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Uncovering Species Diversity in the Subgenus *Lasius* s. str. (Ruzsky, 1913): A Morphological and Molecular Study from Bosnia and Herzegovina

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Abstract

Background and purpose: *Ants are a dominant terrestrial insect group and rank among the most abundant and diverse animals on land, playing significant ecological and economic roles. Due to their global diversity and the high morphological similarity among closely related species, accurate identification of ants can be difficult. The aim of this study was to assess the species status within one of the most important ant groups –* Lasius *genus, subgenus* Lasius *s.str. – in Bosnia and Herzegovina, using a combination of morphological and molecular approaches.*

Materials and methods: *Forty-two individuals were collected from four localities and identified using appropriate morphological keys. DNA barcoding was performed to complement morphological identification, and sequences were deposited in GenBank. A Maximum Likelihood phylogenetic tree was constructed using MEGA11, incorporating both the sequences from this study and selected sequences from the BOLD database.*

Results: *We identified three species -* Lasius niger*,* L. platythorax *and* L. emarginatus*, by applying both approaches. Specific dark-brown mesosoma and a new haplotype were recorded for* L. emarginatus*.*

Conclusions: *The observed morphometric differences and a discovery of a new* Lasius emarginatus *haplotype suggest that the diversity of species within the subgenus in Bosnia and Herzegovina is greater than previously recognized. Further research is warranted to explore the regional diversity and ecological roles of* Lasius *species.*

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INTRODUCTION

Ants, eusocial insects belonging to the family Formicidae (order Hymenoptera), comprise approximately 15-20% of the terrestrial animal biomass and serve as major contributors to energy and organic matter cycling *(1)*. The evolutionary history of ants dates back roughly 120 Mya, making them important model organisms in ecological studies. The diverse ant fauna of Europe has been extensively studied *(2, 3)*, providing valuable insights into their morphology and ecology.

In Bosnia and Herzegovina (B&H), ants have been investigated since the 19th century, when Wasmann *(4)* reported the first data on ant fauna. Subsequent studies have recorded various species, including five from subgenus *Formica* in Central Bosnia *(5)* and species from the genus *Messor* in the Mediterranean part of B&H *(6)*. Investigations after 2011 revealed first records of species *Liometopum microcephalum*, *Bothryomyrmex meridionalis* and the genus *Strongylognathus (7, 8)*. According to the

inventory by Guénard et al. *(9)*, the genus Lasius in Bosnia and Herzegovina is represented by 14 species. Of these, five species belong to the subgenus Lasius, two to the subgenus Cautolasius, two to the subgenus Chthonolasius, two to the subgenus Dendrolasius, and one to the subgenus Austrolasius.

Within the Holarctic realm, the genus *Lasius* Fabricius, 1804 is recognized as one of the most complex and taxonomically challenging ant groups. This genus comprises 125 species *(10)*, divided into five subgenera: *Lasius* s.str. Ruzsky 1913, *Cautolasius* Wilson 1955, *Dendrolasius* Ruzsky 1913, *Chthonolasius* Ruzsky 1913, and *Austrolasius* Faber 1967. Species of the subgenus *Lasius* s.str. inhabit urban centers, rural and natural habitats. The subgenus is characterized by distinctive morphological traits such as elongated maxillary palps, and large-eyed workers that engage in intensive above-ground foraging *(11)*.

Accurate identification of ant species is often challenging and typically requires a combination of morphological and molecular methods. A widely adopted molecular approach involves sequencing the cytochrome oxidase subunit I (*COI*) region, a universal DNA barcode for animals *(12)*. DNA barcoding has become an essential tool for studying species biodiversity *(13)*. Out of 116 species listed on the Barcode of Life Data System (BOLD) *(14) Lasius* taxon page, there are currently more than 8000 sequence records in BOLD database (accessed on October 21, 2025) for 114 *Lasius* species, providing cca 98.3% species coverage (by species with barcodes). Europe, including Central and Western regions, has the highest concentration of *Lasius* barcodes, but coverage still lacks density, especially for rare or difficult-to-identify species. Coverage in the Balkans is typically sparse, often represented by single records or species with disjunct distributions. No sequence records of the *Lasius* genus are available for Slovenia and North Macedonia. There are 17 sequences for specimens from Croatia, three sequences from Serbia, and 40 sequences for Montenegrin specimens. The present study aims to improve our understanding of ant distribution in Bosnia and Herzegovina, with a particular focus on the genus *Lasius* and its subgenus *Lasius* s.str., utilizing both morphological and molecular techniques.

MATERIAL AND METHODS

Samples of *Lasius* species were collected manually with tweezers between April 2016 and May 2018 at four localities in Bosnia and Herzegovina (Figure 1). All samples were stored in 96% ethanol and kept at +4°C until further analysis.

MORPHOLOGICAL ANALYSIS

Morphological analysis included a measurement of dry mounted ant specimens under stereomicroscope equipped

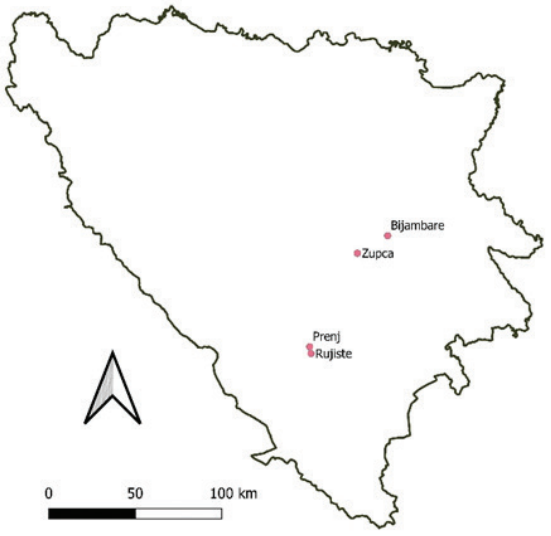

Figure 1. *Outline map of Bosnia and Herzegovina showing sampling sites where ants were collected: Bijambare (lat: 44.081°; lon: 18.509°), Župća (lat: 43.991°; lon: 18.290°), Prenj (lat: 43.503°; lon: 17.947°), Rujište (lat: 43.468°; lon: 17.956°).*

with microoculars cross scale. A led ring light, cold–light source was used for illumination of specimens.

Morphometric analysis was performed on a Reichert (Greenough stereo microscope) binocular loupe with a 10X magnification eyepiece and 10X and 5X objectives. A 100-division scale was fitted on the right eyepiece. Eyepiece calibration was performed on a 100-division calibration slide, 1 division on the calibration slide equals 0.01 mm. At 100X magnification, one division on the eyepiece scale equals 13.66248111 µm, and at 50X magnification, 23.529412 µm. The measurement of morphological characters smaller than one division on the micro-ocular of a binocular loupe under 100X magnification was performed under a microscope. The microscope with micro-ocular PZO K15X and objective 40X, one division of scale on the micro-ocular corresponds to 7.692308 µm. A led ring light, cold–light source was used for illumination of specimens. Dry mounted specimens were attached to a pin holder that allows positioning specimens for measuring and taxonomical character analysis.

The measurement of morphometric characters was carried out for characters listed in the taxonomical key. Fifteen morphometric characters and morphometric indices are defined in the paper and additional definitions of these characters are given in key Seifert *(11, 15, 16, 17)*.

CW – maximum cephalic width; this is either across, behind, or before the eyes.

CL – maximum cephalic length in median line; the head must be carefully tilted to the position with the true maximum. Excavations of posterior head and/or clypeus reduce CL.

SL – maximum straight line scape length excluding the articular condyle.

EL – large diameter of the ecliptic compound eye measured over all structurally visible ommatidiae, also including unpigmented marginal ommatidiae.

EW – small diameter of the elliptic compound eye measured over all structurally visible ommatidiae, also including unpigmented marginal ommatidiae.

PnHL – length of the longest hair on pronotum.

PoOc – postocular distance. Use a cross-scaled ocular micrometer and adjust the head to the measuring position of CL. Caudal measuring point: median occipital margin; frontal measuring point: median head at the level of the posterior eye margin. Note that many heads are asymmetric and average the left and right postocular distance.

nHT – with large diameter of hind tibia in visual plane, unilateral number of setae surpassing its extensor margin by > 10 µm (> 20 µm in subgenus *Lasius* s. str.).

nSc – with small diameter of scape in visual plane, unilateral number of setae on dorsal plane of scape protruding > 10µm from cuticular surface (> 20 µm in subgenus Lasius s. str.).

nSt – setae number on lateral and caudolateral surface of metapleuron. The upper margin of the counting area is an imagined line parallel to the lower straight margin of metapleuron and crossing the lower margin of the cuticular ring of the propodeal spiracle. Protective setae fringing the orifice of the metapleural gland are excluded. The bilateral sum is halved.

GuHL – maximum length of setae on underside of head ("gula").

CS – arithmetic mean of CL and CW as less variable indicator of body size.

EYE – eye-size: the arithmetic mean of the large (EL) and small diameter (EW) of the elliptic compound eye under consideration of all structurally visible ommatidia, including also unpigmented ones.

dCLAN – Torulo-clypeal distance. The minimal distance between the inner margin of the antennal socket and the posterior marginal clypeal suture, in mm.

Molecular-genetic analysis

Two to four specimens per presumed morpho-species were selected for molecular-genetic analyses to complement morphological identification, resulting in nine total samples. These included two specimens of *L. niger* from Bijambare site, two specimens of *L. platythorax* from Rujište and one from Župća sites, as well as two specimens of *Lasius* sp. per locations of Prenj and Rujište. Total genomic DNA (gDNA) from *L. niger* samples was extracted using a modified Taggart protocol *(18)*, while gDNA from specimens of *L. platythorax* and *Lasius* sp. was obtained utilizing the ExtractMe DNA Tissue kit (Biolab Innovative Research Technologies, Poland) following the manufacturer's recommendations. In both cases, whole bodies were used as a starting material. For the modified Taggart protocol, 400 µl of freshly prepared lysis buffer, containing 187 mM EDTA, 0.5% n-lauryl sarcosine, and 0.5 mg/µl proteinase K, was used, and samples were incubated at 37°C overnight. Subsequently, 400 µl of a phenol/chloroform/isoamyl alcohol mixture (25:24:1) was added and samples were centrifuged for 10 minutes at 13,000 rpm. The aqueous phase containing DNA was transferred into clean 1.5 ml tubes and DNA was precipitated by adding cold absolute ethanol (twice the volume of the supernatant). Samples were held at -20°C for 20 minutes, and then centrifuged for 3 minutes at 13,000 rpm. After a second wash step with 600 µl of cold 70% ethanol, samples were air-dried. DNA was resuspended in a low-TE buffer (10 mM TRIS, 0,1 mM EDTA).

The quality of the extracted DNA was assessed using electrophoresis in 1.5% (w/v) agarose gel in 1x SB buffer, pH 8 *(19)*. Genomic DNA was visualized under UV light using a Fusion Solo S imaging system (Vilber, France) after staining with Midori Green Advance DNA Stain (Nippon Genetics Europe, Germany).

Amplification of the 658 bp *COI* barcode region was carried out using LCO1490 and HCO2198 primers *(20)*. PCR was carried out on the GeneAmp® PCR System 9700 (Applied Biosystems, United States) in a three-step cycling protocol: an initial denaturation at 95°C for 5 minutes, followed by 40 cycles comprising denaturation at 94°C for 30 seconds, annealing at progressively decreasing temperatures (56°C for the first 10 cycles, then decreasing to 53°C for 15 cycles and to 50°C for the final 15 cycles), and extension at 72°C for 45 seconds each cycle. A final elongation step was performed at 72°C for 8 minutes. Sanger sequencing was carried out by Macrogen Europe (The Netherlands) in one direction using LCO1490 primer.

The obtained raw nucleotide sequences of the COI gene were analyzed using software tools. These sequences of the mitochondrial protein-coding gene were checked using identity and similarity indices within local databases with the FASTA program and within the GenBank database on the National Center for Biotechnology Information (NCBI) platform using the Basic Local Alignment Search Tool (BLAST) *(21)* for local alignments and sequence homologies. In addition to the NCBI database, the curated sequences were also analyzed in the BOLD Systems database using the Identification tool for additional verification and identification. Manual trimming of the sequences was performed based on the homology results. We downloaded the top 100 matches from each BOLD Identification search. Sequences not determined to the species level were removed, together with the se-

Table 1. *List of sequences used in the construction of the phylogenetic tree.*

BOLD-ID	GenBank	Species	Country
GBAAM19688-25	PQ506279	*L. platythorax*	Switzerland
GBMIN33036-13	GQ503250	*L. platythorax*	–
GBMIN33091-13	GQ503249	*L. platythorax*	–
GBAAM19493-25	PQ506272	*L. platythorax*	Switzerland
GBAAM19573-25	PQ506176	*L. platythorax*	Switzerland
GBAAM19691-25	PQ506175	*L. platythorax*	Switzerland
ANTEU3542-22	–	*L. platythorax*	Italy
ANTEU3582-22	–	*L. platythorax*	Spain
BCHYM3656-14	–	*L. platythorax*	Germany
GBAAM19605-25	PQ506177	*L. platythorax*	Switzerland
GBAHF5308-19	LT977536	*L. platythorax*	-
ACUFI1292-13	MZ608373	*L. platythorax*	Finland
ANTEU3480-22	–	*L. cf. platythorax*	Italy
ANTBG045-10	JN287555	*L. platythorax*	Bulgaria
ANTEU2235-22	–	*L. platythorax*	Bulgaria
ANTEU1594-22	–	*L. platythorax*	Montenegro
GBAAM19491-25	PQ506240	*L. platythorax*	Switzerland
GBAAM19551-25	PQ506183	*L. platythorax*	Switzerland
GBAAM19415-25	PQ506274	*L. platythorax*	Switzerland
ZMBN975-17	–	*L. platythorax*	Norway
GBAAM19435-25	PQ506277	*L. platythorax*	Switzerland
GBAAM19418-25	PQ506278	*L. platythorax*	Switzerland
GBAAM19681-25	PQ506182	*L. platythorax*	Switzerland
GBAAM19449-25	PQ506239	*L. platythorax*	Switzerland
GBAAM19543-25	PQ506280	*L. platythorax*	Switzerland
GBAAM19442-25	PQ506320	*L. platythorax*	Switzerland
ANTEU1288-22	–	*L. emarginatus*	Italy
ANTEU4658-22	OQ025630	*L. emarginatus*	Italy
ANFMB055-16	MH138381	*L. emarginatus*	France
ANFMB064-16	MH138385	*L. emarginatus*	Italy
ANTEU2566-22	–	*L. emarginatus*	France
ANTEU1350-22	–	*L. emarginatus*	France
ANFMB072-16	MH138380	*L. emarginatus*	France
AEANT124-20	MT606326	*L. emarginatus*	Italy
ANTEU1199-22	–	*L. illyricus*	Greece
ANTEU3926-22	–	*L. illyricus*	Greece
ANTEU632-22	–	*L. emarginatus*	Poland
GBAAM3822-25	OR856605	*L. emarginatus*	Poland
ANFMB090-16	MH138382	*L. emarginatus*	France
ANTEU056-21	–	*L. illyricus*	Greece
AEANT122-20	MT606324	*L. emarginatus*	Italy
ANTEU088-21	OQ025622	*L. emarginatus*	Italy
ACUFI1280-13	MZ611152	*L. niger*	Finland
ANTEU1589-22	–	*L. niger*	Ireland
ANTEU2576-22	–	*L. niger*	France
ANTEU631-22	–	*L. niger*	Poland
GBAAM20437-25	PQ507006	*L. niger*	Switzerland
GBAAZ23700-24	–	*L. niger*	
GBAHF5279-19	LT977504	*L. niger*	
GBMIN33092-13	GQ503247	*L. niger*	
NOPRA377-16	–	*L. niger*	Norway
ACUFI1330-13	MZ610655	*L. umbratus*	Finland

Table 2. *Samples collected for morphological analysis.*

Species	Location	Collection date	No of individuals
Lasius niger	Bijambare (lat: 44.081°; lon: 18.509°)	30.04.2016	5 workers
Lasius platythorax	Župća (lat: 43.991°; lon: 18.290°)	21.04.2018	3 workers
	Rujište (lat: 43.468°; lon: 17.956°)	02.05.2018	25 workers
Lasius sp.	Rujište (lat: 43.468°; lon: 17.956°)	02.05.2018	7 workers
	Prenj (lat: 43.503°; lon: 17.947°)	30.07.2017	2 workers

quences containing internal stop codons. The sequence of *L. umbratus* (BOLD ID ACUFI1330-13) was included as an outgroup (Table 1). Multiple sequence alignment (MSA) was performed using the MUSCLE algorithm, and a Maximum Likelihood (ML) phylogenetic tree was constructed based on the Hasegawa-Kishino-Yano model within MEGA11 software *(22)*. For species delimitation we used ASAP (Assemble Species by Automatic Partitioning) *(23)* programme and bPTP server *(24)*.

RESULTS AND DISCUSSION

Morphological analysis identified three species belonging to the subgenus *Lasius* s.str.: *Lasius niger* Linnaeus, 1758, *Lasius platythorax* Seifert, 1991, and *Lasius emarginatus* Olivier, 1792. Specimens of *Lasius sp.* were sent to Dr. Bernhard Seifert for identification as *Lasius emarginatus* (table 2). This was prompted by a discrepancy in the coloration of the mesosoma, which was uniformly brown in the analyzed specimens, deviating from the typical red coloration. Accurate identification of *L. emarginatus* was crucial for our research to avoid confusion with the closely related species *L. illyricus* Zimmermann, 1935.

Species *Lasius niger* and *L. platythorax* corresponded in morphological characters to the description from the taxonomic key (16, 17). Morphological parameters of the *L. emarginatus* sample (N=5, average±standard deviation, measurements in µm): CW 819.3±33.5, CL 874.3±18.6, SL 891.4±29.6, EL 245.7±12.0, EW 188.6±6.4, PnHL 91.4±7.8, GuHL 88.6±12.0, PoOc 206.4±18.6, nHT 11.4±2.4, nSc 7.0±1.6, nSt 4.0±0.71, dCLAN 7.7±0.00, PNHL/CL 0.10±0.01, GuHL/CL 0.09±0.01, SL/CS 0.71±0.04, EYE 0.217±0.02, GuHL/CS 0.105±0.09, PnHL/CS 0.118±0.15.

Morphometric analysis showed that morphometric characters (11, 17) fit into the range corresponding to the species *L. emarginatus:* 0.075*nSc+0.172*nHT+0.33*nSt-46.18*poOc+6.108 = 0.401>0. Other diagnostic patterns based on morphometry also coincide with the diagnosis of the species *L. emarginatus*: 24.63*SL+51.992*dCLAN-55.46*PoOc-7.557*CW+6.521 = 8.07 > 0; EYE/CS >-0.0607*CS+0.269 = 0.256 > 0.218, EYE/CS = 0,256 > 0.170, SL > 0.7692*CW+245 = 891.4 > 874.9.

The findings of the morphometric analysis and comparison with morphometric indices in Seifert *(16, 17)* showed that sample *Lasius sp.* belongs to the southern population of *L. emarginatus*. The European realm of *L. emarginatus* is from 52.6° N in the north (southern England and the Netherlands) to 37° N in the south (across the Balkans, Iberia and Apennine). While populations in

Figure 2. *Lateral view of Lasius emarginatus worker caste. Ectomorphs from Rujište, B&H have dark-brown mesosoma.*

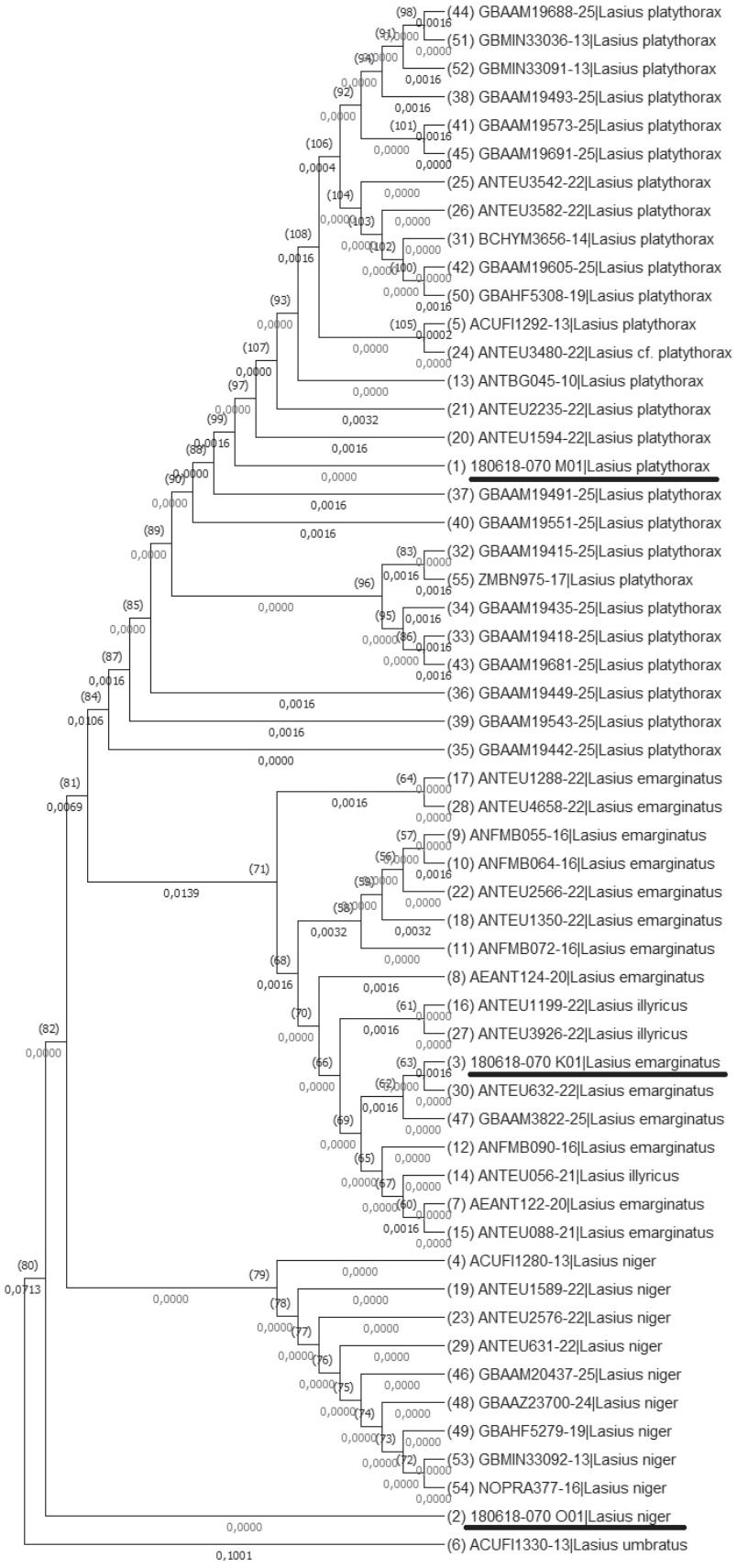

Figure 3. *Maximum Likelihood (ML) phylogenetic tree. Samples from this study are underlined.*

central Europe are common in urban/suburban habitats and open forests, populations in southeastern Europe are mostly found in closed broadleaf forests *(11)*. The occurrence of *L. emarginatus* within wooded regions results in adaptation where ectomorphs have dark-brown mesosoma, which strongly deviate from the typical West and Central European forms (Figure 2).

Variations in body structure within the same caste of a species are a common phenomenon that poses a challenge for morphology-based identification of ants. DNA barcoding confirmed the morphological identification of *L. niger* and *L. platythorax*. For the specimen morphologically identified as *Lasius* sp., DNA barcoding showed ≤99.84% sequence identity with *L. emarginatus.* Obtained sequences were deposited in GenBank (accession numbers PX470043, PX470044 and MT951569). These results demonstrate that DNA barcoding is a reliable tool for resolving ambiguous morphological identification *(3)*. Notably, the *L. emarginatus* haplotype identified in this study has not been previously reported.

Based on the MSA results of sequences from the BOLD database and this study, a Maximum Likelihood (ML) phylogenetic tree was constructed. Results of ML analysis, performed using the Hasegawa-Kishino-Yano model in MEGA software, placed our three samples in the appropriate clades (Figure 3). The best ASAP score suggests two distinct species which was expected, since the outgroup is clearly distinct from the other analyzed species. The second ASAP score relates to more fine-grained partitions corresponding to four presumed species in the analyzed group of sequences (Supplementary material 1). Taking into account morphological and ecological data of the species, as recommended *(23)*, we can consider that the second-best ASAP result actually provides the most accurate representation of the real situation. Results of the bPTP analysis showed similar results, with ML partition results indicating five species and simple heuristic search indicating 13 (Supplementary material 2 and 3). Since bPTP is a purely tree-based program, it is recommended to compare results with morphological characters, behavioral traits, and information on geographic distributions *(25)*. Taking into account the distances between the collection sites of the samples employed in the phylogenetic tree reconstruction (Table 1), the bPTP analysis results align well with the outcomes of the ML and ASAP analyses.

As previously mentioned, the *Lasius* ant group is among those with the most complex and challenging taxonomy. Borowiec and Salata *(26)* noted that Seifert previously synonymized *L. illyricus* with *L. emarginatus*, albeit with some reservations, emphasizing that both species are very similar in overall appearance. However, they also pointed out that certain morphological characters allow these two species to be distinguished. During the BOLD identification, specimens of *L. illyricus* were also matched with *L. emarginatus*, and these specimens clustered with *L. emarginatus* sequences in the phylogenetic tree, ASAP, and bPTP analyses. Altogether, these findings once again demonstrate that integrating both morphological and molecular approaches in taxonomy leads to more consistent species classification and enhances the stability and reliability of the taxonomic workflow.

CONCLUSION

In our study, the combination of morphological and molecular methods enabled the identification of three distinct species within the *Lasius* s. str. subgenus in analyzed samples from Bosnia and Herzegovina. The observed morphometric differences, along with the discovery of a novel haplotype of *Lasius emarginatus*, suggest that the genetic and morphological diversity within the *Lasius* genus in Bosnia and Herzegovina is likely greater than previously recognized. These findings underscore the need for further investigation into the regional distribution and ecological roles of *Lasius* species, as well as the potential for uncovering additional, yet unreported species.

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Supplementary material is available on-line at https://hrcak.srce.hr/pb

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