

Cryptic diversity in the Mediterranean *Temnothorax lichtensteini* species complex (Hymenoptera:Formicidae)

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Received: 6 May 2013 / Accepted: 9 August 2013 / Published online: 4 September 2013
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Abstract In this paper we provide insight into the cryptic diversity and biogeographic patterns of a widely distributed Mediterranean ant species, *Temnothorax lichtensteini* (Bondroit *Annales de la Société Entomologique de France* 87:1–174, 1918), based on evidence from multiple data sources. An exploratory analysis of morphometric data, combined with sequencing of a 652 bp fragment of the mitochondrial gene for the cytochrome *c* oxidase subunit I (CO I), indicates the existence of three distinct lineages. Divergence of two recognised genetic lineages, *Western* and *Eastern Mediterranean* clusters, is not reliably supported by confirmatory analysis of morphological data. We hypothesise that this reflects incomplete speciation in separate glacial refugia and therefore discuss only biogeographic aspects of these two parapatric populations. However, the third, Peloponnese, lineage is divergent in both morphology and CO I sequences and its separation from the other two lineages is convincingly confirmed. For this lineage we assume a completed speciation and describe the taxon formally as *Temnothorax laconicus* sp.n., the sister species of *T. lichtensteini*.

Keywords Taxonomy · Cryptic species · Integrative taxonomy · Biogeography · Morphometrics · Exploratory data analysis

Introduction

Over the past decade, sophisticated morphometric analyses (Seifert 2006, 2011, 2012; Csósz and Schulz 2010), geometric morphometrics (Bagherian et al. 2012), and combinations of various data sources (Seifert and Goropashnaya 2004; Schlick-Steiner et al. 2010; Steiner et al. 2010; Seppä et al. 2011; Ward and Sumnicht 2012) have disclosed the formerly unexpected cryptic diversity of many European ants. In fact, many widely distributed taxa consist of several morphologically similar species (Schlick-Steiner et al. 2006a, b).

The Mediterranean ant *Temnothorax lichtensteini* (Bondroit 1918) is distributed widely in xerothermous open forests across the northern coastline of the Mediterranean region from the Iberian Peninsula to Western Turkey, and from 47°N in Austria to 37°N in Greece. Despite its unusually wide geographic distribution, cryptic diversity and the possible existence of several distinct lineages has never been the subject of focussed research.

Temnothorax species are considered quite challenging for taxonomists. The frequently overlapping characters and hybridisation (Seifert 1984, 1999; Douwes and Stille 1991; Pusch et al. 2006; Steiner et al. 2011) often hamper a safe separation of species, and boundaries of distinct morphological lineages cannot always be recognised reliably by conventional morphological approaches (i.e. recognising morphological entities based on researcher's subjective a priori grouping).

Therefore, in the present study we assessed the cryptic diversity within the *T. lichtensteini* species-complex and geographic patterns of recognised morphological entities using exploratory

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data analyses (EDA, see Tukey 1977). This approach allows the boundaries of morphological entities to be inferred without having a prior hypothesis on either the number of clusters or on classification of a particular sample. The performance of various EDA tools was tested by Seifert et al. (2013) and found to allow the formation of sound hypotheses on formerly unexpected morphologically cryptic entities in ants. They proposed a new clustering technique called NC-clustering. In order to improve the reliability of prior hypotheses, we here apply coalescent runs of NC-clustering to infer boundaries of morphological lineages and test a priori grouping with confirmatory linear discriminant analysis (LDA). To strengthen the consistency of conclusions by the complex morphometric analyses, we also analysed a fragment of the mitochondrial gene for cytochrome *c* oxidase subunit I (CO I) for all morphological clusters considered.

Our study represents one of the first applications of NC-clustering in morphology-based ant taxonomy to study a complex of cryptic species.

Materials and methods

The general appearance of species in the *T. lichtensteini* complex is quite uniform. Workers of this complex can be distinguished from other *Temnothorax* species by the combination of the following salient features: yellowish to light brown mesosomal colour with the head being concolorous or slightly darker; distinct mesopropodeal depression; long to very long propodeal spines [SPST/CS 0.358 (0.294, 0.450)]; petiolar node rather high, in profile with short truncate to slightly convex dorsum, its slightly concave frontal face forms a rounded transition or rounded corner with the dorsal face that slopes down to caudal cylinder without a distinct step. Head oval, much longer than broad [CL/CWb 1.226 (1.147, 1.297)]; frontal carinae parallel to subparallel; dorsum of head longitudinally carinate to rugulose, the transversal component of head sculpture is weaker and reduced to anastomoses between the carinae; sculpture of mesosoma more rugulose, the interspaces between rugulae coriaceous and dull.

In the present study, 22 continuous morphometric traits were recorded in 359 worker individuals belonging to 101 nest samples from 70 localities of nine countries across the Mediterranean region, including the type series of *T. lichtensteini*. For the full list of material investigated see Table 1. Images from the lectotype specimen can be identified uniquely with a specimen-level code affixed to each pin (CASENT0906682). All images presented are available online and can be seen on AntWeb (<http://www.antweb.org>).

Abbreviation of depositories

CAS San Francisco California Academy of Sciences,
San Francisco, CA

HNHM Budapest Hungarian Natural History Museum,
Budapest / Hungary
SMN Görlitz Senckenberg Museum für Naturkunde
Görlitz, Germany

Mitochondrial DNA

We isolated DNA from workers of colonies of *Temnothorax lichtensteini* from France, Croatia, and Greece and several other species of *Temnothorax* using the CTAB method including digestion with proteinase K (Sambrook and Russell 2001) and amplified a 658 bp fragment of the CO I gene using the primers LCO1490 and HCO2198 (Folmer et al. 1994). Other workers from the same colonies were investigated by morphometry (see below).

PCR was carried out with GO-Taq Hot Start Master Mix from Promega (Madison, WI). The final primer concentration was 0.7 μ M and 1 μ l DNA was added. PCR conditions consisted of an initial denaturation at 94 °C for 240 s, 38 cycles of denaturation at 94 °C, 45 s; annealing at 50 °C, 45 s; elongation at 72 °C, 1 min; and a final step of 72 °C, 300 s. For sequencing we used an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA).

Sequences were aligned using Bioedit (Hall 1999) and ambiguous stretches at the 5' and 3' ends of the fragments were removed, resulting in fragments of 652 bp for further analysis.

The models underlying the Bayesian analysis of the data were estimated using MrModeltest GUI (available under <http://genedrift.org>) in conjunction with PAUP 4.0b10 (Swofford 1998), using a sequence from *T. cf. korbi* from Nur Dağları, Turkey, as an outgroup. The analysis was performed with MrBayes 3.2.1 (Ronquist et al. 2012) using HKY + I + G as a model and with the default number of four Markov chains (three heated, one cold) and the default heating parameter set at 0.2 with an MCMC length of 3,000,000 generations. The first 500 generations (burn-in) were discarded and every 100th generation was sampled. The phylogenetic tree was drawn using FigTree version 1.2.2 (available at <http://tree.bio.ed.ac.uk/software>). GenBank accession numbers are given in Table 2.

We checked the most divergent sequence (PELOGRE:Profitis-Ilias-20110501-336) visually for inversions, deletions, or stop codons to exclude that it represents a pseudogene. Its amino acid sequence showed 95 % homology to published sequences of CO I from ants in GenBank.

Protocol of morphometric character recording

All measurements were made in micrometres using a pin-holding stage, permitting rotations around the *x*, *y*, and *z* axes. An Olympus SZX9 stereomicroscope was used at a magnification

Table 1 List of samples investigated by numeric character analysis. Sampling date is given in alpha-numeric format followed after a hyphen by the field sample number (e.g. 19980623–093). Geographic coordinates latitude (LAT) and longitude (LON) are provided in decimal format. ALT Altitude in m a.s.l. Results of various exploratory data analyses (EDA) methods are listed by algorithms for each nest series. Results of confirmatory linear discriminant analysis (LDA) with geometric mean of posterior probabilities for nest samples of “East Mediterranean cluster” (EAST), “West Mediterranean cluster” (WEST) of *T. lichtensteini* and *T. laconicus* sp. n. (PELO) are given

Site	n	LAT	LON	ALT	NMDS kmeans. cluster	kmeans.LDAnest. cluster	NC-ward	LDA-input	LDA-result	EAST	WEST	PELO
Austria	2	46.6452	14.9508	350	E	E	E	E	EAST	0.999	0.001	0.000
Austria	2	46.6452	14.9508	350	E	E	E	E	EAST	0.991	0.009	0.000
Bulgaria	3	43.0630	23.688	400	W	E	E	wildcard	EAST	0.993	0.007	0.000
Bulgaria	5	43.0956	23.5770	300	W	E	E	wildcard	EAST	0.976	0.024	0.000
Bulgaria	5	43.1683	24.0606	150	E	E	E	E	EAST	0.974	0.023	0.002
Croatia	3	43.2722	16.7061	100	E	E	E	E	EAST	0.994	0.006	0.000
Croatia	3	43.2833	16.6167	600–750	E	E	E	E	EAST	0.946	0.054	0.000
Croatia	3	43.3169	16.7031	400	W	W	W	W	WEST	0.153	0.847	0.000
Croatia	2	43.2833	16.6167	600–750	W	W	W	W	EAST	0.909	0.091	0.000
Croatia	5	45.0194	14.6878	50–200	E	E	E	E	EAST	0.979	0.021	0.000
Croatia	5	45.0515	14.5669	50–200	E	E	E	E	EAST	0.994	0.005	0.000
Croatia	3	44.9919	14.7191	50–200	E	E	E	E	EAST	0.998	0.002	0.000
Croatia	5	44.8271	14.9156	500–600	E	E	E	E	EAST	0.998	0.002	0.000
Croatia	5	45.2584	14.8002	1,300	E	E	E	E	EAST	0.987	0.013	0.000
Croatia	6	45.1906	14.8109	600–800	E	E	E	E	EAST	0.996	0.004	0.000
France	3	43.9635	3.8764	300	W	W	W	W	WEST	0.011	0.989	0.000
France	3	45.846	5.7924	250	W	W	W	W	WEST	0.010	0.990	0.000
France	3	43.9635	3.8764	300	W	W	W	W	WEST	0.000	1.000	0.000
France	3	42.3060	9.1654	400	W	W	W	W	WEST	0.005	0.995	0.000
France	2	43.5290	5.5857	700–1,000	W	W	W	W	WEST	0.059	0.941	0.000
France	3	43.7866	5.3517	500	W	W	W	W	WEST	0.018	0.982	0.000
France	3	43.7866	5.3517	500	W	W	W	W	WEST	0.001	0.999	0.000
France	3	41.6283	9.06641	400	W	W	W	W	WEST	0.011	0.989	0.000
France	3	42.8076	9.3708	350	W	W	W	W	WEST	0.184	0.805	0.011
France	3	43.7909	3.727	200	W	W	W	W	WEST	0.000	1.000	0.000
France	3	43.7909	3.727	200	W	W	W	W	WEST	0.006	0.994	0.000
France	2	43.8543	4.9734	200	W	W	W	W	WEST	0.051	0.949	0.000
France	2	43.768	6.3075	1,000	W	W	W	W	WEST	0.080	0.920	0.000
France	3	43.7922	6.1575	500	W	W	W	W	WEST	0.094	0.906	0.000
France	3	43.7922	6.1575	500	W	W	W	W	WEST	0.024	0.976	0.000
France	3	43.7922	6.1575	500	E	E	W	wildcard	WEST	0.322	0.676	0.002
France	2	43.4345	6.1345	200	W	W	W	W	WEST	0.032	0.968	0.000

Table 1 (continued)

Site	n	LAT	LON	ALT	NMDS.kmeans. cluster	kmeans.LDAnest. cluster	NC-ward	LDA-input	LDA-result	EAST	WEST	PELO
France	3	42.6142	2.3332	450	W	W	W	W	WEST	0.402	0.598	0.000
France	3	43.5265	5.6209	600	W	W	W	W	WEST	0.027	0.973	0.000
France	3	44.0801	5.2805	700	W	W	E	wildcard	EAST	0.785	0.215	0.000
France	3	44.080	5.2805	700	W	W	W	W	WEST	0.077	0.923	0.000
France	3	42.484	3.0842	100	W	W	W	W	WEST	0.070	0.930	0.000
France	3	44.500	5.113	400	W	W	W	W	WEST	0.034	0.966	0.000
France	3	43.4365	6.8657	20	W	W	W	W	WEST	0.003	0.997	0.000
France	3	43.6028	7.0078	200	W	W	W	W	WEST	0.023	0.977	0.000
France	3	43.185	2.994	50	W	W	W	W	WEST	0.031	0.969	0.000
France	2	44.1828	5.166	400	W	W	W	W	WEST	0.018	0.981	0.000
Greece	3	36.968	22.404	800	P	P	P	P	PELO	0.000	0.000	1.000
Greece	6	39.3125	21.078	400	W	W	W	W	EAST?	0.978	0.021	0.001
Greece	2	37.6833	22.4333	800	P	P	P	P	PELO	0.004	0.001	0.995
Greece	5	40.5191	22.0851	800-900	W	E	W	wildcard	EAST	0.930	0.070	0.000
Greece	7	37.0715	22.2699	1,100-1,400	P	P	P	P	PELO	0.000	0.000	1.000
Greece	3	36.955	22.358	1,800-2,000	P	P	P	P	PELO	0.000	0.000	1.000
Greece	6	36.9682	22.4045	800	P	P	P	P	PELO	0.004	0.001	0.995
Greece	6	36.9682	22.4045	800	P	P	P	P	PELO	0.000	0.000	1.000
Greece	2	36.9682	22.4045	800	P	P	P	P	PELO	0.005	0.013	0.982
Greece	3	37.6565	22.1491	1,140	P	P	P	P	PELO	0.000	0.000	1.000
Greece	4	37.9982	22.4703	1,050	P	P	P	P	PELO	0.002	0.000	0.998
Greece	3	40.1933	22.3296	700-900	E	E	W	E	EAST	0.954	0.046	0.000
Greece	5	40.1077	22.4741	500-700	E	E	E	E	EAST	0.992	0.008	0.000
Greece	5	40.1077	22.4741	500-700	E	E	W	wildcard	EAST	0.971	0.029	0.000
Greece	3	36.968	22.404	800	P	P	P	P	PELO	0.000	0.000	1.000
Greece	3	36.984	22.262	700-800	P	P	P	P	PELO	0.000	0.000	1.000
Greece	3	39.3967	23.0619	1,000-1,400	E	E	E	E	EAST	0.986	0.013	0.000
Greece	3	39.3967	23.0619	1,000-1,400	E	E	W	wildcard	EAST	0.992	0.006	0.002
Greece	3	39.3967	23.0619	1,000-1,400	W	W	W	W	WEST	0.108	0.892	0.000
Greece	5	39.1356	20.6748	300	E	W	W	wildcard	EAST	0.820	0.180	0.000
Greece	5	40.1075	20.7638	500-600	W	E	W	wildcard	EAST	0.932	0.033	0.035
Greece	5	40.1106	22.5521	1,650	E	E	E	E	EAST	0.993	0.005	0.002
Greece	5	40.1330	23.7676	550	W	W	W	W	EAST	0.901	0.099	0.000
Greece	2	40.03	23.21	280	E	E	W	wildcard	EAST	0.976	0.024	0.000
Greece	5	37.7001	20.800	30	P	P	P	P	PELO	0.010	0.009	0.981

Table 1 (continued)

Site	n	LAT	LON	ALT	NMDS.kmeans. cluster	kmeans.LDAnest. cluster	NC-ward	LDA-input	LDA-result	EAST	WEST	PELO
Greece	5	39.7	19.700	200	P	P	P	P	PELO	0.004	0.000	0.996
Greece	3	39.7	19.700	200	P	P	P	P	PELO	0.055	0.000	0.944
Greece	3	36.968	22.404	800	P	P	P	P	PELO	0.000	0.000	1.000
Greece	3	36.968	22.404	800	P	P	P	P	PELO	0.000	0.000	1.000
Greece	3	36.984	22.262	700–800	P	P	P	P	PELO	0.000	0.000	1.000
Italy	3	46.6981	11.1217	800	E	W	E	wildcard	WEST	0.102	0.898	0.000
Italy	5	46.6981	11.1217	800	E	E	E	E	EAST	0.998	0.002	0.000
Italy	6	46.2675	11.0987	500	E	E	E	E	EAST	0.996	0.004	0.000
Italy	3	40.7999	15.0234	700	W	W	W	W	WEST	0.001	0.999	0.000
Italy	3	38.0476	15.8958	1,100	W	W	W	W	WEST	0.029	0.971	0.000
Italy	3	38.053	15.9273	750	W	W	W	W	EAST	0.956	0.044	0.000
Italy	4	38.9721	16.2573	200–300	E	E	W	wildcard	EAST	0.917	0.083	0.000
Italy	3	38.9721	16.2573	200–300	W	W	W	W	WEST	0.470	0.529	0.000
Italy	6	38.0476	15.8958	1,100	W	W	W	W	WEST	0.021	0.979	0.000
Italy	3	46.2675	11.0987	500	E	E	E	E	EAST	0.983	0.015	0.002
Italy	3	37.6704	14.9393	1,000–1,200	E	E	W	wildcard	EAST	0.849	0.148	0.003
Italy	5	44.5370	8.8476	500	W	W	W	W	WEST	0.002	0.997	0.001
Italy	5	44.5370	8.8476	500	W	W	W	W	WEST	0.087	0.903	0.010
Italy	3	39.965	15.813	200	W	W	W	W	EAST	0.926	0.074	0.000
Slovenia	5	45.5370	13.660455	10	E	E	E	E	EAST	0.947	0.053	0.000
Slovenia	5	45.5558	13.819227	200	E	E	E	E	EAST	0.973	0.027	0.000
Slovenia	6	45.5113	13.900831	200	E	E	E	E	EAST	0.999	0.001	0.000
Slovenia	5	45.5398	15.203569	200	E	E	E	E	EAST	0.999	0.001	0.000
Spain	3	42.2028	1.0965	1,000	W	W	W	W	WEST	0.003	0.997	0.000
Spain	3	42.2028	1.0965	1,000	W	W	W	W	WEST	0.018	0.982	0.000
Spain	3	42.2028	1.0965	1,000	W	W	W	W	WEST	0.010	0.990	0.000
Spain	2	42.3384	1.0670	600	W	W	W	W	WEST	0.114	0.886	0.000
Spain	3	42.3384	1.0670	600	W	W	W	W	WEST	0.018	0.967	0.015
Spain	3	42.3384	1.0670	600	W	W	W	W	WEST	0.007	0.993	0.000
Spain	3	42.2652	2.3805	700	W	W	W	W	WEST	0.006	0.994	0.000
Turkey	3	39.122	30.080	1,120	E	E	E	E	EAST	1.000	0.000	0.000
Turkey	3	39.556	29.588	210	E	E	E	E	EAST	0.999	0.001	0.000
Turkey	3	41.401	27.259	206	E	E	E	E	EAST	0.995	0.005	0.000
France	2	43.610	3.885		W	W	W	wildcard	WEST	0.077	0.923	0.000

Table 2 List of samples from colonies of which separate workers were studied both by morphology and sequencing of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) with accession numbers for gene sequences

Site	Species name	COI cluster	GenBank accession number
Croatia Bol-3 km E-20100714-208	<i>lichtensteini</i>	EAST	KF593820
Croatia Vidova Gora-20100713-237	<i>lichtensteini</i>	EAST	KF593818
Croatia Praznica-20100711-165	<i>lichtensteini</i>	EAST	KF593821
Croatia Vidova Gora-20100713-232	<i>lichtensteini</i>	EAST	KF593819
France Aix-En-Provence-10 km E-20090313-526	<i>lichtensteini</i>	WEST	KF593823
France Apt-Luberon-Mt-10 km S-20090314-559	<i>lichtensteini</i>	WEST	KF593824
France Apt-Luberon-Mt-10 km S-20090314-572	<i>lichtensteini</i>	WEST	KF593822
Greece Profitis-Ilias-20110501-342	<i>laconicus</i>	PELO	KF593825
Greece Profitis-Ilias-20110501-345	<i>laconicus</i>	PELO	KF593816
Greece Pigadia-Canyon-20110501-356	<i>laconicus</i>	PELO	KF593814
Greece Pigadia-Canyon-20110501-358	<i>laconicus</i>	PELO	KF593817
Greece Profitis-Ilias-20110501-336	<i>laconicus</i>	OUTLIER	KF593815
Turkey Hassa-10 km NW-20111108-555	<i>cf. korbi</i>	OUTGROUP	KF593813

of x150 for each character, allowing a precision of $\pm 2 \mu\text{m}$. All measurements were made by the S.C. Due to the lack of sufficient males and female sexuals, morphometric investigation was restricted to workers only. Explanation and abbreviations for measured characters are as follows:

CL	Length of head, measured in a straight line from the anterior-most point of median clypeal margin to the mid-point of the posterior margin of the head.	
CWb	Maximum width of head capsule, measured just posterior of the compound eyes.	
EL	Maximum diameter of the compound eye.	
PoOc	Post ocular distance. Measured from the reference line fitted on the posterior margin of compound eyes to median posterior margin of the head.	
FRS	Distance of the frontal carinae immediately caudal of the posterior intersection points between frontal carinae and the lamellae dorsal of the torulus. If these dorsal lamellae do not laterally surpass the frontal carinae, the deepest point of scape corner pits may be taken as reference line. These pits take up the inner corner of scape base when the scape is fully switched caudad and produce a dark triangular shadow in the lateral frontal lobes immediately posterior of the dorsal lamellae of scape joint capsule.	
SL	Maximum length of the scape excluding the neck of articulatory condyle.	
ML	Diagonal length of mesosoma measured in lateral view from the anterior-most point of the pronotal slope to the posterior (or postero-ventral) margin of the propodeal lobes.	
MW	Maximum width of the pronotum from above.	
PEH	Maximum petiole height measured perpendicular to a reference line, which is, in <i>Leptothorax</i> and <i>Temnothorax</i> , the chord spanning between caudal corner of ventral petiole profile and the caudal end of the subpetiolar process.	
		PL
		NODL
		NOH
		NOL
		PEW
		PPH
		PPW
		SPST
		SPL

corner of ventral petiole profile and the caudal end of the subpetiolar process.

Petiole length measured in dorsal view distance between the dorsalmost point of caudal petiolar margin and the dorsalmost point of anterior petiolar peduncle at the transversal level of its strongest constriction. Both points have to be positioned in the same horizontal plane (focal level).

Measured in dorsal view from the anteriormost hair pit on the petiolar node to articulation condyle with propodeum.

Maximum height of the petiolar node, measured from the uppermost point of the petiolar node perpendicular to a reference line set from the petiolar spiracle to the dorso-caudal corner of caudal cylinder of the petiole.

Length of the petiolar node, measured from petiolar spiracle to dorso-caudal corner of caudal cylinder. (Note: do not erroneously take as the reference point the dorso-caudal corner of the helcium, which is sometimes visible).

Maximum width of petiole.

Maximum height of the postpetiole in lateral view measured perpendicularly to a line defined by the linear section of the segment border between dorsal and ventral petiolar sclerite.

Maximum width of the postpetiole in dorsal view. Distance between the centre of the propodeal stigma and spine tip. The stigma centre refers to the midpoint defined by the outer cuticular ring but not to the centre of stigma opening, which may be positioned eccentrically.

Minimum distance between the center of propodeal spiracle and the propodeal declivity.

PPL	Maximum length of the postpetiole in lateral view.
SPBA	Smallest distance of the lateral margins of the spines at their base. This should be measured in dorso-frontal view, since the wider parts of the ventral propodeum do not interfere with the measurement in this position. If the lateral margins of spines diverge continuously from the tip to the base, a smallest distance at base is not defined. In this case SPBA is measured at the level of the bottom of the interspinal meniscus.
SPTI	Distance of spine tips in dorsal view if spine tips are rounded or thick take the centres of spine tips as reference points.
SPWI	Maximum distance between outer margins of spines measured in same position as SPBA.

Exploratory analyses of morphometric data

Cryptic diversity within the *T. lichtensteini* species-complex was assessed using EDA of continuous morphometric traits. Recently, a new variant of EDA called “Nest Centroid Clustering” (NC clustering) was introduced (Seifert et al. 2013). The characteristic feature of NC clustering is an unconventional application of the preparative LDA in which no species hypothesis is imposed. Instead, each nest sample, composed of individual ant workers, is treated as a separate class. This creates a multidimensional distance matrix between group centroids of nest samples as input data for the clustering methods. In this partition, conclusions on morphological patterns was drawn from the integrated output of three types of EDA algorithms: NC-Ward clustering (an hierarchical, agglomerative nesting method), NC-K-Means clustering (a non-hierarchical agglomerative method) and NC-NMDS-K-Means clustering (a combination of an ordination method with K-Means). For methodological details, we refer to Seifert et al. (2013). Scripts of these methods are written in R and are freely available at: <http://sourceforge.net/projects/agnesclustering/>.

Deriving hypotheses from NC-clustering and checking with confirmative LDA

For the purpose of increased reliability of pattern recognition, parallel runs of NC-Ward, NC-NMDS-K-Means and NC-K-Means clustering were applied. In a confirmative LDA, run on the worker individual level, classification hypotheses were imposed for all samples congruently classified by all three methods while wild-card settings (i.e. no hypothesis imposed) were given to samples whose classification differed between the methods. The confirmative LDA was run as an iterative process and sample means of posterior probabilities formed the basis for the finally accepted classification. There remained no undecided samples (for details see Seifert et al. 2013).

Results

Mitochondrial DNA

DNA sequences were available from only a few representative populations and the interpretation of the phylogenetic tree depicted in Fig. 1 therefore remains provisional. The tree shows three branches, which form an unresolved polyphyly. One well-supported branch contains specimens from various places on the island of Brač, Croatia—the *East Mediterranean cluster*; the second well-supported branch consists of samples from Southern France—the *West Mediterranean cluster*; and the third, more variable lineage comprises specimens from Taygetos Oros in Greece—the *Peloponnese cluster*. One sample from Taygetos Oros, belonging morphologically to the *Peloponnese cluster*, forms the outgroup to all other studied material of *T. lichtensteini*. As there is no evidence of this sequence representing a numt, we interpret the position of this sequence in the phylogeny as caused by incomplete lineage sorting or ancient hybridisation events.

Multivariate analyses of numeric morphology—exploratory data analyses for hypothesis formation

Each EDA algorithm reveals the existence of three clusters within populations of *lichtensteini*: the *East Mediterranean cluster* (Austria, Bulgaria, Croatia, Greece, N-Italy, Turkey); *West Mediterranean cluster* (France, Italy, and Spain) in which the *lichtensteini* type series is nested; and a *Peloponnese cluster* that seems endemic to Peloponnese peninsula (Fig. 2). The three NC-clustering methods arrived at the same grouping hypotheses over the samples of the *Peloponnese cluster*. Accordingly, 84 samples belong to the *lichtensteini* cluster (Eastern and Western clusters combined) and 17 formed the *Peloponnese cluster* (Table 1). While there was no discrepant placement between the *Peloponnese cluster* and the others, a few discrepant placements appeared between samples of *East Mediterranean cluster* and *West Mediterranean cluster*; alternative classification was predicted by one of the three NC-clustering algorithms for 11 (13 %) of the total 84 *lichtensteini* samples (Table 1). These mismatching samples, plus the type series of *T. lichtensteini*, were involved in the confirmatory analysis as wildcards.

Confirmatory data analyses

The LDA confirms the complete separation of the *Peloponnese cluster* from the rest using 22 morphometric characters (Fig. 3) at nest sample mean level. Individual level separation of *East Mediterranean* and *West Mediterranean* clusters of *T. lichtensteini* is not sufficient (94.6 %), but the *Peloponnese cluster* is convincingly (96.8 %) separated from workers of the former clusters using the entire character set. Distance between

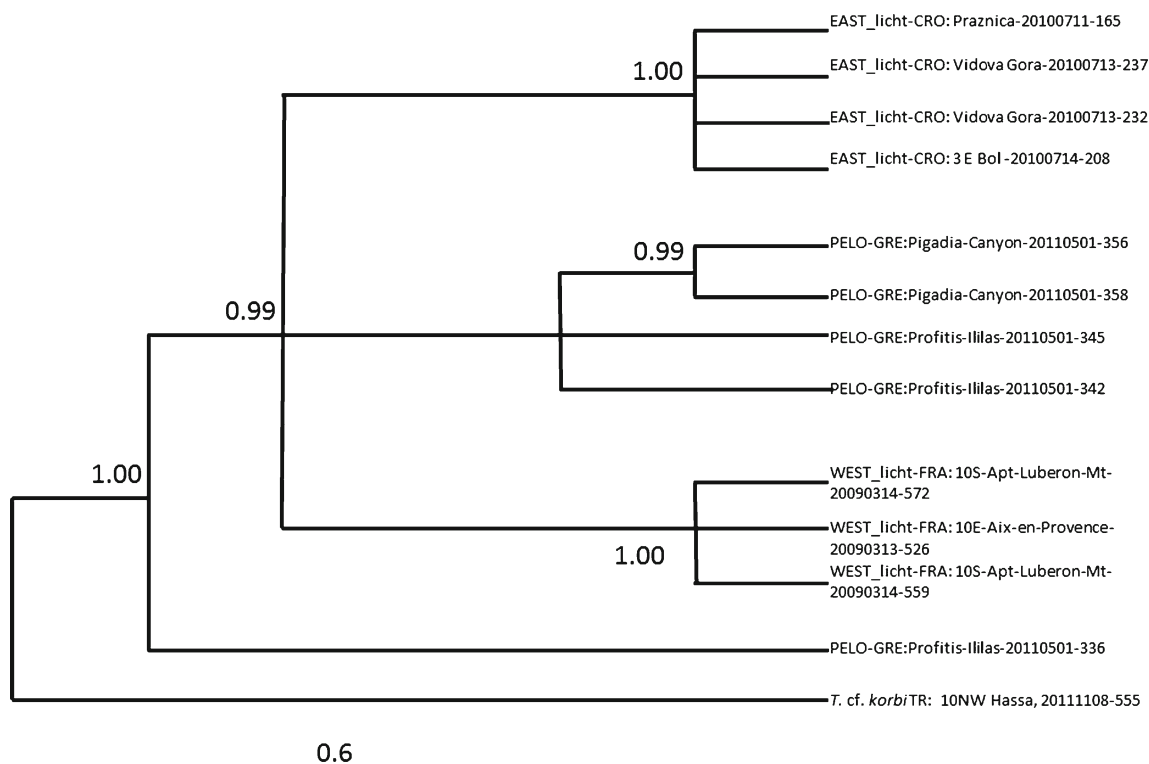


Fig. 1 Bayesian tree based on a 652 bp fragment of the mitochondrial gene for cytochrome *c* oxidase subunit I (CO I) of samples of the *Temnothorax lichtensteini* complex and *T. cf. korbi* as outgroup.

Bayesian posterior probabilities of 0.95 and above are given at the nodes. Nestmate workers from all studied colonies were also investigated by morphometry

groups was calculated with squared Mahalanobis distance (MD): “*Peloponnese cluster*” is the most distantly related of all (MD=21.20, $F_{22,335}=38.806$, $P<0.001$), while “*East Mediterranean cluster*” and “*West Mediterranean cluster*” are closely related to each other by means of morphology (MD=8.18, $F_{22,335}=25.413$, $P<0.001$).

A reduction to 15 characters in LDA yielded 95 % classification success between “*East Mediterranean cluster*” and “*West Mediterranean cluster*”, but further character reduction decreased the classification success.

Description of worker caste of *Temnothorax laconicus* sp.n.

Types

Holotype worker Taygethos Oros, Street to Profitis Ilias (GRE:S_342), N36.968, E22.404, 800 mH, 01.05.2011, leg: A. Schulz, (1♂ / HNHM Budapest);

Paratypes Taygethos Oros, Street to Profitis Ilias (GRE:S_342), N36.968, E22.404, 800 mH, 01.05.2011, leg: A. Schulz, (2♂♂ / CAS San Francisco [unique specimen identifier CASENT0906682], 3♂♂ / HNHM Budapest, 3♂♂ / SMNG); W Taygethos Oros, Pigadia Canyon (GRE:S_358),

N36.984, E22.262, 700–800 mH, 01.05.2011, leg: A. Schulz, (2♂♂ / HNHM Budapest); Taygethos Oros, Street to Profitis Ilias (GRE:2011:0345), N36.968, E22.404, 800 mH, 01.05.2011, leg: A. Schulz, (4♂♂ / HNHM Budapest); W Taygethos Oros, Pigadia Canyon (GRE:2011:0356), N36.984, E22.262, 700–800 mH, 01.05.2011, leg: A. Schulz, (3♂♂ / HNHM Budapest); Taygethos Oros, Street to Profitis Ilias (GRE:2011:0336), N36.968, E22.404, 800 mH, 01.05.2011, leg: A. Schulz, (4♂♂ / HNHM Budapest);

For the full list of material investigated see Table 1.

Etymology This adjective [*laconicus* (masculinum)] refers to Laconia, the region of the Peloponnese peninsula in which the type locality is situated.

Workers (Figs. 5–7)

General colouration of this species somewhat darker than in *T. lichtensteini*; appendages, mesosoma, waist and basis of first gaster tergite dirty yellowish-brown to light-brown. Head dorsum and the posterior surfaces of gaster tergites darker, usually light-brown to brown. Small species (CS 545 μm), but similar in size to its sister species. Head significantly longer than broad (CL/CWb 1.226) and with feebly convex sides.

Fig. 2 Dendrogram of NC-Ward clustering of three cryptic lineages *T. lichtensteini* with two subsets (“*East Mediterranean cluster*” and “*West Mediterranean cluster*”) and *T. laconicus* sp.n. (“*Peloponnese cluster*”). Sequence of information in the string designating the samples: abbreviation of geographic cluster hypothesis inferred from the three exploratory data analyses (EDA methods)—country—locality—date—sample number. Asterisk Position of the *T. lichtensteini* type series

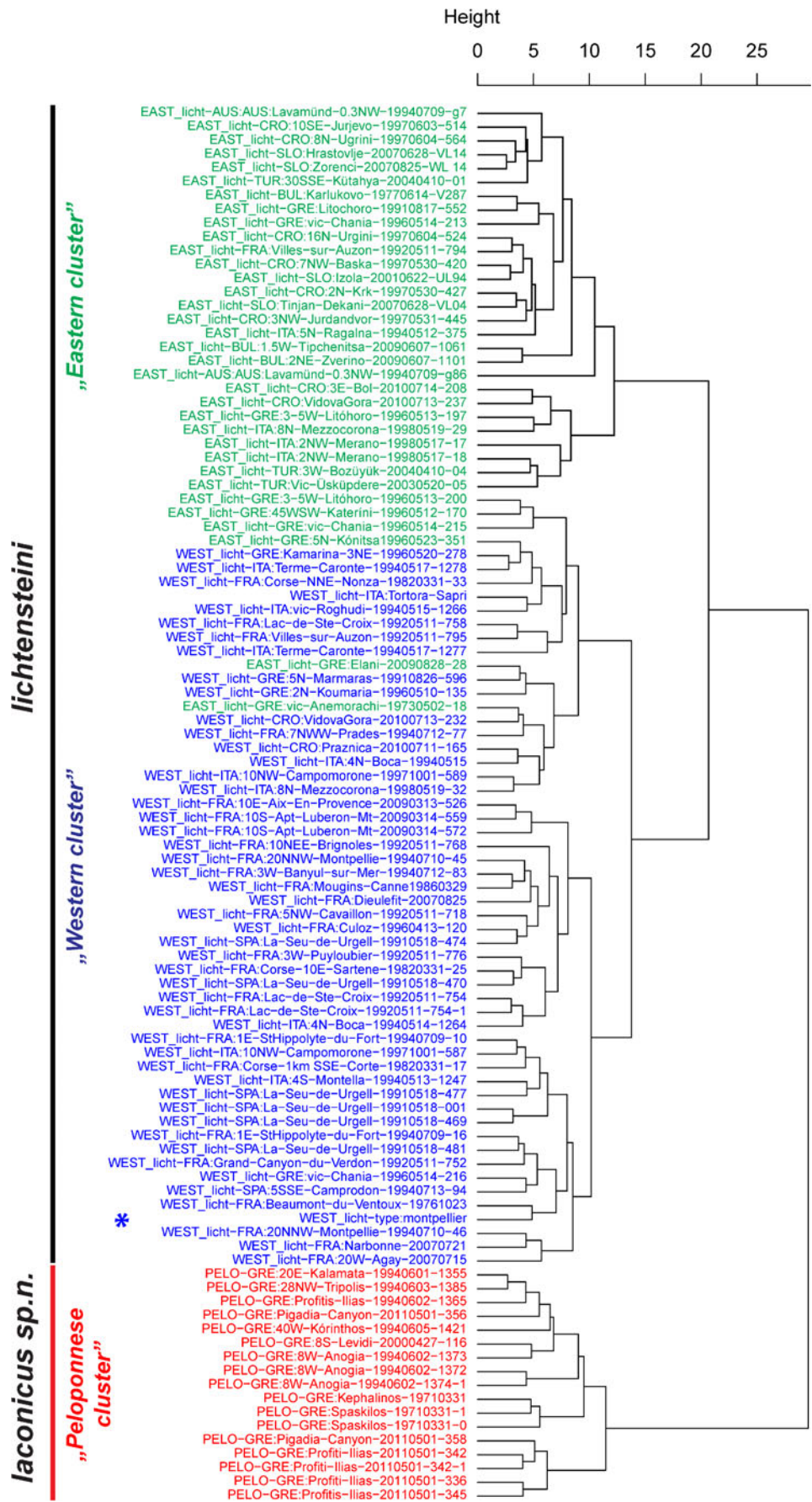
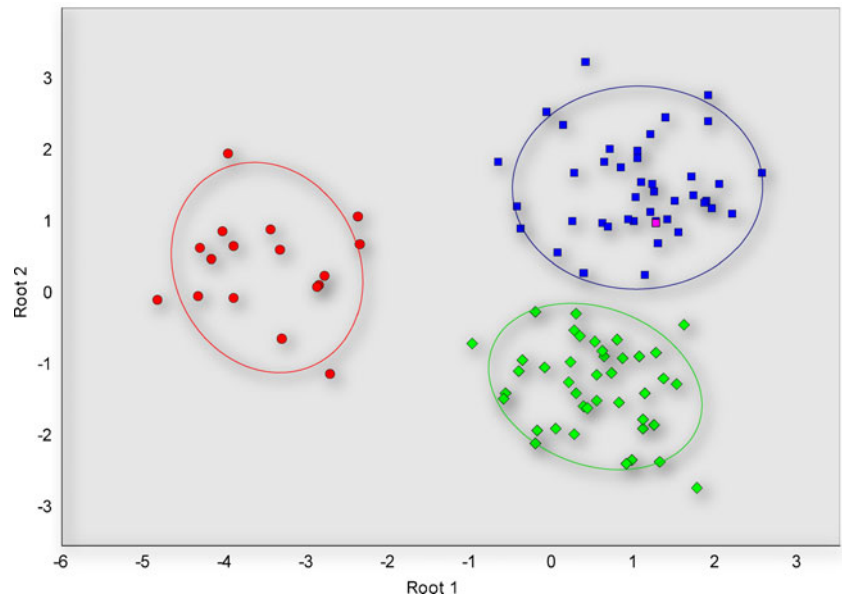


Fig. 3 Scatterplot of discriminant scores for *T. laconicus* sp.n. (red circles), *T. lichtensteini* “East Mediterranean cluster” (green diamonds), *T. lichtensteini* “West Mediterranean cluster” (blue rectangles) and *T. lichtensteini* type material (purple rectangle) is illustrated. Ellipses of 95 % range are given for each group



Vertexal corners rounded, posterior margin of vertex strait. Scape long (SL/CS 0.788) with variable pubescence: adpressed (0–5°) to decumbent (10–15°) on the surface that is adjacent to head surface when the scape is directed fully caudad and subdecumbent (30°) to suberect (35–45°) on the surface that becomes external in the same scape position. Clypeus between sagittal level of frontal carinae with a number of longitudinal carinulae, interstices smooth and shiny. Eyes rather large (EL/CS 0.246) and more approached to hind margin of vertex (PoOc/CL 0.396). Vertex with fine microreticulate sculpture

that is superimposed by a number of longitudinal rugulae. Frontal carinae non-divergent. Frontal lobes widely distant (FRS/CS 0.344). Propodeal spines very long, longer than in *T. lichtensteini* (SPST/CS 0.409) and acute, in lateral view deviating from longitudinal axis of mesosoma by 20–25°. Propodeal spines in dorsal view much more divergent than in related species (SPWI/CS 0.434), spine-tips curving inward (SPTI/CS 0.410). Mesosoma as wide as in related species (MW/CS 0.608). Metanotal depression well visible, moderately deep. Mesosoma with microreticulate sculpture that is

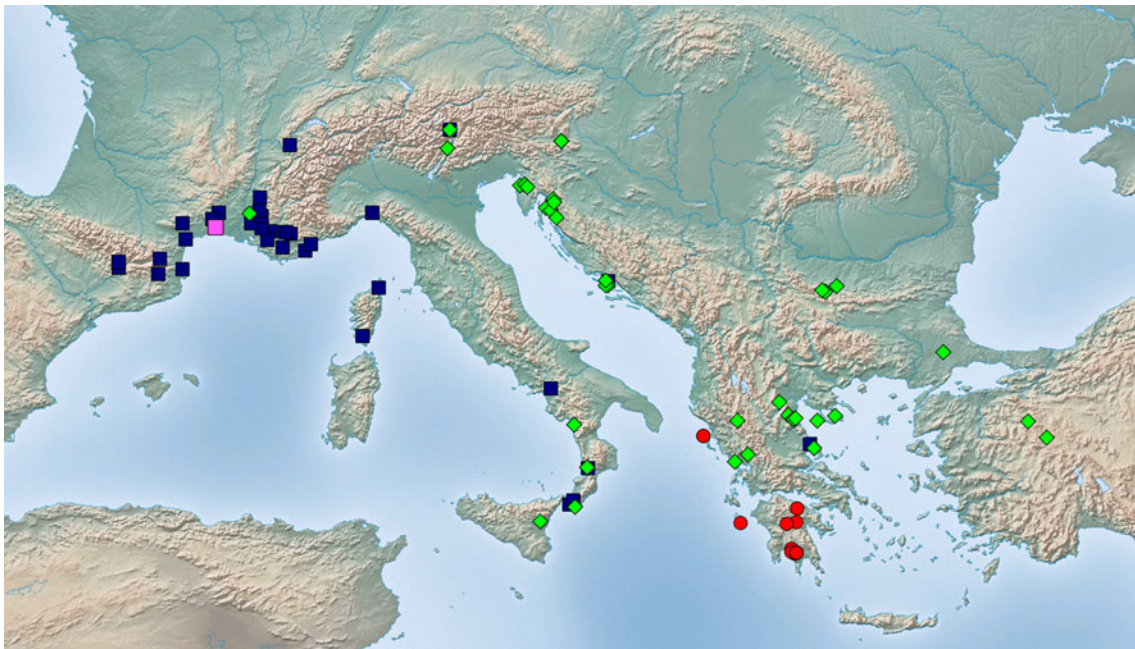


Fig. 4 Sampling sites of *Temnothorax lichtensteini* species-complex. Separate lineages are illustrated as follows: *T. laconicus* sp.n. (red circles), *T. lichtensteini* “East Mediterranean cluster” (green diamonds),

T. lichtensteini “West Mediterranean cluster” (blue rectangles) and *T. lichtensteini* type material (purple rectangle)



Fig. 5 Head of *Temnothorax laconicus* sp.n. paratype worker (Profitis-Ilias-20110501-342 / CAS San Francisco / CASENT0906682) in dorsal view

superimposed by a few irregular or longitudinal rugulae. Petiole and postpetiole covered by a fine reticulate microsculpture that can occasionally be superimposed by a few irregular rugulae in particular on dorsum of petiolar node. Petiole in profile with a high node and a rather straight or slightly concave anterior face; the top of the node forms a short truncate dorsum that slopes down to caudal cylinder without a distinct step.

For morphometric data of 17 nest sample means see Table 3.

Diagnosis

Pairwise analysis confirms nearly complete separation (99.4 %) between workers of *T. lichtensteini* (“Western” and “Eastern clusters” combined) and *T. laconicus* sp. n. Even drastic character reduction up to three characters ($D3 = -0.0498$ PoOc–0.0541 FRS +0.0975 SPST +3.3108) yields 98.6 % classification success at individual level.



Fig. 6 Lateral view of *Temnothorax laconicus* sp.n. paratype worker (Profitis-Ilias-20110501-342 / CAS San Francisco / CASENT0906682)



Fig. 7 Dorsal view of *Temnothorax laconicus* sp.n. paratype worker (Profitis-Ilias-20110501-342 / CAS San Francisco / CASENT0906682)

$D3$ scores for *T. lichtensteini* ($n = 295$) : -0.6671 , $[-4.0905, +1.9182]$
 $D3$ scores for *T. laconicus* sp.n. ($n = 64$) + 3.0748 $[+0.8556, +4.9404]$

The same function yields complete separation at nest sample mean level.

$D3$ scores for *T. lichtensteini* ($n = 84$) : -0.6762 , $[-2.1468, +0.9519]$
 $D3$ scores for *T. laconicus* sp.n. ($n = 17$) + 3.1357 $[+1.9419, +4.2677]$

Application of a simple ratio (SPST/CS) yields non-overlapping ranges of *T. lichtensteini* and *laconicus* sp.n. at nest sample mean level (see Table 3); therefore, it can provide the most simple opportunity for separation.

Redescription of worker caste of *Temnothorax lichtensteini* (Bondroit 1918)

Types Four workers labeled “Montpellier Jean Lichtenstein”, “Leptoth. lichtensteini Type Bondr. ” and “Lectotype Leptothorax lichtensteini Bondroit 1918 Top specimen det. A.Schulz & M.Verhaagh 1999”; IRSNB Bruxelles; lectotype with CS 546.6. 5 workers labeled “Menton de Dalmas” and “Leptoth. lichtensteini Type Bondr. ”; IRSNB Bruxelles.

For the full list of material investigated see Table 1.

Workers

General colouration of this species is somewhat lighter than that of *T. laconicus* sp.n. ; appendages, mesosoma, waist and basis of first gaster tergite-yellow to dirty yellowish-brown. Head dorsum and the posterior surfaces of gaster tergites darker, usually dirty yellowish-brown to light-brown. Small species (CS 535 μ m), but similar in size to its sister species. Head

Table 3 Morphometric data of nest sample means for *Temnothorax laconicus* sp.n. and *T. lichtensteini* (“*East Mediterranean cluster*” and “*West Mediterranean cluster*” are separated). Mean of indices, \pm SD are provided in the upper row, and minimum and maximum values are given in parentheses

	<i>laconicus</i> sp.n. (<i>n</i> =17)	<i>lichtensteini</i> <i>east</i> (<i>n</i> =41)	<i>lichtensteini</i> <i>west</i> (<i>n</i> =43)
CS	545.8 \pm 23.7 [500.3, 589.8]	536.2 \pm 20.1 [474.3, 579.5]	533.3 \pm 21.9 [487.6, 584.9]
CL/CWb	1.227 \pm 0.014 [1.199, 1.258]	1.223 \pm 0.018 [1.181, 1.257]	1.228 \pm 0.016 [1.193, 1.261]
PoOc/CL	0.396 \pm 0.005 [0.383, 0.403]	0.401 \pm 0.006 [0.386, 0.415]	0.400 \pm 0.007 [0.387, 0.418]
FRS/CS	0.343 \pm 0.008 [0.329, 0.360]	0.362 \pm 0.007 [0.350, 0.380]	0.350 \pm 0.007 [0.336, 0.363]
SL/CS	0.787 \pm 0.010 [0.766, 0.809]	0.786 \pm 0.010 [0.763, 0.805]	0.787 \pm 0.010 [0.768, 0.808]
EL/CS	0.245 \pm 0.011 [0.229, 0.265]	0.248 \pm 0.008 [0.232, 0.268]	0.248 \pm 0.007 [0.232, 0.271]
ML/CS	1.233 \pm 0.019 [1.190, 1.258]	1.226 \pm 0.019 [1.190, 1.272]	1.234 \pm 0.015 [1.197, 1.270]
NodL/CS	0.314 \pm 0.024 [0.276, 0.355]	0.302 \pm 0.014 [0.266, 0.331]	0.292 \pm 0.013 [0.248, 0.316]
PEH/CS	0.380 \pm 0.012 [0.358, 0.396]	0.384 \pm 0.011 [0.363, 0.416]	0.370 \pm 0.008 [0.355, 0.394]
PL/CS	0.435 \pm 0.016 [0.402, 0.465]	0.426 \pm 0.010 [0.409, 0.449]	0.426 \pm 0.009 [0.405, 0.448]
NOH/CS	0.170 \pm 0.006 [0.158, 0.185]	0.169 \pm 0.008 [0.156, 0.191]	0.163 \pm 0.007 [0.149, 0.176]
NOL/CS	0.266 \pm 0.010 [0.247, 0.286]	0.255 \pm 0.008 [0.237, 0.271]	0.264 \pm 0.009 [0.246, 0.291]
PPH/CS	0.341 \pm 0.021 [0.275, 0.368]	0.359 \pm 0.012 [0.331, 0.390]	0.342 \pm 0.008 [0.328, 0.362]
PPL/CS	0.250 \pm 0.008 [0.235, 0.262]	0.257 \pm 0.008 [0.240, 0.277]	0.250 \pm 0.008 [0.237, 0.265]
SPST/CS	0.410 \pm 0.012 [0.391, 0.429]	0.349 \pm 0.013 [0.328, 0.377]	0.343 \pm 0.014 [0.324, 0.371]
SPL/CS	0.156 \pm 0.007 [0.144, 0.165]	0.160 \pm 0.008 [0.145, 0.179]	0.161 \pm 0.007 [0.145, 0.174]
MW/CS	0.609 \pm 0.013 [0.587, 0.629]	0.615 \pm 0.013 [0.590, 0.662]	0.602 \pm 0.010 [0.570, 0.619]
PEW/CS	0.262 \pm 0.010 [0.244, 0.274]	0.269 \pm 0.009 [0.250, 0.292]	0.254 \pm 0.007 [0.241, 0.272]
PPW/CS	0.365 \pm 0.009 [0.352, 0.378]	0.375 \pm 0.010 [0.357, 0.401]	0.362 \pm 0.011 [0.345, 0.390]
SPBA/CS	0.283 \pm 0.013 [0.257, 0.311]	0.279 \pm 0.010 [0.259, 0.302]	0.265 \pm 0.009 [0.250, 0.282]
SPWI/CS	0.437 \pm 0.022 [0.401, 0.485]	0.402 \pm 0.013 [0.376, 0.428]	0.379 \pm 0.016 [0.335, 0.421]
SPTI/CS	0.412 \pm 0.022 [0.381, 0.462]	0.383 \pm 0.013 [0.358, 0.411]	0.359 \pm 0.015 [0.318, 0.397]

significantly longer than broad (CL/CWb 1.226) and with feebly convex sides. Vertexal corners rounded, posterior margin of vertex strait. Scape long (SL/CS 0.786) with variable pubescence: adpressed (0–5°) to decumbent (10–15°) on the surface that is adjacent to head surface when the scape is directed fully caudad and subdecumbent (30°) on the surface that becomes external in the same scape position. Clypeus between sagittal level of frontal carinae with a number of longitudinal carinulae, interstices smooth and shiny. Eyes rather large (EL/CS 0.248) and more approached to hind margin of vertex (PoOc/CL 0.401). Vertex with fine microreticulate sculpture that is superimposed by a number of longitudinal rugulae. Frontal carinae non-divergent and widely distant (FRS/CS 0.357). Propodeal spines long, but significantly shorter than in *T. laconicus* sp.n. (SPST/CS 0.347) and acute, in lateral view deviating from longitudinal axis of mesosoma by 20–25°. In dorsal view, propodeal spines less divergent than in the sibling species (SPWI/CS 0.393), spine-tips curving inward (SPTI/CS 0.373). Mesosoma as wide as in related species (MW/CS 0.610). Metanotal depression well visible, moderately deep. Mesosoma with fine microreticulate microsculpture that is superimposed by a few irregular or longitudinal rugulae. Petiole and postpetiole covered by a reticulate microsculpture that can occasionally be superimposed by a few irregular rugulae in particular on dorsum of petiolar node. Petiole in

profile with a rather straight or slightly concave anterior face; top of node forms a short truncate dorsum that continue to posterior slope with a distinct step.

For morphometric data of 84 (“*Eastern cluster*” *n*=41, “*Western cluster*” *n*=43) nest sample means see Table 3.

Diagnosis

Separation between workers of *T. lichtensteini* (“*Western*”, and “*Eastern clusters*” combined) and *T. laconicus* sp. n. is given under description and diagnosis of *T. laconicus* sp. n. (see above).

Temnothorax lichtensteini consist of two parapatric metapopulations, “*Western cluster*”, and “*Eastern cluster*” and their separation based on morphological traits are not sufficient to raise them to species rank, but we do find sufficient morphological characters to diagnose them as evolutionarily distinct metapopulations.

General appearance of these lineages are quite similar. In mesosomal and petiolar traits (MW/CS, PEW/CS, PPW/CS, SPBA/CS, SPWI/CS, SPTI/CS) “*Eastern*” lineage appears more stout than the “*Western*” one ($P < 0.001$, in *t*-test), but these significantly different traits show a broad overlap, therefore, single characters are not available for safe separation.

However, multivariate discriminant analysis provides sufficient separation between the two geographically distinct

clusters. The morphological resemblance of the two clusters allows a little character reduction in LDA as:

$$D15 = -0.0618 PEW + 0.0521 NOL - 0.0315 SPTI + 0.0466 PL - 0.0965 FRS + 0.0512 CWb \\ + 0.0551 EL - 0.0406 SPBA - 0.0418 PPL + 0.0269 ML - 0.0319 PEH - 0.0203 MW - 0.0190 CL \\ - 0.0388 NOH + 0.0335 SPL + 2.3903$$

This combination provides 95.3 % success in classification for worker individuals:

$$\text{“Eastern cluster”}(n = 166) D15 = -1.251 [-3.945, +2.216] \\ \text{“Western cluster”}(n = 129) D15 = +1.609 [-0.890, +3.906]$$

The same function yields complete separation at nest sample mean level.

$$\text{“Eastern cluster”}(n = 41) D15 = -1.245 [-2.508, -0.199] \\ \text{“Western cluster”}(n = 43) D15 = +1.585 [+0.224, +3.463]$$

Discussion

Based on morphometric analyses and mtDNA we arrived at the conclusion that *T. lichtensteini*, which was formerly considered one species, in fact consists of three separate clusters across its distributional range.

The existence of obviously divergent lineages underpins the notion that *T. lichtensteini* is likely a complex of monophyletic lineages, in which each cluster (“*East Mediterranean cluster*”, “*West Mediterranean cluster*” and “*Peloponnese cluster*”) has an independent evolutionary history with rather distinct distributional area.

The biogeographic pattern can be explained by postglacial range expansion from glacial refugia as described in other groups. The currently dominating view is that the present phylogeographic structure results mostly from post-glacial colonisation events with some remolding related to population fluctuations during the Quaternary (Hewitt 1999; Goropashnaya et al. 2007; Litvinchuk et al. 2013). The East Mediterranean population of *T. lichtensteini* most certainly spread from a refuge centre situated in the south Balkans, while the West Mediterranean population spread from an Iberian and/or Apennine refuge centre. Such an East–West separation in the Pleistocene with development of para- and sympatry in the Holocene is known from a large number of closely related pairs of vertebrate and invertebrate species. In ants, it is evident in *Lasius piliferus* Seifert, 1992 and *Lasius psammophilus* Seifert, 1992 (Seifert 1992), *Temnothorax nylanderi* Förster 1850 and *T. crassispinus* Karavajev, 1926 (Seifert 1995; Pusch et al. 2006),

Myrmica spinosior Santschi, 1931 and *M. sabuleti* Meinert, 1861 (Seifert 2005) and two clades of *Tetramorium moravicum* Kratochvil, 1941 (Schlick-Steiner et al. 2007).

The *West Mediterranean* and *East Mediterranean clusters* of *T. lichtensteini* are not convincingly separated from each other by morphology, though both can be distinguished readily from the “*Peloponnese cluster*”. Based on the slight morphological differences it appears that the speciation process of these two clusters is underway. However, there is also a biological difference supporting differentiation of populations: intensive sampling did not reveal a single socially parasitic ant parasitising the West Mediterranean population, while the slave-making ant *Myrmoxenus gordiagini* Ruzsky, 1902 is known to parasitise the much less investigated Eastern population (Buschinger et al. 1983; A. Buschinger, personal communication 2010).

According to our interpretation, these clusters might well be considered as separate subspecies but, because they cannot be distinguished easily in practical use, we refrain from taxonomically describing them. Investigation of the genetic relations of the two entities in their contact zone is desired for better assessment of the case.

Conclusion

The combined evidence from mtDNA and morphometric analyses suggests that the three recognised clusters (“*East Mediterranean cluster*”, “*West Mediterranean cluster*” and “*Peloponnese cluster*”) represent two or more distinct species, even though genetic variation in the “*Peloponnese cluster*” might suggest incomplete lineage sorting. In morphology, the “*Peloponnese cluster*” is perfectly separated from the “*East Mediterranean*” and “*West Mediterranean clusters*” of *T. lichtensteini* (Fig. 3), even though it is less well supported by the genetic analysis. Based on these results, we hereby describe the worker caste of the “*Peloponnese cluster*” as a new species, *Temnothorax laconicus* n.sp. In addition, we re-describe its sister species, *T. lichtensteini*. According to the parapatric distribution (Fig. 4) and the difficult discrimination in morphology, the “*Eastern*” and “*Western*” clusters might be considered as subspecies, but are not formally described here.

Acknowledgments We would like to take this opportunity to thank the anonymous referees who carefully read the manuscript and gave useful suggestions. We wish to thank the following curators of entomological collections for loans of ant specimens: Daniel Burckhardt (NHM Basel); Manfred Verhaagh (SMN Karlsruhe) and Wouter Dekoninck (IRSNB Bruxelles). We are very grateful to Brian Fisher (CAS) for help with imaging. The molecular study was supported by the Deutsche Forschungsgemeinschaft (He 1623/24). S.C. was supported by the Hungarian Eötvös scholarship in 2010.

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