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Endosymbiont-free ants: Molecular biological evidence that neither *Wolbachia, Cardinium* or any other bacterial endosymbionts play a role in thelytokous parthenogenesis in the harvester ant species, *Messor barbarus* and *M. capitatus* (Hymenoptera: Formicidae)

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Key words. *Wolbachia*, *Cardinium*, *Spiroplasma*, endosymbionts, Formicidae, *Messor*, thelytokous parthenogenesis

Abstract. Thelytokous parthenogenesis is a type of sex determination in which females are produced from unfertilized eggs. Genetic and endosymbiont-induced forms of thelytoky have been described in the Hymenoptera. Our study has revealed that *Wolbachia, Cardinium, Spiroplasma* and other endosymbionts are probably absent in Iberian populations of harvester ants, *Messor barbarus* and *Messor capitatus* (Hymenoptera: Formicidae: Myrmicinae) and are thus not involved as factors in the sex determination of these two species. Our results lend weight to previous suggestions that bacterial parthenogenesis induction in Hymenoptera is probably limited to the reproductive systems of chalcidoid and cynipoid parasitoid wasps (Hymenoptera: Apocrita: Chalcidoidea and Cynipoidea, respectively).

INTRODUCTION

Arrhenotoky is a characteristic haplodiploid sex-determining system present in most Hymenoptera species, including ants, bees and wasps, in which females are produced from fertilized eggs and males from unfertilized ones (Fig. 1a–c), whilst thelytokous parthenogenesis, in which females are produced from unfertilized eggs, has been characterized in some 1,500 species, including insects, annelids, reptiles, Urodela and fishes (White, 1984), but has only been described in a few species of Hymenoptera (Fig. 1d–f) (Rabeling & Kronauer, 2013) In most of thelytokous Hymenoptera for instance, unfertilized eggs produce diploid female offspring by fusion of meiotic products, enabling this thelytokous species to survive through several generations of females, without males or sexual reproduction (Fig. 2) (Rabeling & Kronauer, 2013).

In ants (Hymenoptera: Apocrita: Formicidae), thelytoky has been reported in some species like including *Cataglyphis piliscapa* (Forel) (author names needed for all Latin names cited; see http://www.antweb.org/description.do?genus=cataglyphis&name=piliscapa&rank=species) (Lenoir & Cagniant, 1986), *Pristomyrmex pungens* (Mayr) (Itow et al., 1984; Tsuji, 1988), *Cerapachys biroi* (Forel), *Platythyrea punctata* (Smith) (Schilder et al., 1999), *Vollenhovia emeryi* (Wheeler) (Ohkawara et al., 2006, Okamoto & Ohkawara, 2009), *Wasmannia auropunctata* (Roger) (Fournier et al., 2005, Foucaud et al., 2007; Fournier & Aron, 2009; Rey et al., 2011), *Mycocepurus smithii* (Forel) (Himler et al., 2009, Rabeling & Kronauer, 2011), *Cerapachys biroi* (Forel) (Kronauer et al., 2012) and *Messor capitatus* (Latreille) (Grasso et al., 1998). Of these species, there are found to be three thelytokousy reproductive strategies involved (Himler et al., 2009; Rabeling et al., 2011), characterised by: (i) ants species such as *P. pungens*, *C. biroi*, *P. punctate* and *M. capitatus*, whose workers produce females but tend to lose their queen (Pearcy et al., 2004); (ii) ants species such as *V. emeryi*, *W. auropunctata* and *Cataglypsis cursor* (Fonscolombe) whose mated queens produce workers sexually and queens asexually (Pearcy et al., 2004; Fournier et al., 2005; Ohkawara et al., 2006); and (iii) ant species such as *M. smithii* whose workers are sterile and queens are strictly thelytokous individuals (Himler et al., 2009).

Cytogenetic mechanisms leading to thelytokous diploidization can be divided into meiotic and post-meiotic modifications (Vavre et al., 2004; Pearcy et al., 2006; Adachi-Hagimori et al., 2008) (see Fig. 2 for mechanisms). Apart from the parthenogenetic system, the detailed causes and cytological processes involved in sex determination in Hymenoptera are not totally resolved and, in many cases, are species-specific (Wenseleers & Billen, 2000). The best-known model of sex determination in Hymenoptera is the complementary sex determination system (CSD), in which diploid males are only produced by inbreeding. In this model revealed by

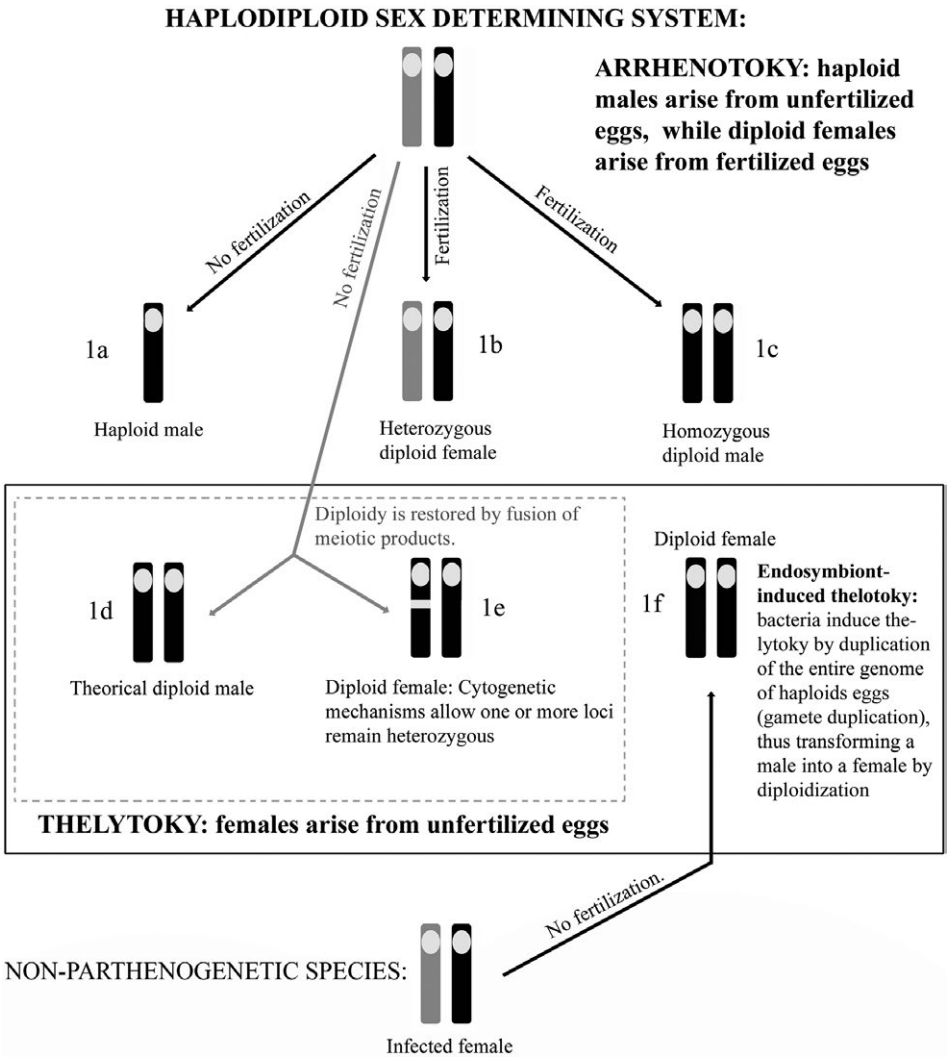

Fig. 1. Haplodiploid sex-determining system types present in most hymenopteran species, including ants, bees and wasps. There are two types of haplodiploid sex-determination system: Arrhenotoky is characterized because haploid males hatch from unfertilized eggs (1a) and diploid females hatch from fertilized eggs (1b). Following complementary sex determination system, theoretically males could appear if all loci remained homozygous (1c and 1d). On the other hand, thelytoky is characterized because females hatch from unfertilized eggs (1e). Microbial induced parthenogenesis (1f) could be considered as an "aberrant" thelytokous sex determination system, in which duplication of the whole genome of the haploid eggs allows the transformation of males into females.

Whiting (1961), sex is determined by the alleles at the sex locus. Diploid heterozygous individuals at this locus become females, homozygous individuals diploid males.

This model is apparently found in many organisms, including Hymenoptera species, with the exception of certain wasps. According to Cook (1993) and Hurst & Peck (1996) the single locus determination model is not applicable in chalcidoid or cynipoid parasitoid wasps (Stouthamer, 1997). Sex determination of the hymenopterous parasitoid, *Nasonia vitripennis* (Walker) (Pteromalidae), for instance, does not involve CDS, but is probably determined by genomic imprinting (Heimpel & de Boer, 2008).

Myrmicine harvester ants of the genus *Messor* are mainly distributed in the Palaearctic region (Bolton, 1982; Agosti, 1987; Cagniant, 1998; Schlick-Steiner et al., 2006), while *M. barbarus* and *M. capitatus* are mainly confined to the Mediterranean basin (Schlick-Steiner et al., 2006). The last Ice Age and post-glacial waves of advancing and retreating ice shaped the phylo-geographic structure of many species, including some ants such as *Formica* sp. (Goropashnaya et al., 2007). Pleistocene glaciations and expansion routes from different refuges have also been proposed to explain the distribution of *Messor* spp. in Central Europe (Schlick-Steiner et al., 2006).

Grasso et al. (2000) found that *M. capitatus* workers can produce female offspring in the absence of a queen. They also confirmed the appearance of males after several laboratory generations. Thelytoky has not been tested in *Messor barbarus* (L.). The taxonomy of the genus *Messor* is poorly resolved in European populations (Lapeva-Gjonova et al., 2010). However, *M. capitatus* and *M. barbarus* belong to the same group (Cagniant, 1998). As far

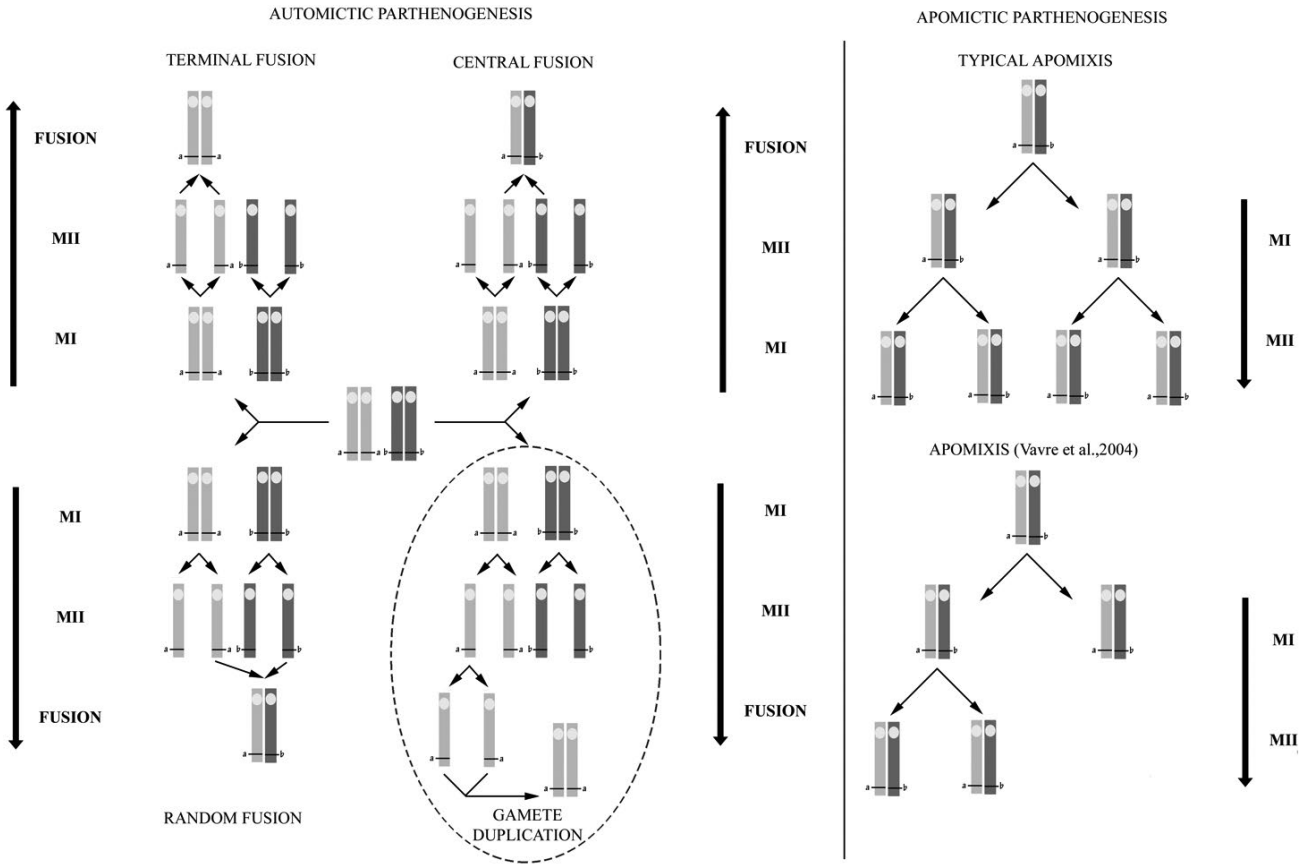

Fig. 2. Cytogenetic mechanisms leading to thelytokous diploidization due to meiotic and post-meiotic modifications. Note the different consequences in homozygosity or heterozygosity of embryos. Automictic parthenogenesis can be induced by terminal fusion, central fusion, random fusion or gamete duplication. A new variant of apomictic parthenogenesis has been described by Vavre et al. (2004). MI – meiosis I; MII – meiosis II.

as we are aware, no data exist about the reproductive system of *M. barbarus.*

The rickettsial bacteria *Wolbachia* is a maternally-inherited endosymbiont that induces reproductive alterations in many arthropods and nematodes (Werren, 1997). It is associated with male killing, male feminisation, cytoplasmic incompatibility (CI) and parthenogenesis induction (Werren et al., 1995, 2008; Serbus et al., 2008). Parthenogenesis induction occurs in distinct parasitoid wasps, where bacterial infection has been linked to their haplodiploid reproduction system (Breeuwer & Werren, 1990; Cordaux et al., 2011). *Wolbachia* induces automictic parthenogenesis in some parasitoid wasps belonging to the genera *Trichogramma, Aphytis, Encarsia, Leptopilina*, and *Muscidifurax*. The bacterium manipulates the wasp's reproduction to produce mostly female offspring and thus guarantees its maternal transmission (Legner, 1987; Stouthamer & Kazmer, 1994; Werren et al., 1995). *Wolbachia* cause "gamete duplication" (Fig. 2) restoring diploidy: a segregation failure in anaphase-I result in a homozygous diploid female (Stouthamer & Kazmer, 1994). However, a new mechanism similar to apomictic parthenogenesis was reported by Vavre et al. (2004), who described *Rickettsia*-induced parthenogenesis in the wasp parasitoid *Neochrysocharis formosa* (Westwood) (Hymenoptera: Eulophidae), with meiosis replaced by a single equatorial division. The traditional cytological differences between bacteria-induced and non-induced thelytokous parthenogenesis should, in light of this, therefore be revised.

Other bacterial symbionts like *Rickettsia* have been proposed as parthenogenesis-inducing or inductors of reproductive distortion. *Cardinium* was the second bacterial lineage discovered to induce CI in arthropods, and the endosymbiotic bacteria *Spiroplasma* spp. are known to cause selective death of male offspring in insects (e.g. Hagimori et al., 2006; Giorgini et al., 2010). Other bacteria such as *Serratia* have also been reported as endosymbionts of wood ants, *Formica cinerea* (Mayr), as well as *Wolbachia* and *Cardinium* (Sirviö & Pamilo, 2010). Endosymbionts that alter reproduction are considered to be highly important in the evolutionary history of social insects (ants, social bees and wasps), potentially interfering in the sex-ratio conflict between workers and queens (Lombardo, 2008; Sirviö & Pamilo, 2010).

Wolbachia infection has been reported in several ant species (Wenseleers et al., 1998; Wenseleers & Billen, 2000; Van Borm et al., 2001; Russell et al., 2009; Frost et al., 2010), although few studies have explored the possible role of bacteria in relation tothelytokous modes of reproduction. For example, Wenseleers & Billen (2000) failed to detect *Wolbachia* in some of the reported thely-

tokous species [*P. punctate, C. biroi, P. pungens, M. capitatus, C. cursor, Cataglyphis piliscapus* (Forel)], and did not test for other endosymbionts. In addition, inherited bacteria such as *Wolbachia* and *Cardinium* have been dismissed as factors inducing asexuality in *M. smithii* (Himler et al., 2009).

Grasso et al. (2000) excluded the influence of *Wolbachia* in thelytokous parthenogenesis in *M. capitatus*, but confirmed the thelytoky of workers, as previously reported in this harvesting ant (Grasso et al., 1998). These authors concluded that in this species, thelytoky has evolved independently of *Wolbachia* (A or B supergroups), certainly in the Italian populations examined. Additional *Wolbachia* supergroups have been reported since then, and further diagnostic bacterial genes have been sequenced, allowing better characterization in many cases, an important aspect given that a different sensitivity of PCR (polymerase chain reaction) primers for detecting *Wolbachia* can lead to false negative results (Hong et al., 2002; Marcon et al., 2011; Simoes et al., 2011). The Multilocus System Typing (MLST) (Baldo et al., 2006b) is a powerful methodology for checking possible *Wolbachia* infection. It allows for the checking of the DNA sequences of five distinct specific bacterial loci simultaneously, reveals the bacterial supergroup, and enables informative phylogenies to be constructed (Frost et al., 2010; Arthofer et al., 2011; Zhang et al., 2011).

Our objective in the present study was to test these new markers in *Messor* sp. in order to confirm the independence of thelytokous parthenogenesis in this ant genus from any kind of endosymbiotic bacterium. We also checked the possible role of other endosymbionts that could alter host reproduction in different ways. *Rickettsia*, *Cardinium* or *Spiroplasma* had not been previously screened in this insect. If present, they might be involved in its peculiar reproductive system (i.e. production of female offspring in the absence of a queen)

The study was conducted on Iberian populations of *M. barbarus* and *M. capitatus* to facilitate their comparison with the Italian populations analyzed by Grasso et al. (1998, 2000) as well as to test geographical differences in the bacterial infection due to the glacial refuge effect.

MATERIAL AND METHODS

Field collections and DNA extraction

More than 250 individuals of *M. capitatus* and *M. barbarus* were collected from different populations in Spain in 2010 (Table 1, Fig. 3). Individuals were stored in 100% ethanol prior to DNA extraction. After careful drying, each sample (a pool of 3–4 individuals from a given location) was homogenized in 300 µL of TNES buffer (50 mM Tris-HCl, pH 8.0; 400 mM NaCl; 20 mM EDTA, pH 8.0; 0.5% SDS) containing proteinase K (0.03% w/v) (Roche Applied Science, Indianapolis, IN, USA) and incubated overnight at 37°C. Standard phenol/chloroform (Acros Organics, Thermo Fisher Scientific, Belgium) extraction followed by ethanol precipitation was used. The samples were dissolved in Tris-EDTA (1 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) and incubated with RNase A (Roche Applied Science) (5 µg/ml) for 2 h at room temperature. The final DNA concentration was 50 ng/µL.

TABLE 1. Sampled populations of *Messor capitatus* and *M. barbarus*, of which a total of *n* individuals were analysed.

Sample	Latitude	Longitude	Altitude	*n*
La Bañeza I	266687	4692040	797	24
La Bañeza III	263733	4684441	788	20
La Bañeza III	253795	4682834	813	20
Villalpando I	305018	4643862	694	23
Villalpando II	299157	4637024	690	20
Villalpando III	294332	4626560	729	20
Rueda I	345498	4585536	762	21
Rueda II	336037	4586225	744	20
Rueda III	328714	4577869	685	20
Sanchidrián I	377110	4527770	1021	23
Sanchidrián II	367258	4529221	927	20
Sanchidrián III	358426	4525813	856	24
Total				255

Bacterial PCR detection

Wolbachia was screened by PCR amplification of the *Wolbachia*'s 16S rRNA gene sequence, using general forward and reverse primers (Zabal-Aguirre et al., 2010) and a second nested PCR amplification with B and F strain-specific primers (nested_FF: 5′ TGA GCC TAT ATT AGA TTA GCT AGT TGG TAA G 3′; nested_FB: 5′ GCC TAT ATT AGA TTA GCT AGT TGG TGG A 3′; nested_reverse: 5′ TAG TCC CCA GGC GGA ATG TT 3′). Reactions were performed in 25 µL containing 2 mM of $MgCl_2$, 0.2 mM dNTPs, 1.2 µM of each primer, 1.25 U BIOTAQ DNA polymerase (Bioline Reagents Ltd, UK) and 2 µL of DNA solution (50 ng/µl). The detection threshold was tested with dilution series in order to determine the optimal genomic DNA concentration for PCR conditions. We estimated that 100 ng of genomic DNA, added to a PCR reaction of 25 µL, allowed the detection of endosymbionts in our positive controls. Thermal cycling conditions were 30 s at 95°C (denaturing), followed by 35 cycles of 30 s at 95°C, 1 min at 54°C (annealing) (for the general 16S rRNA gene primers), 69°C (for the 16S rRNA gene-specific primers) and 1 min at 72°C (extension), and with a final elongation step of 10 min at 72°C.

Multi-locus strain typing (MLST) and *wsp* amplification were performed as described by Baldo et al. (2006a) with modifications: PCR amplification was performed in a 50 µL total volume of reaction mixture containing 2 mM of $MgCl_2$, 0.2 mM dNTPs, 1.2 µM of each primers and 1.25 U of BIOTAQ DNA polymerase. The thermal cycling conditions were incubation at 94°C for 10 min, followed by 36 cycles of 30 s at 94°C, 45 s of 54°C for *hcpA, gatB, ftsZ and coxA*, and 59°C for *fbpA* and *wsp,* and 72°C for 1.5 min, a final elongation step at 70°C for 10 min, and hold at 4°C.

Spiroplasma infection was tested by PCR amplifying the 16S rRNA gene with specific primers Ha-In-1 (Hurst et al., 1999) and SP-ITS-N2 (Jiggins et al., 2000). *Cardinium* were analyzed using specific primers Ch_F and Ch_R (Zchori-Fein & Perlman, 2004). Universal primers 27f and 1492R (Weisburg et al., 1991) were employed as additional controls to verify the presence of any other unknown endosymbionts, including *Rickettsia* sp., in the samples. PCR amplification was here performed in a total volume of 25 µL containing 2 mM of $MgCl_2$, 0.2 mM dNTP, 1.2 µM of each primers, 1.25 U BIOTAQ DNA polymerase and 2 µL of DNA solution (50 ng/µl). Thermal cycling conditions were incubation at 94°C for 10 min, followed by 36 cycles of 30 s at 94°C, 1 min at 55°C (*Spiroplasma* 16S rRNA gene and bacteria universal primers 16S rRNA gene) or 58°C (*Cardinium* and *Spiroplasma* DNAse gene primers) and 1.5 min at 72°C, a

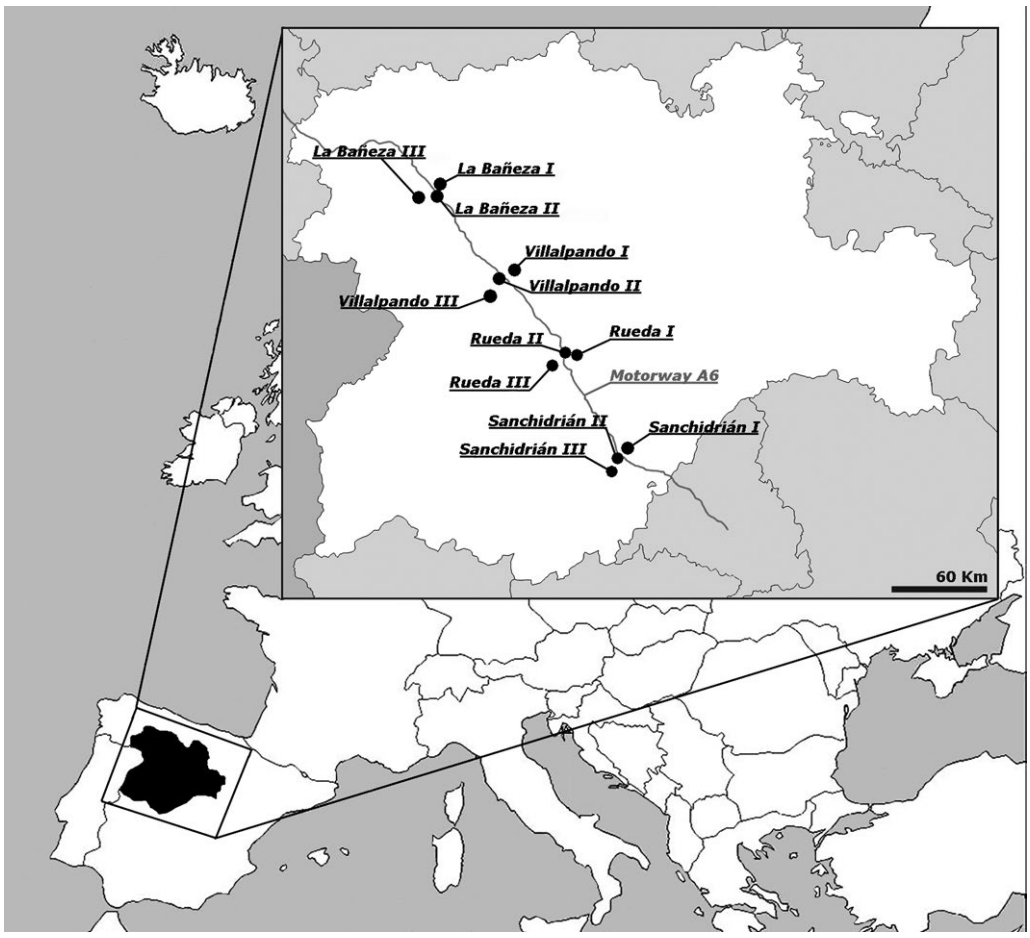

Fig. 3. Geographical distribution of the analyzed populations of *Messor capitatus* and *M. barbarus*.

final elongation step at 70°C for 10 min, and hold at 4°C. 10 µL of each amplification product was separated electrophoretically on 0.7% agarose gel stained with ethidium bromide. Controls were run lacking DNA template. PCR products were purified by illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Sacramento, CA, USA), whilst sequencing was performed by STAB VIDA (www.stabvida.com), Caparica, Portugal.

Positive and negative controls were run: DNA from *Wolbachia* and *Spiroplasma* infected individuals of the meadow grasshopper, *Chorthippus parallelus* (Zetterstedt) (Orthoptera: Acrididae) (Martinez et al., 2009) were employed as templates in positive controls for *Wolbachia*, *Spiroplasma* and Eubacteria PCR reactions. Because DNA from *Cardinium*-infected insects was not available, genomic DNA from a pool of non-identified spiders was instead used as a positive control, given that spiders are usually infected by this bacterium (S. Goodacre, pers. comm.). PCR conditions for *Cardinium* were as described by Zchori-Fein & Perlman (2004). *Cardinium* was not found in any sequencing following Eubacteria PCR reactions.

DNA quality was tested by amplification of the ant's mtDNA gene *Cox-1* (Lunt et al., 1998) and ITS1 from their rRNA gene (Parkin & Butlin, 2004). Reactions were performed in a total volume of 50 µl containing 2 mM of $MgCl_2$, 0.2 mM dNTPs, 1.2 µM of each primers, 1.25 U BIOTAQ DNA polymerase and 2 µl of DNA template (50 ng/µl). Thermal cycling conditions were 30 s at 95°C, followed by 39 cycles of 30 s at 95°C, 1 min at 56°C and 1 min at 72°C, with a a final elongation step of 10 min at 72°C.

RESULTS AND DISCUSSION

Ten individuals from each field colony were examined for *Wolbachia* infection using the above-mentioned PCR assays, but none were found to be infected. However, the amplified PCR products in all positive controls exclude the possibility of technical failure.

Wolbachia diversity has been proposed as a cause for such failures of detection because these bacteria have not always been successfully detected during PCR screening surveys (Sirviö & Pamilo, 2010). However, we tested several pairs of primers (specific for 16S rRNA gene, five MLST loci and the *wsp* gene) to avoid this problem. The primers were designed to amplify any *Wolbachia* supergroup, but none of them detected *Wolbachia*. This result allows us to disregard with some degree of confidence the failure of PCR amplification to be due to an unknown *Wolbachia* strain infecting both *Messor* ant species here tested. These results thereby confirm that *Wolbachia* is not involved in parthenogenesis induction in *Messor* species, and is unlikely to be involved in any of the other previously described thelytokous ant species.

Several reasons have been proposed to explain why *Wolbachia* does not infect thelytokous ants. Hurst & Peck's hypothesis (1996) supports that idea that *Wolbachia*-induced thelytoky would produce homozygous diploid offspring, which in *Messor* ant species

would result in the production of diploid males (Fig. 1d), an impasse for *Wolbachia* maternal transmission (Stouthamer, 1997; Wenseleers & Billen, 2000). This could explain why social Hymenoptera such as *Messor* sp. are not infected by the bacterium.

Wenseleers et al. (1998) proposed that the spread of *Wolbachia* infection could be difficult in monogynous ant colonies (like *M. capitatus*) but easier in polygynous species. In contrast, Shoemaker et al. (2000) argue that monogynous ant species (e.g. red imported fire ant, *Solepnopsis invicta* Buren) favour a high prevalence of *Wolbachia*, inducing cytoplasmic incompatibility. Recently, Sirviö & Pamilo (2010) did not find any correlation between *Wolbachia* infection and the detected polymorphism of the wood ant, *F. cinerea* related to monogynous/polygynous colonies. Our results partially support Wenseleers et al.'s hypothesis. However, more studies are needed to test whether this social behavior is associated with *Wolbachia* infection. In addition, loss of *Wolbachia* infection in ants has been linked to population processes such as expansions and local population bottlenecks (Shoemaker et al., 2000; Reuter et al., 2004). Such stochastic means should be considered in *M. capitatus* and *barbarus*, whose recent natural history has been related to quaternary glaciations (Schlick-Steiner et al., 2006).

In our study, we also tested infection by other bacterial endosymbionts. Neither *Cardinum* nor *Spiroplasma* were detected in any sample, and Eubacteria PCR assays allowed us to disregard other potential endosymbionts. DNA quality assays (successful PCR amplification of ant ribosomal and mitochondrial genes) also allowed us to disregard any false negative PCR amplifications due to degraded DNA.

The bacterial genus *Cardinium* has recently been described, and includes species that manipulate host reproduction (Weeks & Breeuwer, 2001; Hunter et al., 2003; Zchori-Fein & Perlman, 2004). In species such as the false spider mite, *Brevipalpus phoenicis* (Acari: Trombidiformes: Tenuipalpidae), *Cardinium* induces thelytokous reproduction through the feminization of unfertilized eggs. Uninfected unfertilized eggs of *B. phoenicis* develop into haploid males (Weeks & Breeuwer, 2001), thus making this genus a candidate for the induction of parthenogenesis in ants. Recently, Sirviö & Pamilo (2010) detected low prevalence (1–2%) of *Cardinium* in in the wood ant, *F. cinerea*. However, these are not detailed studies about the effect of this endosymbiont in the host. Even so, the low prevalence of this endosymbiont suggests a non-essential role in the biology of *F. cinerea.* Moreover, our own study indicates that *Cardinium* does not induce parthenogenesis in *Messor capitatus*. These data could question previous hypotheses about the role of these bacteria in ant sex ratio alterations.

Spiroplasma is another bacterial genus detected in several arthropods. Its most frequent host phenotype is related to male killing (Gasparich et al., 2004). In our study, *Spiroplasma* was tested with specific primers Ha-In-1 (Hurst et al., 1999) and SP-ITS-N2 (Jiggins et al., 2000), but these bacteria were not detected in our samples. Other *Rickettsia* spp. have been reported as bacteria that could manipulate the reproduction of their host and induce thelytokous parthenogenesis (Adachi-Hagimori et al., 2008). The universal PCR primers used in the present study disallow this possibility, as they should detect any endosymbiont species infecting *Messor* spp. In all samples, positive amplifications matched with environmental bacteria such as *Pseudomonadales*. Neither *Rickettsia* sp. nor any other endosymbionts were detected.

Tests for incidence of other unknown endosymbionts only yielded seven positives samples. While DNA sequences showed a high homology with *Pseudomonas*, *Lactobacillus* and other non-identified bacteria, no endosymbiont sequences were recorded. Environmental and human contamination are the most likely sources of these bacteria.

The incidence of reproductive endosymbionts is probably heavily underestimated in the scientific literature, considering the small number of individuals usually sampled from most species, which may fail to detect relatively rare infections, and also the low prevalence of endosymbionts in several species. For instance, only 1–2% of individuals of *Formica cinerea* were infected by *Cardinium*, including several uninfected populations (Sirviö & Pamilo, 2010). These low proportions could be close to the sample and PCR error (Löfström et al., 2008). Thus, although a large number of individuals were tested in our study, the possibility of an extremely rare endosymbiont infecting *M. capitatus* and/or *M. barbarus* cannot be excluded.

Most species reproduce sexually. Because asexual reproduction is rare (Simon et al., 2003), many theories about the paradox of sex have been proposed to explain the advantages of sex against its cost and inefficiency (Agrawal, 2009; Birky & Barraclough, 2009). Various selected forces have been proposed as explanations for thelytokous parthenogenesis in ants, which appear in different subfamilies. For instance, asexual lineages could colonize new habitats and food sources more easily than related sexual species (Williams, 1975; Maynard, 1978; Bell, 1982). In this case, however, the role of endosymbionts can be excluded definitively.

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