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Molecular and morphological characterisation of Pseudococcidae surveyed on crops and ornamental plants in Spain

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Abstract

Mealybugs (Hemiptera: Pseudococcidae) are common invasive pests in Europe, causing major problems on crops and ornamental plants. However, very few data are available concerning the mealybug fauna of southern Europe. This lack of data and the difficulty of identifying mealybugs morphologically by traditional techniques currently limit the perspectives for efficient specific pest management. The aim of this study was to provide multi-criterion characterization of mealybugs surveyed in eastern Spain in order to facilitate their routine identification through DNA sequencing or the use of derived species-specific molecular tools. We characterised 33 mealybug populations infesting crops and ornamental plants in eastern Spain, using a combination of molecular and morphological techniques, including the sequencing of the universal barcode DNA region cytochrome c oxidase subunit I (COI). This characterisation has led to the identification of ten species and provides sequence data for three previously unsequenced species, contributing to the phylogenetic knowledge of the family Pseudococcidae. In addition, the intraspecific variations found in the populations of five mealybug species provide insight into their invasion history.

Keywords: DNA barcoding, molecular characterization, mealybug, species identification

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Introduction

Invasive species constitute a major threat to biodiversity and agricultural ecosystems and may have a significant ecological and economic impact (Williamson, 1996; Pimentel *et al.*, 2001; Kenis *et al.*, 2009). Scale insects are typical invasive pests; due to their small size and cryptic behaviour, they often remain undetected during quarantine inspections (Miller *et al.*, 2005; Hulme *et al.*, 2008; Pellizzari & Germain, 2010). One particular group of scale insects, mealybugs (Hemiptera: Pseudococcidae), constitutes the third most common family of alien insects in Europe, with about 40 new established species (Roques *et al.*, 2009; Pellizzari & Germain, 2010). Mealybugs are common pests of a wide range of agricultural and ornamental plants (Ben-Dov, 1994) and may cause serious problems if they become established in new environments lacking natural enemies (Miller *et al.*, 2002). They damage the plant by sucking its sap and transmitting viruses. Furthermore, the honeydew they produce may also favour the development of mould fungi and decrease ornamental plant quality (Williams, 1985; Kosztarab & Kozár, 1988; Franco *et al.*, 2000).

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Mealybug management is currently challenged by frequent species misidentification that decreases the efficiency of crop protection methods and increases pesticide use. This situation can be explained by the lack of reliable surveys and characterisations of mealybug species, mainly because their identification has been difficult or even sometimes impossible until recently. Indeed, taxonomy and identification of members of the family Pseudococcidae have generally been based on comparisons of the morphological characters of adult females. However, there are several drawbacks to this method. Firstly, it is a time-consuming process requiring specialised taxonomic knowledge, which is not available on a daily basis for most practitioners. Secondly, some environmental conditions may induce morphological variation in mealybugs, making it impossible in some cases to differentiate between complexes of cryptic species (Cox, 1983; Charles *et al.*, 2000). Thirdly, mealybug morphological identification is generally impossible when specimens are collected at larval stage (a common situation in the field and of special concern in quarantine controls).

These difficulties can be dealt with by taking profit from the complementarities between morphological and molecular characterization to identify the species. Indeed, once a reference specimen is taxonomically identified by morphological examination and characterized by DNA sequencing, any new sample displaying the same DNA sequence can be identified quickly without the need of any competence in taxonomy. For taxonomists, such an approach also avoids repetitive identification of the most common species. This method is the basis of the so-called DNA barcoding international projects (Hebert *et al.*, 2003). The main barcode region used in international projects is a 648 bp region of the cytochrome c oxidase subunit I (COI) (Hebert *et al.*, 2003). However, despite COI having been used in various taxonomic studies of mealybugs (Gullan *et al.*, 2003, 2010; Demontis *et al.*, 2007; Cavalieri *et al.*, 2008; Rung *et al.*, 2008, 2009; Saccaggi *et al.*, 2008; Ashfaq *et al.*, 2010; Pieterse *et al.*, 2010; Park *et al.*, 2011), the universal primers used to amplify this region do not work well in several species of this family (Malausa *et al.*, 2011). Therefore, new primers for this region have been recently designed (Malausa *et al.*, 2011; Park *et al.*, 2011). Moreover, the use of combinations of different DNA markers as nuclear DNA, mitochondrial DNA and endosymbiont DNA (from *Tremblaya Princeps*) proved to be successful not only for DNA barcoding but also to better estimate the genetic distance between species and for disentangle complexes of cryptic taxa (Malausa *et al.*, 2011).

In this study, we coupled the morphological examination of slide-mounted samples and their DNA sequencing at five markers to generate multi-criterion identification of 33 mealybug populations infesting crops and ornamental plants in eastern Spain. This work provides a comprehensive characterisation of ten species found in eastern Spain and will be used as basis for the routine identification of mealybugs in Spain and more generally in southern Europe, by DNA sequencing or with molecular identification tools derived from DNA sequences.

Materials and methods

Sample collections

Thirty-three mealybug populations damaging crops and ornamental plants were sampled in eastern Spain between the years of 2007 and 2009 (table 1). An additional sample of *Phenacoccus peruvianus* Granara de Willink was collected from southern France, for comparison of the populations of this new invasive species in the two countries. The samples consisted mostly of adult females and immature instars, which were just taken when adults were not available. Individuals were checked under a stereoscope and discarded if any parasitoids were detected. The collected insects were preserved in 70% ethanol and stored at −20°C for molecular analysis and morphological identification.

DNA extraction and amplification

DNA was extracted from 239 specimens by using the DNeasy Tissue Kit (QIAGEN). The extraction was performed without crushing the insect body, which enabled us to recover the specimen for its posterior morphological identification. Therefore, the process followed the manufacturer's guidelines with two small variations to improve DNA extraction: cell lysis was carried out over a period of six to eight hours and two elution steps (2 × 50 µl of AE buffer) (Malausa *et al.*, 2011).

DNA was amplified from five different loci, chosen for analysis on the basis of their suitability for DNA barcoding, population genetics and phylogenetic studies: two regions of mitochondrial mealybug DNA (the 2183–2568 and LCO regions of COI), two regions of nuclear DNA (28 s-D2 and the entire ITS2 region) and one region of DNA from the bacterium *Tremblaya princeps* (leuA-16 s) (Malausa *et al.*, 2011). PCR was performed with a 23 µl reaction mixture and 2 µl of diluted DNA (1–20 ng). The reagent concentrations were 1 × Phusion HF buffer (Phusion High-Fidelity DNA polymerase 530 (FINNZYMES, Espoo, Finland)), 0.01 U μl^{-1} Phusion enzyme, 200 µM dNTPs and 0.5 µM of each primer (table 2).

PCR was carried out as follows: initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing for 15 s at a temperature of 48°C–60°C, depending on the primer (table 2), and elongation at 72°C for 5 min. The final products were separated by electrophoresis in a 2% agarose gel, to check their quality. They were then sequenced in both directions, by capillary electrophoresis on an ABI 3130XL automatic sequencer (Applied Biosystems, Foster City, CA, USA) at Genoscreen (Lille, France). Consensus sequences were generated and analysed with Seqscape v2.5 (ABI), and alignments were manually edited with Bioedit (Hall, 1999). When a sequence of a specimen displayed a genetic variation at one or more nucleotide(s), it was considered as a different haplotype. The analysed sequences were deposited in GenBank to ensure future access and use (accession numbers JF714157–JF714210).

Morphological identification

Mealybug populations were identified on the basis of morphological characters. All the individuals sequenced were recovered after the DNA extraction, preserved in 70% ethanol and stored at −20°C. Individuals were posteriorly mounted on slides as described by Williams & Granara de Willink (1992), with the modifications described by Malausa *et al.* (2011) and a few additional changes: a small ventral incision was made behind the back leg, with a micro scalpel (BioQuip Products Inc., Rancho Dominguez, CA, USA). The specimen was heated at 60°C in 10% KOH for 20 min and washed in distilled water for 20 min. It was then stained with a 1:1:1 acid fuchsin (1% solution), lactic acid and glycerol. Specimens were then

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Table 1. List of the material sampled: Population codes, geographic origin and host origin of the samples and number of individuals used for DNA extraction and morphological identification.

Pop #	Region	City	GPS coordinates	Host	Collection date	*N*	Identification
1	C. Valenciana	Valencia	39.480998 N, 0.349395 W	*Coronilla sp.*	03/06/08	8	*Phenacoccus madeirensis*
2	C. Valenciana	Ibi	38.622584 N, 0.575401 W	*Cupressus sempervirens*	10/09/08	6	*Planococcus vovae*
3	C. Valenciana	Valencia	39.472218 N, 0.351524 W	*Cupressus sempervirens*	15/07/08	8	*Planococcus vovae*
4	C. Valenciana	Valencia	39.476822 N, 0.386716 W	*Dyospiros duclouxii*	12/09/08	8	*Planococcus citri* *Phenacoccus madeirensis*
5	C. Valenciana	Valencia	39.476822 N, 0.386716 W	*Erytrina bogotensis*	12/09/08	8	*Phenacoccus madeirensis*
6	C. Valenciana	Altea	38.602324 N, 0.045092 W	*Lantana camara*	23/08/08	8	*Phenacoccus madeirensis*
7	C. Valenciana	Altea	38.602324 N, 0.045092 W	*Unknown host*	23/08/08	8	*Phenacoccus madeirensis*
8	C. Valenciana	Valencia	39.467628 N, 0.344121 W	*Lantana camara*	22/09/08	4	*Phenacoccus madeirensis*
9	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Saccharum officinarum*	02/03/08	8	*Dysmicoccus boninsis*
10	C. Valenciana	Altea	38.608801 N, 0.041615 W	*Ceratonia siliqua*	23/08/08	6	*Planococcus citri*
11	C. Valenciana	Algimia d'Alfara	39.753015 N, 0.360651 W	*Solanum lycopersicum*	21/07/08	8	*Planococcus citri*
12	C. Valenciana	Valencia	39.485907 N, 0.362367 W	*Ocimum basilicum*	22/09/08	8	*Planococcus citri*
13	C. Valenciana	Valencia	39.476822 N, 0.386716 W	*Cleistocactus strausii*	13/11/07	8	*Planococcus citri*
14	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Acalipha wilkesiana*	02/03/08	8	*Planococcus citri*
15	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Aucuba japonica*	18/04/08	8	*Phenacoccus peruvianus*
16	Catalunya	Blanes	41.676872 N, 2.801936 E	*Bougainvillea glabra*	24/09/08	8	*Phenacoccus peruvianus*
17	Catalunya	Blanes	41.676872 N, 2.801936 E	*Cordilyne stricta*	24/09/08	3	*Pseudococcus longispinus*
18	C. Valenciana	Valencia	39.476364 N, 0.357291 W	*Unknown host*	10/06/08	5	*Phenacoccus peruvianus*
19	C. Valenciana	Valencia	39.478956 N, 0.367683 W	*Myoporum sp.*	10/06/08	8	*Phenacoccus peruvianus*
20	C. Valenciana	Altea	38.608801 N, 0.041615 W	*Malva parviflora*	23/08/08	8	*Phenacoccus madeirensis*
21	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Cereus peruvianus*	02/03/08	8	*Hypogeococcus pungens*
22	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Olla carnosa*	03/06/08	5	*Planococcus citri*
23	C. Valenciana	Altea	38.602324 N, 0.045092 W	*Euonymus japonicus*	23/08/08	8	*Pseudococcus longispinus*
24	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Salvia sp.*	15/07/08	8	*Pseudococcus viburni*
25	C. Valenciana	Altea	38.602324 N, 0.045092 W	*Pittosporum tobira*	23/08/08	8	*Pseudoccus longispinus*
26	C. Valenciana	Altea	38.602782 N, 0.047820 W	*Hibiscus rosa-sinensis*	23/08/08	5	*Pseudoccus longispinus*
27	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Bougainvillea glabra*	12/03/08	7	*Phenacoccus peruvianus*
28	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Parietaria judaica*	12/12/08	8	*Planococcus citri*
29	Andalucia	El Ejido	36.719749 N, 2.789198 W	*Capsicum annum*	20/12/08	8	*Phenacoccus solani*
30	Catalunya	Deltebre	40.724924 N, 0.839764 W	*Myoporum laetum*	18/08/08	8	*Phenacoccus peruvianus*
31	C. Valenciana	Valencia	39.482109 N, 0.343475 W	*Chamaedorea sp.*	30/01/08	8	*Pseudococcus longispinus*
32	Alpes Maritimes	Antibes	43.575327 N, 7.125707 E	*Bougainvillea glabra*	25/09/08	8	*Phenacoccus peruvianus*
33	C. Valenciana	Faura	39.732309 N, 0.269403 W	*Citrus reticulata*	19/09/09	4	*Delottococcus aberiae*
34	Illes Balears	Soller	39.764619 N, 2.709765 W	*Justicia suberecta*	22/10/09	4	*Phenacoccus peruvianus*

immersed in acetic acid for one hour and transferred to lavender oil for an additional one hour. Finally, the insects were mounted on a slide in Heinze Mounting Medium (Heinze, 1952) and covered with a coverslip. Slides were then heated at 30°C for 48 h.

Specimens were identified principally with the keys of Williams & Granara de Willink (1992), Gimpel & Miller (1996), Williams (2004) and Granara de Willink & Szumik (2007). For nymph voucher specimen identification, some species for which immature instars have never been described were identified to genus level only. The slides are available from the Polytechnic University of Valencia (Valencia, Spain).

Phylogenetic analysis

Phylogenetic studies were performed by merging our populations with other samples for which the same loci had been sequenced by Malausa *et al*. (2011). Bayesian inference was carried out with BayesPhylogenies (Pagel & Meade, 2004). Interspecific variability was too high for the alignment of ITS2 sequences. Thus, for this region, we inferred the phylogenetic relationships from a mixture model based on the other four loci. Analyses were carried out with nQ+C mixture models, with n varying between one and six independent rate matrices (Qs). The best model was chosen by comparing Bayes factors. We also applied a general time-reversible model, as recommended by Pagel & Meade (2004). Four Markov chains were used for ten million iterations and a print frequency of 1000 iterations. The length of the burn-in period was determined by plotting likelihood across iterations. All iterations corresponding to the burn-in period (around one million iterations) were removed from the output of BayesPhylogenies before subsequent analyses. We used the sump command of MrBayes (Ronquist & Huelsenbeck, 2003) to obtain a

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Table 2. Molecular markers and annealing temperatures used in the study and PCR products obtained.

Locus	Primer name	Primer sequence	Annealing temperature	PCR product length (bp)	Reference
28 s	None(D2) None(D2)	(F) AGAGAGAGTTCAAGAGTACGTG (R) TTGGTCCGTGTTTCAAGACGGG	60°C	~ 320	Belshaw & Quicke (1997)
COI	LCO-M-2d-F LCO-M-2d-R	(F) ATAACTATACCTATYATTATTGGAAG (R) AATAAATGTTGATATAAAATTGG	50°C	491	Malausa *et al.* (2011)
COI	C1-J-2183 C1-N-2568	(F) CAACATTTATTTTGATTTTTTGG (R) GCWACWACRTAATAKGTATCATG	56°C	385	Gullan *et al.* (2003)
ITS2	ITS2-M-F ITS2-M-R	(F) CTCGTGACCAAAGAGTCCTG (R) TGCTTAAGTTCAGCGGGTAG	58°C	~ 800	Malausa *et al.* (2011)
rpS15-16ST	leuA U16S	(F) GTATCTAGAGGNATHCAYCARGAYGGNG (R) GCCGTMCGACTWGCATGTG	60°C	~ 1050	Baumann *et al.* (2002)

Table 3. Summary of the mealybug species identified, populations sampled (see table 1) and different haplotypes obtained for each genetic marker. Haplotype numbers are as in the paper by Malausa *et al.* (2011) and are based on Genbank accession number. The rpS 15-16ST is expected to fail in *Phenacoccus spp.* because *T. princeps* is absent from these species. Different haplotypes obtained for the same species are shown in bold.

Multilocus haplotypes	Species	Populations sampled	LCO COI	2183–2568 COI	28S-D2	ITS2	rpS15-16ST
1	*Delottococcus aberiae*	33	E014	A018	C013	D014	
2	*Dysmicoccus boninsis*	9	E015	A017	C001	D003	B003
3	*Hypogeococcus pungens*	21	E009	A015	C012		
4	*Phenacoccus madeirensis*	1, 4, 5, 6, 7, 8, 20	E012	A013	C004	D013	–
5	*Phenacoccus peruvianus*	15, 16, 18, 19, 27, 30, 32, 34	E007	A010	C002	D008	–
6	*Phenacoccus solani*	29	E010	A011	C003	D009	–
7	*Planococcus vovae H1*	2	E011	**A008**	C006	**D010**	B002
8	*Planococcus vovae H2*	3	E011	**A009**	C006	**D011**	B002
9	*Planococcus citri H2*	10, 11, 28	**E003**	**A001**	C007	D012	B001
10	*Planococcus citri H5*	12	**E004**	**A003**	C007	D012	B001
11	*Planococcus citri H6*	13, 22	**E001**	**A004**	C007	D012	B001
12	*Planococcus citri H7*	14	**E002**	**A002**	C007	D012	
13	*Pseudococcus longispinus H1*	17, 25, 26	E006	A005	C009	**D004**	B006
14	*Pseudococcus longispinus H2*	23	E006	A005	C009	**D005**	
15	*Pseudococcus longispinus H3*	31	E006	A005	C009	**D006**	B006
16	*Pseudococcus viburni H2*	24	E016	A012	C008	D001	B005

summary of BayesPhylogenies outputs and to calculate Bayes factors. Majority rule consensus trees were then drawn with PAUP 4.0b10 (Swofford, 2003) ('contree/Majrule' command) from the output of the BayesPhylogenies analysis (9000 trees) using the best model selected. *Phenacoccus species* was used as outgroups because they are the most divergent taxa of this study (Hardy *et al.*, 2008).

Results

We surveyed a total of 33 mealybug populations from eastern Spain and one from southern France between 2007 and 2009. We identified 239 specimens from these samples morphologically, and DNA was sequenced, when possible, at five loci.

This resulted in the occurrence of 16 multi-locus haplotypes (table 3), which corresponded to ten species in terms of taxonomic identification: *Delottococcus aberiae* (De Lotto), *Dysmicoccus boninsis* (Kuwana), *Hypogeococcus pungens* Granara de Willink, *Phenacoccus madeirensis* Green, *P. peruvianus*, *Phenacoccus solani* Ferris, *Planococcus citri* Risso, *Planococcus vovae* (Nasonov), *Pseudococcus longispinus* (Targioni Tozzetti) and *Pseudococcus viburni* (Signoret). DNA sequences from the species *D. aberiae*, *P. peruvianus* and *P. vovae* were obtained for the first time in this study, and the universal barcode region cytochrome c oxidase subunit I (COI) was also sequenced for the first time from *D. boninsis* and *P. madeirensis* (table S1, supporting information). The genetic markers of the various DNA regions studied generated sequences that distinguished successfully between all the taxa studied.

For five mealybug species, we could sequence more than one population. Among those five species, three species displayed intraspecific variations among or within populations. Four different multi-locus haplotypes were recovered from six populations of *P. citri*, three multi-locus haplotypes were recovered from five populations of *P. longispinus* and two multi-locus haplotypes were recovered from two populations of *P. vovae* (table 3). The distribution of these different haplotypes did not follow any obvious geographic pattern (fig. 1). Other species, such as *P. peruvianus* and *P. madeirensis*, displayed high levels of genetic homogeneity, even though several populations from different hosts and geographic regions were studied. Intraspecific differences were observed

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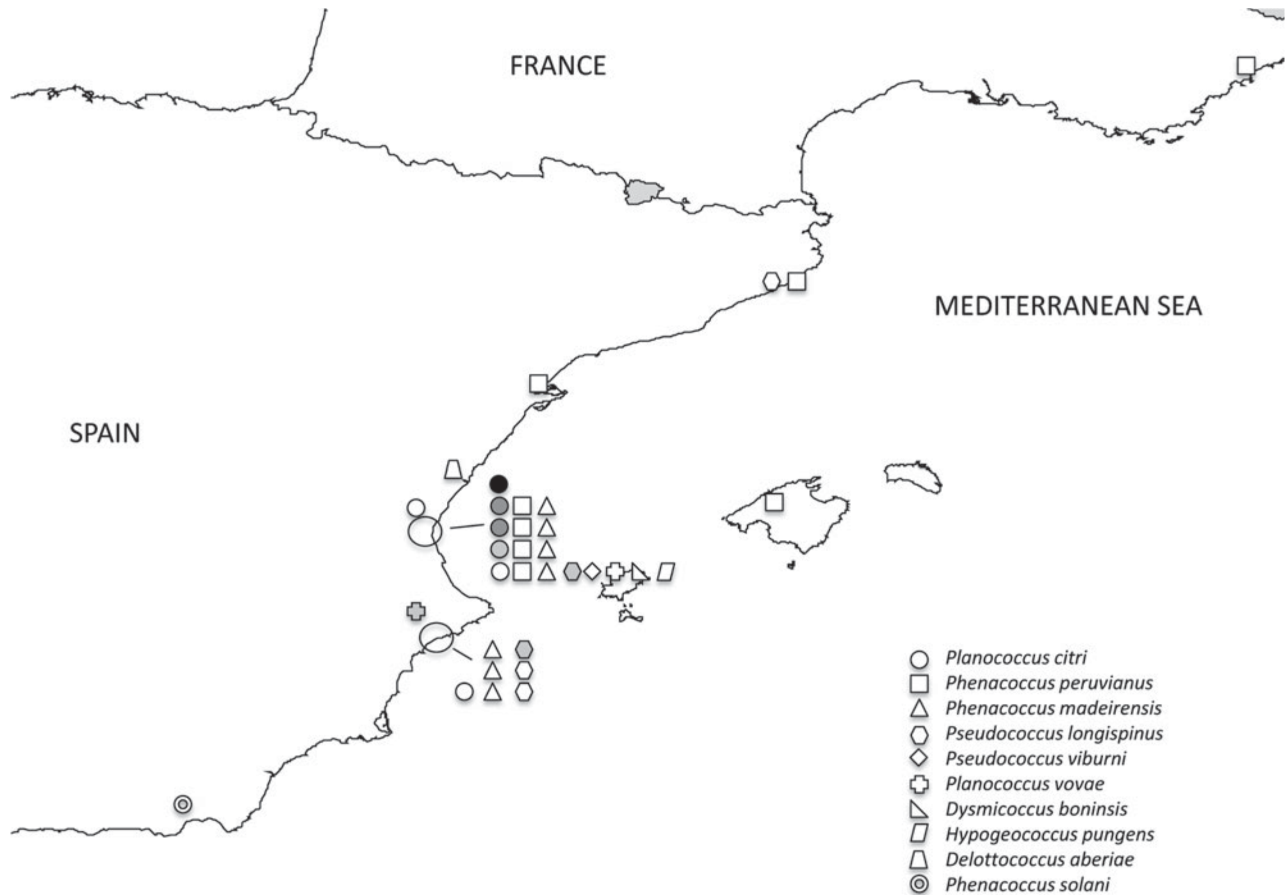

Fig. 1. Distribution of the mealybug populations surveyed in eastern Spain and France. The different symbols indicate the population species and colors denote the haplotypes.

at the sequences obtained from both regions of COI and ITS2. The regions rpS15–16 s and 28sD2 displayed no intraspecific variation.

The phylogenetic tree revealed that the genera *Phenacoccus* and *Planococcus* formed monophyletic groups. On the contrary, the genus *Pseudococcus* appeared paraphyletic. Indeed, in the topology, the *Pseudococcus* species are found in two separate clusters, each containing several *Pseudococcus* species and one *Dysmicoccus* species (fig. 2). In addition, the species for which no DNA sequence was previously available were positioned in the topology with good support: *P. peruvianus* was located close to *Phenacoccus parvus* Morrison populations of Neotropical origin. *Planococcus vovae* differed slightly from the other three species of the genus *Planococcus*. *Delottococcus aberiae* was found in a cluster with *Vryburgia rimariae* Tranfaglia located inside part of the tree corresponding to the tribe Pseudococcini.

Discussion

The main interest of this study is probably to provide a solid basis for further works focusing on mealybug management. For researchers or practitioners with an access to DNA sequencing facilities, our data makes it possible to quickly identify taxa based on simple DNA sequence comparisons. This study also provides the raw data to design rapid identification kits based on the use of species-specific PCR: the large set of sequences available makes it possible to design species-specific PCR primers annealing to regions displaying variations among species but not among populations or individuals of the same species. One additional piece of information directly relevant for pest management is the occurrence of two species (*P. peruvianus* and *D. aberiae*) that represent two cases of recent introductions in Europe.

However, such a survey using multi-criteria sample characterisation also generates valuable data for researches on the evolutionary history of Pseudococcidae.

First, by generating DNA data for various species that had not been sequenced before (*P. peruvianus*, *P. vovae* and *D. aberiae*), this study gives insights into the phylogenetic relationships inside the family Pseudococcidae. *Phenacoccus peruvianus* appears more closely related to *P. parvus* than *P. solani* or *P. madeirensis*. This result is in conflict with the findings of Granara de Willink & Szumik (2007), whose morphological phylogenetic studies placed *P. peruvianus* closer to *P. madeirensis*. *Planococcus vovae* mapped close to the other species of the same genus, but did not come between *P. ficus* and the cryptic species *P. citri* and *P. minor* (Rung *et al.*, 2008; Saccaggi *et al.*, 2008). Moreover, the South African species *D. aberiae* was located close to *V. rimariae* on the

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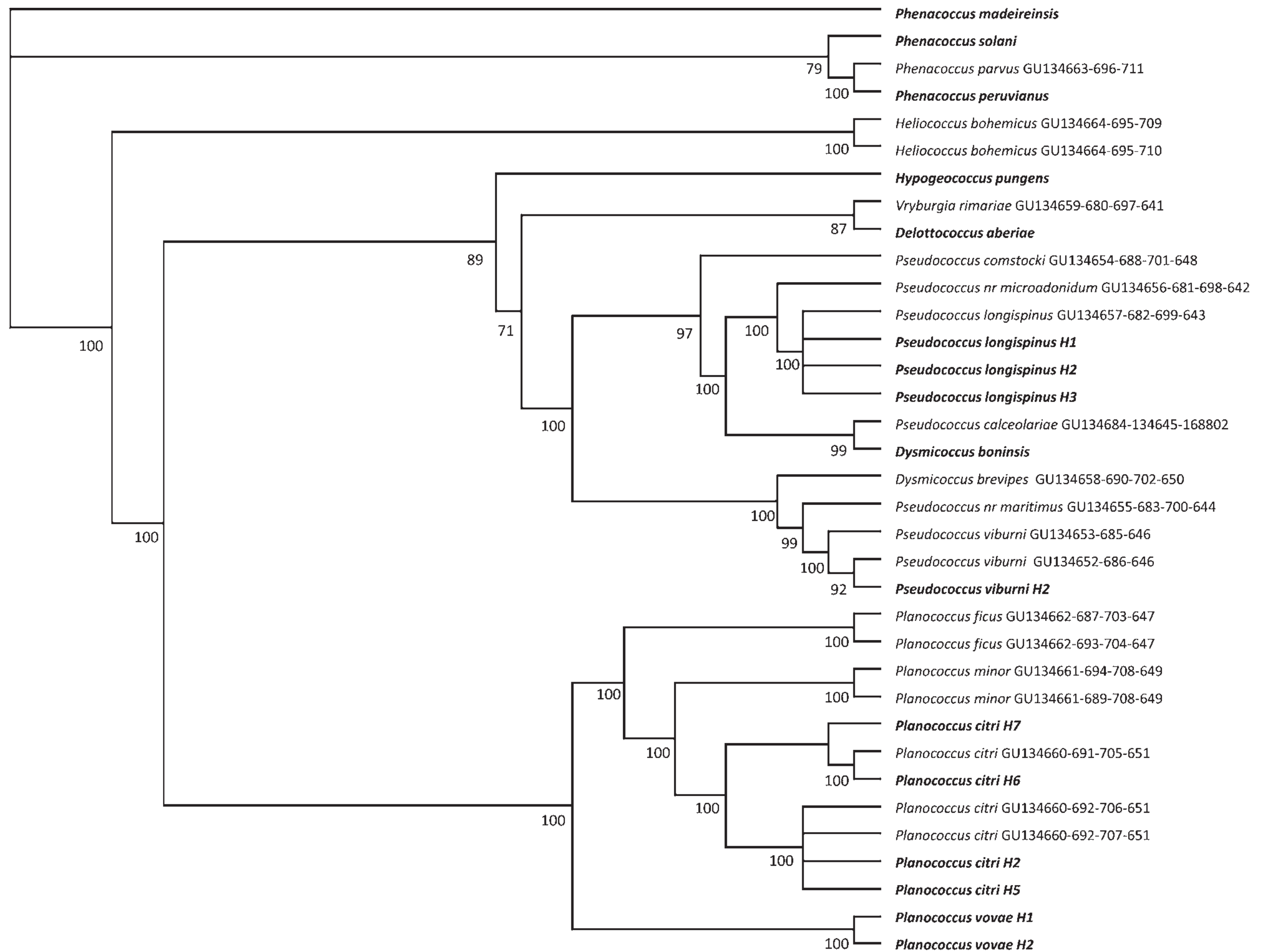

Fig. 2. Bayesian phylogenetic tree of mealybug multilocus haplotypes generated by this study (shown in bold) or by Malausa *et al.* (2011) for multilocus haplotypes including information for at least three loci: 2183–2568 and LCO regions of COI, 28 s-D2 and rpS15-16 s when possible. The majority-rule consensus tree was calculated from the Bayesian analysis, based on the best selected mixture model (three matrices). Bayesian posterior probabilities are represented beyond the nodes (9000 trees, values <70% not shown). Haplotypes are named according to Genbank accession numbers.

phylogenetic tree, providing further evidence for the existence of a southern African clade, as proposed by Hardy *et al.* (2008). In addition, the phylogenetic tree computed in this study confirms several trends observed in previous studies (Hardy *et al.*, 2008; Malausa *et al.*, 2011): (i) the genera *Phenacoccus* and *Planococcus* were found monophyletic, although few closely related species of other genera were in this study; (ii) the presence of two *Dysmicoccus* species among the *Pseudococcus* species suggests that these two genera are paraphyletic, as proposed by Downie & Gullan (2004), Hardy *et al.* (2008) and Malausa *et al.* (2011).

Second, the contrasted patterns of intraspecific variability found in *P. vovae*, *P. citri*, *P. longispinus*, *P. peruvianus* and *P. madeirensis* may be explained by the species histories. Indeed, the extent of intraspecific variation observed in those species does not display any geographic pattern and may rather be accounted for by the time elapsed since these species first began their invasion of Europe. Substantial divergences were observed in the populations of the native species *P. vovae*, as well as in the exotic species *P. citri* and *P. longispinus*. These two exotic species have been present in the Mediterranean Basin for more than a century (Pellizzari & Germain, 2010), long enough for population divergence to have occurred in the new area or for repeated introductions from different regions of the world (Thompson, 1998; Dlugosch & Parker, 2008). By contrast, the invasive species *P. peruvianus* and *P. madeirensis* displayed little or no DNA variability in the multilocus analysis. This suggests (i) that the populations experienced a genetic bottleneck, probably caused by their recent introduction into Europe and specifically in Spain (Marotta & Tranfaglia, 1990; Beltrà *et al.*, 2010; Beltrà & Soto, 2011), and (ii) that the invasive populations came from the same geographic region or spread in Spain and France from a single introduced population.

In conclusion, this study provided a molecular characterisation at several DNA markers and a taxonomic identification for a set of 239 mealybug samples from 33 populations of eastern Spain. Among them, ten different species were identified, and this study provided the first molecular data for three species. In addition, this multi-criteria characterization

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produced new data for the study of the Pseudococcidae phylogeny and revealed various patterns of intraspecific variations among populations of five mealybug species that may be related to their invasion histories.

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Supplementary material

The online table can be viewed at http://journals.cambridge.org/ber.

References

Ashfaq, M., Noor, A.R. & Mansoor, S. (2010) DNA-based characterization of an invasive mealybug (Hemiptera: Pseudococcidae) species damaging cotton in Pakistan. *Applied Entomology and Zoology* **45**, 395–404.

Baumann, L., Thao, M.L., Hess, J.M., Johnson, M.W. & Baumann, P. (2002) The genetic properties of the primary endosymbionts of mealybugs differ from those of other endosymbionts of plant sap-sucking insects. *Applied and Environmental Microbiology* **68**, 3198–3205.

Belshaw, R. & Quicke, D.L.J. (1997) A molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae). *Molecular Phylogenetics and Evolution* **7**, 281–293.

Beltrà, A. & Soto, A. (2011) New records of mealybugs (Hemiptera: Pseudococcidae) from Spain. *Phytoparasitica* **39**, 385–387.

Beltrà, A., Soto, A., Germain, J.F., Matile-Ferrero, D., Mazzeo, G., Pellizzari, G., Russo, A., Franco, J.C. & Williams, D.J. (2010) The Bougainvillea mealybug *Phenacoccus peruvianus*, a rapid invader from South America to Europe. *Entomologia Hellenica* **19**, 137–143.

Ben-Dov, Y. (1994) *A Systematic Catalogue of the Mealybugs of the World: (Insecta; Homoptera; Coccoidea; Pseudococcidae and Putoidae) with Data on Geographical Distribution, Host Plants, Biology and Economic Importance*. Andover, MA, USA, Intercept Ltd.

Cavalieri, V., Mazzeo, G., Garzia, G.T., Buonocore, E. & Russo, A. (2008) Identification of *Planococcus ficus* and *Planococcus citri* (Hemiptera: Pseudococcidae) by PCR-RFLP of COI gene. *Zootaxa* **1816**, 65–68.

Charles, J.G., Froud, K.J. & Henderson, R.C. (2000) Morphological variation and mating compatibility within the mealybugs *Pseudococcus calceolariae* and *P. similans* (Hemiptera: Pseudococcidae), and a new synonymy. *Systematic Entomology* **25**, 285–294.

Cox, J.M. (1983) An experimental study of morphological variation in mealybugs (Homoptera: Coccoidea: Pseudococcidae). *Systematic Entomology* **8**, 361–382.

Demontis, M.A., Ortu, S., Cocco, A., Lentini, A. & Migheli, Q. (2007) Diagnostic markers for *Planococcus ficus* (Signoret) and *Planococcus citri* (Risso) by random amplification of polymorphic DNA-polymerase chain reaction and species-specific mitochondrial DNA primers. *Journal of Applied Entomology* **131**, 59–64.

Dlugosch, K.M. & Parker, I.M. (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular Ecology* **17**, 431–449.

Downie, D.A. & Gullan, P.J. (2004) Phylogenetic analysis of mealybugs (Hemiptera: Coccoidea: Pseudococcidae) based on DNA sequences from three nuclear genes, and a review of the higher classification. *Systematic Entomology* **29**, 238–259.

Franco, J.C., Silva, E.B. & Carvalho, J.P. (2000) *Mealybugs (Hemiptera, Pseudococcidae) Associated with Citrus in Portugal*. Lisbon, Portugal, ISA Press.

Gimpel, W. & Miller, D.R. (1996) *Systematic Analysis of the Mealybugs in the Pseudococcus Maritimus Complex (Homoptera: Pseudococcidae)*. Gainesville, FL, USA, Associated Publishers.

Granara de Willink, M.C. & Szumik, C. (2007) Phenacoccinae de Centro y Sudamérica (Hemiptera: Coccoidea: Pseudococcidae): Sistemática y Filogenia. *Revista de la Sociedad Entomológica Argentina* **66**, 29–129.

Gullan, P.J., Downie, D.A. & Steffan, S.A. (2003) A new pest species of the mealybug genus *Ferrisia* Fullaway (Hemiptera: Pseudococcidae) from the United States. *Annals of the Entomological Society of America* **96**, 723–737.

Gullan, P.J., Kaydan, M.B. & Hardy, N.B. (2010) Molecular phylogeny and species recognition in the mealybug genus *Ferrisia* Fullaway (Hemiptera: Pseudococcidae). *Systematic Entomology* **35**, 329–339.

Hall, T. (1999) Bioedit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Hardy, N.B., Gullan, P.J. & Hodgson, C.J. (2008) A subfamily-level classification of mealybugs (Hemiptera: Pseudococcidae) based on integrated molecular and morphological data. *Systematic Entomology* **33**, 51–71.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society, Series B: Biological Sciences* **270**, 313–321.

Heinze, K. (1952) Polyvinylalkohol-lactophenol-gemisch als einbettungsmittel fur blattlause. *Die Naturwissenschaften* **39**, 285–286.

Hulme, P.E., Bacher, S., Kenis, M., Klotz, S., Kühn, I., Minchin, D., Nentwig, W., Olenin, S., Panov, V., Pergl, J., Pyšek, P., Roques, A., Sol, D., Solarz, W. & Vilà, M. (2008) Grasping at the routes of biological invasions: a framework for integrating pathways into policy. *The Journal of Applied Ecology* **45**, 403–414.

Kenis, M., Auger-Rozenberg, M., Roques, A., Timms, L., Péré, C., Cock, M.J.W., Settele, J., Augustin, S. & Lopez-Vaamonde, C. (2009) Ecological effects of invasive alien insects. *Biological Invasions* **11**, 21–45.

Kosztarab, M. & Kozar, F. (1988) *Scale Insects of Central Europe*. Dordrecht, Netherlands, Dr W. Junk Publishers.

Malausa, T., Fenis, A., Warot, S., Germain, J.-F., Ris, N., Prado, E., Botton, M., Vanlerberghe-Masutti, F., Sforza, R., Cruaud, C., Couloux, A. & Kreiter, P. (2011) DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). *Journal of Applied Entomology* **135**, 142–155.

Marotta, S. & Tranfaglia, A. (1990) New and little known species of Italian scale insects (Homoptera: Coccoidea). pp. 107–112 in *Proceedings of the Sixth International Symposium of Scale*

https://doi.org/10.1017/S0007485311000514 Published online by Cambridge University Press

Insect Studies, Part II. 6–12 August 1990, Crackow, Poland, Agricultural University Press.

Miller, D.R., Miller, G.L. & Watson, G.W. (2002) Invasive species of mealybugs (Hemiptera: Pseudococcidae) and their threat to US agriculture. *Proceedings of the Entomological Society of Washington* **104**, 825–836.

Miller, D.R., Miller, G.L., Hodges, G.S. & Davidson, J.A. (2005) Introduced scale insects (Hemiptera: Coccoidea) of the United States and their impact on US agriculture. *Proceedings of the Entomological Society of Washington* **107**, 123–158.

Pagel, M. & Meade, A. (2004) A phylogenetic mixture model for detecting pattern-heterogeneity in gene sequence or character-state data. *Systematic Biology* **53**, 571–581.

Park, D.S., Suh, S.J., Hebert, P.D.N., Oh, H.W. & Hong, K.J. (2011) DNA barcodes for two scale insects families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). *Bulletin of Entomological Research* **101**, 429–434.

Pellizzari, G. & Germain, J. (2010) Scales (Hemiptera, Superfamily Coccoidea). *BioRisk* **4**, 475–510.

Pieterse, W., Muller, D. & van Vuuren, B. (2010) A molecular identification approach for five species of mealybug (Hemiptera: Pseudococcidae) on citrus fruit exported from South Africa. *African Entomology* **18**, 23–28.

Pimentel, D., McNair, S., Janecka, J., Wightman, J., Simmonds, C., O'Connell, C., Wong, E., Russel, L., Zern, J., Aquino, T. & Tsomondo, T. (2001) Economic and environmental threats of alien plant, animal, and microbe invasions. *Agriculture Ecosystems & Environment* **84**, 1–20.

Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Roques, A., Rabitsch, W., Rasplus, J., Lopez-Vaamonde, C., Nentwig, W. & Kenis, M. (2009) Alien Terrestrial Invertebrates of Europe. pp. 63–79 *in* DAISIE (*Ed.*) *Handbook of Alien Species in Europe*. Dordrecht, Netherlands, Springer.

Rung, A., Scheffer, S.J., Evans, G. & Miller, D. (2008) Molecular identification of two closely related species of mealybugs of the genus *Planococcus* (Homoptera: Pseudococcidae). *Annals of the Entomological Society of America* **101**, 525–532.

Rung, A., Miller, D.R. & Scheffer, S.J. (2009) Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method to Distinguish Three Mealybug Groups Within the *Planococcus citri-P. minor* Species Complex (Hemiptera: Coccoidea: Pseudococcidae). *Journal of Economic Entomology* **102**, 8–12.

Saccaggi, D.L., Pietersen, G. & Kruger, K. (2008) A multiplex PCR assay for the simultaneous identification of three mealybug species (Hemiptera: Pseudococcidae). *Bulletin of Entomological Research* **98**, 27–33.

Swofford, D. (2003) Paup*: phylogenetic analysis using parsimony (* and other methods). Version 4. Sunderland, UK, Sinauer Associates.

Thompson, J.N. (1998) Rapid evolution as an ecological process. *Trends in Ecology & Evolution* **13**, 329–332.

Williams, D.J. (1985) *Australian Mealybugs*. London, UK, British Museum (Natural History).

Williams, D.J. (2004) *Mealybugs of Southern Asia*. London, UK, The Natural History Museum and Southern SDN.

Williams, D.J. & Granara de Willink, M.C. (1992) *Mealybugs of Central and South America*. London, UK, CAB International.

Williamson, M. (1996) *Biological Invasions*. London, UK, Chapman Hall Ltd.

https://doi.org/10.1017/S0007485311000514 Published online by Cambridge University Press