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# Intraspecific diversity of *Myrmecophilus acervorum* (Orthoptera: Myrmecophilidae) indicating an ongoing cryptic speciation

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*Myrmecophilus acervorum*, previously considered a parthenogenetic species widely-distributed in Europe, has been observed to have both sexes in populations inhabiting the central part of the distribution range. Specimens from those heterosexual populations have been found being infected with *Wolbachia*. New mitochondrial data (COI and 16S markers) revealed the well-supported differentiation of *M. acervorum* populations inhabiting western Polesie (Poland) and southern Europe. In turn, analyses of EF1α marker support the hypothesis on the unfinished lineage sorting at the nuclear DNA level. Interestingly, we found that parthenogenetic populations inhabiting western Polesie are infected with *Wolbachia* belonging to supergroup A, while endosymbionts occurring in sexual populations of *M. acervorum* observed in Romania belong to supergroup B. Furthermore, new and potentially diagnostic characteristics in the external structures of the eyes of *M. acervorum* were identified. The surface of ommatidia in specimens occurring in southern Europe was smooth. In contrast, the ommatidia surface of individuals collected in Poland was visibly sculptured. To sum up, the significant genetic variability found in the present case, and the differentiating morphological character, are almost certainly effects of cryptic species being present within *M. acervorum*. This is indicative of ongoing speciation within the populations of this insect, and of simultaneous unfinished lineage sorting at the nuclear DNA level.

**Keywords** *Wolbachia*, Supergroup, COI, 16S, EF1α, Microstructures

The advent of the DNA barcoding concept initiated the widespread application of mitochondrial DNA (mtDNA) in taxonomic research[1]. Mitochondrial markers swiftly became integral components in diverse research areas, including the systematics, phylogeography, population genetics, ecology, and evolutionary biology of animals[2]. The versatility of these markers is closely linked to the characteristics of the mitochondrial genome, i.e. its general uniparental inheritance (Ref.[3] but see[4]), high rate of mutagenesis[5] due to the lack of protective histones and exposure to the impact of free radicals[6], as well as fourfold smaller effective population size compared to the nuclear genome[7,8]. Consequently, intraspecific variation of mitochondrial DNA may surpass that observed in nuclear DNA, making analyses based on mitochondrial markers valuable for resolving population divergences that remain elusive using coding nuclear markers[9].

Divergent mtDNA lineages detected within species may be considered as distinct species according to the value of the genetic distance between them[10]. However, not every divergent mitochondrial DNA lineage should be regarded as a separate species[11–14]. The divergence detected at the mitochondrial level is not always supported by nuclear DNA data[15–17]. Discrepancies between mitochondrial and nuclear DNA patterns of diversity may

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arise from various factors. Analyses based on mitochondrial markers may be more susceptible to false results owing to the effective amplification of pseudogenes (e.g. NUMTs) instead of the functional target genes[18]. Even after this factor has been eliminated in laboratory and bioinformatic analyses, mito-nuclear discordance may persist as a result of biological processes like hybridization and the subsequent introgression of the mitochondrial genome[15,16,19], incomplete lineage sorting of ancestral polymorphisms[19,20] or selection acting directly or indirectly on mitochondrial genes[21]. The presence of bacterial endosymbionts can also indirectly influence genetic diversity at the mitochondrial level, with correlations observed between infection patterns and distinct phylogenetic clades or specific haplotypes of hosts[22–25]. Furthermore, endosymbiotic bacteria can be associated with both greater and smaller genetic variation of hosts at the mitochondrial level[26–28] or deep divergences in mitochondrial phylogenies[29,30]. Therefore, analyses based on mitochondrial markers should incorporate tests for the presence of endosymbiotic bacteria, e.g. *Wolbachia*[31].

*Wolbachia* is a well-known endosymbiotic bacterium, infecting a broad range of insects, crustaceans and nematode species[32–34]. It is estimated that approximately 66% of insect species are infected by *Wolbachia*[35]. The bacterium has been identified in species belonging to several major insect orders, including Coleoptera, Diptera, Hymenoptera, Lepidoptera and Orthoptera[36,37].

Orthopterans, one of the largest hemimetabolic insect orders, inhabit diverse terrestrial habitats worldwide, even the nests of social insects[38]. Ant-loving crickets within the genus *Myrmecophilus* (Orthoptera: Myrmecophilidae) are commonly associated with various ant species[39–41]. The genus currently comprises 63 valid species, with 10 described in Europe. Most of the latter species occur regionally in southern and south-eastern parts of the continent[42]. All *Myrmecophilus* species are morphologically similar, with subtle diagnostic characters based mainly on body colour and the number of spurs on the hind leg, and also the male genitalia and the variability of the female ovipositor's shape[43–46]. However, species identification based on these diagnostic characteristics may be challenging, particularly as nymphs lack the full set of characters of adult specimens. Thus, molecular tools serve as effective means for individual identification, the discovery of potential new species, and the determination of phylogenetic relationships[47–49].

*Myrmecophilus acervorum* (Panzer, 1799) (Fig. 1) is distributed across Europe and inhabits the nests of approximately 20 host ant species from at least eight genera[41,50]. Although it is thought to be thelytokous parthenogenetic species, recent surveys in the central part of its distribution range have revealed the presence of both sexes[42]. Nonetheless, the phylogenetic and phylogeographic relationships among *M. acervorum* populations



**Fig. 1.** Adult female of *Myrmecophilus acervorum* with its host, *Lasius platythorax* worker ant (specimens collected 18. August 2021 in Orzechów, West Polesie, Poland) (phot. G. K. Wagner).

remain enigmatic. Initial attempts to elucidate these relationships using molecular markers identified several haplotypes of *M. acervorum* in Europe, with six forming a parthenogenetic clade in populations distributed west of the Carpathians[42]. Additionally, testing all collected samples for *Wolbachia* infection revealed its presence only in populations where both sexes were observed.

In the present study, we employ morphological and molecular approaches to assess the diversity of parthenogenetic *M. acervorum* occurring in the western Polesie region of Poland and compare the observed patterns with previous data. All the specimens collected were examined morphologically to identify potentially diagnostic traits for routine identification. Subtle differences in the external structure of cricket eyes were noted, appearing to be congruent with interspecific diversity in *M. acervorum*. At the same time, similar patterns were revealed for mitochondrial markers (COI and 16S), though not fully detectable for the nuclear marker (EF1α). Additionally, all specimens were tested for the presence of endosymbiotic *Wolbachia*. Analyses based on the *Wolbachia* 16S gene fragment confirmed infection in all but one of the tested samples, representing the first report of the presence of *Wolbachia* in parthenogenetic populations of *M. acervorum*.

## Results

### Morphological characteristics

The external structure of the *M. acervorum* eyes was tested for 20 individuals collected in Poland and 3 specimens from Romanian populations (Fig. 2). The surface of ommatidia in members of the Romanian population of ant crickets was smooth (Fig. 3a, b). In contrast, the surface of the ommatidia in the specimens collected in Poland was visibly sculptured (Fig. 3c, d). Two types of appendages can be distinguished: larger ones, ranging from a dozen to over twenty on one ommatidium, and numerous small protuberances (Fig. 3d).

### Mitochondrial markers—COI and 16S

Mitochondrial markers were amplified for a subgroup of collected *M. acervorum* specimens (up to 5 individuals per sample). As a result, high-quality sequences of the *cox1* gene fragment were obtained for 83 specimens (Table 1). In the case of 16S mitochondrial marker, only 50 sequences could be generated. Alignment of COI sequences (619 bp) included three variable sites and all of them were parsimony informative. In turn, alignment of 16S sequences (415 bp) included only one variable site, which was not parsimony informative. COI marker sequences were more variable and four unique sequences were identified, while in the case of 16S only two



**Fig. 2.** Field work and collections of ant cricket specimens in the laboratory. (**A,B**) The preferred habitats of *Myrmecophilus acervorum* in the study area; (**C**) selection of material in the field; (**D**) sampling tray (photos **A,B,D**—G. K. Wagner, photo **C**—B. Staniec).
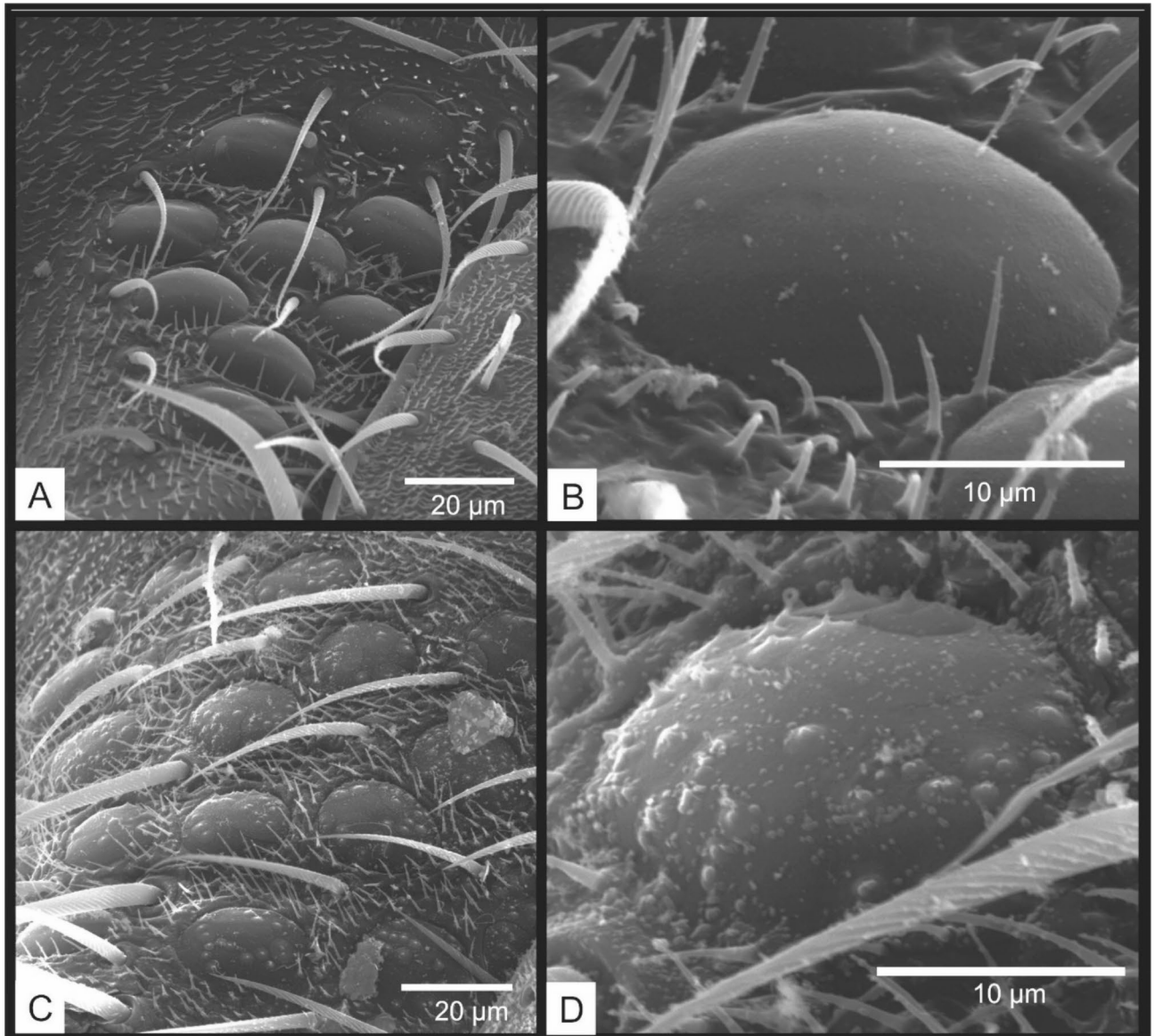
**Fig. 3.** Eyes of *Myrmecophilus acervorum* specimens collected in Romania (**A,B**) and Poland (**C,D**) (phot. G. K. Wagner).

haplotypes were noted (Table 1). Overall haplotype diversity (*Hd*) was 0.2421 for COI and 0.0400 for 16S. In turn, nucleotide diversity ($\pi$) was 0.0004 for COI and 0.0001 for 16 S.

Phylogenetic analyses were performed on a combined alignment (1034 bp) in which we identified five haplotypes (H20-H24; Table 1). Haplotype H20 was common and occurred in all but two of the samples tested (not observed in samples *WW-1* and *P-1*). Subsequent analyses included sequences determined by Iorgu et al.[42] for *M. acervorum* and further 11 congeneric individuals. The genetic distance among selected *Myrmecophilus* species varied between 0.028 and 0.246 (the lowest value of *K2P* distance was between *M. fuscus* and *M. gallicus* and the highest between *M. fuscus* and *M. manni*) (Table 2). In turn, the average intraspecific variation ranged from $0.006 \pm 0.002$ for *M. nonveilleri* to $0.012 \pm 0.002$ for *M. acervorum*. Thus, we additionally calculated the values of the *K2P* distance separately for the *M. acervorum* samples studied by Iorgu et al.[42] and those collected in this study (Table 3). In this analysis, the distance between the two *M. acervorum* subgroups was estimated at 0.020. Distances within the subgroups were smaller ($0.005 \pm 0.001$ for the samples studied by Iorgu et al.[42] and $0.002 \pm 0.001$ for the samples collected in this study). We also calculated the *K2P* distances among all the concatenated haplotypes (Supplementary Table S1). The highest value (0.028) was recorded between haplotype H7 and haplotypes H21-H24. In addition, we calculated the *K2P* genetic distances based only on the barcode fragment of the *cox1* mitochondrial gene. The overall *K2P* distance within *M. acervorum* was $0.035 \pm 0.004$, but after separation into two groups according to their geographic origin, these values were $0.002 \pm 0.001$ for the samples collected in the present study and $0.041 \pm 0.005$ for the samples collected by Iorgu et al.[42]. The genetic distance between these two groups was $0.035 \pm 0.005$.

| Sample | Code | Mitochondrial markers | | | Nuclear marker |
| | | COI | 16 S | Concatenated | EF1α |
|---|---|---|---|---|---|
| Zienki 1 | Z-1 | $H_C1(2)$ | $H_S1(2)$ | H20(2) | $H_E3(1)$ |
| Zienki 2 | Z-2 | $H_C1(4)$ | $H_S1(1)$ | H20(1) | $H_E3(1)$ |
| Zienki 3 | Z-3 | $H_C1(4)$ | $H_S1(1)$ | H20(1) | $H_E3(1)$ |
| Brus 1 | B-1 | $H_C1(4)$ | $H_S1(2)$ | H20(2) | $H_E2(1)$, $H_E3(1)$ |
| Brus 2 | B-2 | $H_C1(2)$ | $H_S1(1)$ | H20(1) | $H_E2(1)$ |
| Brus 3 | B-3 | $H_C1(5)$ | $H_S1(1)$ | H20(1) | $H_E3(1)$ |
| Lake Czarne 1 | CL-1 | $H_C1(4)$ | – | – | – |
| Lake Czarne 2 | CL-2 | $H_C1(1)$ | – | – | – |
| Lake Czarne 3 | WW-1 | $H_C1(1)$, $H_C2(2)$ | $H_S1(1)$ | H21(1) | $H_E3(1)$ |
| Lake Czarne 4 | WW-2 | $H_C1(5)$ | $H_S1(2)$, $H_S2(1)$ | H20(1), H22(1) | $H_E1(1)$, $H_E2(1)$ |
| Wielkopole | WP | $H_C1(4)$ | $H_S1(3)$ | H20(3) | – |
| Łańcuchów | LN | $H_C1(1)$ | $H_S1(1)$ | H20(1) | – |
| Łowiszów | LW | $H_C1(1)$ | $H_S1(1)$ | H20(1) | – |
| Wólka Wytycka | DB | $H_C1(2)$ | $H_S1(2)$ | H20(2) | – |
| Pieszowola 1 | P-1 | $H_C3(2)$ | $H_S1(1)$ | H23(1) | – |
| Pieszowola 2 | P-2 | $H_C1(4)$, $H_C3(1)$ | $H_S1(3)$ | H20(2), H23(1) | – |
| Pieszowola 3 | P-3 | $H_C3(4)$ | $H_S1(4)$ | H23(4) | – |
| Orzechów 1 | O-1 | $H_C1(3)$ | $H_S1(3)$ | H20(1) | $H_E2(1)$, $H_E3(1)$ |
| Orzechów 2 | O-2 | $H_C1(5)$ | $H_S1(5)$ | H20(5) | $H_E3(2)$ |
| Orzechów 3 | O-3 | $H_C1(3)$ | $H_S1(2)$ | H20(2) | – |
| Orzechów 4 | O-4 | $H_C1(5)$ | $H_S1(5)$ | H20(5) | – |
| Rogóźno 1 | R-1 | $H_C1(2)$, $H_C4(2)$ | $H_S1(5)$ | H20(2), H24(2) | $H_E3(1)$ |
| Rogóźno 2 | R-2 | $H_C1(5)$ | $H_S1(3)$ | H20(3) | – |

**Table 1.** Haplotypes identified for mitochondrial and nuclear markers.

| | M. acervorum | M. gallicus | M. fuscus | M. cf. nonveilleri | M. nonveilleri | M. balcanicus | M. manni (outgroup) |
|---|---|---|---|---|---|---|---|
| M. acervorum | **0.012 ± 0.002** | 0.013 | 0.016 | 0.013 | 0.012 | 0.012 | 0.017 |
| M. gallicus | 0.141 | – | 0.007 | 0.013 | 0.012 | 0.012 | 0.018 |
| M. fuscus | 0.138 | 0.028 | – | 0.019 | 0.017 | 0.016 | 0.023 |
| M. cf. nonveilleri | 0.153 | 0.141 | 0.182 | – | 0.006 | 0.013 | 0.018 |
| M. nonveilleri | 0.143 | 0.128 | 0.155 | 0.035 | **0.006 ± 0.002** | 0.012 | 0.017 |
| M. balcanicus | 0.134 | 0.127 | 0.132 | 0.142 | 0.130 | **0.007 ± 0.002** | 0.016 |
| M. manni (outgroup) | 0.225 | 0.245 | 0.246 | 0.239 | 0.227 | 0.218 | – |

**Table 2.** Genetic distance among *Myrmecophilus* taxa based on K2P distances for concatenated mitochondrial markers (COI and 16S). Standard error estimates are shown above the diagonal (bold). Values of the average intraspecific distance are shown on the diagonal.

| | M. acervorum (Iorgu et al. (2023)) | M. acervorum (this study) | M. gallicus | M. fuscus | M. cf. nonveilleri | M. nonveilleri | M. balcanicus | M. manni (outgroup) |
|---|---|---|---|---|---|---|---|---|
| M. acervorum (Iorgu et al. (2023)) | **0.005 ± 0.001** | 0.004 | 0.013 | 0.016 | 0.014 | 0.013 | 0.013 | 0.017 |
| M. acervorum (this study) | 0.020 | **0.002 ± 0.001** | 0.013 | 0.016 | 0.013 | 0.013 | 0.012 | 0.016 |
| M. gallicus | 0.140 | 0.145 | – | 0.007 | 0.013 | 0.012 | 0.012 | 0.018 |
| M. fuscus | 0.137 | 0.1408 | 0.028 | – | 0.019 | 0.017 | 0.016 | 0.023 |
| M. cf. nonveilleri | 0.153 | 0.153 | 0.141 | 0.182 | – | 0.006 | 0.012 | 0.017 |
| M. nonveilleri | 0.143 | 0.143 | 0.128 | 0.155 | 0.035 | **0.006 ± 0.002** | 0.012 | 0.017 |
| M. balcanicus | 0.135 | 0.132 | 0.127 | 0.132 | 0.142 | 0.130 | **0.007 ± 0.002** | 0.016 |
| M. manni (outgroup) | 0.225 | 0.226 | 0.245 | 0.246 | 0.239 | 0.227 | 0.218 | – |

**Table 3.** Genetic distance among *Myrmecophilus* taxa based on K2P distances for concatenated mitochondrial markers (COI and 16S), after the separation of *Myrmecophilus acervorum* subgroups. Standard error estimates are shown above the diagonal (bold). Values of the average intraspecific distance are shown on the diagonal.

Additionally, we used concatenated haplotypes as an input data for a sequences-based species delineation attempt according to the protocol described by Iorgu et al.[42] (Supplementary Table S2). At the 3% value of the barcode gap, we determined six initial and seven recursive partitions. The obtained pattern of separation was similar to previously determined, i.e. the distinctiveness of *M. balcanicus*, *M. gallicus*, and *M. nonveilleri* was supported, as well as internal split within *M. nonveilleri*. Interestingly, we also observed the clear separation within *M. acervorum* and as a result we determined three separate inner groups, including: (1) haplotypes H1-H6, H8, and H10 determined by Iorgu et al.[42] for *M. acervorum* samples collected in Romania, Hungary, Bulgaria, and Germany; (2) haplotype H7 determined for *M. acervorum* sample collected in Pobit Kamak (Bulgaria); and (3) haplotypes H20-H24 determined for samples collected in western Polesie (Poland).

The phylogenetic trees constructed for the concatenated data according to the BI and ML approaches had similar topologies with high branch support. The topology was congruent with that previously determined by Iorgu et al.[42] and revealed well-supported groups (Fig. 4). Additionally, two well-separated clades were observed within *M. acervorum*. These clades grouped haplotypes determined for the previously studied samples and those identified for the samples collected in this study. The populations of *M. acervorum* from Poland are most probably parthenogenetic[41]. Males were only historically recorded in Poland, but in a different region (the Pomeranian Lakeland in northern Poland)[51]. Samples collected from parthenogenetic populations of *M. acervorum* occurring in Germany (with haplotypes H1-H3 and H8-H10) were also included in the previous study. However, this feature was not reflected in the pattern of clade separation (Fig. 4).

Median-joining network (Fig. 5) was determined for dataset of concatenated mitochondrial sequences. Thus, two haplotypes determined previously for samples collected from parthenogenetic populations occurring in Germany (i.e. haplotypes H8 and H9) were not included in this analysis. In turn, haplotypes H1 and H10 had the same sequence (marked with an asterisk in Fig. 5). The topology of determined network revealed the presence of two well-separated haplogroups (Fig. 5). The pattern of this separation was congruent with that previously observed in the ML tree topology, and the *M. acervorum* reproductive system (sexual vs. parthenogenetic) was not the grouping factor.

### Nuclear marker—EF1α

This nuclear marker was successfully amplified for 15 individuals of *M. acervorum* collected from 11 sampling sites (Table 1). Analysis of sequence variation (483 bp) revealed 3 unique variants marked as $H_E1$-$H_E3$. Sequence $H_E3$ was common and occurred in all but two of the samples (was not found in Brus 2 and Lake Czarne 4; Table 1). Further analyses included sequences determined by Hsu et al.[45] for another 7 congeners, as well as sequences determined for two specimens collected in Romania and previously analysed by Iorgu et al.[42] (specimen MA4.1 from Lepşa and specimen MA59 from Magura Baita).

The genetic distance among the *Myrmecophilus* species varied between 0.024 and 0.417 (Table 4), and the average intraspecific variation ranged from $0.001 \pm 0.001$ (*M. antilucanus*) to $0.009 \pm 0.004$ (*M. mayaealberti*). The intraspecific distance calculated for *M. acervorum* was $0.004 \pm 0.002$ and the pairwise *K2P* distance calculated for this species was from 0.002 to 0.006. The highest value was noted for $H_E1$-$H_E2$, $H_E1$-$H_E4$, and $H_E2$-$H_E5$ haplotype pairs (Supplementary Table S3). The value calculated for two *M. acervorum* subgroups (this study vs. previous study) was $0.005 \pm 0.003$.

The maximum-likelihood phylogeny reconstructed on the nuclear marker showed well-supported groups but a weak structure within *M. acervorum* (Fig. 6). The pattern of separation revealed at the mitochondrial level was not fully supported by nuclear data.

### *Wolbachia* prevalence in the samples of *Myrmecophilus acervorum*

A positive sign of *Wolbachia* infection was present in all but one of the *M. acervorum* samples tested (in 101 out of 141 individuals tested collected in 22 out of 23 samples). Only in the sample collected at Łowiszów (LW), the endosymbiont was not detected (Table 5).

Four high-quality PCR products were sequenced and 561 bp long reads were obtained. Among them, two haplotypes were identified ($H_W1$ was observed at Zienki 3, Brus 3, and Lake Czarne 1; haplotype $H_W2$ was observed only at Lake Czarne 1). These haplotypes were analysed with sequences obtained from the GenBank, revealing a high percentage of identity with the previously determined 16 S haplotypes of known *Wolbachia* endosymbionts infecting other insects (Supplementary Table S4).

The identified 16S haplotypes differed from a single haplotype determined previously for *Wolbachia* infecting sexual populations of *M. acervorum*. The values of the *p*-distance determined for $H_W1$-$H_W2$ and $H_W1$-$H_W3$ pairs were 0.016 and 0.018, respectively (Supplementary Table S5). Similar values of the genetic distance were also found e.g. between haplotypes belonging to supergroups A and E (e.g. 0.011 for KT319093-AJ575104 pair) or between haplotypes representing supergroups C and J (0.014 for AJ010276- AJ548802 pair) (Supplementary Table S5). The ML tree topology revealed that *Wolbachia* haplotypes $H_W2$ and $H_W3$ determined for bacteria infecting parthenogenetic populations of *M. acervorum* occurring in western Polesie grouped together with sequences identified for *Wolbachia* belonging to supergroup A (Fig. 7). In turn, haplotype $H_W1$ determined previously for endosymbionts identified in sexual populations of *M. acervorum* was found within clade including sequences of *Wolbachia* belonging to supergroup B (Fig. 7).

### Discussion

Knowledge of the biology, ecology and also the morphological and genetic diversity of the myrmecophilous cricket *M. acervorum* remains limited. Our analyses indicated three unusual phenomena observed in populations inhabiting *Lasius* nests located in western Polesie (Poland) compared to previously tested samples collected in southern Europe: (i) the presence of potentially diagnostic characteristics in the external structures of *M. acervorum* eyes differentiating these two groups of samples, (ii) genetic distinctiveness identified between given
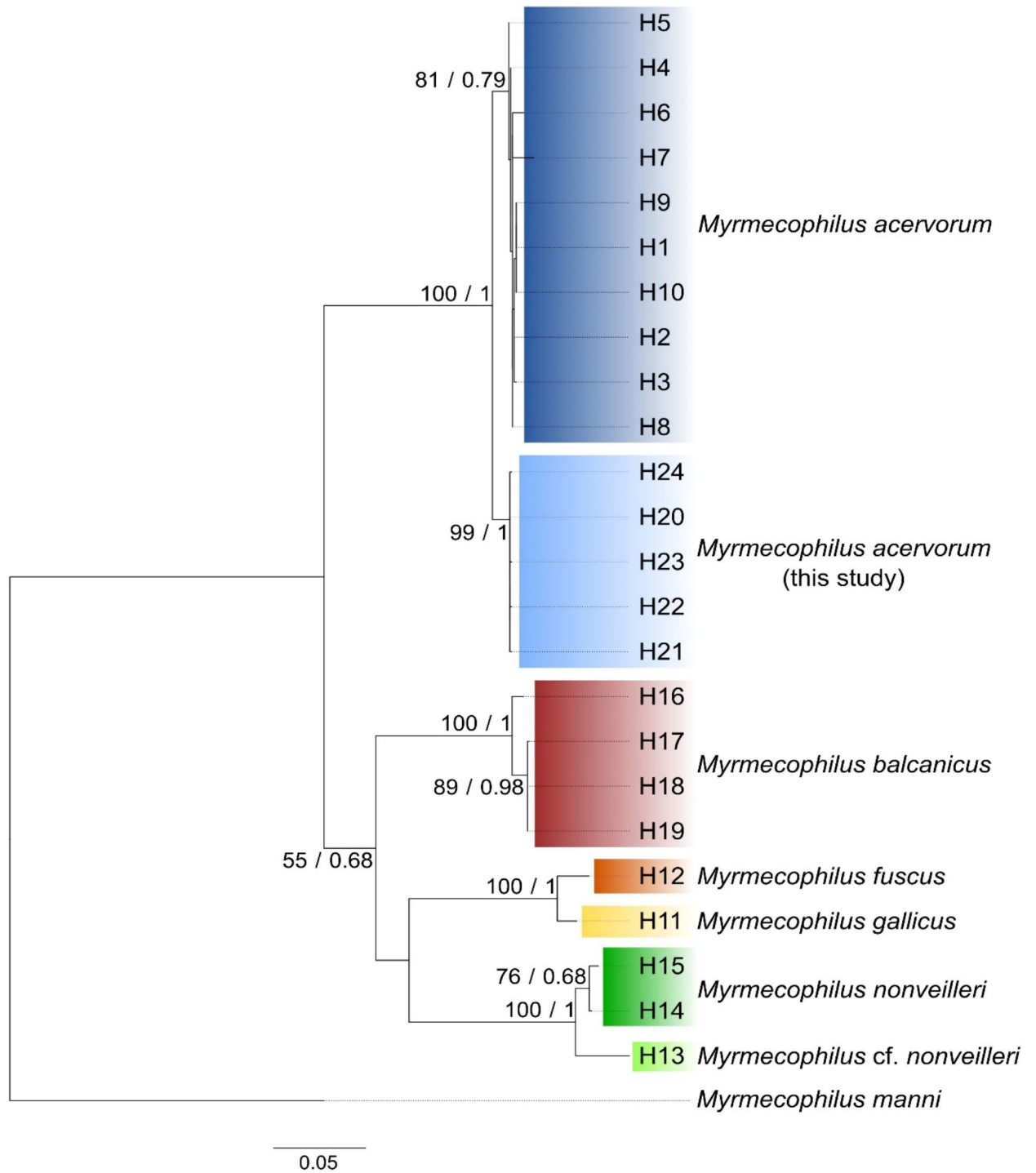
**Fig. 4.** Maximum likelihood tree constructed with the concatenated COI and 16S mitochondrial markers of *Myrmecophilus acervorum* and selected species belonging to the same genus. The numbers at the nodes indicate bootstrap (values < 50 are not shown) and posterior probabilities values.

groups of samples determined at the mitochondrial level with subtle support by data determined at the nuclear level, and (iii) the presence of endosymbiotic *Wolbachia* bacteria infecting the parthenogenetic populations of *M. acervorum* in western Polesie.

### Morphological characteristics
The taxonomic diagnostics of insects is based on their external morphology. For this reason, ever since the early development of the systematics of these arthropods, the classic ways to identify their systematic position were
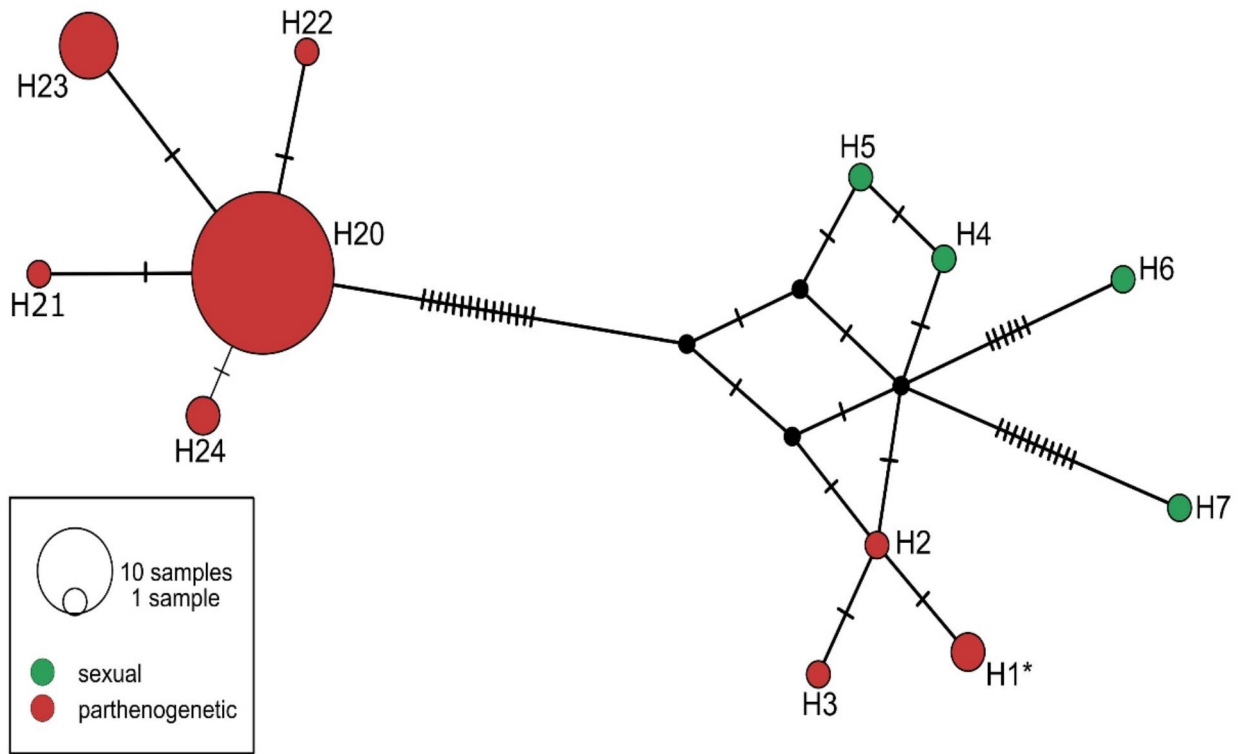
**Fig. 5**. Median-joining network of *Myrmecophilus acervorum*, based on concatenated sequences of COI and 16S mitochondrial markers. The circle size reflects haplotype frequency (for the haplotypes determined in this study). The black circles represent intermediate haplotypes not encountered in the sampling. The small perpendicular lines represent mutational steps. Haplotypes H1 and H10 had the same sequence. Thus, haplotype H1 is marked with an asterisk.

| | *M. quadrispinus* | *M. hebardi* | *M. antilucanus* | *M. mayaealberti* | *M. albicinctus* | *M. dubius* | *M. americanus* | *M. acervorum* | *G. orientalis* |
|---|---|---|---|---|---|---|---|---|---|
| *M. quadrispinus* | **0.002 ± 0.001** | 0.020 | 0.020 | 0.021 | 0.038 | 0.021 | 0.040 | 0.031 | 0.043 |
| *M. hebardi* | 0.169 | **0.005 ± 0.002** | 0.019 | 0.007 | 0.039 | 0.009 | 0.038 | 0.032 | 0.045 |
| *M. antilucanus* | 0.158 | 0.144 | **0.001 ± 0.001** | 0.019 | 0.038 | 0.020 | 0.039 | 0.031 | 0.043 |
| *M. mayaealberti* | 0.177 | 0.024 | 0.147 | **0.009 ± 0.004** | 0.038 | 0.009 | 0.037 | 0.032 | 0.044 |
| *M. albicinctus* | 0.398 | 0.394 | 0.393 | 0.391 | **0.002 ± 0.002** | 0.038 | 0.011 | 0.037 | 0.038 |
| *M. dubius* | 0.184 | 0.036 | 0.148 | 0.037 | 0.399 | – | 0.038 | 0.032 | 0.044 |
| *M. americanus* | 0.417 | 0.392 | 0.399 | 0.388 | 0.047 | 0.396 | – | 0.036 | 0.040 |
| *M. acervorum* | 0.325 | 0.321 | 0.322 | 0.319 | 0.391 | 0.314 | 0.374 | **0.004 ± 0.002** | 0.041 |
| *G. orientalis* | 0.502 | 0.513 | 0.504 | 0.507 | 0.412 | 0.521 | 0.419 | 0.495 | – |

**Table 4**. Genetic distance among *Myrmecophilus* taxa and *Gryllotalpa orientalis* (outgroup) based on K2P distances for the EF1α nuclear marker. Standard error estimates are shown above the diagonal (bold), values of the average intraspecific distance are shown on the diagonal.

constructed on the basis of selected morphological characters[52–54]. Nowadays, morphological analysis remains a relevant approach in studies on integrative taxonomy and systematics of insects, including Orthoptera[55–57]. Important diagnostic characters in members of the genus *Myrmecophilus* include the coloration, the number, size and shape of spines on the tibia and the hindleg pretarsus, the body chaetotaxy and the appearance of the female subgenital plate[42,46,58].

Comparative analysis of the morphological structure of 23 specimens of *M. acervorum* from Poland (20) and Romania (3) generally revealed subtle differences in a few key structures, e.g. the structure of the components of the female's ovipositor, the structure of the spines on the tibia and the hindleg pretarsus, and the chaetotaxy of the insect's body. However, since only a small number of specimens from Romania were studied, the data obtained to date cannot be binding, which suggests the need for further analyses based on a much larger sample
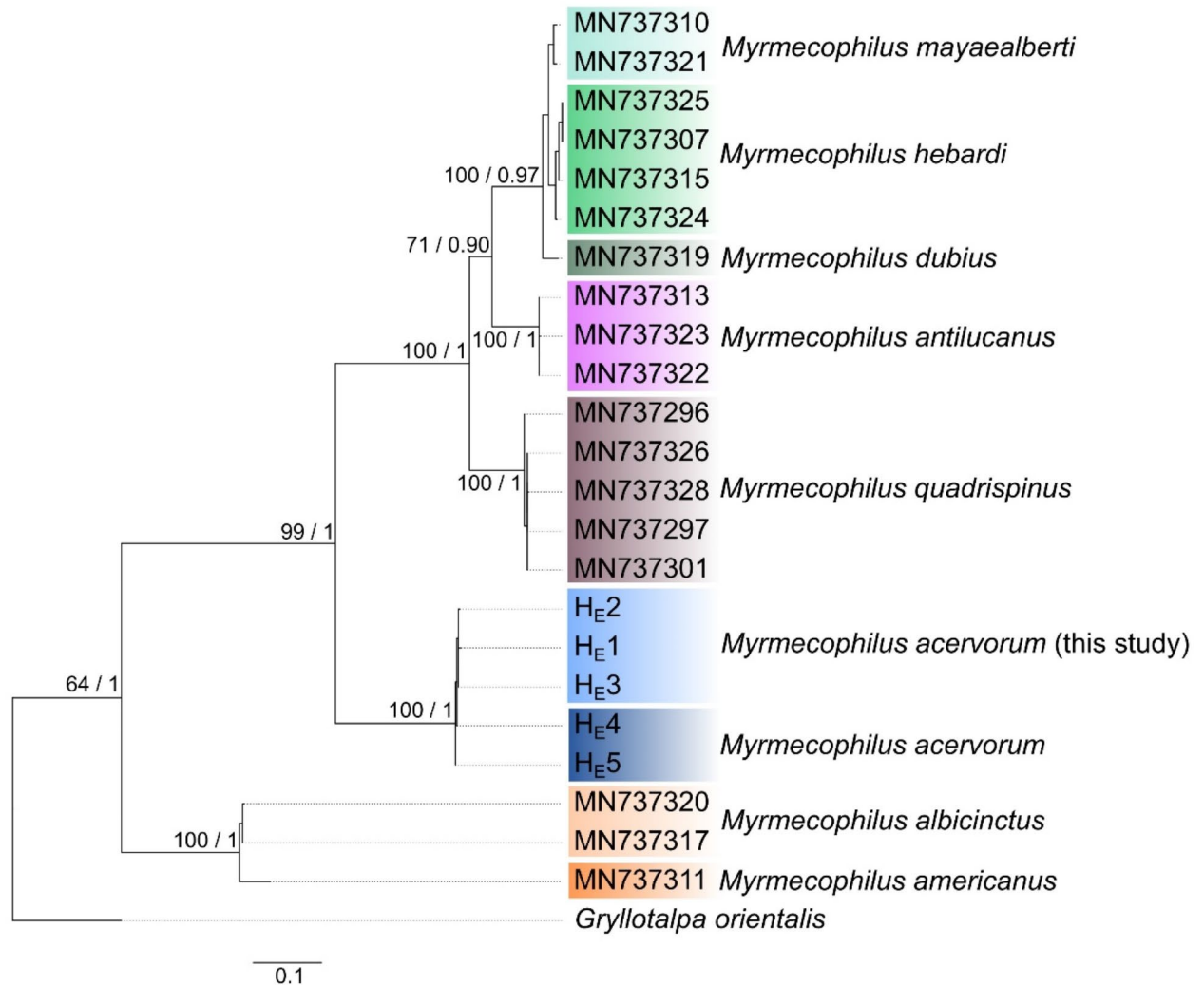
**Fig. 6**. Maximum likelihood tree constructed with the EF1α nuclear marker of *Myrmecophilus acervorum* and selected species belonging to the same genus. The numbers at the nodes indicate bootstrap (values < 50 are not shown) and posterior probabilities values.

of research material. In contrast, distinct differences were found in the eye structure. The appearance of the eyes in the Romanian specimens corresponds with a character described by Panzer (1799) in the original description of the species *M. acervorum* (then *Blatta acervorum*): *oculi parvi subprominuli nitidi*, which should be translated as "inconspicuous, shining eyes". In the Polish specimens, the eyes are matt and covered with a distinctive microstructure with characteristic tubercules. The contemporary literature makes no mention of this character, and what references there are to the structure of the eyes concern solely their presence in a partially reduced form[53] and sometimes their coloration[59].

### Patterns of the genetic diversity at the mitochondrial and nuclear levels

Molecular markers (e.g. mitochondrial fragments) have been frequently applied in studies on orthopteran taxonomy to resolve problems arising from observed phenotypic plasticity, the lack of descriptions of immature stages, and cryptic species complexes[60]. Some molecular markers are considered to be neutral, i.e. their variants confer no fitness advantage (e.g. microsatellites). In the past, also mtDNA was considered as a source of neutral markers. However, performed studies showed that mtDNA is not always neutrally evolving[61]. More recently, genome-wide analyses depicted that most nucleotide sites experience fluctuating selection with mean selection coefficients near zero[62]. However, combining mtDNA and nuclear markers, as well as a better understanding their properties, can improve the power of molecular data.

DNA barcoding is one of the most emblematic contemporary techniques for molecular taxonomy. This approach involves analyses of the genetic distance among sequences of the 5' end-region of the mitochondrial gene cytochrome *c* oxidase subunit I with diagnosing and delimiting species[63]. According to this concept, distinct species can be identified based on the difference between the maximum intraspecific and the minimum interspecific genetic distance, i.e. the barcode gap[64]. The majority of genera within the order Orthoptera exhibit

| Sample | Code | Number of individuals screened | Number of positive results/Wolbachia prevalence [%] |
|---|---|---|---|
| Zienki 1 | Z-1 | 2 | 1/50 |
| Zienki 2 | Z-2 | 2 | 2/100 |
| Zienki 3 | Z-3 | 8 | 6/75 |
| Brus 1 | B-1 | 3 | 3/100 |
| Brus 2 | B-2 | 2 | 1/50 |
| Brus 3 | B-3 | 9 | 7/78 |
| Lake Czarne 1 | CL-1 | 4 | 3/75 |
| Lake Czarne 2 | CL-2 | 1 | 1/100 |
| Lake Czarne 3 | WW-1 | 3 | 3/100 |
| Lake Czarne 4 | WW-2 | 14 | 13/93 |
| Wielkopole | WP | 20 | 18/90 |
| Łańcuchów | LN | 1 | 1/100 |
| Łowiszów | LW | 1 | –/0 |
| Wólka Wytycka | DB | 2 | 2/100 |
| Pieszowola 1 | P-1 | 2 | 1/50 |
| Pieszowola 2 | P-2 | 10 | 6/60 |
| Pieszowola 3 | P-3 | 6 | 4/67 |
| Orzechów 1 | O-1 | 3 | 2/67 |
| Orzechów 2 | O-2 | 5 | 3/60 |
| Orzechów 3 | O-3 | 4 | 3/75 |
| Orzechów 4 | O-4 | 10 | 4/40 |
| Rogóźno 1 | R-1 | 19 | 12/63 |
| Rogóźno 2 | R-2 | 10 | 5/50 |

**Table 5**. *Wolbachia* prevalence determined for *Myrmecophilus acervorum* samples.

interspecific distances from 7 to 15% and intraspecific distances of less than 5%[60]. In the case of the genus *Myrmecophilus*, the interspecific distance based on COI sequences exceed 13%, and intraspecific distances are less than 1%[42]. In the present study, the COI-based genetic distance within *M. acervorum* was 3.5%. After data separation based on a tree topology, the genetic distance between the two groups took the same value ($0.035 \pm 0.005$), while the values within the groups were 0.24% for samples tested by Iorgu et al.[42] and 0.2% for samples tested in the present study. The genetic distance between groups raises further questions about the reasons for the observed divergence within *M. acervorum*. Firstly, the value obtained is higher than the interspecific genetic distance determined for *M. fuscus* and *M. gallicus* (2.66%), two morphologically well-supported species. Thus, taking into account the morphological distinctiveness of the two groups of *M. acervorum* samples based on external eye structures, it is possible that the two identified lineages belong to different species. On the other hand, an alternative explanation should also be considered. Although morphological characteristics of orthopteran eyes are included in species descriptions[65–67], the references to microstructures present or absent on their surface are often unavailable. Thus, we cannot state unequivocally that this trait is diagnostic within the order Orthoptera or within its genera. However, even if these eye characteristics are not diagnostic, the level of genetic difference may suggest the existence of cryptic species within *M. acervorum*. A similar case has been described for *M. nonveilleri*. There, two separate lineages were identified, and the distance between them was estimated at 5.03% on the COI sequences and 3.38% on the concatenated COI and 16S markers. In the present study, we also analysed concatenated mitochondrial data (COI + 16S), and the distance between the two *M. acervorum* groups was 2%. However, it is worth noting that the genetic distances among haplotypes belonging to these two groups were even higher (up to 2.8%).

Observed genetic differences within and among *Myrmecophilus* species were also confronted with the result of the preliminary sequence-based delineation test based on concatenated haplotypes. Interestingly, the test confirmed the clear separation within *M. acervorum* and the presence of three separate groups including: (1) haplotypes determined by Iorgu et al.[42] for *M. acervorum* samples collected in Romania, Hungary, Bulgaria, and Germany; (2) haplotype H7 determined for *M. acervorum* sample collected in Pobit Kamak (Bulgaria); and (3) haplotypes determined for samples collected in the present study in western Polesie (Poland). We suspect that *M. acervorum* could be recognized as a complex of cryptic species. Evidences for such phenomena were identified e.g. for *Myrmecophilus* species associated with *Anoplolepis gracilipes*[45]. Nevertheless, in the case of diversity observed in *M. acervorum*, more samples and specimens collected across its distribution range are needed to perform detailed analyses.

In turn, the nuclear marker (EF1α) is a conserved protein-coding gene that has previously been reported to be useful not only in phylogenetic analyses at the intra- and interordinal levels, but also for resolving relationships among congeneric insect species[68–70]. Moreover, EF1α was analysed together with the mitochondrial marker *cytb* in order to resolve phylogenetic relationships among *Myrmecophilus* species associated with *Anoplolepis gracilipes* ants in the Indo-Pacific region[45]. The intraspecific genetic distance calculated for the nuclear marker was comparable to values determined for other *Myrmecophilus* species. Although the haplotypes determined
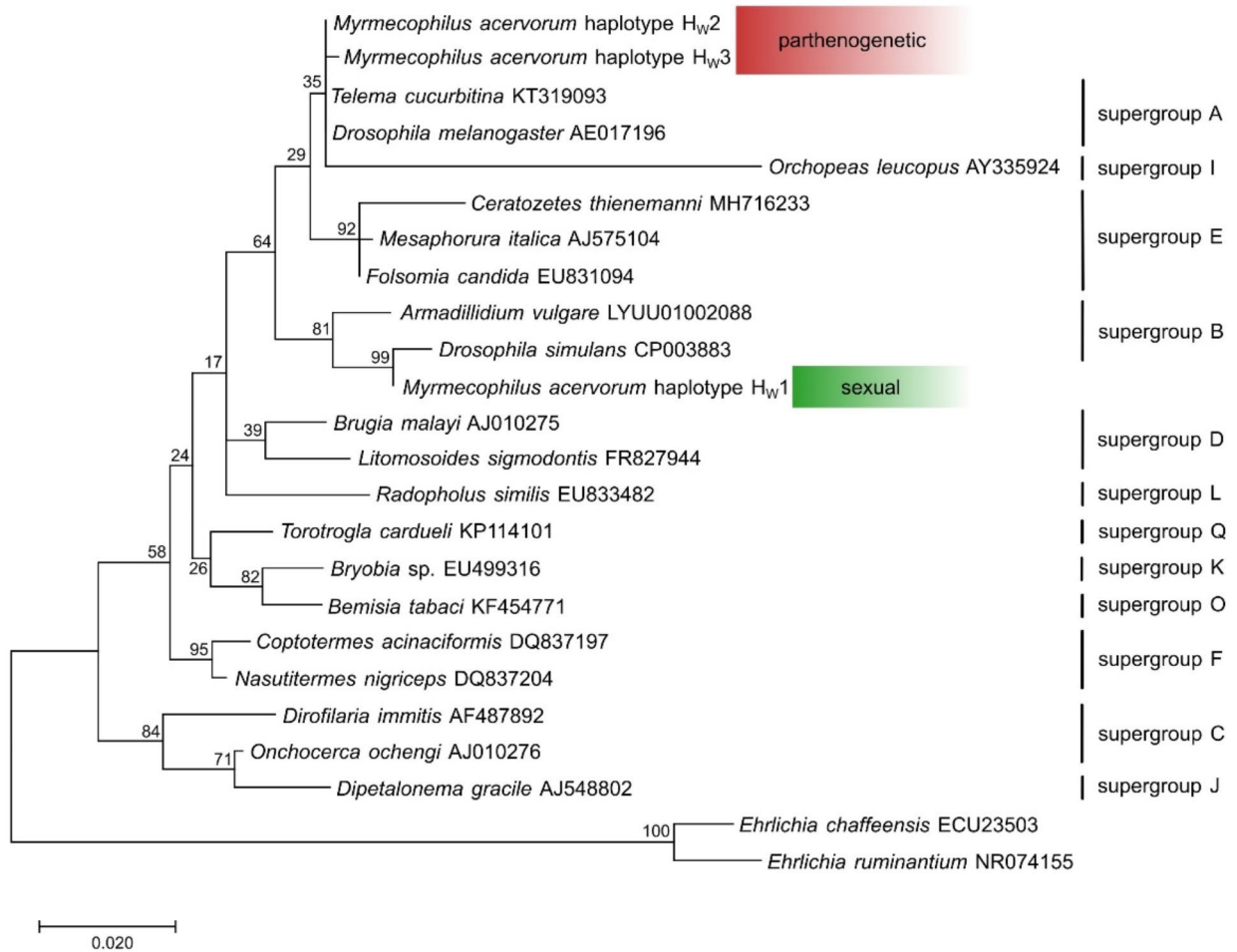
**Fig. 7**. Maximum likelihood tree constructed with the 16S molecular marker of *Wolbachia* infecting *Myrmecophilus acervorum* and other host species. The references of both *M. acervorum* reproductive system and corresponding *Wolbachia* supergroups were added. The numbers at the nodes indicate bootstrap values based on 1000 replicates. The 16S sequences obtained for *Ehrlichia ruminantium* and *E. chaffeensis* were used as the outgroup.

for specimens collected in Romania and Poland did not form distinct clades, as was earlier the case for mitochondrial data, the haplotypes identified for specimens collected in western Polesie seem to have originated from those identified for samples from southern Europe. However, taking into account the low bootstrap values within the identified clade, our explanation should be treated with caution, pending more detailed analysis. Nevertheless, we suggest that the observed phylogenetic pattern may be interpreted as a result of the incomplete sorting of nuclear lineages, potentially indicating an ongoing speciation process. On the other hand, it could also be an effect of introgression resulting from secondary contact of formerly peripatric or allopatric mitochondrial lineages. A similar complex pattern of the genetic diversity at both mitochondrial and nuclear levels has been described for the water louse *Asellus aquaticus*, widely distributed across Europe, suggesting its possible cryptic speciation[71]. However, in the case of *M. acervorum*, more samples collected at a wider spatial scale should be included in any future study to test the proposed hypotheses.

### *Wolbachia* prevalence and its genetic diversity

All 141 individuals of *M. acervorum* collected in western Polesie (Poland) were tested for *Wolbachia* infections: the endosymbiont was found in 101 of them (72%). *Wolbachia* were identified in all but one of the samples obtained from parthenogenetic populations of ant crickets (22 out of 23). This result contradicts the recently published observations described by Iorgu et al.[42], who found *Wolbachia* only in biparental populations of *M. acervorum*. In that group, the endosymbiont was present at a lower frequency (5 out of 30 specimens tested; 17% of the endosymbiont's prevalence) than determined here for parthenogenetic populations. A lower frequency in sexual populations of *M. acervorum* could be associated with *Wolbachia*-mediated mechanisms induced in its host (for instance, endosymbionts causing male-killing are expected to occur at lower frequencies)[72]. In turn, a

high *Wolbachia* prevalence in the parthenogenetic population of *M. acervorum* tested in the present study may be strictly related to its vertical transmission via egg cytoplasm[73].

Although our study was focused primarily on testing *M. acervorum* samples for the presence of *Wolbachia*, the results shed light on the genetic diversity of this endosymbiont. A comparative analysis with data deposited in GenBank confirmed the > 99% similarity of *Wolbachia* found in *M. acervorum* to endosymbionts infecting other insects (e.g. the flies *Stomoxys calcitrans* and *Melieria omissa*, the moth *Schoenobius gigantella* or the hoverfly *Microdon myrmicae*). Moreover, *Wolbachia* infecting parthenogenetic populations of *M. acervorum* differed significantly from endosymbionts previously identified in sexual populations (1.6% difference between the sequences $H_W1$ (found in sexual populations) and $H_W2$ (found in parthenogenetic populations), and 1.8% between the sequences $H_W1$ and $H_W3$ (also found in parthenogenetic populations)). In turn, both *Wolbachia* haplotypes identified in the parthenogenetic populations tested in the present study differ from each other to a lesser extent (0.2%).

The phylogeny reconstruction including also sequences representing *Wolbachia* from various supergroups clearly confirmed a genetic differentiation between endosymbionts infecting populations of *M. acervorum* occurring in western Polesie and Romania. Haplotype $H_W1$ determined previously clustered with sequences identified for *Wolbachia* belonging to supergroup B, while newly found haplotypes $H_W2$ and $H_W3$ grouped with sequences representing supergroup A. These two *Wolbachia* supergroups are frequently observed in insects[37], also among species representing order Orthoptera[74]. For example, species belonging to genus *Hemiandrus* (Family Anostostomatidae) are infected with supergroup A or B but none of the tested species was identified to simultaneously harbour bacteria from both supergroups. Thus, we suggest that both groups of *M. acervorum* populations (i.e. parthenogenetic from western Polesie and sexual from Pașcani, Coșereni and Lepșa sampling sites in Romania) were independently infected with genetically divergent endosymbionts. Moreover, we cannot exclude that *Wolbachia* infecting *M. acervorum* and belonging to separate supergroups differ in rates of transfer or induced mechanisms manipulating host's reproduction. Those characteristics might explain differences in endosymbiont frequency described above. Interestingly, the parthenogenetic populations belonging to the western clade determined by Iorgu et al.[42] remain uninfected with *Wolbachia*. The background of this phenomenon remains unsolved. Thus, more comprehensive analyses are needed to investigate both the genetic diversity of *Wolbachia* infecting *M. acervorum* within its distribution range, as well as endosymbiont transmission among host's parthenogenetic and sexual populations (e.g. based on MLST system or phylogenomic approach)[75,76].

### Proposed scenarios inducing observed diversity within *Myrmecophilus acervorum*

*Myrmecophilus acervorum* is an Orthopteran highly adapted to co-existence with ants. Inhabiting the distinctive habitat of an ant's nest, it possesses a series of adaptive morphological characters, e.g. partially reduced eyes, the lack of wings and small body size[53,59], which account for its poor dispersal abilities. Another important feature of this species is its reproductive strategy based on thelytokous parthenogenesis, which occurs in populations over a wide area of its distribution range[42]. Parthenogenesis enables insects to survive in unpropitious conditions and facilitates the faster colonization of new terrain[77]. Speciation after the transition to parthenogenesis is considered to be very rare, and so are reversals to sexuality[78].

Parthenogenesis is rare among Orthoptera[79,80], and known cases relate not only to species living in highly distinctive habitats, such as cave crickets of the genus *Hadenoecus* Scudder, 1863[81], but also to those inhabiting less specialized niches, such as the flightless bush cricket *Poecilimon intermedius* Fieber, 1853, which is associated with grassy habitats[82] or grasshopper *Saga pedo* Pallas, 1771 inhabiting various habitats (preferably areas with dry summers and mild winters, e.g. grasslands, meadows, pastures, or gorges)[83]. According to Kirkendall and Normark[84], its inability to fly and its consequent poor dispersal abilities might be recompensed by parthenogenesis.

It is known that following the last glaciation, parthenogenesis enabled many species to colonize available terrain faster, which is probably why they are now more widely distributed than their heterosexual counterparts[82,85,86]. *Myrmecophilus acervorum* is by a large margin the most widely distributed member of the genus in Europe[87], far more than other, heterosexual *Myrmecophilus* spp. that inhabit only the Balkans and other regions in southern Europe. This ant cricket is therefore a perfect example of the crucial role of parthenogenetic reproduction in the effective colonization of new terrain by living organisms. Moreover, there are further examples of this phenomenon among other orthopterans. The distribution of most members of the genus *Poecilimon* Fischer, 1853, with populations in which males are present, is restricted to the southern Balkans, Turkey and the Caucasus, whereas the parthenogenetic *P. intermedius* occurs from central Europe all the way to western China[82,88]. Likewise, the parthenogenetic *Saga pedo* Pallas, 1771, is widely distributed in southern Europe, and also in Russia and China, whereas the ranges of the other heterosexual species from this genus are limited to the Balkans[83].

Analysis of the present-day distribution of *M. acervorum* in the context of the postglacial migration history of the European fauna suggests that the Balkan region was probably the centre of occurrence and the refugial area from which Europe became colonized by *M. acervorum*. This hypothesis is also supported by data given by Varga[89], who regards the areas to the south and east of the Carpathians as key to the survival and evolution of autochthonous lineages of poorly mobile species. In this part of Europe, the Carpathians was the only region during the last glaciation that was not covered by the ice sheet, which enabled the survival of many species of flora and fauna[90,91].

On the basis of literature data describing the directions of post-glacial migrations of species of flora and fauna from their glacial refugia in southern Europe[92], one can set up a probable hypothesis regarding the directions of northwards dispersal of *M. acervorum* during the post-glacial period and assume that recolonization routes were generally congruent with the well-known migration corridors. Nevertheless, the exact locations of glacial refugia of *M. acervorum* remain unknown and more comprehensive, phylogeographic approach is needed to determine

them. On the one hand, we may suggest that the glacial refugium was likely located in Carpathian region, where sexual populations of *M. acervorum* have been identified[42]. In turn, parthenogenetic populations have been broadly observed across species distribution range (i.e. in Bulgaria, western Romania, Hungary, Germany, and Poland). Parthenogenetic forms are better colonizers and thus, could efficiently colonize new areas available during interglacial cycles[93,94]. Postglacial history could support the divergence among populations of *M. acervorum*, giving rise to the separate phylogenetic lineages recorded in this study, and which, moreover, differ in the external structure of the eye. The division at the level of mitochondrial DNA, not explicitly supported by the results of nuclear marker analysis, could be associated with the dynamics of lineage sorting, which is quicker in the case of the mitochondrial genome. Furthermore, the possibility cannot be ruled out that the phylogenetic lineages of *M. acervorum* identified here are in fact hitherto undescribed cryptic species defined as genetically distinct units, which on the basis of their evident morphological similarity, are classified as a single species[95]. This hypothesis is supported by the result of the sequences-based species delineation but needs more data to be confirmed.

On the other hand, the observed intraspecific divergence (observed primarily at the mitochondrial level) could be linked with the presence of *Wolbachia* infecting *M. acervorum*. Endosymbionts detected in sexual populations group with *Wolbachia* belonging to supergroup B, while those found in parthenogenetic populations cluster with *Wolbachia* from supergroup A. The identified diversity could explain the difference in *Wolbachia* frequency within sexual and parthenogenetic populations of *M. acervorum*. We detected a high percentage of endosymbionts in parthenogenetic populations inhabiting western Polesie. That may suggest that in these populations *Wolbachia* is predominantly vertically transmitted, while in sexual populations could induce one of the well-known mechanisms of manipulation of host's reproduction. However, *Wolbachia* has not been found in parthenogenetic populations from Romania and thus, further studies are needed to explain this phenomenon (especially in the context of induced parthenogenesis).

Nevertheless, the presence of endosymbionts infecting *M. acervorum* and belonging to distinct supergroups is in line with the pattern of host's genetic divergence. Like mtDNA, transmission of *Wolbachia* is effected through the maternal lineage and can be associated with the correlated transmission of the symbiont together with its associated mitogenome haplotype[96]. Nonetheless, in the case of *M. acervorum*, we also discovered a distinctive morphological character, the state of which differentiates ant cricket populations in accordance with the separation pattern at the mitochondrial DNA level. To date, no influence on the part of *Wolbachia* has been reported on the variability of diagnostic morphological characters, so the alternative hypothesis outlined above appears to be less probable.

## Conclusions

A range of factors are known, e.g. the isolation of particular populations, climatic conditions, phenotypic plasticity associated with the type of habitat, and genetic drift, which can affect the genetic and morphological diversity of constantly evolving organisms. But where intraspecific diversity is concerned, particular individuals usually differ only in the morphometrics of specific body parts[97,98] or in their coloration[99]. Hence, the significant genetic variability at the mitochondrial DNA level (COI and 16S) found in the present case, and the differentiating morphological character (i.e. eye structure), are almost certainly effects of cryptic species being present within *M. acervorum*. This, in turn, is indicative of ongoing speciation within the populations of this insect, and of simultaneous unfinished lineage sorting at the nuclear DNA level. However, the absence of males evidently limits the effectiveness of a morphological analysis performed solely on the basis of available female specimens as regards establishing their actual taxonomic status. It is therefore essential to continue this research and to establish genetic variability in populations of the ant cricket from all parts of its distribution range, based on both mitochondrial and nuclear markers. In addition, further studies need to focus on detecting and analysing the genetic variability in *Wolbachia* bacteria in both heterosexual and parthenogenetic ant cricket populations. In this way, the hypothesis regarding the independent infection of identified lineages of *M. acervorum* by endosymbionts from separate groups can be tested.

## Methods

### Sample collection

The 141 specimens of *M. acervorum* from Poland used in this research were collected from May to October 2021 in western Polesie (Table 6). The main habitats of the ant crickets in this area were dry pine forests with decaying birch trunks lying on the forest floor, colonized by *Lasius platythorax* or *Formica fusca* ants (Fig. 2). Some specimens were found in different habitats (Table 6). Sampling included preliminary screening of selected nests for the presence of *M. acervorum* individuals. Once ant-loving crickets were found in a particular nest, a sample of nest material (about 1 dm³) was collected and transported to the laboratory. Then, all samples were separately searched and each *M. acervorum* specimen found was preserved in the appropriate dilution of ethanol (i.e. 75% EtOH for analyses of morphology and 99% EtOH for further genetic analyses).

### Microscopic methods

Material selection and a general analysis of specimens (20 from Poland and 3 from Romania) were carried out using an OLYMPUS SZ51 stereoscopic microscope. For the SEM techniques, samples were dried at the critical point of $CO_2$ using an Emitech K850 Critical Point Dryer. The 23 dried specimens were then coated with a gold layer using an Emitech K550X Sputter Coater, after which the samples were placed directly in the VEGA3 TESCAN SEM chamber for examination and visualization. The SEM parameters were as follows: HV 30.00 kV, magnification 3.00 kx (Fig. 3a, c) and 9.00 kx (Fig. 3b, d). WD 18.68–18.86 mm.

| Sample | Code | Sampling date | Coordinates | Host | Habitat |
|---|---|---|---|---|---|
| Zienki 1 | Z-1 | 25.08.2021 | N51° 30′ 30.1″ E023° 05′ 39.5″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Zienki 2 | Z-2 | 25.08.2021 | N51° 30′ 30.1″ E023° 05′ 39.5″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Pinus* |
| Zienki 3 | Z-3 | 01.10.2021 | N51° 30′ 34.9″ E023° 05′ 42.4″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Pinus* |
| Brus 1 | B-1 | 25.08.2021 | N51° 31′ 05.8″ E023° 03′ 01.1″ | *Formica fusca* | Dry pine forest, decaying trunk of *Betula* |
| Brus 2 | B-2 | 03.09.2021 | N51° 29′ 43.6″ E023° 16′ 12.8″ | *Formica fusca* | Dry pine forest, decaying trunk of *Betula* |
| Brus 3 | B-3 | 01.10.2021 | N51° 29′ 34.0″ E023° 16′ 14.8″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Lake Czarne 1 | CL-1 | 25.08.2021 | N51° 31′ 06.2″ E023° 03′ 00.9″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Pinus* |
| Lake Czarne 2 | CL-2 | 25.08.2021 | N51° 31′ 06.2″ E023° 03′ 00.9″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Pinus* |
| Lake Czarne 3 | WW-1 | 03.09.2021 | N51° 31′ 05.8″ E023° 03′ 01.1″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Pinus* |
| Lake Czarne 4 | WW-2 | 03.09.2021 | N51° 31′ 05.8″ E023° 03′ 01.1″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Wielkopole | WP | 01.10.2021 | N51° 20′ 44.4″ E023°15′ 39.8″ | *Lasius platythorax* | Wet birch forest, decaying trunk of *Betula* |
| Łańcuchów | LN | 07.05.2021 | N51° 15′ 58.8″ E022° 56′ 12.9″ | *Lasius brunneus* | Riparian forest, hollow in living *Alnus* |
| Łowiszów | LW | 14.05.2021 | N51° 26′ 31.0″ E023° 13′ 33.8″ | *Lasius* sp. | Mixed forest, decaying trunk of *Quercus* |
| Wólka Wytycka | DB | 06.07.2021 | N51° 26′ 52.6″ E023° 11′ 45.0″ | *Lasius platythorax* | dry pine forest, decaying trunk of *Betula* |
| Pieszowola 1 | P-1 | 21.05.2021 | N51° 29′ 39.0″ E023° 10′ 11.2″ | *Lasius brunneus* | OAK forest, decaying trunk of *Quercus* |
| Pieszowola 2 | P-2 | 23.09.2021 | N51° 29′ 55.6″ E023° 11′ 03.1″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Pieszowola 3 | P-3 | 23.09.2021 | N51° 30′ 11.2″ E023° 10′ 48.9″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Orzechów 1 | O-1 | 25.08.2021 | N51° 19′ 31.3″ E023° 18′ 37.8″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Orzechów 2 | O-2 | 18.08.2021 | N51° 19′ 39.5″ E023° 18′ 43.3″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Orzechów 3 | O-3 | 18.08.2021 | N51° 19′ 39.5″ E023° 18′ 43.3″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Orzechów 4 | O-4 | 19.08.2021 | N51° 19′ 39.5″ E023° 18′ 43.3″ | *Formica fusca* | Dry pine forest, decaying trunk of *Betula* |
| Rogóźno 1 | R-1 | 23.09.2021 | N51° 23′ 32.1″ E022° 57′ 08.7″ | *Lasius platythorax* | Dry pine forest, decaying trunk of *Betula* |
| Rogóźno 2 | R-2 | 23.09.2021 | N51° 23′ 38.3″ E022° 57′ 27.7″ | *Lasius* sp. | Wet mixed forest, decaying trunk of *Quercus* |

**Table 6**. Sampling sites of *Myrmecophilus acervorum* with reference to the host ant species and environmental conditions in the different localities.

### DNA extraction and amplification of selected molecular markers of *Myrmecophilus acervorum*

Genomic DNA was extracted from 141 collected specimens using the Sherlock AX kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's protocol. Genetic material was extracted from whole body of all the specimens collected.

Both mitochondrial (COI and 16S) and nuclear (EF1α) markers were chosen to examine the genetic diversity of *M. acervorum* (for up to 5 specimens per sample). Selected mitochondrial markers were previously examined in *M. acervorum* samples collected in south-eastern and western Europe[42]. Thus, for further comparative analyses of sequence data, we applied the PCR protocols in our study. The COI fragment was amplified using the LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2189 (5'-TAAACTTCAGGGTGACCAA AAAATCA-3') primer pair[100]. In turn, the 16S fragment was amplified using 16Sar-Dr (5'-CGCCTGTTTAAC AAAAACAT-3') and 16Sbr (5'- CCGGTCTGAACTCAAGATCACGT-3') primers[101,102]. The nuclear marker (EF1α) was amplified using the EF-alpha-1 F (5'-ATCGAGAGGTTCGAGAARGARGC-3') and EF-alpha-1R (5'-CCAYCCCTTRAACCANGGCAT-3') primer pair[45]. PCR reactions were performed in a 20 µL volume containing 0.8x JumpStart RED*Taq* ReadyMix (1 U of JumpStart RED*Taq* DNA polymerase, 4 mM Tris-HCl, 20 mM KCl, 0.6 mM MgCl$_2$, 0.08 mM of dNTP; Sigma-Aldrich, Germany), 0.4 µM of forward and reverse primers and ~100 ng of DNA. The volume was made up to 20 µL with autoclaved water. The amplification of these molecular markers involved the same conditions – 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 46.9 °C (COI and 16S) or 52.9 °C (EF1α), 1 min at 72 °C; a final extension at 72 °C for 5 min. All molecular markers were amplified for the same individuals.

The amplified fragments were separated by 1% agarose gel electrophoresis in a 1x SB buffer and visualized with SimplySafe™ (ethidium bromide replacement; EURx, Poland) in UV light. All PCR products were purified by Eppic Fast (A&A Biotechnology, Poland) treatment according to the relevant protocols and sequenced using the BigDye™ terminator cycle sequencing method. All the newly obtained sequences were deposited in the GenBank database (accession nos.: PP896881-PP896884 for COI, PP886506-PP886507 for 16 S, PP915641-PP915645 for EF1α).

### Testing for *Wolbachia* infections

The *M. acervorum* samples were tested for *Wolbachia* infections. All 141 specimens were screened using primers specific for the *Wolbachia* 16S fragment (WF: 5'-CGGGGGGAAAATTTATTGCT-3' and WR: 5'- AGCTGTA ATACAGAAAGGAAA-3')[103]. Amplifications were performed with a total volume of 25 µl using DreamTaq Hot Start Green PCR Master Mix (Thermo Scientific, UK), in accordance with the manufacturer's instructions. Amplifications were performed with positive and negative controls. For the positive control, the DNA template

isolated from *D. melanogaster* individuals infected with the *wMel Wolbachia* strain was used, while the negative control was undertaken without the DNA template.

The amplified fragments were separated by 1% agarose gel electrophoresis in a 1x SB buffer and visualized with SimplySafe™ (ethidium bromide replacement; EURx, Poland) in UV light. Selected good-quality products were purified by Eppic Fast (A&A Biotechnology, Poland) treatment according to the relevant protocols and sequenced using the BigDye™ terminator cycle sequencing method. All the newly obtained sequences were deposited in the GenBank database (accession nos.: PP897406-PP897407).

### Bioinformatic analyses

The BLAST application (Altschul et al. 1990) was used to compare the sequences with those deposited in the NCBI database. Alignments were prepared using Geneious Prime 2023.2.1 (https://www.geneious.com) with Clustal Omega algorithm[104]. Mitochondrial sequences were analysed separately and as a concatenated alignment. Haplotypes were determined using DnaSP v.6.12.03[105].

In Mega X[106], we estimated pairwise genetic distances using the Kimura 2-parameter (*K2P*) nucleotide substitution model with 1,000 bootstrap replicates. Reconstructions of phylogenetic relationships among the identified haplotypes and genotypes with a median-joining algorithm (MJ[107]) were performed in PopART 1.7 software[108]. The best-fit models for nucleotide substitution (for mitochondrial and nuclear data) were estimated using jModelTest 2.1.10[109] with both the Akaike and Bayesian information criterions (AIC and BIC, respectively).

Phylogenetic analyses were generated using the maximum likelihood (ML) and Bayesian inference (BI) methods. ML analyses were performed in RAxML-v.8 software[110] implemented online on the CIPRES Science Gateway[111], using 1,000 bootstrap resampling and other parameters as default. In turn, MrBayes v.3.2.7[112] was used for BI analyses. The Markov Chain Monte Carlo (MCMC) search was run with four chains for 10 million generations with a sampling frequency of 1/1000 trees, with a burn-in of 10%. The DNA sequence-based species delineation approach was applied on concatenated sequences of COI and 16S, as described by Iorgu et al.[42]. Mentioned analysis was performed using ABGD web platform[113].

In turn, maximum likelihood reconstruction of *Wolbachia* phylogeny based on 16S rRNA gene sequences were performed in Mega X software[106] with 1,000 bootstrap replicates. Additional 16S sequences determined for *Wolbachia* belonging to various supergroups, as well as sequences identified for *Ehrlichia ruminantium* and *E. chaffeensis* (served as the outgroup), were added to the analysis[114]. Final phylogenetic trees were edited in FigTree v.1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/).

### Data availability

### References

1. DeSalle, R. & Goldstein, P. Review and interpretation of trends in DNA barcoding. *Front. Ecol. Evol.* **7**, 302 (2019).
2. Harrison, R. G. Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Trends Ecol. Evol.* **4**, 6–11 (1989).
3. Birky, C. W. The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu. Rev. Genet.* **35**, 125–148 (2001).
4. Breton, S. & Stewart, D. T. Atypical mitochondrial inheritance patterns in eukaryotes. *Genome* **58**, 423–431 (2015).
5. Brown, W. M., George, M. & Wilson, A. C. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1967–1971 (1979).
6. Shigenaga, M. K., Hagen, T. M. & Ames, B. N. Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci.* **91**, 10771–10778 (1994).
7. Birky, C. W., Fuerst, P. & Maruyama, T. Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics* **121**, 613–627 (1989).
8. Hudson, R. R. & Turelli, M. Stochasticity overrules the three-times rule: genetic drift, genetic draft, and coalescence times for nuclear loci versus mitochondrial dna. *Evolution* **57**, 182–190 (2003).
9. Lin, C. P. & Danforth, B. N. How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from bayesian analyses of combined datasets. *Mol. Phylogenet. Evol.* **30**, 686–702 (2004).
10. Petrusek, A., Thielsch, A. & Schwenk, K. Mitochondrial sequence variation suggests extensive cryptic diversity within the western palearctic *Daphnia longispina* complex. *Limnol. Oceanogr.* **57**, 1838–1845 (2012).
11. Dasmahapatra, K. K., Elias, M., Hill, R. I., Hoffman, J. I. & Mallet, J. Mitochondrial DNA barcoding detects some species that are real, and some that are not. *Mol. Ecol. Resour.* **10**, 264–273 (2010).
12. Hernández-López, A. et al. Host tracking or cryptic adaptation? Phylogeography of *Pediobius saulius* (Hymenoptera, Eulophidae), a parasitoid of the highly invasive horse-chestnut leafminer. *Evol. Appl.* **5**, 256–269 (2012).
13. Mardulyn, P., Othmezouri, N., Mikhailov, Y. E. & Pasteels, J. M. Conflicting mitochondrial and nuclear phylogeographic signals and evolution of host-plant shifts in the boreo-montane leaf beetle *Chrysomela lapponica*. *Mol. Phylogenet. Evol.* **61**, 686–696 (2011).
14. Thielsch, A., Knell, A., Mohammadyari, A., Petrusek, A. & Schwenk, K. Divergent clades or cryptic species? Mito-nuclear discordance in a *Daphnia* species complex. *BMC Evol. Biol.* **17**, 227 (2017).
15. Gompert, Z., Forister, M. L., Fordyce, J. A. & Nice, C. C. Widespread mito-nuclear discordance with evidence for introgressive hybridization and selective sweeps in Lycaeides. *Mol. Ecol.* **17**, 5231–5244 (2008).
16. Linnen, C. R. & Farrell, B. D. Mitonuclear discordance is caused by rampant mitochondrial introgression in *Neodiprion* (Hymenoptera: Diprionidae) sawflies. *Evolution* **61**, 1417–1438 (2007).

17. Shu, X. X. et al. Rapid genetic divergence and mitonuclear discordance in the Taliang knobby newt (*Liangshantriton taliangensis*, Salamandridae, Caudata) and their driving forces. *Zool. Res.* **43**, 129–146 (2022).
18. Song, H., Buhay, J. E., Whiting, M. F. & Crandall, K. A. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 13486–13491 (2008).
19. Funk, D. J. & Omland, K. E. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annu. Rev. Ecol. Evol. Syst.* **34**, 397–423 (2003).
20. Franco, F., Lavagnini, T., Sene, F. & Manfrin, M. Mito-nuclear discordance with evidence of shared ancestral polymorphism and selection in cactophilic species of *Drosophila*. *Biol. J. Linn. Soc.* **116**, 1 (2015).
21. Pavlova, A. et al. Perched at the mito-nuclear crossroads: divergent mitochondrial lineages correlate with environment in the face of ongoing nuclear gene flow in an Australian bird. *Evol. Int. J. Org. Evol.* **67**, 3412–3428 (2013).
22. D. D Hurst, G. & M Jiggins, F. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proc. R. Soc. B Biol. Sci.* **272**, 1525–1534 (2005).
23. Kambhampati, S., Rai, K. S. & Verleye, D. M. Frequencies of mitochondrial DNA haplotypes in laboratory cage populations of the mosquito, *Aedes albopictus*. *Genetics* **132**, 205–209 (1992).
24. Sun, X. J., Xiao, J. H., Cook, J. M., Feng, G. & Huang, D. W. Comparisons of host mitochondrial, nuclear and endosymbiont bacterial genes reveal cryptic fig wasp species and the effects of *Wolbachia* on host mtDNA evolution and diversity. *BMC Evol. Biol.* **11**, 86 (2011).
25. Wendt, M., Kulanek, D., Varga, Z., Rákosy, L. & Schmitt, T. Pronounced mito-nuclear discordance and various *Wolbachia* infections in the water ringlet *Erebia pronoe* have resulted in a complex phylogeographic structure. *Sci. Rep.* **12**, 5175 (2022).
26. Cariou, M., Duret, L. & Charlat, S. The global impact of *Wolbachia* on mitochondrial diversity and evolution. *J. Evol. Biol.* **30**, 2204–2210 (2017).
27. Jäckel, R., Mora, D. & Dobler, S. Evidence for selective sweeps by *Wolbachia* infections: phylogeny of *Altica* leaf beetles and their reproductive parasites. *Mol. Ecol.* **22**, 4241–4255 (2013).
28. Zhu, X. et al. Diversity of *Wolbachia* infection and its influence on mitochondrial DNA variation in the diamondback moth, *Plutella xylostella*. *Mol. Phylogenet. Evol.* **182**, 107751 (2023).
29. Kodandaramaiah, U., Simonsen, T. J., Bromilow, S., Wahlberg, N. & Sperling, F. Deceptive single-locus taxonomy and phylogeography: *Wolbachia*-associated divergence in mitochondrial DNA is not reflected in morphology and nuclear markers in a butterfly species. *Ecol. Evol.* **3**, 5167–5176 (2013).
30. Ritter, S. et al. Wolbachia infections mimic cryptic speciation in two parasitic butterfly species, *Phengaris teleius* and *P. nausithous* (Lepidoptera: Lycaenidae). *PLoS ONE* **8**, e78107 (2013).
31. Sucháčková Bartoňová, A. et al. *Wolbachia* affects mitochondrial population structure in two systems of closely related Palaearctic blue butterflies. *Sci. Rep.* **11**, 3019 (2021).
32. Sazama, E. J., Ouellette, S. P. & Wesner, J. S. Bacterial endosymbionts are common among, but not necessarily within, insect species. *Environ. Entomol.* **48**, 127–133 (2019).
33. Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P. & Wesner, J. S. Incidence of *Wolbachia* in aquatic insects. *Ecol. Evol.* **7**, 1165–1169 (2017).
34. Zug, R. & Hammerstein, P. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE* **7**, e38544 (2012).
35. Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. & Werren, J. H. How many species are infected with *Wolbachia*?—a statistical analysis of current data. *FEMS Microbiol. Lett.* **281**, 215–220 (2008).
36. De Oliveira, C. D. et al. Broader prevalence of *Wolbachia* in insects including potential human disease vectors. *Bull. Entomol. Res.* **105**, 305–315 (2015).
37. Werren, J. H. & Windsor, D. M. Wolbachia infection frequencies in insects: evidence of a global equilibrium?. *Proc. R. Soc. B Biol. Sci.* **267**, 1277–1285 (2000).
38. Naskrecki, P. Grasshoppers and their relatives. In *Encyclopedia of Biodiversity* 722–736. https://doi.org/10.1016/B978-0-12-384719-5.00168-4 (2013).
39. Komatsu, T., Maruyama, M. & Itino, T. Nonintegrated host association of *Myrmecophilus tetramorii*, a specialist myrmecophilous ant cricket (Orthoptera: Myrmecophilidae). *Psyche J. Entomol.* **2013**, e568536 (2013).
40. Komatsu, T. & Maruyama, M. Taxonomic recovery of the ant cricket *Myrmecophilus albicinctus* from *M. americanus* (Orthoptera, Myrmecophilidae). *ZooKeys* **589**, 97–106 (2016).
41. Żurawlew, P. et al. Distribution of *Myrmecophilus acervorum* (Panzer, 1799) (Orthoptera: Myrmecophilidae) in Poland. *Fragm. Fauna* **65**, 55–68 (2022).
42. Iorgu, I. Ş et al. Ant crickets and their secrets: *Myrmecophilus acervorum* is not always parthenogenetic (Insecta: Orthoptera: Myrmecophilidae). *Zool. J. Linn. Soc.* **197**, 211–228 (2023).
43. Desutter-Grandcolas, L. First record of ant-loving crickets (Orthoptera: Myrmecophilidae: Myrmecophilinae) in New Caledonia. *Aust J. Entomol.* **36**, 159–163 (1997).
44. Hebard, M. Revision of the north American species of the genus *Myrmecophila* (Orthoptera; Gryllidae; Myrmecophilinae). *Trans. Am. Entomol. Soc.* **46**, 91–111 (1920).
45. Hsu, P. W. et al. Ant crickets (Orthoptera: Myrmecophilidae) associated with the invasive yellow crazy ant A*noplolepis gracilipes* (Hymenoptera: Formicidae): evidence for cryptic species and potential co-introduction with hosts. https://doi.org/10.25849/MYRMECOL.NEWS_030:103 (2020).
46. Stalling, T. & Birrer, S. Identification of the ant-loving crickets, *Myrmecophilus* Berthold, 1827 (Orthoptera: Myrmecophilidae), in Central Europe and the northern Mediterranean Basin. *Articulata* **28**, 1–11 (2013).
47. Komatsu, T., Maruyama, M. & Itino, T. Differences in host specificity and behavior of two ant cricket species (Orthoptera: Myrmecophilidae) in Honshu, Japan. *J. Entomol. Sci.* **45**, 227–238 (2010).
48. Komatsu, T., Maruyama, M., Ueda, S. & Itino, T. mtDNA phylogeny of Japanese ant crickets (Orthoptera: Myrmecophilidae): diversification in host specificity and habitat use. 52 (2008).
49. Ortega-Morales, A. I. et al. First record of the ant cricket *Myrmecophilus (Myrmecophilina) americanus* (Orthoptera: Myrmecophilidae) in Mexico. *Zootaxa* **4258**, 195–200 (2017).
50. Franc, V., Majzlan, O., Krištín, A. & Wiezik, M. On the distribution and ecology of the ant cricket (Myrmecophilus Acervorum) (Orthoptera: Myrmecophilidae) in Slovakia. *Matthias Belivs Univ. Proc.* **5**, 40–50 (2015).
51. Bazyluk, W. Nowe dla Polski lub rzadsze gatunki z rzędów Blattodea, Mantodea, Orthoptera i Dermaptera. *Fragm. Fauna* **7**, 263–282 (1957).
52. Dominik, J. *Klucze Od Oznaczania Owadów Polski. Cz. XIX Chrząszcze—Coleoptera. Zeszyt 41 Kołatki—Anobiidae* (Państwowe Wydawnictwo Naukowe, 1955).
53. Bazyluk, W. *Prostoskrzydłe—Orthoptera (Saltatoria)* (Warszawa, 1956).
54. Nunberg, M. *Obumierki—Rhizophagidae* Vol. 64 (Warszawa, 1967).
55. Bellman, H. *Szarańczaki. Łatwe Oznaczanie Gatunków Europy Środkowej* (MULTICO Oficyna Wydawnicza, 2009).
56. Louveaux, A., Amédégnato, C., Poulain, S. & Desutter-Grandcolas, L. Catalogue and keys of the Acridomorpha (Insecta, Orthoptera) from north West Africa. *Zoosystema* **35**, 175–181 (2013).
57. Smith, T., Froeba, J. & Capinera, J. Key to the grasshoppers (Orthoptera: Acrididae) of Florida. *Fla. Entomol.* **87**, 537–550 (2009).

58. Stalling, T. A new species of ant-loving cricket from Mallorca, Balearic Islands, Spain (Orthoptera, Myrmecophilidae). *Graellsia* **69**, 153–156 (2013).

59. Stalling, T. *MyrmecopGallicusllicus*, a new species of ant cricket from France (Orthoptera: Myrmecophilidae). *Articulata* **32**, 1–7 (2017).

60. Timm, V. F., Gonçalves, L. T., Valente, V. L. D. S. & Deprá, M. The efficiency of the COI gene as a DNA barcode and an overview of Orthoptera (Caelifera and Ensifera) sequences in the BOLD system. *Can. J. Zool.* **100**, 710–718 (2022).

61. Dong, Z., Wang, Y., Li, C., Li, L. & Men, X. Mitochondrial DNA as a molecular marker in insect ecology: current status and future prospects. *Ann. Entomol. Soc. Am.* **114**, 470–476 (2021).

62. Lynch, M., Wei, W., Ye, Z. & Pfrender, M. The genome-wide signature of short-term temporal selection. *Proc. Natl. Acad. Sci.* **121**, e2307107121 (2024).

63. Hebert, P. D. N., Cywinska, A., Ball, S. L. & deWaard, J. R. Biological identifications through DNA barcodes. *Proc. R Soc. B Biol. Sci.* **270**, 313–321 (2003).

64. Meyer, C. P. & Paulay, G. DNA barcoding: error rates based on comprehensive sampling. *PLoS Biol.* **3**, e422 (2005).

65. K Tan, M. & Robillard, T. Rugabinthus, a new genus of Lebinthina (Orthoptera, Gryllidae, Eneopterinae) from New Guinea. *J. Orthoptera Res.* **31**, 9–40 (2022).

66. Cao, C., Rong, H. & Naveed, H. Two new species of the genus Xya Latreille, 1809 (Orthoptera, Tridactyloidea, Tridactylidae) from Yunnan with a key to all Xya species in China. *ZooKeys* **947**, 103–112 (2020).

67. Gu, J. J., Zhou, Y. & Yuan, W. New Genera and species of trigonidiidae (Orthoptera: Grylloidea) from the mid-cretaceous of Myanmar with a redescription of *Birmaninemobius hirsutus*. *Insects* **15**, 442 (2024).

68. Kim, S., Lee, W. & Lee, S. Estimation of a new molecular marker of the genus Stathmopoda (Lepidoptera: Stathmopodidae): comparing EF1a and COI sequences. *J. Asia-Pac. Entomol.* **20**, 269–280 (2017).

69. Marques, J. F., Winde, I., Jönsson, A. M. & Anderbrant, O. Taxonomic relationship among four European *physokermes* species (Hemiptera: Coccomorpha) based on nuclear and mitochondrial DNA. *Front. Glob. Change* **6**, 1 (2023).

70. Simon, S., Schierwater, B. & Hadrys, H. On the value of elongation factor-1α for reconstructing pterygote insect phylogeny. *Mol. Phylogenet. Evol.* **54**, 651–656 (2010).

71. Sworobowicz, L. et al. Revisiting the phylogeography of *Asellus aquaticus* in Europe: insights into cryptic diversity and spatiotemporal diversification. *Freshw. Biol.* **60**, 1824–1840 (2015).

72. Hurst, G. & Jiggins, F. M. Male-killing bacteria in insects: mechanisms, incidence, and implications. *Emerg. Infect. Dis.* **6**, 329–336 (2000).

73. Huigens, M. E., de Almeida, R. P., Boons, P. A. H., Luck, R. F. & Stouthamer, R. Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proc. R. Soc. B Biol. Sci.* **271**, 509–515 (2004).

74. Bridgeman, B., Morgan-Richards, M., Wheeler, D. & Trewick, S. A. First detection of *Wolbachia* in the New Zealand biota. *PLoS ONE* **13**, e0195517 (2018).

75. Wang, X. et al. Phylogenomic analysis of *Wolbachia* strains reveals patterns of genome evolution and recombination. *Genome Biol. Evol.* **12**, 2508–2520 (2020).

76. Baldo, L. et al. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Appl. Environ. Microbiol.* **72**, 7098–7110 (2006).

77. O'Woma, O. O., Chigozirim, U. P., Emmanuel, O. & Chukwuebuka, E. M. Reproductive and survival strategies utilized by insect. A review. *Am. J. Zool. Res.* **4**, 1–6 (2016).

78. Bell, G. *The Masterpiece of Nature: The Evolution and Genetics of Sexuality* (Routledge, 2019).

79. Normark, B. Modes of reproduction. In *The Evolution of Insect Mating Systems* Vol. 352 (eds Shuker, D. M. & Simmons, L. W.) (Oxford University Press, 2014).

80. Fernandes, M. L., Zacaro, A. A. & Serrão, J. E. First report of a parthenogenetic Grylloidea and new genus of Neoaclini (Insecta: Orthoptera: Grylloidea: Phalangopsidae: Phalangopsinae). *Zootaxa* **4032**, 407–416 (2015).

81. Lamb, R. Y. & Willey, R. B. The First parthenogenetic populations of orthoptera saltatoria to be reported from North America. *Ann. Entomol. Soc. Am.* **68**, 721–722 (1975).

82. Borissov, S. B., Hristov, G. H. & Chobanov, D. P. Phylogeography of the *Poecilimon ampliatus* species group (Orthoptera: Tettigoniidae) in the context of the Pleistocene glacial cycles and the origin of the only thelytokous parthenogenetic phanopterine bush-cricket. *Arthropod. Syst. Phylogeny* **79**, 401–418 (2021).

83. Dutrillaux, A. M. et al. Origin of the complex karyotype of the polyploid parthenogenetic grasshopper *Saga pedo* (Orthoptera: Tettigoniidae). *Eur. J. Entomol.* **106**, 477–483 (2009).

84. Kirkendall, L. & Normark, B. Parthenogenesis. In *Encyclopedia of Insects* (Resh, V. H. & Cardé, R.) 851–856 (Academic Press, 2003).

85. Vandel, A. P. La parthénogenèse géographique: contribution á l'étude biologique et cytologique de la parthénogenèse naturelle. *Bull. Biol. Fr. Belg.* **62**, 164–281 (1928).

86. Cosendai, A. C., Wagner, J., Ladinig, U., Rosche, C. & Hörandl, E. Geographical parthenogenesis and population genetic structure in the alpine species *Ranunculus kuepferi* (Ranunculaceae). *Heredity* **110**, 560–569 (2013).

87. de Jong, Y., Fauna Europaea Consortium. Checklist dataset. *Fauna Europaea*. https://doi.org/10.15468/ymk1bx (2016).

88. Wu, C. & Liu, C. New record of a *Poecilimon* species (Orthoptera: Tettigoniidae: Phaneropterinae; Barbitistini) from China. *Zootaxa* **4612**, 296–300 (2019).

89. Varga, Z. *Post-glacial Dispersal Strategies of Orthoptera and Lepidoptera in Europe and in the Carpathian Basin* 93–105 (2003).

90. Hewitt, G. M. Post-glacial re-colonization of European biota. *Biol. J. Linn. Soc.* **68**, 87–112 (1999).

91. Zasadni, J. & Kłapyta, P. The tatra mountains during the last glacial maximum. *J. Maps* **10**, 440–456 (2014).

92. Taberlet, P., Fumagalli, L., Wust-Saucy, A. & Cosson, J. Comparative phylogeography and postglacial colonization routes in Europe. *Mol. Ecol.* **7**, 453–464 (1998).

93. Morgan-Richards, M., Trewick, S. A. & Stringer, I. A. N. Geographic parthenogenesis and the common tea-tree stick insect of New Zealand. *Mol. Ecol.* **19**, 1227–1238 (2010).

94. Hoffmann, A. A., Reynolds, T., Nash, K. & Weeks, A. M. A. R. A high incidence of parthenogenesis in agricultural pests. *Proc. R. Soc. B Biol. Sci.* **275**, 2473–2481 (2008).

95. Pfenninger, M. & Schwenk, K. Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. *BMC Evol. Biol.* **7**, 121 (2007).

96. Oliveira, D. C. S. G., Raychoudhury, R., Lavrov, D. V. & Werren, J. H. Rapidly evolving mitochondrial genome and directional selection in mitochondrial genes in the parasitic wasp *Nasonia* (Hymenoptera: Pteromalidae). *Mol. Biol. Evol.* **25**, 2167–2180 (2008).

97. Agrawal, A. A. Phenotypic plasticity in the interactions and evolution of species. *Science* **294**, 321–326 (2001).

98. Pan, Z., Hong, F. & Jiang, G. Morphometrics reveal correlation between morphology and bioclimatic factors and population mixture in *Tetrix japonica* (Orthoptera: Tetrigidae). *Acta Zool.* **99**, 199–210 (2018).

99. Yadav, S., Stow, A. J., Harris, R. M. B. & Dudaniec, R. Y. Morphological variation tracks environmental gradients in an agricultural pest, *Phaulacridium vittatum* (Orthoptera: Acrididae). *J. Insect Sci. Online* **18**, 13 (2018).

100. Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **3**, 294–299 (1994).

101. Palumbi, S. et al. *The Simple Fool's Guide to PCR, Ver. 2* (Department of Zoology and Kewalo Marine Laboratory, University of Hawaii, 1991).
102. Palumbi, S. R. *Molecular Systematics* 2nd edn, 205–247 (Sinauer, 1996).
103. Singh, S. T., Kumar, J., Thomas, A., Ramamurthy, V. V. & Rajagopal, R. Detection and localization of *Rickettsia* sp. in Mealybug. *Environ. Entomol.* **42**, 711–716 (2013).
104. Sievers, F. et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
105. Rozas, J. et al. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* **34**, 3299–3302 (2017).
106. Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).
107. Bandelt, H. J., Forster, P. & Röhl, A. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* **16**, 37–48 (1999).
108. Leigh, J. W. & Bryant, D. Popart: full-feature software for haplotype network construction. *Methods Ecol. Evol.* **6**, 1110–1116 (2015).
109. Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* **9**, 772 (2012).
110. Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313 (2014).
111. Miller, M. A., Pfeiffer, W. & Schwartz, T. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 Gateway Computing Environments Workshop (GCE)* 1–8. https://doi.org/10.1109/GCE.2010.5676129 (IEEE, 2010)
112. Ronquist, F. et al. MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **61**, 539–542 (2012).
113. Puillandre, N., Lambert, A., Brouillet, S. & Achaz, G. ABGD, automatic barcode gap discovery for primary species delimitation. *Mol. Ecol.* **21**, 1864–1877 (2012).
114. Konecka, E. & Olszanowski, Z. Phylogenetic analysis based on the 16S rDNA, *gltA*, *gatB*, and *hcpA* gene sequences of *Wolbachia* from the novel host *Ceratozetes thienemanni* (Acari: Oribatida). *Infect. Genet. Evol.* **70**, 175–181 (2019).

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## Author contributions

A.K.Z.—conceptualization; data curation; resources; methodology; formal analysis; writing—original draft preparation; writing—review and editing; visualization; G.K.W.—conceptualization; methodology; resources; writing—original draft preparation; visualization; B.S—resources; supervision; project administration; funding acquisition; writing—review and editing; M.Z.—resources; writing—review and editing; E.P.T.—resources; writing – review and editing; E.I.I.—resources; writing—review and editing; I.S.I.—resources; writing—review and editing.

## Declarations

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-75335-y.

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