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Ophelimus mediterraneus sp. n. (Hymenoptera, Eulophidae): a new *Eucalyptus* gall wasp in the Mediterranean region

N. Borowiec[1]*, J. La Salle[2], L. Brancaccio[1–3], M. Thaon[1], S. Warot[1], M. Branco[4], N. Ris[1], J.-C. Malausa[1] and R. Burks[5]

[1]UMR INRA-CNRS-Université Côte d'Azur 'Sophia Agrobiotech Institute', 400 Route des Chappes, 06903 Sophia Antipolis, France: [2]Atlas of Living Australia, CSIRO Ecosystem Sciences, GPO Box 1700, Canberra, ACT 2601, Australia: [3]Bioline Agroscience, R&D Division, 1306 route de Biot, 06560 Valbonne, France: [4]Forest Research Center, Institute Superior of Agronomy, Technical University of Lisbon, Tapada da Ajuda 1349-017 Lisbon, Portugal: [5]University of California, Riverside, CA 92521, USA

Abstract

We report here for the first time the presence of *Ophelimus mediterraneus* sp. n. in Mediterranean Europe. This species appears to be closely related to *Ophelimus maskelli*, a well-known invasive pest of *Eucalyptus*. Based on molecular (cytochrome oxidase I, 28S), morphological (multivariate ratio analysis) and bio-ecological investigations, our study gives unambiguous relevant criteria that allow the discrimination between these two species. A full description of *O. mediterraneus* sp. n. is also provided. The geographic distribution of *O. mediterraneus* sp. n. as well as its impact on *Eucalyptus* species needs to be more widely assessed since its presence may have been confused with *O. maskelli* in their sympatric introduced areas. Further investigations of potential parasitoids in the native area may thus be welcomed to evaluate classical biological control achievability.

Keywords: Gall wasp, eucalypts, Chalcidoidea, integrative characterization, *Ophelimus mediterraneus*, *Eucalyptus globulus*, invasive pest

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Introduction

With more than 700 species, *Eucalyptus* is a wide plant genus originating almost exclusively from Australia where these trees are key component for many natural ecosystems (Ladiges *et al*., 2003). Following James Cook expeditions, several species of *Eucalyptus* were described for the first time in the late 18th century (Doughty, 2000). As a consequence of their interest for timber production or ornamental purposes, eucalypts were then introduced in more than 90 countries worldwide and, at present, almost 20 million hectares of *Eucalyptus* are cultivated in the world, mainly in Brazil, India and China (Doughty, 2000; Iglesias Trabado & Wilstermann, 2008). Current plantations are dominated by nine species among which some (e.g. *E. globulus*) spread beyond cultivated areas and became invasive (Gordon *et al*., 2012).

In Australia, up to 20,000 species of phytophagous insects are associated with *Eucalyptus* among which the most abundant guilds are leaf feeders (Coleoptera and Lepidoptera) and sap-suckers (Hemiptera, Psyllidae) (Majer *et al*., 1997). Several species of these guilds are now worldwide invaders such as the psyllids *Ctenarytaina* spp., *Glycaspis brimblecombei* Moore and *Blastopsylla occidentalis* Taylor, as well as the eucalyptus snout beetles *Gonipterus* spp. (Curculionidae). Gall-inducers are also main pests for *Eucalyptus* species (Paine *et al*., 2011; Hurley *et al*., 2016). Contrary to the Holarctic region where most of

*Author for correspondence
Phone: (+33)4 92 38 65 00
Fax: (+33)4 92 38 64 01
E-mail: nicolas.borowiec@inra.fr

gall-inducers are gall midges (Diptera, Cecidomyiidae) and cynipid gall wasps (Hymenoptera, Cynipidae) (Csoka *et al*., 2005; Yukawa & Rohfritsch, 2005), gall-inducers associated with *Eucalyptus* trees in Australia are chalcid wasps (Chalcidoidea), and especially species from the family Eulophidae, the largest family among chalcidoid wasps (Noyes, 2002). Since Eulophid gall wasps represent a major radiation in Australia (Austin *et al*., 2004), it is not surprising that some of those developing on eucalypts became invasive recently such as *Epichrysocharis burwelli* Schauff, *Leptocybe invasa* Fisher & La Salle, *Moona spermophaga* Kim & La Salle, *Leprosa milga* Kim & La Salle and *Selitrichodes globulus* La Salle & Gates (Schauff & Garrison, 2000; Mendel *et al*., 2004; Kim *et al*., 2005; Kim & La Salle, 2008; La Salle *et al*., 2009). Other Eulophid pests belong to the genus *Ophelimus*.

Firstly described by Haliday (1844), this genus belongs to the subfamily Opheliminae and contains almost 50 known species, mainly native from Australia (Bouček, 1988; Burks *et al*., 2011). The precise biology of most species is unknown but all may be associated with galls, and probably as gall-inducers (La Salle, 2005). *Ophelimus eucalypti* (Gahan) was the first invasive species reported outside its native area. In New Zealand, it was reported to induce economic damage on *Eucalyptus* species of both the *Transversaria* and *Maidenaria* sections and this pest may have contributed to the decline of some *Eucalyptus* species (Withers *et al*., 2000; Withers, 2001). Currently, the most largely distributed invader is however *Ophelimus maskelli* (Ashmead). Originally described by Ashmead in 1900 as *Pteroptrix maskelli*, it was transferred to the genus *Ophelimus* by Bouček in 1988. Outside its native area, *O. maskelli* was firstly reported in Italy (Arzone & Alma, 2000) and in Spain (Pujade-Villar & Riba-Flinch, 2004), where it has been misidentified as *O. eucalypti* (Protasov *et al*., 2007*b*). From then, it rapidly disseminated throughout the Mediterranean region: Israel (Mendel *et al*., 2005), Greece (Kavallieratos *et al*., 2006), France (EPPO, 2006), Turkey (Doğanlar & Mendel, 2007), Portugal (Branco *et al*., 2009), Tunisia (Dhahri *et al*., 2010), Algeria (Caleca, 2010), Malta (Mifsud, 2012) and probably in the UK (Tilbury & Jukes, 2006). It has also been recently reported from Indonesia, South Africa, Vietnam, Mauritius and, for the first time in the Nearctic Region, in California (Lawson *et al*., 2012; Burks *et al*., 2015*a*). Males of *O. maskelli* have only been mentioned by Ashmead (1900) in its original description. However, in all the invaded areas, this species seems to be represented only by females that thus reproduce by thelytoky. The pervasiveness of asexuality in invasive strains has been repeatedly observed for other Hymenoptera for which both sexual and asexual reproductions coexist in the native areas (see Fusu (2017) for details). This pattern may be explained by the increased probability of establishment of asexual individuals in new environments (absence of Allee effects linked to the necessity of meeting and mating). The biology of *O. maskelli* was studied by Protasov *et al*. (2007*b*). Females are able to oviposit around 100 eggs on midribs of leaves of several *Eucalyptus* species among which *E. camaldulensis* Dehnh. seems to be the most common host. In the case of high population levels, the entire leaf surface can be covered with galls. *O. maskelli* develops on a single-cell gall and its entire development (from oviposition to adult emergence) takes around 90 days in ventilated greenhouses (temperature: 18–25°C) and, according to the local climatic conditions, several generations can be completed within a year (e.g. three generations in Israel, Protasov *et al*., 2007*b*).

As for other invasive species (Simberloff, 2009), the eradication of *O. maskelli* in particular is almost impossible once established. Since part of its success as invader may be due to the absence of natural enemies (enemy release hypothesis: Torchin *et al*., 2003; Colautti *et al*., 2004), its regulation may nevertheless be achieved through classical biological control, i.e. the deliberate introduction of an adapted natural enemy for its permanent establishment and the long-term control of the target pest (Eilenberg *et al*., 2001). *Closterocerus chamaeleon* (Girault), a chalcid wasp belonging to the family Eulophidae and the subfamily Entedoninae, was hence identified as relevant candidate for biological control. In its native range, it is indeed widely distributed and mostly reported to parasitize *O. maskelli* on *Eucalyptus* species although it was found associated with other gall-inducing Eulophids on the same host-plants (Protasov *et al*., 2007*a*). Biological control programmes using *C. chamaeleon* were thus implemented in Israel and in Italy where it successfully established (Mendel *et al*., 2007; Caleca *et al*., 2011). The high dispersal ability of this parasitoid observed in these countries was confirmed by its quick spread in other neighbouring countries without reported intentional releases: Turkey (Doğanlar & Mendel, 2007), Spain (Borrajo *et al*., 2008), Portugal (Branco *et al*., 2009), Algeria (Caleca, 2010), Tunisia (Lo Verde *et al*., 2010) and France (Borowiec *et al*., 2012). In only a few years, *C. chameleon* extremely reduced the populations of *O. maskelli* in Israel (Protasov *et al*., 2007*a*), Italy (Caleca *et al*., 2011), Tunisia and Portugal (Branco *et al*., 2014).

Within this context of an overall control of *O. maskelli* by *C. chamaeleon*, severe damages on foliage were nevertheless reported on *Eucalyptus* plantations located in South-eastern France. First observations indicated that the gall-inducer should belong to the genus *Ophelimus* but both the galls' shape as well as the intensity of damages were intriguing.

From these observations, we thus decided to more precisely investigate the identity of *Ophelimus* species present in different Mediterranean countries using simultaneously molecular, morphological and bio-ecological information.

Materials and methods

Wasp samplings

All the specimens used in this study were collected on *Eucalyptus* species.

Field sampling were conducted between 2010 and 2012 in South-eastern France (Alpes-Maritimes, Bouches-du-Rhône and Var) and Western Italy (Liguria) and, in 2013, in Portugal (in a *Eucalyptus* arboretum in Tapada da Ajuda, Lisbon). To collect living specimens of *Ophelimus*, small branches with mature galls were collected and placed in small plastic bags at INRA Sophia Antipolis (for samples collected in France and Italy), and at Laboratory of Forest Entomology of Instituto Superior de Agronomia, Lisbon (for samples collected in Portugal). Emergences were checked daily and all emerged adults were killed in 95% ethanol to allow molecular and morphological characterizations. The presence of parasitoids in the samples was checked to provide information on natural parasitism.

We also used additional *Ophelimus* specimens kept in 95% ethanol and collected in the native area of this genus: Australia (dry specimens from the Australian National Insect Collection, Canberra) and New Zealand (from North and South Islands). Detailed information about specimens used in this study is given in table 1.

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Table 1. Information about specimens used in this study.

Species	Host	Country	Locality	Parasitoids in samples	Number of specimens used in the study	Number of specimens used for observation of Sm setae	Number of specimens used for morphometric measures	Number of specimens used for molecular characterization		Molecular id.
								COI	*28S*	
***Ophelimus eucalypti* 'Maid.'**	*Eucalyptus globulus*	New Zealand (South Island)	Port Chalmers	no data	4	4	2	0	4	6580–6583
***Ophelimus eucalypti* 'Trans.'**	*Eucalyptus saligna*	New Zealand (North Island)	Whangapoua Forest	no data	8	8	3	0	4	6584–6587
Ophelimus maskelli	*Eucalyptus camaldulensis*	France	Cannes	*Closterocerus chamaeleon*	9	9	4	2	2	3753–3754
			Frejus	*Closterocerus chamaeleon*	6	6	3	2	2	3759–3760
			Golf Juan	*Closterocerus chamaeleon*	1	0	0	0	1	3765
			Mandelieu (Casino)	*Closterocerus chamaeleon*	19	18	7	11	12	3755–3756/ 6552–6561
			Plascassier	*Closterocerus chamaeleon*	6	6	1	2	2	3747–3748
			Theoules	*Closterocerus chamaeleon*	6	6	6	0	0	–
			Villeneuve-Loubet	*Closterocerus chamaeleon*	2	2	0	0	0	–
		Portugal	Lisbon	No data	15	15	6	5	0	11015–11019
	Eucalyptus rudis	Portugal	Lisbon	No data	9	9	4	5	0	11010–11014
***Ophelimus mediterraneus* sp. n.**	*Eucalyptus globulus*	Australia	–	No data	52	50	6	2	0	6797–6797
		France	Mandelieu (Casino)	No parasitoids	41	41	7	10	10	6562–6571
			Mandelieu (Pelazza)	No parasitoids	9	9	5	9	9	6533–6541
		Italy	Savone	No parasitoids	18	18	4	5	0	11020–11024
		Portugal	Lisbon	No data	11	11	4	10[1]	0	11000–11009
	Eucalyptus gunnii	France	La Ciotat	No data	9	9	0	8	8	6572–6579
			Mandelieu (Pelazza)	No parasitoids	34	34	5	9	10	6542–6551
			Tanneron	No parasitoids	8	7	5	8	8	3739–3746
Closterocerus chamaeleon	*O. maskelli (E. camaldulensis)*	France	Cannes	–	1	–	–	1	0	3774
Closterocerus chamaeleon	*O. maskelli (E. camaldulensis)*	France	Puget/Argens	–	1	–	–	0	1	3763
				Total	269	262	72	89	73	

[1]For two specimens, PCR failed; one was contaminated (blast with Penicillium).

Molecular characterization

Two molecular markers were used: the cytochrome oxidase subunit I (*COI* – mitochondrial coding marker) and the 28s rDNA region (*28S* – nuclear non-coding marker). Among all the *Ophelimus* specimens collected in the field, 99 were selected for molecular characterization in order to adequately cover geographical and host-plant ranges. Two specimens of *C. chamaeleon* collected in France on *O. maskelli* were also selected and used as outgroup (information about molecular methods used for each specimen is given in table S1).

DNA extraction

Genomic DNA was extracted from frozen (individuals previously kept in 95% ethanol at −20°C) tissues using a prepGEM® Insect kit (ZYGEM, PIN0500). In order to allow subsequent examination of morphology, specimens were placed individually in different extraction volumes (methods 'zygem3 h_20 µL', 'zygem3 h_30 µL', 'zygem3 h_40 µL'; table S2) of mix without destruction of the specimens and incubated 3 h at 75°C. The DNA extracts were then stored at −20°C. For specimens collected in Australia that were kept dry for more than 10 years, we decided to use a destructive method to extract sufficient quantity of DNA (methods 'Chitinase_QiaBT' and 'Chitinase_QiaAmp'; table S2).

Amplification by polymerase chain reaction (PCR)

The *COI* barcoding fragment was amplified using either the primers LCO1490 (5′-GTCAACAAATCATAAAGAT ATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACC AAAAAATCA-3′) (Folmer *et al*., 1994) or LCO1490puc (5′-TTTCAACWAATCATAAAGATATTGG-3′) and HCO 2198puc (5′-TAAACTTCWGGRTGWCCAAARAATCA-3′) (Cruaud *et al*., 2010).

The *28s* gene was amplified using the primers D2-3551F (5′-CGTGTTGCTTGATAGTGCAGC-3′) and D2-4057R (5′-TCAAGACGGGTCCTGAAAGT-3′) (Gillespie *et al*., 2005). Detailed PCR conditions are described in table S3. All the PCRs were performed on a GeneAmp 9700 thermocycler. PCR products were visualized using the QIAxcel Advanced System and QIAxcel DNA Fast Analysis Kit (Qiagen, Germany). They were sent to Genoscreen (Lille, France) or to Beckman Coulter Genomics (Stansted, UK) for sequencing in both directions.

Morphological characterization

Terminology of morphological characters follows Gibson *et al*. (1997). Except for the study published by Girault (1913*c*) that included only eight species, no useful identification key is available for the genus *Ophelimus*. However, according to Protasov *et al*. (2007*b*), *O. maskelli* can be discriminated from other *Ophelimus* species by the presence of only one seta on the submarginal vein of forewings. Using a Leica M205C microscope (×140 magnification), we thus investigated the number of submarginal setae on forewings of a total of 262 specimens of *Ophelimus* collected on various species of *Eucalyptus* (*E. camaldulensis*, *E. globulus* Labill., *E. gunnii* Hook., *E. rudis* Endl., *E. saligna* Sm.) and in different countries (Australia, France, Italy, New Zealand, Portugal) (table 1).

For 72 of these 262 specimens (all females), we also did measurements of five morphological characters. Since adults of *Ophelimus* tend to collapse, in particular after storage in alcohol, we only used characters from wings and from hind legs: length of hind tibia (T3L), length of forewing (FwL), length of forewing submarginal vein (SmvL), length of forewing marginal vein (MvL) and length of forewing stigma vein (StvL). The end of forewing post-marginal vein was difficult to locate unambiguously and since this character seems to present wide interspecific variation (Bouček, 1988), we did not measure this vein. To avoid variation due to asymmetry, we did all these measurements and observations on left hand side of the specimens. For this work, all left forewings and left hind tibias were removed for all specimens and embedded in Hoyer's medium on slides prior to measurements using a Leica M205C microscope coupled with image processing software Leica Application Suite version 4.0 (Leica Microsystems GmbH) (fig. 1). Images presented in fig. 9 were taken using a Keyence VHX-5000 digital microscope, available at INRA CBGP, Montpellier.

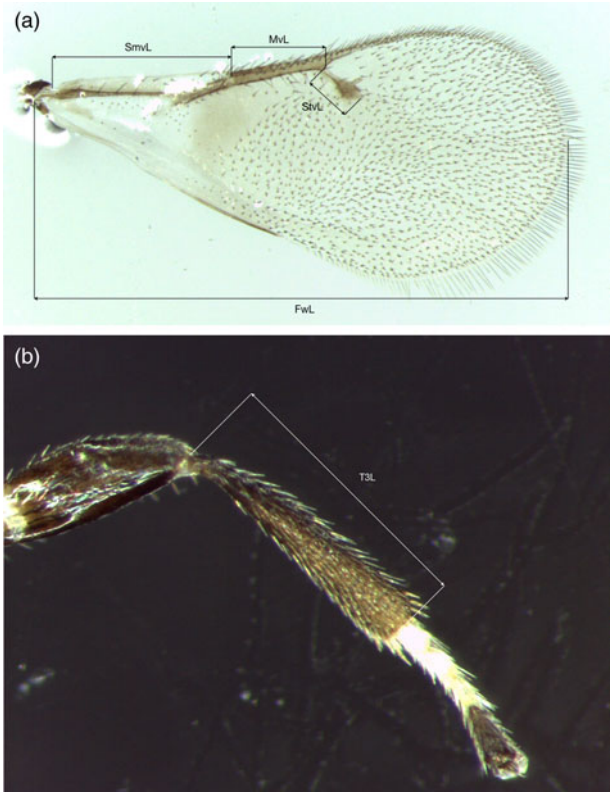

Fig. 1. *O. mediterraneus*: measurements of forewings (a) and hind tibia (b) for morphometrics analysis. T3L: hind tibia length; FwL: forewing length; SmvL: submarginal vein length; MvL: marginal vein length; StvL: stigmal vein length.

Bio-ecological investigations

We investigated the size of galls induced by both *O. maskelli* and *O. mediterraneus* sp. n. by collecting leaves with fully developed galls on *E. camaldulensis* and *E. globulus* respectively. Since the presence of galls on some species of *Eucalyptus* seems to be seasonal (Borowiec *et al*., 2013), leaves with mature galls were collected in May 2011 in Mandelieu (France) and in the

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arboretum of Lisbon in April 2013 (Portugal). Diameter of galls was measured on an *x*–*y* basis. Measurements were performed:

(i) using a Leica M205C stereomicroscope (100× magnification) coupled with Leica Application Suite software (version 4.0) for galls collected in France: *E. camaldulensis* (100 galls); *E. globulus* (95 galls) and

(ii) using an Olympus SZX-ZB12 stereomicroscope (80× magnification) equipped with ocular micrometre for galls collected in Portugal: *E. camaldulensis* (100 galls); *E. globulus* (100 galls).

We also investigated the biological cycle of *O. mediterraneus* sp. n. on *E. gunnii* by carrying out a field survey in a *Eucalyptus* plantation in Mandelieu (Pelazza) between January 2011 and January 2012. Eight samples were collected in 2011 (January, March, April, May, June, August, October and December) and one sample was collected in January 2012. For each of these samples, 20–30 leaves were randomly collected on young trees of *E. gunnii*. Leaves were brought to the laboratory where galls were dissected using a Leica M205C microscope. Gall's content was categorized as follows:

- 'larva': *Ophelimus* larva was observed feeding within the gall;
- 'nymph': nymph of *Ophelimus* was observed within the gall;
- 'adult': adult of *Ophelimus* was observed;
- 'exit holes': the gall was empty and characterized by the presence of an adult's exit hole on the surface of the gall;
- 'empty': no *O. mediterraneus* sp. n. inside nor exit holes were visible but, often, some organic remains were observed that were attributed to *O. mediterraneus* corpse. We used this item to estimate the mortality of this species in natural conditions, even though we were not able to determine its causes (phytotoxicity, predation, viruses, etc.).

Abbreviations: The following abbreviations were used for depositions of voucher specimens (paratypes and holotypes):

- ANIC: Australian National Insect Collection, Canberra (Australia)
- BNHM: British Natural History Museum, London (UK)
- MNHN: Muséum National d'Histoire Naturelle, Paris (France)
- NMNH: National Museum of Natural History, Washington (USA)
- UCRC: University of California, Riverside Entomology Research Museum (USA)

Data analysis

Phylogenetic reconstruction

Sequences were aligned using MEGA 7 (Kumar *et al.*, 2016). Alignments were corrected manually and, for *COI*, sequences were translated to amino acids to detect frame-shift mutations and premature stop-codons that may indicate the presence of pseudogenes. Sequences were analysed using the neighbour-joining method and the Kimura's two-parameters evolutionary model (Kimura, 1980). Bootstrap support was evaluated with 1000 replicates starting with a random tree.

Morphological characterization

To analyse morphometric measures, we used multivariate ratio analysis (MRA; Baur & Leuenberger, 2011) that allows the interpretation of results from principal component analysis (PCA) and linear discriminant analysis (LDA) in terms of body ratios. These tools have been recently used to disentangle cryptic species complex in several groups of hymenoptera (Laszlo *et al.*, 2013; Baur *et al.*, 2014; Fusu, 2017; Gebiola *et al.*, 2017). According to Baur & Leuenberger (2011), we first defined an isometric size axis (isosize), calculated as the geometric mean of all variables, and then performed a shape PCA (i.e. a PCA in the space of all ratios) to evaluate how well the morphometric pattern fitted groups obtained with molecular analysis. The most important shape components are then plotted against isosize axis to visualize the correlation between size and shape and evaluate data allometry. We also performed a PCA ratio spectrum – a graphical tool that aims to explain shape PCA components in term of body ratios – and an allometry ratio spectrum that allows checking allometry behaviour of characters used for calculation of ratios. Finally we used the LDA ratio extractor to find ratios that best discriminate groups previously identified by shape PCA.

We performed statistical analyses in R version 3.3.3 (R Core Team, 2017) using R-scripts provided by Baur & Leuenberger (2011). Because of the very low number of individuals (five), specimens of *O. eucalypti* were not included in the analyses.

Gall size

Area of galls induced by both *O. maskelli* and *O. mediterraneus* sp. n. was estimated using both radii measured on an *x*–*y* basis with the formula of an ellipse area (Area = $\pi \times r1 \times r2$, with $r1$ the longest radius and $r2$ the smallest radius). After an exploratory step, we decided to fit linear model (function *lm*) using the galls' area as the dependent variable and the species of *Ophelimus* and the country as possible explanatory variables. A transformation ($1/\mathrm{sqrt}(x)$) was applied to the dependent variable to reach homogeneity of variances. We then applied a two-way analysis of variance to the model.

Results

Molecular characterization

Among the 99 specimens of *Ophelimus*, 61 were used for both *COI* and *28S*, 27 were used only for *COI* and 11 were used only for *28S*. For *28S*, the amplification was successful for all the specimens, the 72 sequences of *Ophelimus* ranging from 550 to 609 bp. For *COI*, three sequences were not amplified so that we obtained 85 sequences of *Ophelimus* ranging from 540 to 583 bp. For both *COI* and *28S*, the minimum interspecific distance exceeds the maximum intraspecific distance (table 2).

Both phylogenetic analyses confirmed the presence of two distinct clusters of *Ophelimus* in Europe (figs 2 and 3). The first one encompasses individuals collected in several localities of South-eastern France, in Italy and in Portugal as well as previously identified *O. maskelli* collected in Italy by Burks *et al.* (2011) (accession number: HM365046 for *COI* and HM364944 for *28S*). In our samples, *O. maskelli* was recovered on two different species of *Eucalyptus* belonging to *Exertaria* section (*E. camaldulensis* and *E. rudis*). The intra-cluster

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Table 2. Estimates of evolutionary divergence between sequences (*COI* and *28S*) expressed as mean number of base substitutions per site between sequences × 100 (min–max) using Kimura two-parameters model. The analysis involved 87 nucleotide sequences for *COI* and 74 nucleotide sequences for *28S*.

	O. mediterraneus		*O. maskelli*		*O. eucalypti* 'Maid.'	*O. eucalypti* 'Trans.'
	COI	*28S*	*COI*	*28S*	*28S*	*28S*
O. mediterraneus	0.37% (0–1.16%)	0.017% (0–0.39%)	8.53% (8.09–9.61%)	0.40% (0.40–0.79%)	1.00% (0.99–1.39%)	2.32% (2.21–2.82%)
O. maskelli	8.31% (8.09 9.61%)	–	0.33% (0.0–1.74%)	0.0% (0.0–0.0%)	0.99% (0.99–0.99%)	2.31% (2.21–2.41%)
O. eucalypti 'Maid.'	–	–	–	–	0.0% (0.0–0.0%)	1.70% (1.59–1.80%)
O. eucalypti 'Trans.'	–	–	–	–	–	0.13% (0–0.20%)

distance in *O. maskelli* is 0.0% for *28S*, and ranged from 0.0 to 1.7% for *COI* (table 2).

The second cluster of *Ophelimus* encompasses individuals collected on *E. globulus* and *E. gunnii* (section *Maidenaria*) in South-Eastern France, in Italy and in Portugal as well as two specimens collected on *E. globulus* in Western Australia (Victoria state) in 2003 (molecular codes: 6796 and 6797) (figs 2 and 3). This cluster is clearly genetically differentiated from *O. maskelli* both with *COI* and *28S*. The inter-cluster distance between this species and *O. maskelli* ranged from 8.1 to 9.6% for *COI* and from 0.4 to 0.8% for *28S*.

Finally, on the *28S* tree, we have some other specimens that are differentiated from both previously discussed *Ophelimus* species (fig. 2). These were collected in New Zealand on *E. globulus* and *E. saligna* and were identified as two 'biotypes' according to their host-plants: *O. eucalypti* 'Maid.' collected on *E. globulus* and *O. eucalypti* 'Trans.' collected on *E. saligna*. For the first one, the interspecific distance is 1% with *O. maskelli*, and ranged from 1 to 1.4% with *O. mediterraneus* sp. n. For *O. eucalypti* 'Trans.', interspecific distance ranged from 2.2 to 2.4%, and from 2.2 to 2.8% with *O. maskelli* and *O. mediterraneus* sp. n. respectively. Interestingly, the distance between each of these two 'biotypes' (ranging from 1.6 to 1.8%) is higher than the interspecific distance found for *O. maskelli* and *O. mediterraneus* sp. n., suggesting that these two 'biotypes' may be two distinct species.

Morphological investigations

Observations of the submarginal vein of the forewings of 262 specimens of *Ophelimus* collected on five different *Eucalyptus* species and in five countries showed results that are congruent with those obtained with molecular tools (fig. 4). Indeed, we confirm that *O. maskelli* is the only species that presents no variability of this character, having always only one submarginal seta on the forewings, be it developing on *E. camaldulensis* or *E. rudis*. On the other hand, *O. mediterraneus* sp. n. and the two 'biotypes' of *O. eucalypti* present at least two submarginal setae. *O. mediterraneus* sp. n., collected on *E. globulus* and *E. gunnii* in Australia, France, Italy and Portugal present from two to four submarginal setae on the forewings but mostly three (60% of the total number of specimens observed). Interestingly, differences in the number of submarginal setae were found between the two 'biotypes' of *O. eucalypti*: two to four submarginal setae for *O. eucalypti* 'Maid.' and five to six submarginal setae for *O. eucalypti* 'Trans.'.

For the shape PCA, we used only the two first components that accounted for 81% of the total variance (43 and 38% for the first and second component respectively). Specimens were assigned to species based on qualitative characters (number of submarginal setae). The shape PCA showed that *O. maskelli* and *O. mediterraneus* sp. n. cannot be separated using the quantitative characters used for this study, with a strong overlap between individuals of these two species. Along the second component, the overlap is also almost total with only two specimens of *O. maskelli* that are separated from *O. mediterraneus* sp. n. (not shown). The first component is a bit more discriminant with the separation of five (among 37) *O. mediterraneus* sp. n. and five (among 30) *O. maskelli* (fig. 5a). Scatterplot of isosize against first (fig. 5b) and second (not shown) shape PC showed that size ranges of these two species are largely overlapping but *O. mediterraneus* sp. n. seems on average slightly larger than *O. maskelli*. Size variation for both species is high.

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Fig. 2. Neighbour-joining tree figuring relationships between *Ophelimus* species based on the nuclear gene *28S*. *C. chamaeleon* is used as outgroup. Kimura two-parameters analysis with 1000 bootstrap replicates. Each line represents a sequenced individual with information in the following order: molecular code, molecular marker, species (host species), country and (locality).

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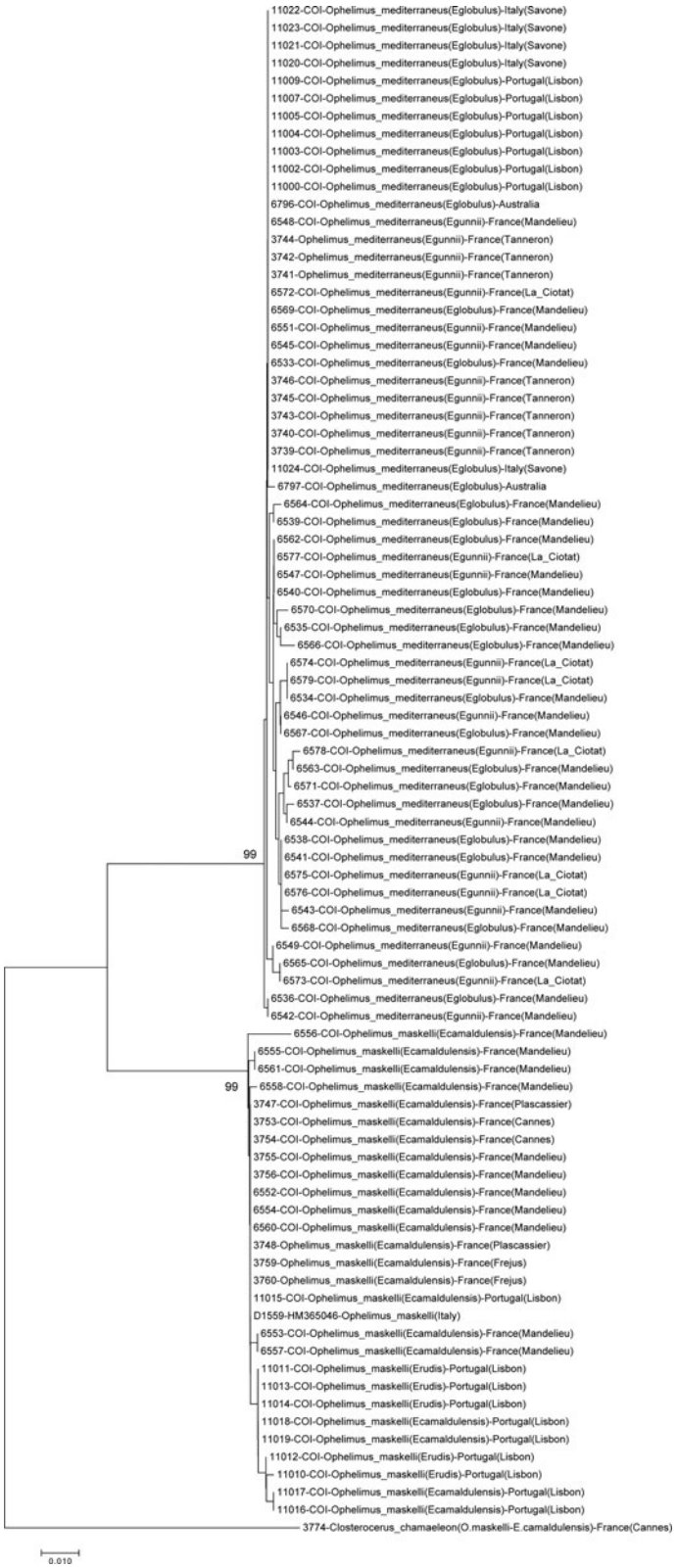

Fig. 3. Neighbour-joining tree figuring relationships between *Ophelimus* species based on the mitochondrial gene *COI*. *C. chamaeleon* is used as outgroup. Kimura two-parameters analysis with 1000 bootstrap replicates (supports >70% are indicated at nodes). Each line represents a sequenced individual with information in the following order: molecular code, molecular marker, species (host species), country and (locality).

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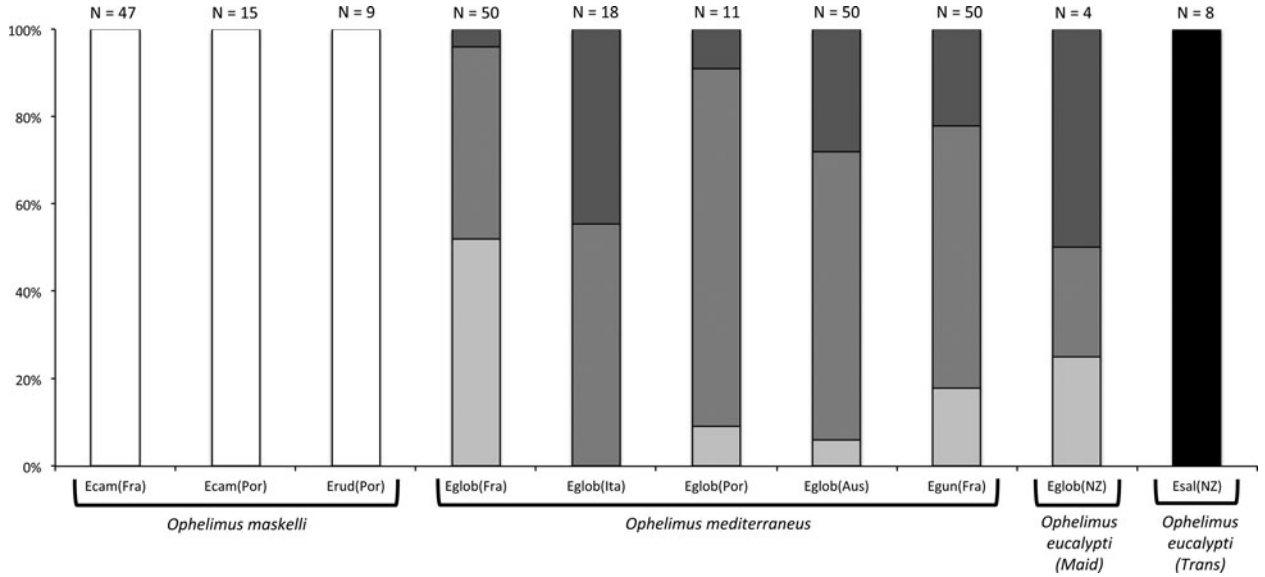

Fig. 4. Number of submarginal setae observed in different species of *Ophelimus* collected on different species of *Eucalyptus* (Ecam: *E. camaldulensis*; Eglob: *E. globulus*; Egun: *E. gunnii*, Erud: *E. rudis*; Esal: *E. saligna*) and in different countries (Aus: Australia; Ita: Italy; Fra: France; NZ: New-Zealand; Por: Portugal). Numbers in square bracket (top) represent the number of specimens observed. White: one submarginal seta; light grey: two submarginal setae; medium grey: three submarginal setae; dark grey: four submarginal setae; black: >4 submarginal setae.

PCA ratio spectrum (fig. 5c) of the first component of the shape PCA showed that most of the variation along this axis is explained by characters that are lying to the opposite ends of the spectrum (such as 'SmvL:StvL') whereas ratios composed of characters lying close to each other in the spectrum (such as 'FwL:T3L') contributed very little to this component. The PCA ratio spectrum ranged from −0.41 to 0.45 for the variable coefficients. In the same way, the allometry ratio spectrum (fig. 5d) showed that variables lying close to each other are those showing the lowest amount of allometry (such as 'MvL:StvL') whereas the most distant variable are those showing the greatest amount of allometry (such as such as 'T3L: StvL').

Finally the LDA ratio extractor gives us the two ratios that best discriminate groups previously identified: 'FwL:MvL' and 'FwL:SmvL' (table 3). For these two ratios, the theta value is 0.36 and 0.40 respectively, indicating that discrimination between these two species of *Ophelimus* is mainly due to shape rather than size. However, ranges of the best two ratios are mostly overlapped indicating that they cannot be used to discriminate these two species. This is confirmed by the value of standard distance of these two ratios that are 1.86 and 1.60 respectively. Interestingly, the ratio 'T3L:FwL', that is not useful to discriminate between *O. maskelli* and *O. mediterraneus* sp. n., may be used to distinguish *O. eucalypti* 'Maid.' from *O. eucalypti* 'Trans.' as the range of this ratio is not overlapping (table 3).

Bio-ecological investigations

Gall area

Results of gall size measurements showed that both in France and in Portugal, there is a significant effect of the species of *Ophelimus* ($F_{1,391} = 781$, $p < 2e\text{-}16$) and of the country ($F_{1,391} = 10.59$, $p = 0.0012$) on galls' area. Indeed, in both countries, galls induced by *O. maskelli* (on *E. camaldulensis*) are larger than those induced by *O. mediterraneus* sp. n. (on *E. globulus*) (fig. 6). There is no significant effect of the interaction between 'species of *Ophelimus*' and 'country' ($F_{1,391} = 1.02$, $p = 0.31$).

Biological cycle

A total of 651 galls of *O. mediterraneus* sp. n. collected on *E. gunnii* were dissected between January 2011 and January 2012. As shown in fig. 7, the emergence period of *O. mediterraneus* sp. n. is in late spring–early summer. After, no sign of gall development was visible on leaves collected between August and October. Using a transmitted light source, it was nevertheless possible to observe oviposition marks. Attempts to observe eggs by dissection were however not conclusive. Following this 3–4 months period without sign of gall development, reddish flat marks appeared on leaves in early winter (December) (fig. 8a) with young larval instars of *O. mediterraneus* sp. n. present inside. This period of early stage of gall development was followed by the development of first galls in January. These galls are brown coloured, ellipsoid shaped, with a crackled surface and are visible only on the upper surface of the leaf (fig. 8b). After (January–March), these fully developed galls were occupied by larval instars but in March around 12% of the total number of the galls were empty indicating mortality of the larvae. In spring, the presence of larval instars became scarce (from 0 to 1.6% of the dissected galls) whereas nymphal instars became predominant (from 88 to 94% of the dissected galls). During this stage, few adults were observed within these galls and the mortality ranged from 3 to 9%. In June, 60% of the total number of dissected galls was characterized by the presence of exit holes of

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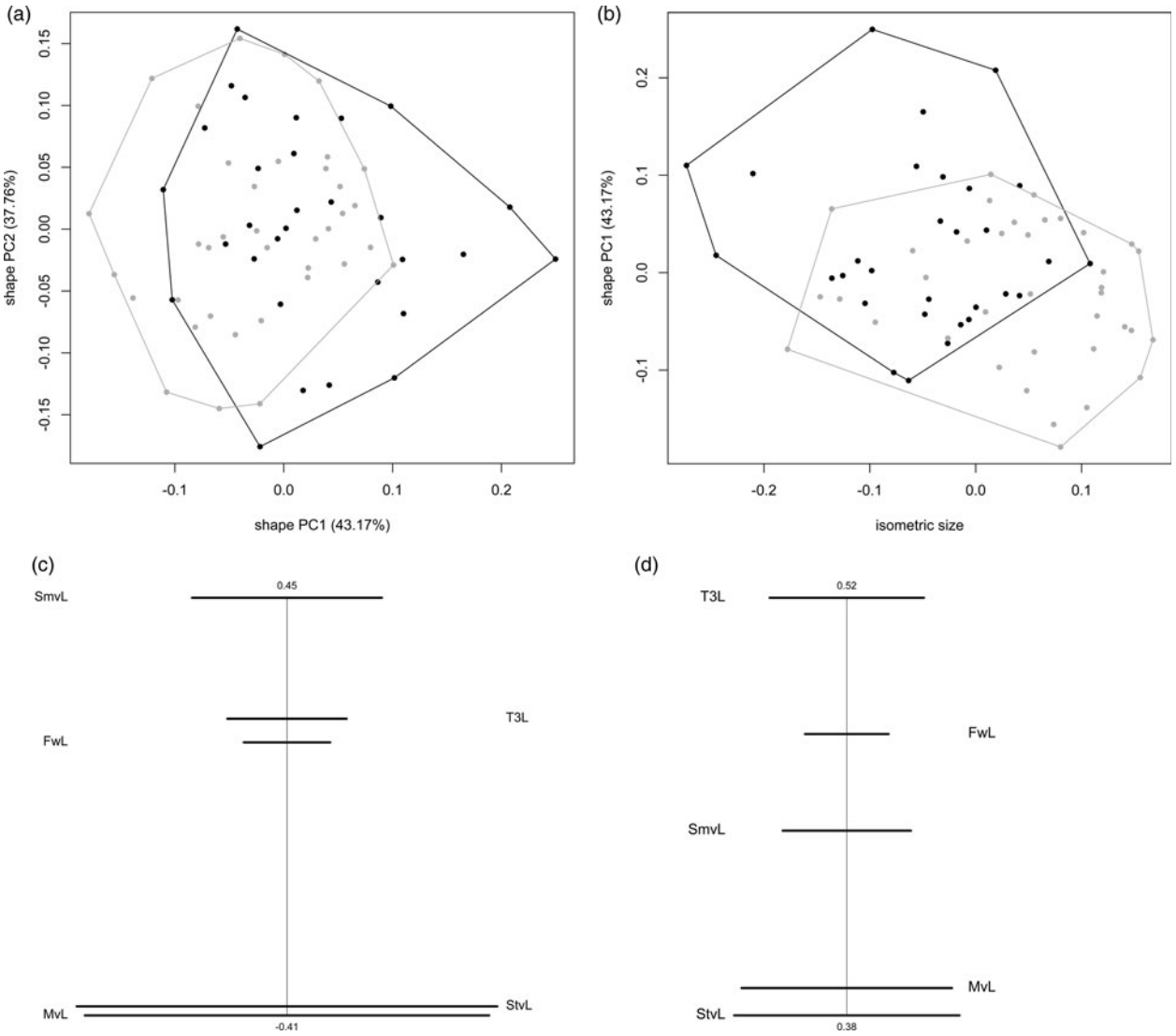

Fig. 5. Results of MRA for females of *O. maskelli* and *O. mediterraneus* sp. n. (a, b) Scatterplot of PCA in shape space: scatterplot of first against second shape PC (a), scatterplot of isosize against first shape PC (b). Symbols: black dots, *O. maskelli*; grey dots, *O. mediterraneus* sp. n. (c, d) Ratio spectra: PCA ratio spectrum (c), allometry ratio spectrum (d). Horizontal bars in the ratio spectra represent 68% bootstrap confidence intervals based on 500 replicates.

emerged *O. mediterraneus* sp. n. whereas around 7% of the galls was at the nymphal stage and 7% of the galls contained adults not yet emerged. It is during this emergence period that the mortality rate is the most important with 26% of the total number of the galls being empty.

Parasitism

C. chamaeleon emerged from the galls induced by *O. maskelli* on *E. camaldulensis* in all the samples we collected in France and Portugal indicating that this parasitoid is well established in these countries. On the contrary, no parasitoid emerged from the galls induced by *O. mediterraneus* sp. n., in France, Italy and Portugal despite suitable conditions and an important sampling (more than 2000 *O. mediterraneus* emerged).

Species description

Ophelimus Haliday

Ophelimus Haliday, 1844: 300. Type species *Eulophus ursidius* Walker, designated by Ashmead, 1904: 353.
Chrysoatomus Ashmead, 1904: 342. Type species *Chrysoatomus zealandicus* Ashmead
Rhicnopeltella Girault, 1913*a*: 109. Type species *Rhicnopeltella immaculatipennis* Girault
Cirrospilomyia Girault, 1913*b*: 76. Type species *Cirrospilomyia magniventris* Girault
Brachychrysocharella Girault, 1913*c*: 168. Type species *Brachychrysocharella dubia* Girault
Omphalomorphella Girault and Dodd, in Girault, 1913*c*: 178. Type species *Omphalomorphella auripes* Girault

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Table 3. Summarized data of body measurements (in µm) for each species of *Ophelimus* and best ratios.

	O. maskelli ($n = 30$)			*O. mediterraneus* ($n = 37$)			*O. eucalypti* 'Maid'		($n = 2$)	*O. eucalypti* 'Trans.' ($n = 3$)		
Characters	min	max	mean	SD	min	max	mean	SD	min	max	mean	SD	min	max	mean	SD
T3L	154	280	226.7	25.04	191	307	250.5	29.14	306	355	330.5	34.65	345	494	420.7	74.53
FwL	646	882	782.5	64.46	715	1012	893.1	83.01	1325	1368	1346.5	30.40	1200	1666	1449.7	234.78
MvL	104	143	120.3	10.93	93	153	128.5	14.49	242	249	245.5	4.95	217	321	271.3	52.16
SmvL	224	322	276.1	28.35	250	352	308.4	29.12	451	497	474	32.53	418	600	516.7	91.96
StvL	51	82	68.4	8.01	60	88	72.7	6.60	118	119	118.5	0.71	104	146	127.3	21.38
Best ratios																
FwL/MvL	5.458	7.50	6.531	0.542	6.176	8.107	6.977	0.447	5.321	5.653	5.487	0.235	5.190	5.530	5.364	0.170
FwL/SmvL	2.628	3.676	2.845	0.198	2.713	3.181	2.898	0.097	2.753	2.938	2.845	0.131	2.777	2.871	2.812	0.051
T3L/FwL	0.238	0.317	0.289	0.015	0.237	0.311	0.280	0.014	0.224	0.268	0.246	0.031	0.285	0.297	0.290	0.001

Elachertetrastichus Girault, 1913*c*: 264. Type species *Elachertetrastichus purpureus* Girault
Omphalomorphoides Dodd, in Girault, 1915: 214. Type species *Omphalomorphoides violescens* Dodd
Deciana Girault, 1925: 92. Type species *Deciana aenoviridis* Girault

Ophelimus mediterraneus *Borowiec & Burks, n. sp.*

Material examined (all females)
Holotype. France: Bouches-du-Rhône: La Ciotat, jardin, 24.v.2010, H. Dumas [MNHN: UCRCENT00485453], deposited in MNHN (GenBank accession numbers: *COI*, JX096442; *28S*, MH651684).
Paratypes. 34 paratypes with depositions indicated by coden: France: *Alpes-Maritimes*: Mandelieu, casino; alt: 7 m; 43°31′56″N, 6°55′53″E; 3.v.2011; N. Borowiec [MNHN: UCRCENT00485484, GenBank accession numbers: *COI*, MH651533; *28S*, MH651673 – NMNH: UCRCENT00485485, GenBank accession numbers: *COI*, JX096435; *28S*, MH651675]. Mandelieu, Pelazza; alt: 217 m; 43°33′05″N, 6°54′51″; 3.v.2011; N. Borowiec [MNHN: UCRCENT00485482, GenBank accession numbers: *COI*, MH651528; *28S*, MH651653 – UCRC: UCRCENT00485483, GenBank accession numbers: *COI*, JX096426; *28S*, MH651652]. *Bouches-du-Rhône*: La Ciotat, jardin; 24.v.2010; H. Dumas [MNHN: UCRCENT00485454–55, GenBank accession numbers: *COI*, JX096440 and JX096441; *28S*, MH651682 and MH651683]. *Var*: Tanneron, Courrin; 43°35′00″N, 6°50′06″E; 27.iv.2010; N. Borowiec [MNHN: UCRCENT00485456; MNHN: UCRCENT00485464; MNHN: UCRCENT00485466–73; MNHN: UCRCENT00485473–81; BMNH: UCRCENT00485474]. Italy: Savona Prov.: Loano; alt: 15 m; 44°08′34″N, 8°16′05″E; 11.iv.2012 ; M. Thaon [ANIC: UCRCENT00485495–96, GenBank accession numbers: *COI*, MH651520 and MH651521 – MNHN: UCRCENT00485497, GenBank accession number: *COI*, MH651522].
Other material examined (not paratypes because locality is uncertain): Australia: Victoria?; 8.x.2003; Z. Mendel [MNHN: UCRCENT00499638; MNHN: UCRCENT00499641].
Etymology. The names *mediterraneus* refers to the large geographical distribution of this species in the Mediterranean region.
Diagnosis. Mostly with three (from two to four) setae on the submarginal vein dorsally (fig. 1a). Ventral half of face with long setae. Mesoscutum at most only slightly longer than mesoscutellum (mesoscutum 0.93–1.13× mesoscutellum length; Fig. 9c). Otherwise similar to *O. maskelli*. The mesoscutum in *O. maskelli* is noticeably longer: about 1.3× the mesoscutellum length, and *O. maskelli* has only one dorsal submarginal vein seta. *O. eucalypti*, the other well-known invasive species of the genus, differs in having more than three submarginal vein setae, in having far more callar setae on the propodeum (over five, instead of only two), and in having a body about twice as long (nearly 2 mm, vs. about 1 mm or less).
Description: Female.

Body length: 0.8–1.0 mm.

Colour: Head and body brown with variable metallic luster (fig. 9a, c). Eyes and ocelli grey (fig. 9b). Antenna light brown (fig. 9b). Legs brown, but tips of femora and tibiae and first three tarsomeres pale brown (fig. 9a).

Head (fig. 9b): Dorsally imbricate and ventrally becoming reticulate; interantennal elevation distinctively reticulates, scrobal depressions broad and smooth. With antennal scrobe

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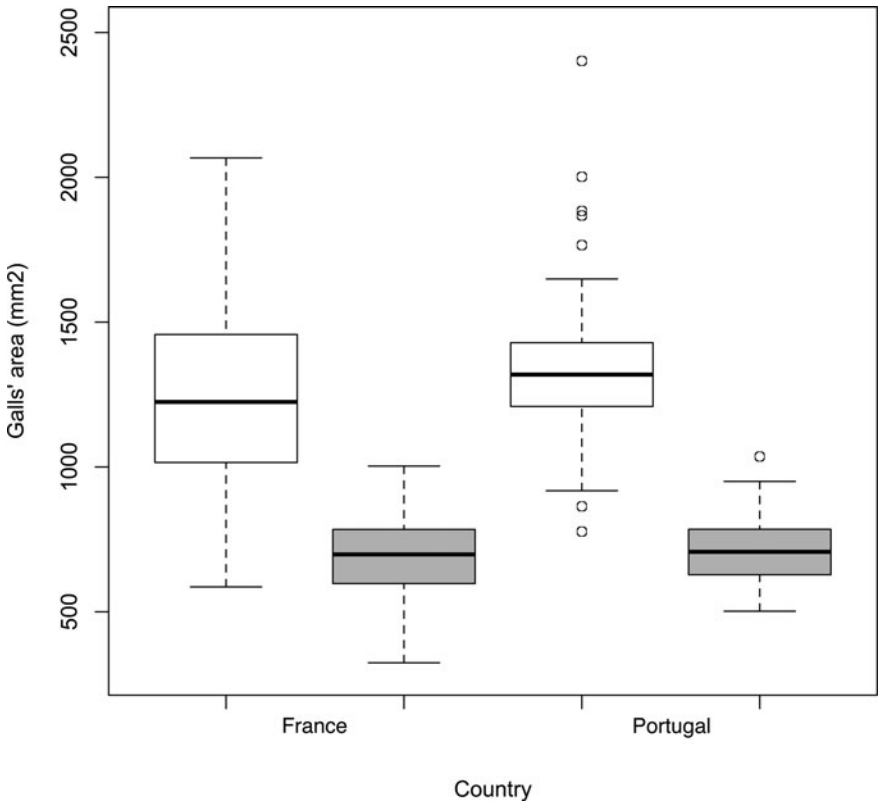

Fig. 6. Boxplots of gall's area (mm^2) induced by *O. maskelli* (white boxes) and *O. mediterraneus* sp. n. (grey boxes) in France and Portugal. Boxes represent the 25–75th percentile range and the horizontal line within each box represents the median value. The range lines correspond to the highest and lowest values, with outliers marked with circles.

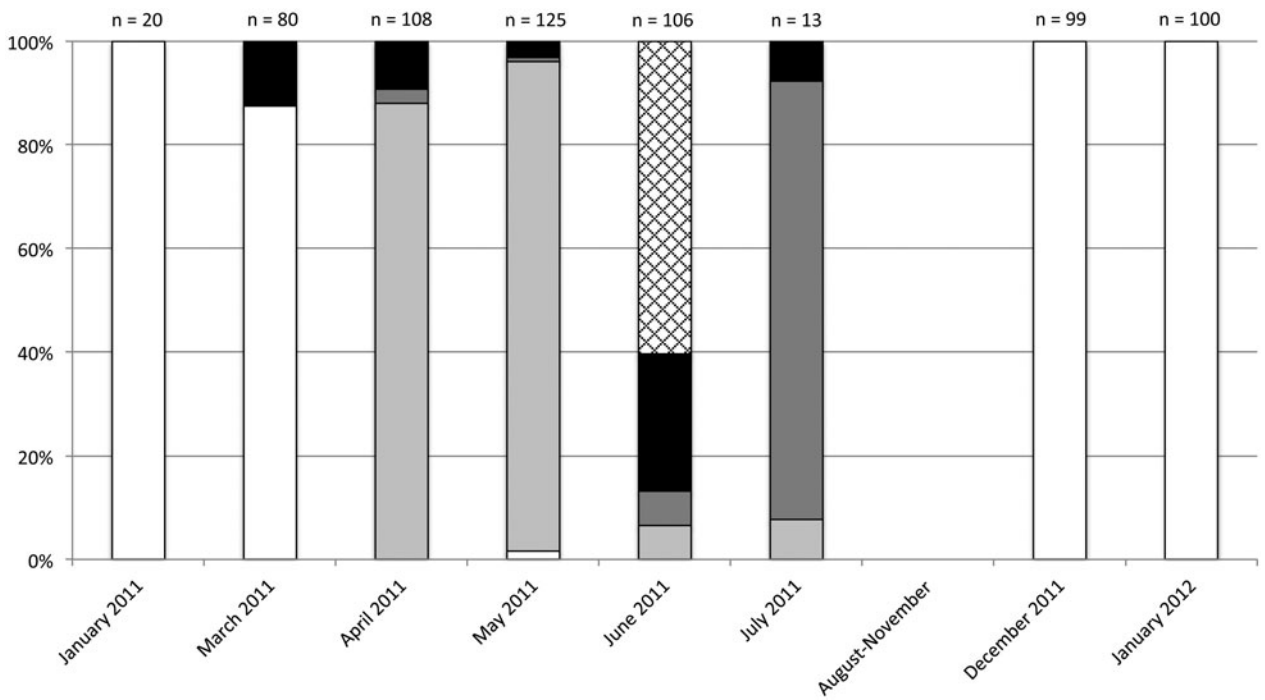

Fig. 7. Seasonal evolution of the relative frequency (%) of the different 'life stages' within dissected galls induced by *O. mediterraneus* sp. n. on *E. gunnii* in France. White: 'larvae'; light grey: 'nymphs'; dark grey: 'adults'; black: 'empty'; hatched: 'exit holes'.

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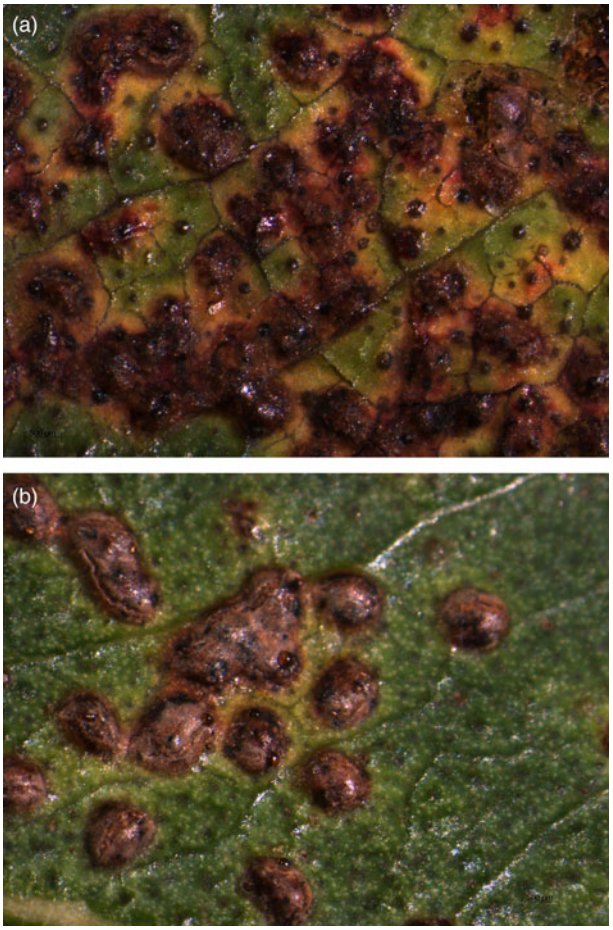

Fig. 8. Galls of *O. mediterraneus* sp. n. on *E. globulus*: (a) newly formed galls and (b) fully developed galls.

hardly visible in some specimens, but united immediately below centre of face, reaching oblique grooves at ocellar triangle. Interantennal elevation, parascrobal area, and most of ventral half of face with long setae, but scrobal area bare. Eyes with setae that are visible at high magnifications. Toruli at lower eye margin. Malar sulcus distinct. Clypeal margin transverse and hardly distinguished from adjacent parts of mouth margin.

Antennae (fig. 9b): Scape reaching only slightly above mid eye height. Pedicel longer than funicle. First four flagellomeres anelliform, the last also transverse but larger and bearing multiporous plate sensilla. Clava longer than the other flagellomeres together, with three distinct clavomeres, the apical one bearing a long terminal seta.

Mesosoma (fig. 9c): Pronotum medially vertical, but laterally with collar. Mesoscutum 0.93–1.13× mesoscutellum length along midline. Mesoscutal midlobe anteriorly imbricate, posteriorly becoming reticulate to coriaceous, with two pairs of setae; mesoscutal sidelobe reticulate. Axilla coriaceous, advanced for about half its length. Mesoscutellum coriaceous, 1.07–1.33× as broad as long, with two pairs of setae, the anterior pair in anterior half near scutoscutellar sulcus, the posterior pair near posterior rim of mesoscutellum; mesoscutellar rim not carinate, but overhanging metascutellum slightly; axillular groove present but not visible from dorsal view. Metascutellum very shallowly reticulate, slightly overhanging propodeum. Propodeum medially not longer than metascutellum, posterior margin deeply excavated medially; in some specimens there is a broad and shallow median carina that splits to form a nuchal triangle.

Wings (figs 1a and 9d): Forewing about as long as body, about twice as long as broad. Submarginal vein about 1/3 forewing length, with three dorsal setae (these often broken off, but their sockets are easily seen). Parastigma with slight extension at basal fold, with distinct parastigmal break at base of marginal vein; two parastigmal sensilla present. Costal cell and basal cell with a few extremely short dorsal setae. Speculum present. Marginal vein slightly longer than stigmal vein, but shorter than postmarginal vein; three distinctively long setae present along anterior edge of marginal vein. Stigmal vein with dorsal setae only on expanded stigmal area; uncus present. Apex of postmarginal vein indistinct. Wing disc densely setose; admarginal setae only present near along stigmal vein and apical half of marginal vein. Longest fringe setae as long or slightly longer than marginal vein. Hindwing with dark spot near base of the single parastigmal seta.

Legs (figs 1b and 9a): Coxa shallowly reticulate. Femora and tibiae imbricate. Mesotibial spur about as long as the first two mid tarsomeres together. Hind tibia densely setose, with one apical spur.

Metasoma: About as long as mesosoma. Petiole transverse and not easily visible without removing metasoma. Terga easily collapsing and in some areas with faint reticulation. First gastral tergum the longest. Very small, semi-translucent apical tergum present that may be an epipygium. Ovipositor short, stylets slightly down-turned apically. Hypopygium reaching about 0.8× gaster length.

Male. Unknown

Distribution: France, Italy, Portugal, Australia. Probably present in more countries, in particular in the Mediterranean region, since the presence of *O. maskelli* may have dissimulated its presence.

Hosts: Exclusively associated with *Eucalyptus* species and to date reported only on *Eucalyptus* species from the *Maidenaria* Section. Mainly found on *E. globulus* in Italy, France and Portugal. In a context of outbreaks in France, it was also found on *E. cinerea*, *E. gunnii* and *E. parvula*, as well as on *E. cypellocarpa* in Portugal (Garcia *et al*., submitted).

Comments: Univoltine, the adults' flying period is around late spring. Fully developed galls are visible from January (see above for details). In native (Australia) or invaded (Italy, France, Portugal) areas, only females of *O. mediterraneus* were collected, this species being supposed to reproduce by thelytoky.

Discriminating between *Ophelimus* species using previous work is quite a hard task since Girault's descriptions (42 descriptions among a total of 51 known species of *Ophelimus*; Noyes, 2018) are always poor and the types are in very bad conditions. The genus *Ophelimus* would definitively need a taxonomic revision to evaluate the status of all species and provide an identification key.

Identification key for some *Ophelimus* species of agricultural interest

1. Small species, <1.2 mm long . . . . . (2)
1′. Larger species, >1.5 mm long . . . . . (3)

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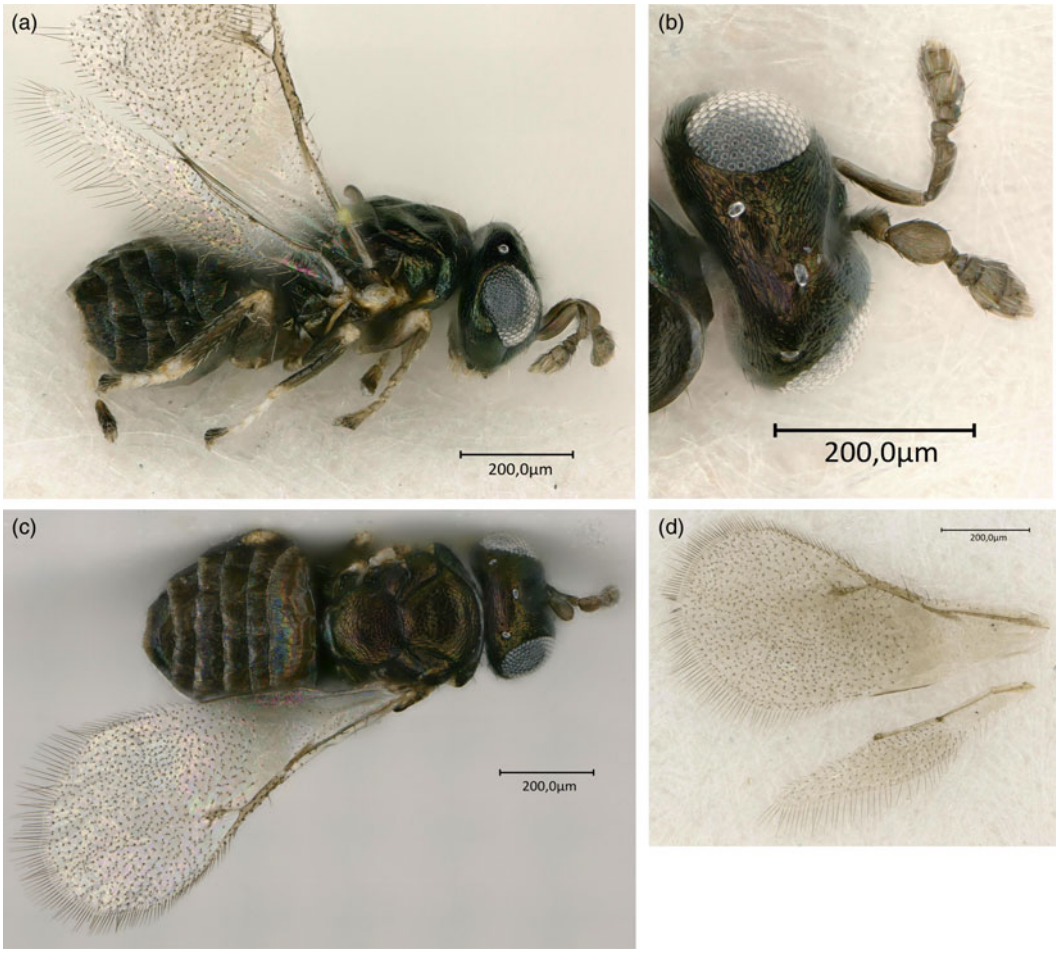

Fig. 9. *O. mediterraneus* sp. n. female: (a) lateral view; (b) head and antennae in dorso-lateral view; (c) dorsal view and (d) wings.

2(1). Only one submarginal seta on forewings; induces large green round-shaped galls, visible on both sides of the leaves, that become reddish with maturation. Mainly found on *Eucalyptus* species belonging to *Transversaria* section (e.g. *E. camaldulensis* and *E. rudis*). At least two generations per year . . . . . ***maskelli***

2′. Two—four (mostly three) submarginal setae on forewings. Induces small ellipsoid green galls, only visible on upper side of leaves, that become brownish with maturation. On *Eucalyptus* species belonging to the *Maidenaria* section (mainly *E. globulus*). Univoltine. . . . . ***mediterraneus sp. n.***

3(1′). Two—four submarginal setae on forewings. Develops on *Eucalyptus* from section *Maidenaria* . . . . . ***eucalypti* 'Maid.'**

3′. More than four submarginal setae on forewings. Develops on *Eucalyptus* from section *Transversaria*. . . . . ***eucalypti* 'Trans.'**

Discussion

Using an integrative taxonomy approach (Al Khatib *et al.*, 2014; Baur *et al.*, 2014; Fusu, 2017; Gebiola *et al.*, 2017), our study unambiguously evidences the presence in the Mediterranean region (France, Italy, Portugal) of a third species of *Ophelimus* that threatened *Eucalyptus* plantations. This new species was also found in its supposed native area (Australia) where it was not until now described. A full description of this new species, finally named *O. mediterraneus*, is provided here.

From a methodological point of view, both molecular markers (*COI* and *28S*) showed unambiguous differentiation between the four species of *Ophelimus* sampled: *O. eucalypti* 'Maid.', *O. eucalypti* 'Trans.', *O. maskelli* and *O. mediterraneus* sp. n. The capacity of discrimination of the morphological characters used depends upon the characters and species considered. We hence confirm here that, to date, *O. maskelli* is the only species that present only one submarginal seta on its forewings. Even if few individuals were examined, *O. eucalypti* 'Trans.' seems to be the only one with at least five submarginal setae. This character was however not discriminant for the two last species that nevertheless differ in their overall size, *O. eucalypti* 'Maid.' being clearly bigger than *O. mediterraneus* sp. n. Finally, ecological criteria proved to be also relevant in distinguishing *O. maskelli* and *O. mediterraneus* sp. n. Indeed, the galls induced by both species are easily distinguishable, *O. maskelli* producing round and smooth, green reddish galls visible on both sides of the leaves while *O. mediterraneus* sp. n. produces ellipsoidal, conical shaped galls, brown coloured with rough and cracked surface on just the upper side of the leaves. Moreover, unlike *O. maskelli* that have several

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generations per year, *O. mediterraneus* sp. n. appears to be univoltine.

As *O. maskelli* was misidentified as *O. eucalypti* when it was firstly discovered in Europe (Protasov *et al.*, 2007*b*), *O. mediterraneus* sp. n. may have been confused with *O. maskelli* in their sympatric introduced areas. Now, the discovery of *O. mediterraneus* sp. n. rather speaks for an ecological specialization in the different *Ophelimus* species, at least in Europe. Indeed, *O. mediterraneus* sp. n. may be strictly associated with *Eucalyptus* species from the section *Maidenaria* while *O. maskelli*, although generalist (Protasov *et al.*, 2007*b*; Branco *et al.*, 2014), develops mostly on *E. camaldulensis* and related species.

By the way, our study also evidences a structuration within *O. eucalypti*. Based on molecular, morphological (number of submarginal setae) as well as ecological (host range) features, we indeed confirm that the two biotypes, *O. eucalypti* 'Trans.' and *O. eucalypti* 'Maid.', may be two distinct species that are closely related morphologically. Because some *Eucalyptus* species have a restricted distribution in Australia and more than 50 species of *Ophelimus* are described, intra- or inter-specific variations in *Eucalyptus–Ophelimus* interactions are not so surprising (Withers *et al.*, 2000). However, the consequences of such eco-evolutionary processes (ecological specialization and speciation) on the management of *Eucalyptus* pests may have been under-estimated.

In the frame of biological control, the accurate identification of the pest species targeted and its biological control agents is the first indispensable pre-requisite for the implementation of a biological control programme (Rosen & DeBach, 1973). If this is not the case, this could lead to the failure of the programme and could have consequences such as the non-establishment of the biological control agent released as well as unintentional effects such as non-target impacts on native communities (Kenis *et al.*, 2009; Kenis & Branco, 2010). About the control of *Ophelimus* species, *C. chamaeleon* was proved to be successful against *O. maskelli* in several countries (this study and Branco *et al.*, 2009; Caleca *et al.*, 2011; Burks *et al.*, 2015*b*; Mendel *et al.*, 2017). However, neither *C. chamaeleon* nor any other species emerged from *O. mediterraneus* sp. n. even in case of very close proximity between *E. camaldulensis* infested by *O. maskelli* and *E. globulus* infested by *O. mediterraneus*. This may be the consequence of an asynchronous life cycle between *O. mediterraneus* sp. n. and *C. chamaeleon* (Garcia *et al.*, submitted). *E. globulus* covers about 3 million hectares worldwide with more than 50% of the total cultivated area localized in the Mediterranean region (Potts *et al.*, 2004). One can fear a rapid expansion and outbreaks of *O. mediterraneus* sp. n. Specific investigations on parasitoids of *E. mediterraneus* sp. n. in the native area should thus be welcomed to evaluate the feasibility of a classical biological control.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485318001037.

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