Title: Comprehensive analysis of male-free reproduction in *Monomorium triviale* (Formicidae: Myrmicinae)

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Abstract

We report comprehensive evidence for obligatory thelytokous parthenogenesis in an ant *Monomorium triviale*. This species is characterized by distinct queen–worker dimorphism with strict reproductive division of labor: queens produce both workers and new queens without mating, whereas workers are completely sterile. We collected 333 nests of this species from 14 localities and three laboratory-reared populations in Japan. All wild queens dissected had no sperm in their spermathecae. Laboratory observation confirmed that virgin queens produced workers without mating. Furthermore, microsatellite genotyping showed identical heterozygous genotypes between mothers and their respective daughters, suggesting an extremely low probability of sexual reproduction. Microbial analysis detected no bacterial genera that are known to induce thelytokous parthenogenesis in Hymenoptera. Finally, the lack of variation in partial sequences of mitochondrial DNA among individuals sampled from across Japan suggests recent rapid spread or selective sweep. *M. triviale* would be a promising model system of superorganism-like adaptation through comparative analysis with well-studied sexual congeners, including the pharaoh ant *M. pharaonis*.

Introduction

Hymenopteran insects are characterized by haplo-diploid sex determination systems, in which females are derived from fertilized diploid eggs and males are derived from unfertilized haploid eggs via arrhenotokous parthenogenesis [1]. However, the production of diploid females from unfertilized eggs—known as thelytokous parthenogenesis—has been reported sporadically across the Hymenoptera [2]. Thelytoky has provided an opportunity for empirical testing of evolutionary hypotheses to account for the near-ubiquity of sex among eukaryotes [3]. The last decade has seen an increasing number of known thelytokous Hymenoptera, especially in eusocial species [4]. In studies taking advantage of this simplified mode of reproduction, thelytokous social insects have been acknowledged as a model system in behavioral ecology (e.g., [5–8]) and sociogenomics (e.g., [9]).

Previous studies of thelytokous parthenogenesis of ants have identified three distinct categories [10, 11]: (type I) queens produce workers sexually but daughter queens via thelytoky; (type II) the queen caste is usually lost, males are absent, and workers produce workers via thelytoky; and (type III) queens produce both workers and queens via thelytoky, males are usually absent, and workers are sterile.

In this paper, we report comprehensive evidence of the third type of thelytokous parthenogenesis, namely obligatory thelytoky by queens of *M. triviale* [12]. The genus *Monomorium* is one of the most species-rich genera among ants [13] and has a worldwide distribution [14]. With the marked exception of tramp species such as the pharaoh ant *M. pharaonis* [15], most *Monomorium* species remain to be investigated [16]. *M. triviale* is reported from East Asia, encompassing Japan, South Korea and mainland China [14]. Neither males nor inseminated females have been found to date; this has been referred to in several

studies [1, 4, 17–23]. Surprisingly, however, direct evidence of thelytoky is lacking. We examined the reproductive system of *M. triviale* through multiple approaches, namely dissection of spermathecae of field-collected queens, direct observation of virgin queen reproduction, and microsatellite genotyping of mothers and daughters. Furthermore, we analyzed for the presence of parthenogenesis-inducing microsymbionts and within-species phylogenetic relationships among populations in Japan.

Materials & Methods

Colony sampling

A total of 333 nests of *M. triviale* were collected from 14 local populations in Japan (Fig 1, Table 1). In the field, nests were found in small gaps in dead plant bodies such as rotten roots, dead twigs, hollow bamboo sticks, and acorns. Because of the small nest size (about 200 workers on average) it was easy to collect the whole nest. During the field investigation, males of *M. triviale* were never found. Colonies were transferred into artificial nests in the laboratory as soon as possible and were kept at 25 °C until the following experiments. In addition, laboratory-reared nests and ethanol-preserved samples originating from three other sites and provided by other researchers were examined (Fig 1).

Fig 1. Localization of the 14 sampling sites (1 to 14) and the three sites of origin of laboratory-reared populations (15 to 17) in Japan. Open circles indicate site locations. Site names, numbers of nests collected, and types of analyses are described in **Table 1**.

83 **Table 1. Sampling sites of *Monomorium triviale* populations and experiments performed**

	Locality	Latitude	Longitude	Collection dates	No. of nests	Experiments
1	Kyoto City, Kyoto Prefecture	35.060087	135.788488	19/04/2017 to 27/11/2017 (24 times)	176	SP, VR, SEQ
2	Tsuchiura City, Ibaraki Prefecture	36.077927	140.165473	20/06/2017, 09/10/2018	28	SP, VR, SEQ
3	Chofu City, Tokyo Metropolis	35.668182	139.549061	16/06/2017, 27/09/2018	28	SP, VR, SEQ
4	Tsukuba City, Ibaraki Prefecture	36.100415	140.101297	19/06/2017, 08/10/2018	26	SP, VR, SEQ
5	Otsu City, Shiga Prefecture	34.970262	135.956026	10/06/2017	26	SP, VR, SEQ
6	Matsudo City, Chiba Prefecture	35.774787	139.899362	18/06/2017, 28/09/2018	22	SP, VR, SEQ
7	Higashiosaka City, Osaka Prefecture	34.665748	135.671267	09/11/2017	5	SP, SEQ
8	Okazaki City, Aichi Prefecture	34.941529	137.175594	03/09/2017	1	SP, SEQ
9	Sanda City, Hyogo Prefecture	34.914575	135.165764	09/05/2017	1	VR, SEQ
10	Kaizu City, Gifu Prefecture	35.22	136.63	06/06/2018	1	SP
11	Tobishima Island, Sakata City, Yamagata Prefecture	39.19	139.55	20/09/2018	2	SP
12	Takamatsu City, Kagawa Prefecture	34.364349	134.098205	17/06/2017	16	SEQ
13	Matsuyama City, Ehime Prefecture	33.845539	132.765722	19/09/2018	2	SEQ
14	Takashima City, Shiga Prefecture	35.3677	135.9168	30/05/2017	1	SEQ
15	Kanonji City, Kagawa Prefecture	34.12	133.66	Reared in Laboratory	–	SEQ
16	Kagamihara City, Gifu Prefecture	35.40	136.85	Reared in Laboratory	–	SEQ
17	Matsue City, Shimane Prefecture	35.47	133.05	Reared in Laboratory	–	SEQ
				Total	**333**	

84 The nests from Kaizu City (locality no. 10) and Tobishima Island (no. 11) were provided by Dr. K.

85 Ohkawara. Samples from Higashiosaka City (no. 7) and Takashima City (no. 14) were provided by Mr.

K. Sadahiro and Dr. T. Nozaki, respectively. Ethanol-preserved samples from Kanonji City, Kagamihara City, and Matsue City (nos. 15 to 17) were maintained in the laboratory for several years and were provided by Dr. F. Ito. Experiments on the samples from the 17 sites are abbreviated as follows: SP: dissection of wild queens' spermathecae; VR: observation of virgin queen reproduction; SEQ: phylogenetic analysis based on mtDNA sequencing.

Dissection of wild queens

To confirm the reproductive status of the queens from wild nests, we dissected 63 individuals from 38 nests of 10 *M. triviale* populations within 6 months after collection (Table 2). First, the queen was immobilized by being soaked in 70% ethanol for 3 min. The body was then transferred to a 30-mm petri dish filled with distilled water, and the internal reproductive organs were pulled out from the end of the abdomen by using precision forceps under a binocular microscope (SZ40; OLYMPUS Optical, Tokyo, Japan). The mating status of the queen was determined by the presence or absence of sperm in the spermatheca. As an indicator of oviposition, the ovary's yellow body was checked. As a positive control [18], 11 queens of a congeneric species, *M. intrudens*, were dissected in the same manner.

Table 2. Dissected queens from each sampling site

	Locality	No. of nests	No. of queens	Insemination		Yellow body	
				yes	no	yes	unclear
1	Kyoto City, Kyoto Prefecture	3	10	0	10	9	1
2	Tsuchiura City, Ibaraki Prefecture	8	8	0	8	7	1
3	Chofu City, Tokyo Metropolis	8	8	0	8	5	3
4	Tsukuba City, Ibaraki Prefecture	8	8	0	8	3	5

5	Otsu City, Shiga Prefecture	2	9	0	9	9	0
6	Matsudo City, Chiba Prefecture	4	8	0	8	8	0
7	Higashiosaka City, Osaka Prefecture	1	1	0	1	1	0
8	Okazaki City, Aichi Prefecture	1	8	0	8	8	0
10	Kaizu City, Gifu Prefecture	1	1	0	1	1	0
11	Tobishima Island, Sakata City, Yamagata Prefecture	2	2	0	2	1	1
	Total	38	63	0	63	52	11
–	*Monomorium intrudens*, Kyoto City, Kyoto Prefecture	4	11	10	1	11	0

The numbers of individuals possessing a yellow body and presumed to have experienced oviposition are also shown. As a positive control, data for the sexual congener *M. intrudens* are shown in the bottom row.

Rearing experiment

Each wild nest was put into a plastic container (68 × 39 × 15 mm) with gypsum on the bottom. The nests were kept in the laboratory at 25 °C, and water and food were replenished every 3 days. The nests were fed mealworms, *Tenebrio molitor*, cut into approximately 5-mm lengths. During July and August 2017, a total of 44 queen broods (larvae or pupae) from 21 nests of seven populations were produced (Table 3). Each queen was isolated with 10 nestmate workers in a plastic container (36 × 36 × 14 mm). These nests were kept under the same conditions as the source nests. Reproduction by these virgin queens was observed for 6 months. Finally, all remaining queens were dissected and confirmed to have no sperm in their spermathecae (Table 4).

Table 3. Thelytokous worker production in the rearing experiment

Locality	No. of nests	No. of queens isolated	No. of queens surviving for 6 months	No. of workers produced

1	Kyoto City, Kyoto Prefecture	6	10	6	17
2	Tsuchiura City, Ibaraki Prefecture	3	7	2	11
3	Chofu City, Tokyo Metropolis	3	5	5	26
4	Tsukuba City, Ibaraki Prefecture	3	5	5	28
5	Otsu City, Shiga Prefecture	3	8	8	52
6	Matsudo City, Chiba Prefecture	2	7	8	28
9	Sanda City, Hyogo Prefecture	1	2	2	15
	Total	**21**	**44**	**36**	**177**

Table 4. Dissection of virgin queens from the rearing experiment

		No. of queens dissected	**Insemination**		**Yellow body**	
	Locality		**yes**	**no**	**yes**	**unclear**
2	Tsuchiura City, Ibaraki Prefecture	2	0	2	2	0
3	Chofu City, Tokyo Metropolis	5	0	5	3	2
4	Tsukuba City, Ibaraki Prefecture	5	0	5	2	3
5	Otsu City, Shiga Prefecture	8	0	8	3	5
6	Matsudo City, Chiba Prefecture	7	0	7	2	5
9	Sanda City, Hyogo Prefecture	2	0	2	1	1
	Total	**29**	**0**	**29**	**13**	**16**

Microsatellite analysis

To provide genetic evidence of thelytoky, 33 queens (from 17 nests of seven populations; all used in the rearing experiment) were examined (Table 5). They were genotyped at microsatellite locus *Mp-1* (f: GCCAATGGTTTAATCCCTCA; r: TCATACTGCGTGTGCCTTTC), originally developed from *M. pharaonis* [24]. Daughter workers produced from these virgin queens (174 individuals in total) were also genotyped and were compared with their mothers. The thorax of each individual was crushed and placed in a 0.2-mL microtube filled with 100 μL of a DNA extraction reagent (PrepMan Ultra Reagent, Applied Biosystems, Foster City, CA, USA). The polymerase chain reaction (PCR) cocktail contained 1 μL of template DNA, 0.3 μL of 25 mM $MgCl_2$, 0.3 μL of 10 mM dNTPs, 1.5 μL

130 of 10× PCR Buffer, 0.1 μL of 5 U/μL Taq DNA Polymerase (QIAGEN, Valencia, CA, USA),
131 0.2 μL of U19 fluorescent dye, and 1.0 μL of each primer pair, to which distilled water was
132 added to make a total volume of 15.2 μL. The PCR program consisted of an initial step of
133 94 °C for 180 s, followed by 35 cycles of 94 °C for 30 s, 57 °C for 60 s and 72 °C for 60 s,
134 with a final step of 72 °C for 10 min. PCR products were mixed with Hi-Di formamide and
135 GS-600 LIZ size standard and were analyzed by using a 3500 Series Genetic Analyzer and
136 GeneMapper 5.0 software (Applied Biosystems).

137 **Table 5. Microsatellite analysis data**

	Locality	No. of nests	No. of queens	No. of workers	Genotype	Prob. that all offspring were heterozygous
1	Kyoto City, Kyoto Prefecture	4	4	15	222/234	$(1/2)^{15} = 3.05 \times 10^{-5}$
2	Tsuchiura City, Ibaraki Prefecture	1	2	11	220/232	$(1/2)^{11} = 4.88 \times 10^{-4}$
3	Chofu City, Tokyo Metropolis	3	5	26	222/232	$(1/2)^{26} = 1.49 \times 10^{-8}$
4	Tsukuba City, Ibaraki Prefecture	3	5	28	220/232	$(1/2)^{28} = 3.73 \times 10^{-9}$
5	Otsu City, Shiga Prefecture	3	8	52	220/228	$(1/2)^{52} = 2.22 \times 10^{-16}$
6	Matsudo City, Chiba Prefecture	2	7	27	220/234	$(1/2)^{27} = 7.45 \times 10^{-9}$
9	Sanda City, Hyogo Prefecture	1	2	15	222/238	$(1/2)^{15} = 3.05 \times 10^{-5}$
	Total	**17**	**33**	**174**	–	$\mathbf{(1/2)^{174} = 4.18 \times 10^{-53}}$

138 Genotypes at the *Mp-1* locus are shown. Under the assumption of sexual reproduction, the probability that
139 all daughter workers would be heterozygous was calculated.

140

141 # Microbial analysis

142 To assess potential infection of *M. triviale* by thelytoky-inducing bacteria, we
143 performed high-throughput amplicon sequencing by using whole bodies of adults. In August
144 2020, three *M. triviale* nests were collected in Takaragaike Park, Kyoto, Japan (lat 35.060087,

145 long 135.788488). All colonies were transported to our laboratory and moved to plastic
146 containers (68 × 39 × 30 mm) with gypsum on the bottom. They were maintained at 25 °C,
147 and water replenishment and mealworm-feeding were performed every 3 days. In October
148 2020, ten queens and 100 workers were picked up from each nest. The queens or workers
149 from each nest were stored as a group in acetone at –30 °C. The pooled individuals
150 represented one biological replicate (i.e., three replicates per caste). Whole bodies in each
151 replicate were air-dried and pooled in a 1.5-mL plastic tube, and DNA was extracted with a
152 QIAamp DNA Micro Kit (QIAGEN). We followed the manufacturer's instructions and added
153 extra steps of thermal cycling and lysozyme to the protocol to ensure the lysis of
154 Gram-positive bacterial cell walls: After being crushed in 180 μL ATL buffer, samples in
155 plastic tubes first underwent two cycles of −80 °C for 30 min and 50 °C for 5 min; then, under
156 room temperature, we added 2 μL of lysozyme from egg white (Nakalai Tesque, Kyoto,
157 Japan; 20 μg/μL TE buffer) to each sample and incubated the samples at 37 °C for 30 min.

158 16S rRNA amplicon sequencing and the subsequent data analysis were performed according
159 to in-house workflow by Bioengineering Lab. Co., Ltd. (Sagamihara, Kanagawa, Japan). The
160 V4 region was amplified from 1 ng of template DNA by using ExTaq HS (Takara Bio, Otsu,
161 Shiga, Japan) polymerase and the 515f–806r primer pair. Sequences were determined by
162 using a MiSeq system with a MiSeq reagent kit v3 (Illumina, San Diego, CA, USA), which
163 generated 2 × 300-bp paired-end reads.

164 Demultiplexing (on the basis of a perfect match with the primer sequences used), adapter
165 trimming, and quality filtering (phred score ≥ 20; primer sequences, 50 bp on both 3′-ends,
166 noise and chimeric reads were removed) of the paired-end reads were performed by using a
167 Fastx toolkit (ver. 0.0.14, http://hannonlab.cshl.edu/fastx_toolkit/) and dada2 plugin for

168 Qiime2 (ver. 2020.8) [25], resulting in representative sequences and operational taxonomic
169 unit (OTU) tables. Assignment of appropriate taxa (confidence level > 0.7) was performed by
170 using the default setting of the feature-classifier plugin for Qiime2 against the EzBioCloud
171 16S reference database (https://www.ezbiocloud.net/).

172

173 **Phylogenetic analysis**

174 To investigate intraspecific diversity and determine the phylogenetic position of *M.*
175 *triviale*, phylogenetic analysis was performed. A 639-bp region of the *cytochrome oxidase I*
176 (*COI*) sequence was determined by using PCR with a combination of primers, namely
177 LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198
178 (TAAACTTCAGGGTGACCAAAAAATCA) [26]. One worker randomly chosen from each
179 of 15 local populations (Table 1) of *M. triviale* and a single worker of the sexual species, *M.*
180 *intrudens*, collected in Kihoku-cho, Mie, Japan (lat 34.21, long 136.33), were sequenced.
181 Protocols for DNA extraction and the PCR mix were the same as for the microsatellite
182 analysis. The thermal cycle consisted of an initial denaturation at 94 °C for 1 min, 35 cycles
183 of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 60 s, and
184 a final extension at 72 °C for 10 min. PCR products were ethanol-precipitated and sequenced
185 in both directions by using a BigDye Terminator v3.1 cycle sequencing kit on a 3500 Series
186 Genetic Analyzer (Applied Biosystems). A total of 16 sequences were submitted to the DNA
187 Data Bank of Japan under accession numbers LC592050 to LC592065; see Fig 3). To identify
188 the phylogenetic position of *M. triviale* after recent advances in systematics of the subfamily
189 Myrmicinae [27], a congeneric species, *M. pharaonis* (GenBank accession number
190 GU710434) and two former *Monomorium* species, *Syllophopsis sechellensis* (EF609858) and

191 *Erromyrma latinodis* (GU709833) were added to the analysis. The red imported fire ant,
192 *Solenopsis invicta* (HQ928672), was used as an outgroup. The 16 sequences, together with
193 those of the above four species, were aligned by using ClustalW [28] in MEGA 10.0 [29].
194 Maximum likelihood analyses were conducted as implemented in MEGA 10.0 by using a
195 GTR+I+Γ model with node support assessed with 1000 bootstrap replicates.

196

197 Results

198 Dissection of wild queens

199 All 63 dissected queens of *M. triviale* had no sperm in their spermathecae (Fig 2,
200 Table 2). Yellow bodies, suggestive of queen oviposition experience, were identified in 52
201 individuals across all 10 sampling sites. In contrast, 10 out of 11 *M. intrudens* queens
202 dissected as positive controls had sperm in their spermathecae.

203

204 **Fig 2. Reproductive organs of (a) *Monomorium triviale* and (b) *M. intrudens* queens. (a)**
205 *M. triviale*: Translucent (empty) spermatheca (spt), ovarioles with oocytes and obvious
206 yellow bodies (yb) indicating that virgin queens were reproductively mature and laid eggs. **(b)**
207 *M. intrudens*: Opaque (filled with sperm) spermatheca, well-developed ovarioles, and yellow
208 bodies indicating that the queen is sexually reproducing. Yellow arrowheads indicate yellow
209 bodies.

210

211 Rearing experiment

Eight queens died before producing daughter workers (Table 3). Thirty-six virgin queens from seven local populations survived, and each produced at least one worker. In total, 177 workers emerged during the experimental period; no males were produced. All dissected queens had empty spermathecae (Table 4). Despite all the queens producing workers, only 13 of 29 queens dissected had obvious yellow bodies.

Microsatellite analysis

Analysis of 33 queens and 174 workers showed that all individuals were heterozygous at *Mp-1* and that the genotypes of all the daughter workers were identical to those of their mothers. In addition, all individuals within the same local population had the same genotype. Comparison of genotypes between mothers and their daughters enables us to infer the occurrence of sexual reproduction. A potential male mate of a heterozygous mother (genotype AB) either shares or does not share the same allele (A or B) of *Mp-1* as the mother. We can rule out the latter possibility on the basis of the daughter genotypes. In the former case, the mother's sexually produced daughters should have two genotypes in the expected ratio of 1:1. One is the same as their mother's (AB) and the other is homozygous at one of the two mother alleles (AA when the father's genotype is A, or BB when the father's genotype is B). Under sexual reproduction, the observed bias toward the daughter's same heterozygous genotypes as their mother would be extremely rare. (The exact binomial probabilities are given in Table 5.) Therefore, we can reject the possibility of sexual reproduction among the individuals that we genotyped.

Microbial analysis

235 MiSeq sequencing and Qiime2-based analysis yielded 8364 to 46,817 reads per
236 biological replicate and a total of 267 OTUs (summarized in S1 Table). Among them, 263
237 OTUs (covering 99.9% to 100% of the reads) were classified as bacterial, and 117 OTUs
238 (92.4% to 99.5% of the reads, excepting 54.7% of the reads from one queen replicate from
239 nest B, see below) were classified at least to the phylum level. No reads were assigned to
240 bacterial genera that are known to induce thelytokous parthenogenesis in Hymenoptera, i.e.,
241 *Cardinium*, *Rickettsia*, and *Wolbachia* [1].

242 In two nests (A and C) out of the three replicates for both queens and workers, the
243 most abundant reads were classified as from *Spiroplasma platyhelix* (88.4% and 67.4% of
244 total reads from queen and worker replicates, respectively, of nest A; 91.5% and 91.1%,
245 respectively, for nest C). The replicates obtained from nest B showed a different pattern of *S.*
246 *platyhelix* abundance: 0% from the queen replicate and 13.2% from the worker replicate. The
247 absence of the *S. platyhelix* sequence from the nest B queen replicate was likely associated
248 with the relatively small number of total reads (8364 vs. >30,000, S1 Table).
249

250 **Phylogenetic analysis**

251 The 639-bp partial sequences of *COI* were completely identical among all
252 individuals representing 15 populations of *M. triviale* (Fig 3). These individuals were placed
253 in the monophyletic group with *M. intrudens* and *M. pharaonis*. Although the genus
254 *Monomorium* has recently been revealed to be a polyphyletic group [26, 30], our result
255 suggests that *M. triviale* belongs to the genus *Monomorium sensu stricto*.

256

Fig 3 Maximum likelihood tree of 15 populations of *M. triviale* and related species, based on *COI* sequences. The number at each branch of the phylogenetic tree represents the bootstrap percentage (1000 replicates). GenBank accession codes follow the taxon names. Scale bar: 0.1 substitutions per site.

Discussion

Our comprehensive investigation allows us to add *M. triviale* to the list of parthenogenetic ants. Production of daughters by unmated queens (indicative of type III thelytoky) was corroborated on the basis of multiple lines of evidence: (i) absence of males and inseminated queens in the field-collected nests; (ii) worker production by laboratory-reared virgin queens; (iii) unfilled spermathecae of queens that produced workers; and (iv) identical genotypes of mother queens and daughter workers. These features were common to all the locations we tested, suggesting that thelytoky is a dominant mode of reproduction across Japanese populations of *M. triviale*.

It should be noted here that rare occurrences of males have been reported in other parthenogenetic ants (type II), such as *Ooceraea biroi* [19] and *Pristomyrmex punctatus* [31]. In addition, queens of *M. triviale* retain undegenerated spermathecae [18], suggesting that they have low level of specialization to male-free reproduction. Moreover, geographic variation in sexual and asexual reproduction has been reported in some thelytokous ant species (types II and III), such as *Mycocepurus smithii*, *Myrmecina nipponica*, and *Platythyrea punctata* [17, 21, 32]. Whether and how often sexual reproduction occurs in *M.*

278 *triviale*, especially in populations outside Japan, would be an interesting topic for future
279 study.

280 As major cytological mechanisms of thelytoky, terminal fusion automixis and central
281 fusion automixis have usually been considered [33]. Under terminal fusion automixis, cells
282 derived from the same cell at the first meiosis are fused to a diploid after the second meiosis.
283 If there is no recombination between chromosomes, the offspring have a homozygous
284 genotype. Under central fusion automixis, different cells at the first meiosis are fused. If
285 recombination does not occur, the offspring have the same genotype as the mother. Many
286 thelytokous species of social Hymenopteran insects adopt the central fusion type of automixis
287 [1]. The identical heterozygous genotypes that we observed here between the mother queen
288 and her daughter workers in *M. triviale* are consistent with central fusion automixis.

289 Our exploratory analysis of bacterial communities in *M. triviale* provides basic
290 information for future studies of the host–symbiont relationship. No evidence was found for
291 infection with thelytoky-inducing bacteria in this species, confirming previous reports of no
292 cases of microorganism-induced thelytoky in ants [10, 24, 34, 35]. *Spiroplasma* has been
293 detected in various ant species [36], including another thelytokous species, *Mycocepurus*
294 *smithii* (type III thelytoky) [37]. In *Solenopsis*, a genus closely related to *Monomorium*,
295 *Spiroplasma* has been detected as a dominant bacterial taxon in whole bodies of adult workers
296 of *S. geminata* [38], thus drawing parallels with our finding. It is noteworthy that type I
297 thelytoky has recently been found in polygynous colonies of this species [39]. *Spiroplasma* is
298 well known as a sex ratio distorter in *Drosophila* [40], and its role in the host's reproductive
299 biology deserves further study in ants.

In our phylogenetic analysis, the extremely low levels of diversity observed in the *COI* sequences suggest the possibility of rapid spread or selective sweep in the recent past [41]. Our results support the concept that this species is a member of the genus *Monomorium sensu stricto*. This phylogenetic status is advantageous in that the study designs established in *M. pharaonis*, a well-studied model system (e.g., [42–47]), will be applicable to future comparative analyses.

Thelytokous parthenogenesis is often considered as a trait overrepresented among introduced or invasive ant species [48]. Although some studies have categorized *M. triviale* as invasive [1, 19], no exotic distribution has been reported so far [14] and there is insufficient information to support the possibility of an introduced origin of the Japanese population of *M. triviale*. The known distribution range of this species is far more limited than those of successful invasive congeners such as *M. pharaonis* and *Monomorium floricola* [15, 49]. A preference for disturbed and urban habitat is shared widely among invasive ants [50]. Ito et al. [50] listed *M. triviale* along with *Strumigenys membranifera*, *P. punctatus*, and *O. biroi* as thelytokous ants found in "open, disturbed areas.'' Among these species, the life history of *M. triviale* is most poorly known. In our study, *M. triviale* nests were found even in a thicket dominated by deciduous broad-leaved trees, which is not typical of "open, disturbed areas." Nevertheless, the above information does not rule out the possibility that *M. triviale* will become invasive in the future. Future studies of this species' social structure, such as queen numbers and the mode of colony foundation, will help to evaluate the potential invasion risk of this species as an ecological consequence of its life history.

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Supporting information

S1 Table. Microbial taxa and their relative abundances (% of total reads) per each *M. triviale* sample

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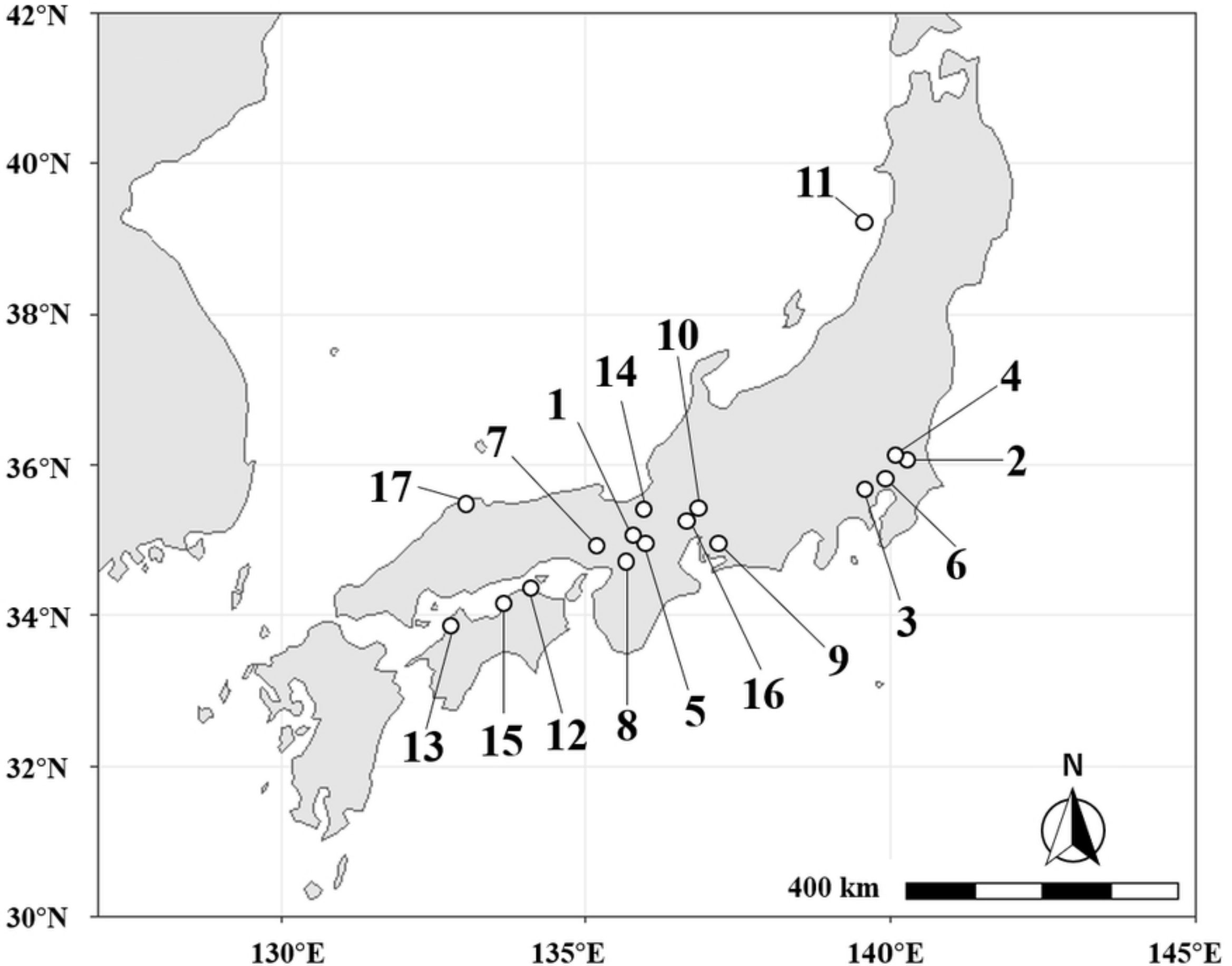
42°N
40°N
38°N
36°N
34°N
32°N
30°N
130°E
135°E
140°E
145°E
11
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N
400 km

Figure_1

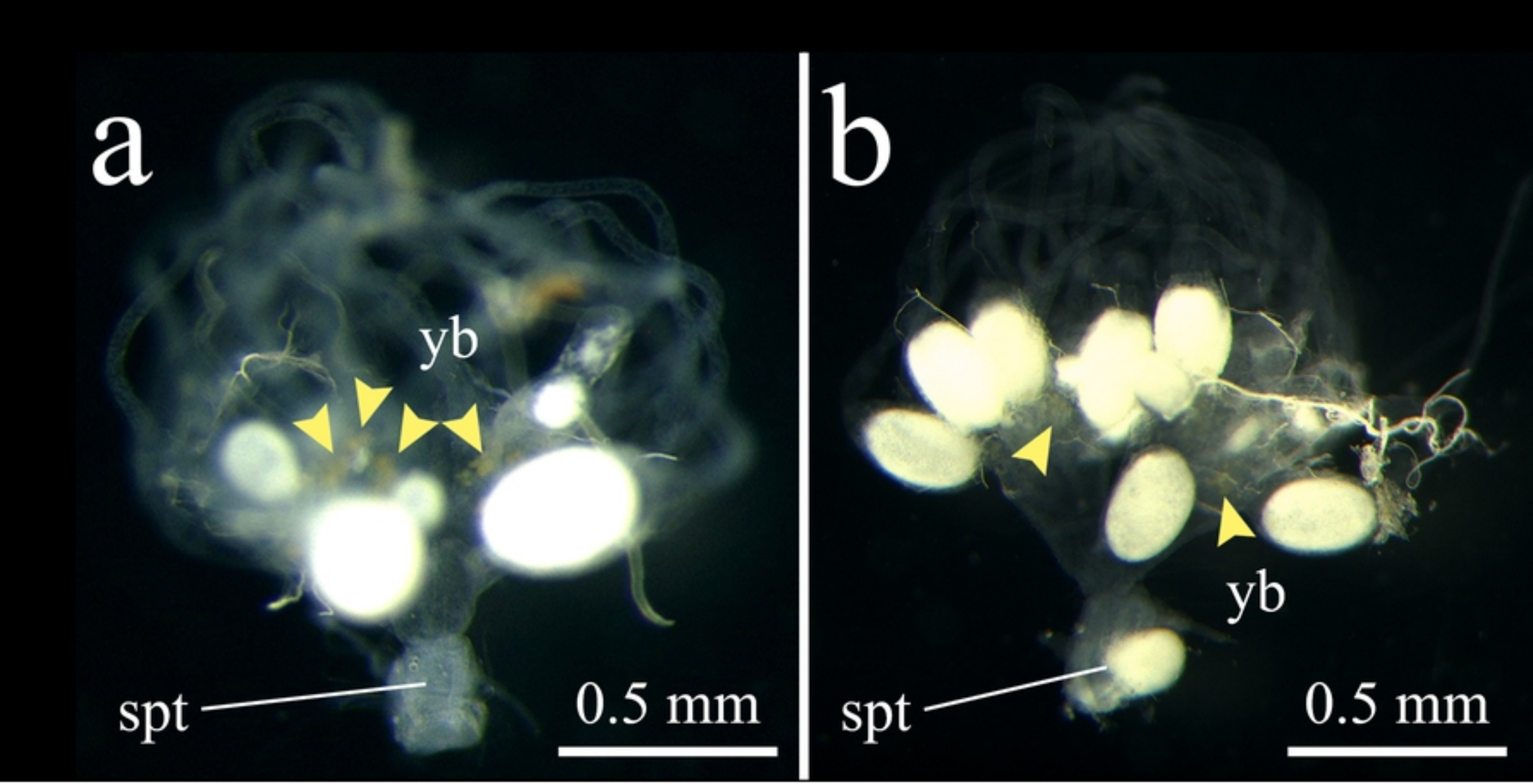

Figure_2

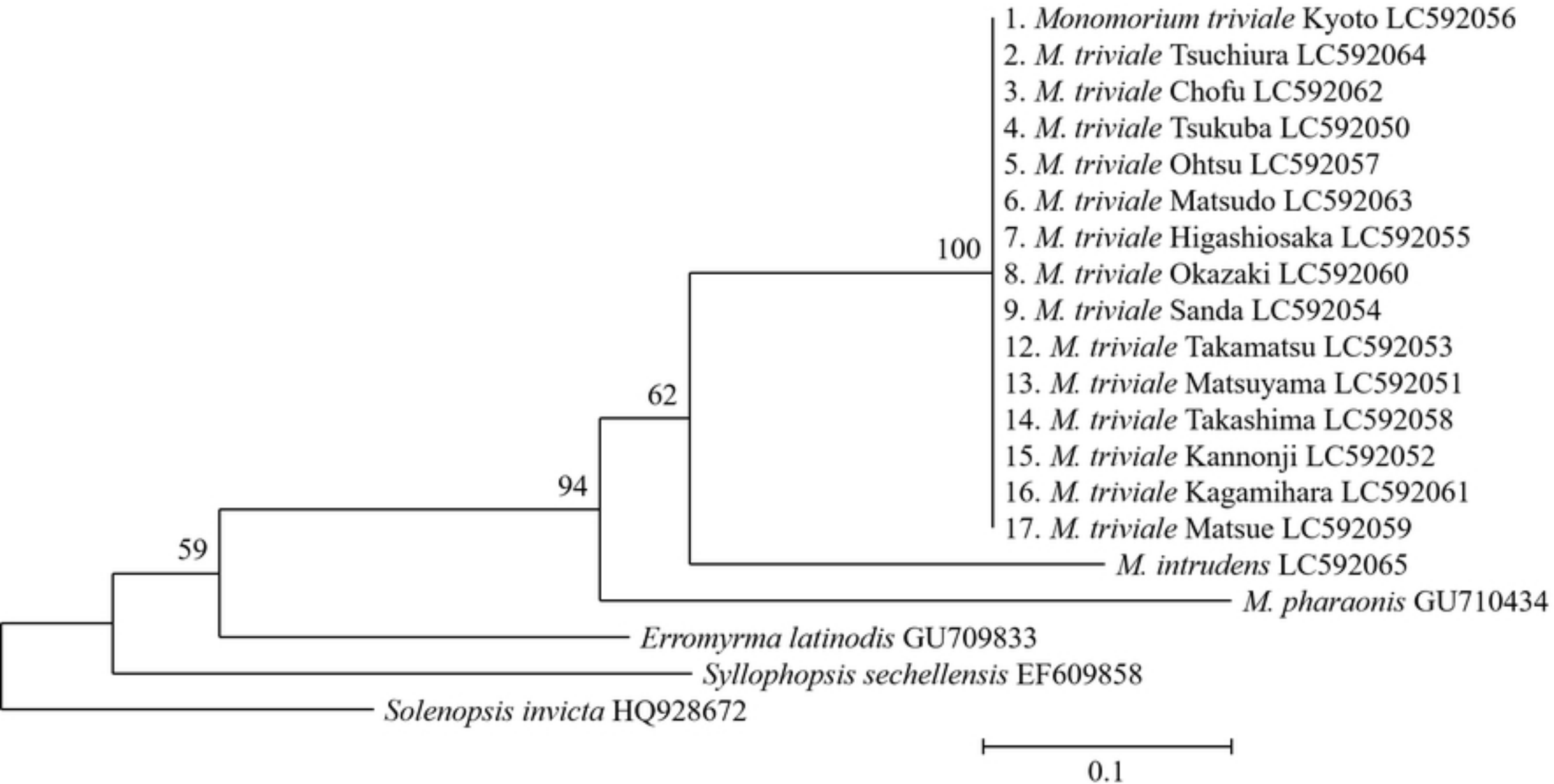
1. Monomorium triviale Kyoto LC592056
2. M. triviale Tsuchiura LC592064
3. M. triviale Chofu LC592062
4. M. triviale Tsukuba LC592050
5. M. triviale Ohtsu LC592057
6. M. triviale Matsudo LC592063
7. M. triviale Higashiosaka LC592055
8. M. triviale Okazaki LC592060
9. M. triviale Sanda LC592054
12. M. triviale Takamatsu LC592053
13. M. triviale Matsuyama LC592051
14. M. triviale Takashima LC592058
15. M. triviale Kannonji LC592052
16. M. triviale Kagamihara LC592061
17. M. triviale Matsue LC592059
M. intrudens LC592065
M. pharaonis GU710434
Erromyrma latinodis GU709833
Syllophopsis sechellensis EF609858
Solenopsis invicta HQ928672
100
62
94
59
0.1

Figure_3