that males were not macerated before incubation in proteinase K and they were removed from the microtube after incubation, and then cleaned and point-mounted.

We sequenced fragments of ten nuclear genes: 18S ribosomal DNA (rDNA), 28S rDNA, wingless (Wg), abdominal-A (AbdA), long-wavelength rhodopsin (LW Rh), elongation factor 1-alpha F1 copy (EF1aF1), elongation factor 1-alpha F2 copy (EF1aF2), arginine kinase (ArgK), triosephosphate isomerase (TPI), and ultrabithorax (Ubx). Amplification and sequencing procedures are described in WARD & DOWNIE (2005), BRADY & al. (2006), and WARD & al. (2010). Primers for two genes new to this study (TPI and Ubx) are given in Table 2. Newly generated sequences have been deposited in GenBank under accession numbers JN967806-JN967895.

As outgroups in phylogenetic analyses we used the following leptanilline species from the Ant AToL (Assembling the Tree of Life) Project: *Leptanilla* TH01, *Leptanilla* ZA01, and *Protanilla* JP01. In an earlier study (BRADY & al. 2006) the last two species were referred to as *Leptanilla* RSA01 and *Protanilla* JAP01, respectively. *Protanilla* was used to root the tree.

Sequences of each gene were straightforwardly aligned using ClustalX v2.0 (LARKIN & al. 2007) and concatenated in MacClade v4.08 (MADDISON & MADDISON 2000). For maximum likelihood (ML) and Bayesian inference of phylogenetic relationships the data set was partitioned by gene, codon position, and intron / exon boundaries. We combined codon positions 1 and 2 into a single partition because of limited information available in the second codon position alone. Two of the gene fragments, ArgK and LW Rh, each included an intron, whereas the other six protein-coding fragments comprised exon sequence only, giving a total of 20 partitions (Tab. 3). MrModelTest v2.3 (NYLANDER 2004) was used to choose a substitution model for each partition, based on the Akaike Information Crite-

Tab. 2: Newly designed primers used for sequencing triosephosphate isomerase (prefixed by "TP") and ultrabithorax (prefixed by "UB") in ants. Coordinates are based on GenBank sequences XM_396203.3 (*Apis mellifera*, TPI) and NM_001168700.1 (*Apis mellifera*, Ubx). This list includes primers employed generally for the Ant AToL (Assembling the Tree of Life) Project. The principal primers are in bold font; the others are used for more recalcitrant samples where it is necessary to amplify smaller overlapping fragments.

Primer	Sequence (5' to 3')	Coordinates
TP1293EF	TKCAG G TGG GAR GAR GAR AAG AA	~ 1293 - 1310
TP1339F	GAR CAY AAR GGA CCK GTR TTY GCA CC	1339 - 1364
TP1506F	C AAC TTY TTC CAY GAY TGG CGR GA	1506 - 1529
TP1525F	CGA GAR GTG ATG ACY GAR TCD GAR CG	1525 - 1550
TP1705F	ATC GAC GGY CAY AAR GAR AAR ATY GG	1705 - 1730
TP1729F2	GGY AAC TTY AAR ATY GAG CCD CCV GG	1729 - 1757
TP1901F2	CY AAT GTY ACD TGG CTH GCR TCH TGG AC	1901 - 1928
TP1987F	GGH GAA AAR GAY TGG CAR AAR TAY GA	1987 - 2012
TP2065F	GAR GAY TGG AAR AGY AAR GAR ATG CG	2065 - 2090
TP1598R	GC RTG CAT CTC YTT RAA GTT RCA	1598 - 1576
TP1793R	TT RCC CAT YTT RGG RTG CTC RCC RCG	1793 - 1762
TP1805R	CG CYT CTT YAR YTT RCC CAT YTT RGG	1805 - 1780
TP2167R	G ATC YTC RTC CTT YTC RTT RCC RGC	2167 - 2143
TP2192R	GA RCA RCA RCC YAC DGT RTC HGC YTG	2192 - 2167
TP2266ER2	GTTAC C TAA RAA RTC RAA YAC RAC BAC	~ 2266 - 2245
TP2266ER3	GTYAC C TAA RAA RTC RAA BAC RAC	~ 2266 - 2248
UB1F6	GATYCAARRT ACCCGGGRTA ATG AAC	~ 1 - 6
UB1F	GGRTA ATG AAC TCG TAY TTY GAR CAG	~ 1 - 21
UB214F	CCR CCY CAR GAY TCR CCR TAY GAY GC	214 - 239
UB259F	AAG CTT TAY TCG ACG ACR CCH GAR GC	259 - 284
UB289F	GGT CAY ACY ACR TCV TCR TAY TCR A	289 - 314
UB323F	CR AAR GAC TGT AAR CAR CAR GAY CA	323 - 347
UB278R	C DGG YGT CGT YGA RTA VAG YTT RCA	278 - 256
UB314R	GT YGA RTA YGA DGA YGT RGT RTG RCC	314 - 289
UB389R	GC TGC CAT YAC CGC VGC RTA DCC	389 - 367
UB412R	G CCA MAC GTC YTT GAC SGC YGC	412 - 391
UB671R	AT RGC CAT CCA RGG RTA GAA SGT RTG	671 - 646
UB676ER	CTYAC C TGC TAT RGC CAT CCA RGG	~ 676 - 658

rion (AIC). For Bayesian estimation of phylogeny we used MrBayes v3.1.2 (RONQUIST & HUELSENBECK 2003), with branch lengths and topology linked between partitions and other parameters unlinked. The MCMC Bayesian analysis ran for 40 million generations, with a generous burn-in of 15 million generations. That stationarity had been achieved was indicated by stable and near-identical harmonic mean likelihoods for the two sub-runs and by PSRF (Potential Scale Reduction Factor) values close to 1.0 for all parameters. ML analyses were carried out with GARLI v2.0 (ZWICKL 2006), with comparable parameter linkage, and 100 bootstrap replicates. The data matrix for these analyses has been deposited at TreeBASE (study accession S12022).

Tab. 3: Gene partitions, their characteristics, and substitution models chosen by AIC in MrModelTest. PIS = parsimony-informative sites; VS = variable sites.

Partition	No. of sites	PIS	VS	Model
18S	1852	4	21	GTR + I
28S	1835	72	194	GTR + I
Wg, pos 1 + 2	274	7	19	GTR + I
Wg, pos 3	138	41	90	HKY
AbdA, pos 1 + 2	402	5	21	HKY + I
AbdA, pos 3	201	35	80	K80 + G
ArgK, pos 1 + 2	448	13	22	HKY + I
ArgK, pos 3	225	75	127	HKY + G
ArgK, intron	228	62	140	HKY
EF1aF1, pos 1 + 2	239	5	6	HKY + I
EF1aF1, pos 3	120	56	93	GTR
EF1aF2, pos 1 + 2	344	5	9	GTR + I
EF1aF2, pos 3	173	42	96	HKY + I
LW Rh, pos 1 + 2	305	12	42	HKY + I
LW Rh, pos 3	153	31	74	HKY
LW Rh, intron	113	24	50	HKY + I
TPI, pos 1 + 2	586	16	36	GTR + I
TPI, pos 3	294	73	166	GTR
Ubx, pos 1 + 2	420	3	22	HKY
Ubx, pos 3	210	30	88	HKY + I
All genes	8560	611	1396	GTR + I + G

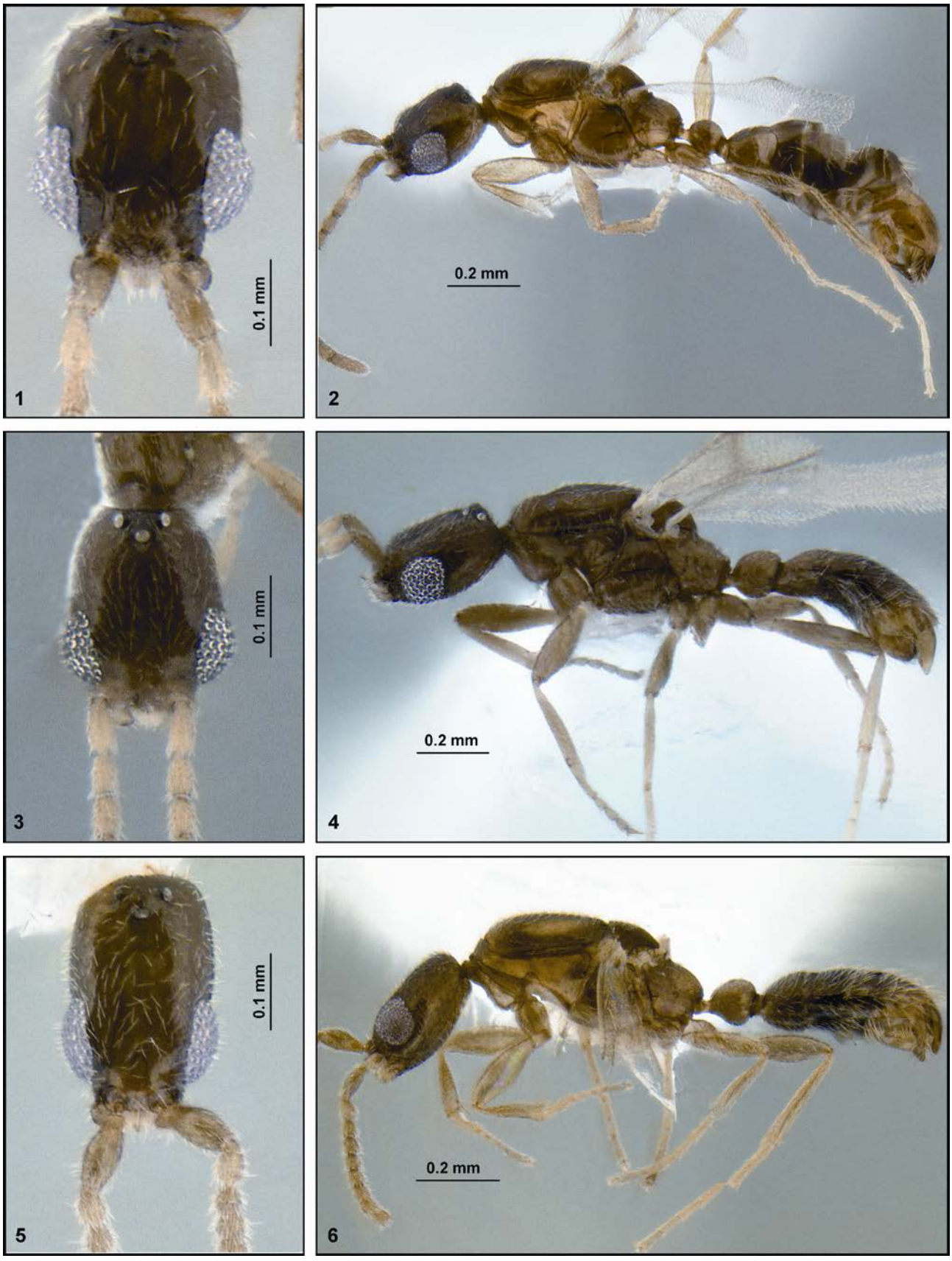
Results

Description of morphotypes

Three distinctive morphotypes were observed in the sample of 14 males, each exhibiting little variation within, but substantial variation between, each type. The three taxa can be diagnosed as follows.

***Leptanilla* GR01** (Figs. 1 - 2): Larger species (HW 0.24, HL 0.29, WL 0.57; n = 1) with relatively broad head (CI 0.83); scape length about one-half head width (SI 0.51), scape slender (SI2 0.42), its maximum width about one-third of length; remaining antennal segments relatively elongate (ALI 0.66), all segments longer than wide; profemur relatively robust (FI 0.37); aedeagus shield-shaped, tapering to a rounded apex that is medially cleft; harpago (distal portion of paramere) expanded apically into a subtriangular lobe, with an acute outer (ventral) tooth and a rounded, obtuse inner (dorsal) tooth.

***Leptanilla* GR02** (Figs. 3 - 4): Smaller species (HW 0.18 - 0.20, HL 0.25 - 0.28, WL 0.43 - 0.48; n = 7) with moderately elongate head (CI 0.68 - 0.74); scape length more than one-half head width (SI 0.57 - 0.66), scape slender (SI2 0.41 - 0.48), its maximum width about one-third of length; remaining antennal segments relatively elongate (ALI 0.63 - 0.72), all segments longer than wide; profemur slender (FI 0.28 - 0.33); aedeagus extended distally as a tongue-like lobe, bluntly rounded apically with a slight medial indentation; harpago expanded apically



Figs. 1 - 6: Automontage images of *Leptanilla* males, full-face (dorsal) view of head (1, 3, 5), and lateral view of body (2, 4, 6). 1 - 2: *Leptanilla* GR01 (CASENT0106236); 3 - 4: *Leptanilla* GR02 (CASENT0106064); 5 - 6: *Leptanilla* GR03 (CASENT0106058).

into a bifurcate process, both teeth acute, the inner (dorsal) tooth subtended proximally by a third, smaller obtuse tooth.

***Leptanilla* GR03** (Figs. 5 - 6): Intermediate-sized species (HW 0.19 - 0.20, HL 0.30 - 0.32, WL 0.50 - 0.57; n = 3) with very elongate head (CI 0.59 - 0.64); scape length more than one-half head width (SI 0.56 - 0.59), scape short and robust (SI2 0.36 - 0.37), apically enlarged, its maximum width about two-fifths of length; remaining antennal segments relatively short (ALI 0.47 - 0.49), almost moniliform, segments 3 - 9 about as wide as long; eye with conspicuous pilosity; profemur robust (FI 0.40 - 0.42); aedeagus extended distally in the form of a broad triangular plate; harpago a simple slender digitiform lobe, neither expanded apically nor with teeth or angles.

Males of all three forms are medium-brown in color, with lighter appendages. Standing or subdecumbent pilosity is common on the head, mesosoma dorsum, and metasoma. The mandibles are small, short and essentially edentate. The petiole is subglobular, and longer than wide in dorsal view. The forewing wing venation is much reduced, consisting of Sc + R and its continuation R1, which together extend about two-thirds of the distance along the wing margin, and a vestigial Rs + M that is directed towards a faint anal (A) vein. The basimeres are large, contiguous ventrally, and well separated dorsally. The harpagones are bent mesad in most specimens examined but this could be a function of the mode of preservation. KUGLER (1987) noted a similar inward deflection of the harpagones in dry specimens of *Leptanilla israelis* whereas those treated in KOH had the harpagones extended caudad, in line with the basimere.

Relationship to previously described taxa

The state of *Leptanilla* taxonomy makes positive identification of the Greek male specimens difficult. The descriptions provided by SANTSCHI (1907, 1908), PETERSEN (1968), BARONI URBANI (1977), KUGLER (1987) and SCUPOLA & BALLARIN (1994) suggest, however, the following tentative associations.

1. *Leptanilla* GR01 = sp. near *L. tanit* SANTSCHI, 1907 and / or *L. israelis* KUGLER, 1987: Similarities between these three forms include the relatively broad head (CI ~ 0.80), elongate antennal segments, robust profemur, and the shapes of the aedeagus and harpago (the latter weakly bifurcate, with a strong outer tooth and obtuse inner tooth). KUGLER (1987) distinguished *Leptanilla israelis* from *L. tanit* on the basis of petiolar shape: strongly convex with a vertical anterior face in *L. tanit*, and with a more gently sloping anterior face in *L. israelis*. In this regard *Leptanilla* GR01 corresponds more closely to *L. tanit*, although the difference in petiolar shape needs to be more fully evaluated.

2. *Leptanilla* GR02 = sp. near *Leptanilla* SIC-1 (of SCUPOLA & BALLARIN 1994): These two forms share the following features: comparable body size (HW 0.17 - 0.21, HL 0.24 - 0.29), elongate head (CI ~ 0.70), slender scape and succeeding antennal segments (the latter all longer than wide), slender profemur, bluntly rounded aedeagus, and apically bidentate harpago. There are differences, however, in the shape of the harpago: In *Leptanilla* GR02 the inner (dorsal) apical tooth is subtended proximally by a smaller obtuse tooth whereas this is apparently absent in *Leptanilla* SIC-1. In addition the volsellae are better developed in *Leptanilla* SIC-1 than in *Leptanilla* GR02 (slender, short and inconspicuous in the latter).

3. *Leptanilla* GR03 = sp. near *L. exigua* SANTSCHI, 1908: Features in common between these two include the very elongate head (CI ~ 0.60), short broad scape, quadrate flagellar segments (PETERSEN 1968), and the simple non-bifurcate harpago. For clarification it should be noted that the legend for Figures 1 - 2 in SANTSCHI (1908) is in error: Figure 1 is *Leptanilla exigua*, while Figure 2 is *L. minuscula*, not the opposite as stated in the paper. PETERSEN (1968) previously corrected the errors in SANTSCHI (1907): In that paper Figure 2 depicts *L. tanit* (not *L. minuscula*) while Figure 3 illustrates *L. minuscula* (not *L. tanit*). Taking into account these corrections, as well as the original descriptions by SANTSCHI (1907, 1908) and the careful reanalysis of Santschi's material by PETERSEN (1968), we provisionally associate *Leptanilla* GR03 with *L. exigua* or a related species.

The other male-based *Leptanilla* species described from the Mediterranean region – *L. tenuis* SANTSCHI, 1907, *L. minuscula* SANTSCHI, 1907, *L. bifurcata* KUGLER, 1987, *Leptanilla* sp. SIC-2 of SCUPOLA & BALLARIN (1994), and *Leptanilla* sp. A and *Leptanilla* sp. B of BARONI URBANI (1977) – all differ from the Rhodes specimens in various combinations of head shape, antennal lengths, and configurations of the male genitalia.

Phylogeny and molecular characterization

Maximum likelihood (ML) and Bayesian phylogenetic analyses of the concatenated 10-gene data set yielded similar topologies and support levels (Fig. 7). Individuals of the same *Leptanilla* morphotype consistently clustered with one another, separately from other individuals, with maximum support values: Bayesian posterior probabilities (PP) of 1.00 and ML bootstraps of 100. Thus the molecular data strongly support their status as distinct species. Among the taxa sampled for this study *Leptanilla* GR01 and *Leptanilla* GR03 are one another's closest relatives. The three Greek species of *Leptanilla* form a weakly supported clade (PP 0.64) that is increasingly more distantly related to *Leptanilla* ZA01, *Leptanilla* TH01 and *Protanilla* JP01.

As is true for morphology, there is very little within-species sequence variation, the mean uncorrected intraspecific genetic distances being 0.0001, 0.0002, and 0.0003 for *Leptanilla* GR01, *Leptanilla* GR02, and *Leptanilla* GR03, respectively, across all ten genes. Mean interspecific genetic distances among the three species are two orders of magnitude greater: 0.0107 (*L.* GR01 versus *L.* GR03), 0.0377 (*L.* GR02 versus *L.* GR03) and 0.0372 (*L.* GR01 versus *L.* GR02). For the two most closely related species (*L.* GR01 and *L.* GR03), the largest interspecific genetic distances were manifested by the genes Wg (0.0269), ArgK (0.0255), EF1aF1 (0.0176), EF1aF2 (0.0174), and LW Rh (0.0165). All eight protein-coding genes exhibited one or more amino acid substitutions among the three species. For five genes (ArgK, LW Rh, TPI, Ubx, and Wg) such non-synonymous changes were observed even between *Leptanilla* GR01 and *Leptanilla* GR03. These genes should be particularly useful for future work on *Leptanilla* taxonomy.

With our current data set we were able to identify numerous potential molecular apomorphies of the three *Leptanilla* species, i.e., substitutions unique to each of the three taxa. More extensive sampling of species and populations

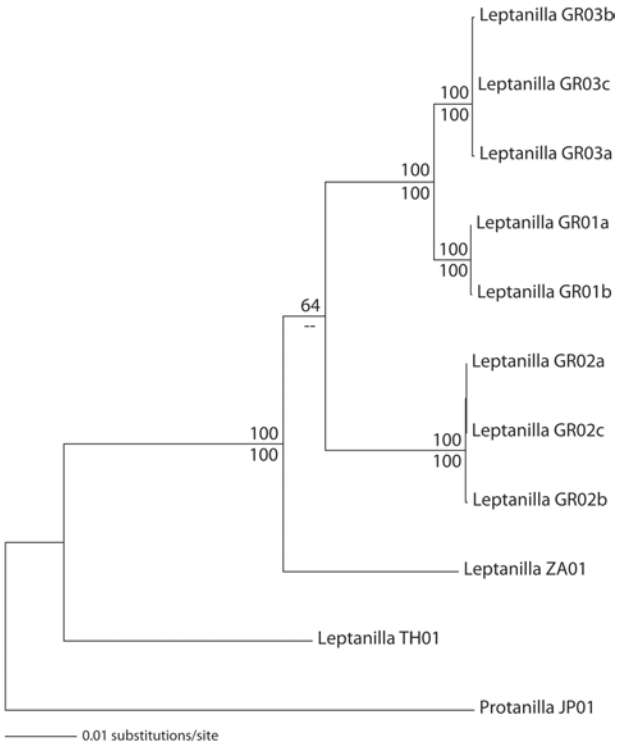


Fig. 7: Majority-rule consensus tree of *Leptanilla* relationships from partitioned Bayesian analysis of 10-gene data set (8560 bp of sequence data). Support values are posterior probabilities × 100 (above nodes) and maximum likelihood bootstraps from a separate GARLI analysis (below nodes).

would be required, however, to determine which of these are true species-level apomorphies. Similarly, our measures of intraspecific genetic distance should be considered minimum estimates since all collections derive from a single pan trap sample.

Discussion

Based on corroborative evidence from male morphology and DNA sequence data we demonstrate the co-occurrence of three *Leptanilla* species on the island of Rhodes. This is consistent with other studies showing the coexistence of multiple species of *Leptanilla* in Tunisia (SANTSCHI 1907, 1908, PETERSEN 1968), Israel (KUGLER 1987), Spain (LOPÉZ & al. 1994) and Sicily (SCUPOLA & BALLARIN 2009). Unfortunately it remains difficult to reconcile the identities of these species across the larger Mediterranean region, because they are based on disparate sexes (workers in Spain, males in Tunisia, Israel and Sicily), and there are no DNA sequence data for the other populations.

The *Leptanilla* males from Rhodes apparently represent the only record of this genus from Greece (LEGAKIS 2011), highlighting our incomplete knowledge of the subterranean ant fauna in this and other parts of the Mediterranean region. There is an obvious need for more comprehensive sampling of *Leptanilla* workers to complement the more extensive collections of males. The "lavage de terre" technique, which involves washing and filtering large quantities of subsurface soil, was employed productively by LOPÉZ & al. (1994) to detect *Leptanilla* workers in Spain, and this method could be usefully applied elsewhere.

Future collections of *Leptanilla* males and workers should include preservation of some specimens in media, such as 95% ethanol, that preserve DNA. This will afford the opportunity to link different sexes and eventually overcome the parallel male / worker taxonomy that has developed for the Leptanillinae. This approach has been successfully applied in other Hymenoptera where there is strong morphological divergence between the sexes and a tendency for them to be collected in different situations (PILGRIM & PITTS 2006, SCHÖNING & al. 2008, WILSON & PITTS 2008).

Since *Leptanilla* queens are entirely wingless this leads to an expectation of reduced dispersal and more pronounced population differentiation than in ants with winged queens, especially with respect to maternally inherited traits such as mitochondrial genes. How to delimit species in closely related and variably differentiated allopatric populations is a long-standing problem in systematics (MAYR 1969). But if closely related forms are sympatric and maintain their distinctness, i.e., they do not show evidence of interbreeding, this provides a compelling basis for their treatment as separate species. The documentation of multiple co-occurring species of *Leptanilla* in Spain, Sicily, Tunisia, Israel, and now Rhodes (Greece) suggests an opportunity to develop criteria for the recognition of allospecies, based on the differences shown by closely related but indubitably separate sympatric species (TOBIAS & al. 2010).

Coda

In the 7-gene ant phylogeny presented in BRADY & al. (2006) the taxon referred to as "*Leptanilla* GRE01" is a chimera, based on sequences generated from two specimens that were assumed (incorrectly) to be conspecific. The sequences for 18S, 28S, Wg, and EF1aF1 are derived from *Leptanilla* GR01a, while those of AbdA, LW Rh, and EF1aF2 are derived from *Leptanilla* GR02a (see Tab. 1). These seven sequences were previously listed under a single species in GenBank, but the identifications have now been updated.

Acknowledgements

We are grateful to Michael Ohl who supplied the *Leptanilla* males to the Ant AToL (Assembling the Tree of Life) Project, and to fellow Ant AToL collaborators, Seán Brady, Brian Fisher and Ted Schultz, for helpful discussions. Bonnie Blaimer provided assistance with DNA sequencing and phylogenetic analyses, and Michael Branstetter took the Automontage images. We thank the editors and an anonymous reviewer for constructive comments that improved the manuscript. This research was supported by NSF grant EF-0431330.

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Corrigendum:

The authors wish to publish the following correction:

The gene that was referred to as "triosephosphate isomerase" (TPI) is actually DNA topoisomerase 1 (Top1). The following corrections to the article are required:

Page 6, Table 1. Change "TPI" to "Top1".
Page 6, column 2, line 15. Change "triosephosphate isomerase" to "DNA topoisomerase 1".
Page 6, column 2, line 18. Change "TPI" to "Top1".
Page 7, Table 2. Change "triosephosphate isomerase" to "DNA topoisomerase 1".
Page 7, Table 2. Change "TPI" to "Top1".
Page 7, Table 3. Change the two "TPI" to "Top1".
Page 9, column 2, line 55. Change "TPI" to "Top1".

This corrigendum was not part of the Online Earlier publication.