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ORIGINAL ARTICLE

Distinct barcodes for the Cereal leaf beetles *Oulema melanopus* and *Oulema duftschmidi* (Coleoptera: Chrysomelidae), two syntopical sibling species

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Abstract. *Oulema melanopus* (Linnaeus, 1758) and *Oulema duftschmidi* (Redtenbacher, 1874) (Coleoptera: Chrysomelidae) are two native West Palaearctic species developing on various cultivated and wild grasses. Along with *O. obscura* they are considered to be secondary pests of cereal crops. However, local outbreaks have been recorded recently and their status as secondary pests may evolve, especially as the use of broad-spectrum insecticides is now greatly reduced. *Oulema melanopus* and *O. duftschmidi* are considered to be sibling species. They are morphologically very close and difficult to distinguish from each other, which makes it difficult to study them. We tested the reliability of the standard barcode fragment (*COI*) for distinguishing between these species. A total of 92 samples of the two species, covering the majority of their natural range, was sequenced for the barcode fragment and inter- and intraspecific genetic distances were estimated. Our results confirm those of Bezděk & Baselga (2015, *Acta Entomol. Mus. Nat. Prag.* **55**: 273–304) in that this marker cannot differentiate between all the species of the *Oulema melanopus* complex, which in the Mediterranean basin contains several described and possibly some undescribed cryptic species. However, this marker may be useful in an agricultural context in areas where only *O. melanopus* and *O. duftschmidi* occur (such as in cereal crops in France) where it can be used to reliably and rapidly separate all stages of these two taxa and can therefore help in studying their ecology and dynamics.

INTRODUCTION

Cereal leaf beetles belong to Coleoptera of the subfamily Criocerinae within the family Chrysomelidae and their larvae feed and develop on various cultivated or wild grasses (Venturi, 1942; Jolivet, 1997). Nine species are recognized in France, one belonging to the genus *Lema* [*Lema cyanella* (Linnaeus, 1758)] and 8 to the genus *Oulema* [*Oulema duftschmidi* (Redtenbacher, 1874), *O. erichsonii* (Suffrian, 1841), *O. obscura* (Stephens, 1831), *O. hoffmannseggii* (Lacordaire, 1845), *O. melanopus* (Linnaeus, 1758), *O. rufocyanea* (Suffrian, 1847), *O. septentrionis* (Weise, 1880) and *O. tristis* (Herbst, 1786)]. The genus *Oulema* comprises about 130 species worldwide, of which 19 are Nearctic and 21 Palearctic. Currently, 11 species are known from Europe (Bezděk & Schmitt, 2017; Rilet et al., 2003), among which two new species were recently described from Italy (*Oulema mauroi* Bezděk & Baselga, 2015) and Spain (*Oulema verae* Bezděk & Baselga, 2015). Only two species of *Oulema* are frequently cited as cereal pests in the Palearctic region: *O. melanopus* and *O. obscura* (Balachowsky & Mesnil, 1935; Bonnemaison, 1962; Labeyrie, 1963; Chambon et al., 1983; ACTA, 2016). The history of the taxonomy of *O. obscura* is very confusing: early works mention *O. obscura* as *Lema lichenis* Weise, 1882 or *Lema lichenis* Voet, 1806 (an invalid name according to White, 1981), then as *O. gallaeciana* (Heyden, 1870), before it was synonymized with *O. obscura* (Stephens, 1831) (Cox, 2000; Bezděk & Schmitt, 2017). Labeyrie (1963) further cites *O. tristis* as a pest, as does Feytaud (1924). With the exception of *O. tristis*, which has not been reported on crops since then, three species of *Oulema* are regularly observed in crops and are likely to damage them in France and Europe: *O. obscura*, *O. melanopus*, but also *O. duftschmidi*, which was confused with the previous species until 1989 and is still not included in plant protection manuals. *O. melanopus* and *O. duftschmidi* are considered to be sibling species, as they are very similar both in their internal (genitalia) and external morphology. Their identification requires the dissection of the flagellum located in the penis of the males (Berti, 1989; Bezděk & Baselga,

2015; Chapelin-Viscardi & Maillet-Mezeray, 2015; Leroy & Chapelin-Viscardi, 2018), which makes their study particularly difficult for non-specialists, especially as it is not possible to identify females and the immature stages. The existence of both species in France was reported by Berti (1989), who provides reliable morphological criteria for identifying them (Fig. 2) and reports the existence of specimens of *O. duftschmidi* identified as *O. melanopus* in the collections of the French National Museum of Natural History (MNHN, Paris). Berti (1989) also states that both species are sympatric and widely distributed in France. More recently, Bezděk & Baselga (2015) revised the *Oulema melanopus* species complex in Western Europe. They recognize five species, including two new ones and review the taxonomy of the group.

Oulema melanopus and *O. duftschmidi* can damage crops, especially the larvae that feed on leaves of cereal plants (Philips et al., 2011). These sibling species could potentially harm cereal crops in France (Bonnemaison, 1962; Labeyrie, 1963; Anglade et al., 1976; Chambon et al., 1983; ACTA, 2016 etc.) and other European countries (Labeyrie, 1963): Romania (Knechtel & Monolache, 1936), Hungary (Sajó, 1893), Spain (Urquijo, 1940), Greece (Pélécassis, 1951) and Italy (Bechini et al., 2013). Most of these publications refer only to *O. melanopus* whereas *O. duftschmidi* may be also involved (Chapelin-Viscardi & Maillet-Mezeray, 2015). Recently, high population densities of larvae of cereal leaf beetles were recorded in various parts of mainland France, such as the Ille-et-Vilaine, Loiret and Allier departments (pers. obs.). In addition, extreme climatic events, which are becoming increasingly frequent, are conducive to pest outbreaks, particularly of species that are highly dependent on the climate, such as cereal leaf beetles (Guppy & Harcourt, 1978; Olfert & Weiss, 2006; Bechini et al., 2013). The greatly reduced use of neonicotinoid insecticides, and more generally that of pesticides, along with the increase in organic farming in Europe, could also provide suitable conditions for future outbreaks of these pests. Their status could shift from secondary to major pests, as has occurred in the United States and Asia (Philips et al., 2011).

Due to the impossibility of identifying the larvae of these species little is known about the life history traits and relative abundance of these two species. Preliminary agricultural monitoring indicates that *O. duftschmidi* is the more common in several French agricultural landscapes (Chapelin-Viscardi & Maillet-Mezeray, 2015). These surveys also reveal that the flight activity of the adults of both these species is synchronous, which indicates simultaneous larval development. A more recent biogeographical study provides clear evidence that both species are sympatric and coexist throughout France (Leroy & Chapelin-Viscardi, 2018).

In order to better understand the structure of the *O. melanopus*/*O. duftschmidi* species complex it is crucial to have a reliable and routine method for identifying all the development stages of the species. Kubisz et al. (2012) used the standard barcode fragment of the mitochondrial COI gene (Hebert et al., 2003a) to successfully distinguish between and identify several species of Criocerinae in the genus *Crioceris*. Similarly, Bezděk & Baselga (2015) use this DNA fragment for identifying European species of *Oulema*, but their results, based on a small number of specimens, indicate that the COI barcode is not appropriate for the molecular identification of these species as there are discrepancies between the species boundaries revealed by morphology and DNA barcodes. In this study the effectiveness of this gene for identifying the species of *Oulema* feeding on cereals in Europe is re-evaluated using a larger number of specimens.

MATERIALS AND METHODS

Sampling and morphological identification

Specimens were collected between 2005 and 2017 in France, Portugal (Madeira), Greece (Crete), Spain and Italy (Table 1 and Fig. 1). At each site sampled, one to three adults or larvae were collected and killed directly in 96.5% ethanol. The adult specimens were identified to species, based on external morphological characters (Warchałowski, 2003; Bezděk & Mlejnek, 2016), except for specimens belonging to the *Oulema melanopus/duftschmidi* species pair (Fig. 2) for which the identification was based on the structure of dissected male genitalia as only by examination of the flagellum can the two species in this complex be reliably separated (Chapelin-Viscardi & Maillet-Mezeray, 2015). *O. duftschmidi* has a thin, elongated flagellum (Fig. 2d, f) whereas *O. melanopus* has a short, stocky flagellum (Fig. 2e, g) (Bukejs & Ferenca, 2010; Bezděk & Baselga, 2015). For this reason, only males were used to validate our DNA sequences for *O. melanopus* and *O. duftschmidi*. Three species of *Oulema* were sequenced, including 92 male specimens belonging to the *O. melanopus/duftschmidi* complex (44 *O. melanopus* ♂, 48 *O. duftschmidi* ♂) and 43 specimens of both sexes of *O. obscura*. To account for these species in an evolutionary context and validate our means of identification, 25 additional specimens of 8 different species belonging to the subfamily Criocerinae were also sampled. The species *Epitrix pubescens* (Chrysomelidae: Galerucinae) was used as an outgroup to root the phylogenetic analysis reported below. After validation of the barcoding method, we tested the molecular identification of 17 females and 7 larvae of the *O. melanopus/duftschmidi* complex. As a result, the sample for molecular analyses included a total of 185 specimens (Table 1).

DNA sequencing and analysis of sequences

The extraction and amplification protocol was that used by Streito et al. (2018): extraction of the total genomic DNA was carried out in a non-destructive manner, on whole specimens, using the DNeasy 96 Blood & Tissue extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The standard barcode fragment (Hebert et al., 2003a) was amplified using a mixture of tailed primers (based on Cruaud et al., 2010; Germain et al., 2013 and Ivanova et al., 2007) (Table 2). PCRs were carried out in 25 µl of reagents with 2 µl of matrix DNA, 0.07 mM of each primer, 2.5 mM of $MgCl_2$, 0.05 mM of dNTPs and 0.025 U/µl Dreamtaq DNA Polymerase (Thermo Scientific, Waltham, USA). The PCR conditions used were 94°C for 3 min followed by 5 cycles at 94°C for 30 s, 45°C for 40 s, 72°C for 60 s, then 35 cycles at 94°C for 30 s, 51°C for 40 s, 72°C for 60 s, with a final extension phase at 72°C for 10 min. PCR products were purified, then sequenced directly by Eurofins MWG Operon according to their protocol using M13 sequencing primers (M13F and M13R). Forward and reverse overlapping strands were assembled

Table 1. List of sequenced specimens and the sequence accession numbers available in Bold and GenBank. For presentation purposes, COI sequences of specimens highlighted in grey were not included in the phylogenetic trees (Figs 3 and S1).

Species	Specimens	Phylogenetic tree codes	Life stage (sex)	Country	Locality	Geographic coordinates Lon./Lat., decimal degr.	Sampling date	BOLD ID	GenBank Access no.
Crioceris asparagi (L., 1758)	CCOC09462_0101	–	adult	France	La Chapelle-sur-Loire	47.238952/0.185079	15/07/2010	BCELB001-19	MT456383
	CCOC09463_0101	–	adult	France	La Chapelle-sur-Loire	47.238952/0.185079	15/07/2010	BCELB002-19	MT456384
	CCOC09464_0101	–	adult	France	La Chapelle-sur-Loire	47.238952/0.185079	15/07/2010	BCELB003-19	MT456382
	CCOC00752_0101	C007520101	adult	France	La Chapelle-sur-Loire	47.249645/0.210105	10/07/2005	BCELB004-19	MT456385
	CCOC02373_0101	C023730101	adult	France	Vernou-sur-Brenne	47.400450/0.863481	19/05/2007	BCELB005-19	MT456386
Crioceris bicruciata (Sahlberg, 1823)	JSTR00373_0101	R003730101	adult	Greece	Askinou (Crete)	35.291880/24.178090	25/05/2013	BCELB006-19	MT456388
	JSTR00373_0103	R003730103	adult	Greece	Askinou (Crete)	35.291880/24.178090	25/05/2013	BCELB007-19	MT456387
Crioceris duodecim-punctata (L., 1758)	CCOC00757_0101	–	adult	France	La Chapelle-sur-Loire	47.249645/0.210105	13/07/2005	BCELB008-19	MT456390
	CCOC02404_0101	–	adult	France	La Chapelle-sur-Loire	47.248332/0.194144	13/05/2007	BCELB009-19	MT456391
	JSTR01304_0101	–	adult	France	Villandry	47.340936/0.500805	18/06/2012	BCELB010-19	MT456392
	CCOC00555_0101	C005550101	adult	France	Montauroux	43.617652/6.807396	18/09/2005	BCELB011-19	MT456389
	CCOC01914_0101	C019140101	adult	France	Saint-Martin-en-Ré	46.198628/–1.344658	12/08/2007	BCELB012-19	MT456393
Crioceris paracenthesis (L., 1767)	JSTR01291_0102	–	adult	France	Saint-Raphaël	43.417100/6.859020	10/07/2005	BCELB013-19	MT456395
	JSTR01296_0101	–	adult	France	Sainte-Croix-de-Quintillargue	43.775419/3.907606	07/05/2005	BCELB014-19	MT456396
	JSTR01291_0101	R012910101	adult	France	Saint-Raphaël	43.417100/6.859020	10/07/2005	BCELB015-19	MT456394
	JSTR01294_0101	R012940101	adult	France	Mireval	43.531030/3.802800	30/04/2005	BCELB016-19	MT456397
Lilioceris lilii (Scopoli, 1763)	JSTR01305_0101	R013050101	adult	France	Montlouis-sur-Loire	47.370624/0.837422	26/04/2012	BCELB024-19	MT456406
	JSTR01305_0102	R013050102	adult	France	Montlouis-sur-Loire	47.370624/0.837422	26/04/2012	BCELB025-19	MT456407
Lema aenea (Lacordaire, 1845)	JSTR01223_0101	–	adult	France	Salazie (Reunion Island)	–21.052480/55.525760	29/03/2014	BCELB017-19	MT456399
	JSTR00629_0101	R006290101	adult	France	Saint-Benoît (Reunion Island)	–21.096460/55.653440	25/03/2014	BCELB018-19	MT456400
	JSTR00629_0102	R006290102	adult	France	Saint-Benoît (Reunion Island)	–21.096460/55.653440	25/03/2014	BCELB019-19	MT456401
Lema borboniae (Jolivet, 1979)	JSTR01222_0101	–	adult	France	Salazie (Reunion Island)	–21.052480/55.525760	29/03/2014	BCELB020-19	MT456402
	JSTR00629_1301	R006291301	adult	France	Saint-Benoît (Reunion Island)	–21.096460/55.653440	25/03/2014	BCELB021-19	MT456403
	JSTR00629_1302	R006291302	adult	France	Saint-Benoît (Reunion Island)	–21.096460/55.653440	25/03/2014	BCELB022-19	MT456404
Lema cyanella (L., 1758)	JSTR04552_0201	R045520201	adult (♀)	Spain	Villafranca de Ebro	41.540200/–0.565540	6/8/2017	BCELB023-19	MT456405
Oulema duftschmidi (Retdenbacher, 1874)	JDCH00002_0302	–	adult (♂)	France	Mirecourt	48.286220/6.106403	25/03/2014	BCELB026-19	MT456451
	JDCH00003_0302	–	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB027-19	MT456452
	JDCH00003_0303	–	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB028-19	MT456453
	JDCH00004_0302	–	adult (♂)	France	Houville-la-Branche	48.447991/1.625547	18/07/2014	BCELB029-19	MT456454
	JDCH00005_0302	–	adult (♂)	France	Treffendel	48.037544/–1.983165	06/06/2014	BCELB030-19	MT456455
	JDCH00006_0302	–	adult (♂)	France	Saint-Aubin-du-Désert	48.322195/–0.203475	15/05/2014	BCELB031-19	MT456456
	JDCH00009_0302	–	adult (♂)	France	Querrieu	49.945034/2.424406	25/07/2014	BCELB032-19	MT456457
	JDCH00010_0302	–	adult (♂)	France	Thiverval-Grignon	48.846643/1.951940	01/06/2014	BCELB033-19	MT456458
	JDCH00015_0302	–	adult (♂)	France	Orléans	-	15/06/2013	BCELB034-19	MT456459
	JDCH00018_0302	–	adult (♂)	France	Tilloy-lès-Mofflaines	50.277301/2.806932	15/05/2014	BCELB035-19	MT456460
	JDCH00018_0303	–	adult (♂)	France	Tilloy-lès-Mofflaines	50.277301/2.806932	15/05/2014	BCELB036-19	MT456461
	JDCH00020_0302	–	adult (♂)	Portugal	Porto Santo Island (Madeira)	33.083000/–16.334400	04/04/2015	BCELB038-19	MT456463
	JDCH00021_0301	–	adult (♂)	Portugal	Porto Santo Island (Madeira)	33.083000/–16.334400	04/04/2015	BCELB039-19	MT456464
	JDCH00025_0302	–	adult (♂)	France	Montargis	47.995572/2.730073	12/07/2015	BCELB040-19	MT456465
	JDCH00026_0302	–	adult (♂)	Greece	Chania (Crete)	35.476613/23.933068	31/05/2016	BCELB041-19	MT456466
	JSTR01261_0101	–	adult (♂)	France	Neuville-Vitasse	50.255900/2.809390	15/05/2014	BCELB037-19	MT456462
	JSTR03883_0102	–	adult (♂)	Italy	Collemeto	40.203419/18.093920	16/11/2016	BCELB042-19	MT456467
	JDCH00002_0301	H000020301	adult (♂)	France	Mirecourt	48.286220/6.106403	25/03/2014	BCELB043-19	MT456468
	JDCH00003_0301	H000030301	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB044-19	MT456469
	JDCH00004_0301	H000040301	adult (♂)	France	Houville-la-Branche	48.447991/1.625547	18/07/2014	BCELB045-19	MT456470
	JDCH00005_0301	H000050301	adult (♂)	France	Treffendel	48.037544/–1.983165	06/06/2014	BCELB046-19	MT456471
	JDCH00006_0301	H000060301	adult (♂)	France	Saint-Aubin-du-Désert	48.322195/–0.203475	15/05/2014	BCELB047-19	MT456472
	JDCH00007_0301	H000070301	adult (♂)	France	Cossé-le-Vivien	47.947795/–0.921883	15/05/2014	BCELB048-19	MT456473
	JDCH00008_0301	H000080301	adult (♂)	France	Beugnâtre	50.129035/2.874760	14/05/2014	BCELB049-19	MT456474
	JDCH00009_0301	H000090301	adult (♂)	France	Querrieu	49.945034/2.424406	25/07/2014	BCELB050-19	MT456475
	JDCH00010_0301	H000100301	adult (♂)	France	Thiverval-Grignon	48.846643/1.951940	01/06/2014	BCELB051-19	MT456476
	JDCH00012_0301	H000120301	adult (♂)	France	Bruxerolles	46.611001/0.386810	18/05/2014	BCELB052-19	MT456477
	JDCH00013_0301	H000130301	adult (♂)	France	Mouzeuil-Saint-Martin	46.465235/–0.983798	15/06/2014	BCELB053-19	MT456478
	JDCH00014_0301	H000140301	adult (♂)	France	Saint-Priest	45.687722/4.967944	30/06/2014	BCELB054-19	MT456479
	JDCH00015_0301	H000150301	adult (♂)	France	Orléans	–	15/06/2013	BCELB055-19	MT456432
	JDCH00016_0301	H000160301	adult (♂)	France	Parcieux	45.917306/4.835083	07/07/2014	BCELB056-19	MT456433
	JDCH00017_0302	H000170302	adult (♂)	France	Plaisir	48.793955/1.942767	03/05/2014	BCELB057-19	MT456434
	JDCH00018_0301	H000180301	adult (♂)	France	Tilloy-lès-Mofflaines	50.277301/2.806932	15/05/2014	BCELB058-19	MT456435
	JDCH00019_0301	H000190301	adult (♂)	France	Neuville-Vitasse	50.255900/2.809400	23/07/2014	BCELB059-19	MT456436
	JDCH00020_0301	H000200301	adult (♂)	Portugal	Porto Santo Island (Madeira)	33.083000/–16.334400	04/04/2015	BCELB064-19	MT456441
	JDCH00021_0302	H000210302	adult (♂)	Portugal	Porto Santo Island (Madeira)	33.083000/–16.334400	04/04/2015	BCELB065-19	MT456450
	JDCH00022_0301	H000220301	adult (♂)	France	Lectoure	43.933604/0.623621	12/05/2015	BCELB066-19	MT456449
	JDCH00024_0301	H000240301	adult (♂)	France	Ladon	48.004543/2.533953	05/07/2015	BCELB067-19	MT456448
	JDCH00025_0301	H000250301	adult (♂)	France	Montargis	47.995572/2.730073	12/07/2015	BCELB068-19	MT456447
	JDCH00026_0301	H000260301	adult (♂)	Greece	Chania (Crete)	35.476613/23.933068	31/05/2016	BCELB069-19	MT456446
	JDCH00026_0303	H000260303	adult (♂)	Greece	Chania (Crete)	35.476613/23.933068	31/05/2016	BCELB070-19	MT456445
	JSTR00666_0102	R006660102	adult (♂)	France	Arles	43.510278/4.781944	27/04/2014	BCELB060-19	MT456437
	JSTR01258_0101	R012580101	adult (♂)	France	Lorgies	50.554260/2.793420	15/05/2014	BCELB061-19	MT456438
	JSTR01297_0102	R012970102	adult (♂)	France	Sainte-Croix-de-Quintillargue	43.775419/3.907606	23/04/2006	BCELB062-19	MT456439
	JSTR01302_0101	R013020101	adult (♂)	France	Assay	47.062201/0.270064	12/05/2006	BCELB063-19	MT456440
	JSTR03882_0101	R038820101	adult (♂)	Italy	Tricase	39.945573/18.352039	17/11/2016	BCELB071-19	MT456444
	JSTR03888_0101	R038880101	adult (♂)	Italy	Cascina Litta	40.091173/18.167204	16/11/2016	BCELB072-19	MT456443
	JSTR04562_0101	R045620101	adult (♂)	Spain	Aguillar-de-Segarra	41.738710/1.610790	6/9/2017	BCELB073-19	MT456442

Table 1 (continued).

Species	Specimens	Phylogenetic tree codes	Life stage (sex)	Country	Locality	Geographic coordinates Lon./Lat., decimal degr.	Sampling date	BOLD ID	GenBank Access. no.
Oulema melanopus (L., 1758)	JDCH00001_0202	–	adult (♂)	France	Berstett	48.682758/7.649488	07/07/2014	BCELB141-19	MT456518
	JDCH00002_0202	–	adult (♂)	France	Mirecourt	48.286220/6.106403	25/03/2014	BCELB142-19	MT456517
	JDCH00003_0202	–	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB143-19	MT456516
	JDCH00003_0203	–	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB144-19	MT456515
	JDCH00005_0202	–	adult (♂)	France	Treffendel	48.037544/–1.983165	06/06/2014	BCELB145-19	MT456514
	JDCH00006_0202	–	adult (♂)	France	Saint-Aubin-du-Désert	48.322195/–0.203475	15/05/2014	BCELB146-19	MT456513
	JDCH00007_0202	–	adult (♂)	France	Cossé-le-Vivien	47.947795/–0.921883	15/05/2014	BCELB147-19	MT456512
	JDCH00012_0202	–	adult (♂)	France	Bruxerolles	46.611001/0.386810	18/05/2014	BCELB148-19	MT456511
	JDCH00013_0202	–	adult (♂)	France	Mouzeuil-Saint-Martin	46.465235/–0.983798	15/06/2014	BCELB149-19	MT456510
	JDCH00014_0202	–	adult (♂)	France	Saint-Priest	45.687722/4.967944	30/06/2014	BCELB150-19	MT456509
	JDCH00015_0202	–	adult (♂)	France	Orléans	–	15/06/2013	BCELB151-19	MT456508
	JDCH00016_0202	–	adult (♂)	France	Parcieux	45.917306/4.835083	07/07/2014	BCELB152-19	MT456507
	JDCH00019_0202	–	adult (♂)	France	Neuville-Vitasse	50.255900/2.809400	23/07/2014	BCELB153-19	MT456506
	JDCH00019_0203	–	adult (♂)	France	Neuville-Vitasse	50.255900/2.809400	23/07/2014	BCELB154-19	MT456505
	JDCH00020_0202	–	adult (♂)	France	Verchain-Maugré	50.266552/3.474370	19/04/2015	BCELB156-19	MT456503
	JDCH00024_0202	–	adult (♂)	France	Ladon	48.004543/2.533953	05/07/2015	BCELB157-19	MT456502
	JDCH00025_0202	–	adult (♂)	France	Montargis	47.995572/2.730073	12/07/2015	BCELB158-19	MT456501
	JDCH00025_0203	–	adult (♂)	France	Montargis	47.995572/2.730073	12/07/2015	BCELB159-19	MT456520
	JSTR00657_0102	–	adult (♂)	France	Clapiers	43.650652/3.873110	16/04/2014	BCELB155-19	MT456504
	JDCH00001_0201	H000010201	adult (♂)	France	Berstett	48.682758/7.649488	07/07/2014	BCELB160-19	MT456499
	JDCH00002_0201	H000020201	adult (♂)	France	Mirecourt	48.286220/6.106403	25/03/2014	BCELB161-19	MT456500
	JDCH00003_0201	H000030201	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB162-19	MT456519
	JDCH00004_0201	H000040201	adult (♂)	France	Houville-la-Branche	48.447991/1.625547	18/07/2014	BCELB163-19	MT456521
	JDCH00005_0201	H000050201	adult (♂)	France	Treffendel	48.037544/–1.983165	06/06/2014	BCELB164-19	MT456522
	JDCH00006_0201	H000060201	adult (♂)	France	Saint-Aubin-du-Désert	48.322195/–0.203475	15/05/2014	BCELB165-19	MT456480
	JDCH00007_0201	H000070201	adult (♂)	France	Cossé-le-Vivien	47.947795/–0.921883	15/05/2014	BCELB166-19	MT456523
	JDCH00009_0201	H000090201	adult (♂)	France	Querrieu	49.945034/2.424406	25/07/2014	BCELB167-19	MT456481
	JDCH00012_0201	H000120201	adult (♂)	France	Bruxerolles	46.611001/0.386810	18/05/2014	BCELB168-19	MT456482
	JDCH00013_0201	H000130201	adult (♂)	France	Mouzeuil-Saint-Martin	46.465235/–0.983798	15/06/2014	BCELB169-19	MT456483
	JDCH00014_0201	H000140201	adult (♂)	France	Saint-Priest	45.687722/4.967944	30/06/2014	BCELB170-19	MT456484
	JDCH00015_0201	H000150201	adult (♂)	France	Orléans	–	15/06/2013	BCELB171-19	MT456485
	JDCH00016_0201	H000160201	adult (♂)	France	Parcieux	45.917306/4.835083	07/07/2014	BCELB172-19	MT456486
	JDCH00018_0201	H000180201	adult (♂)	France	Tilloy-lès-Mofflaines	50.277301/2.806932	15/05/2014	BCELB173-19	MT456487
	JDCH00019_0201	H000190201	adult (♂)	France	Neuville-Vitasse	50.255900/2.809400	23/07/2014	BCELB174-19	MT456488
	JDCH00020_0201	H000200201	adult (♂)	France	Verchain-Maugré	50.266552/3.474370	19/04/2015	BCELB179-19	MT456493
	JDCH00024_0201	H000240201	adult (♂)	France	Ladon	48.004543/2.533953	05/07/2015	BCELB180-19	MT456494
	JDCH00025_0201	H000250201	adult (♂)	France	Montargis	47.995572/2.730073	12/07/2015	BCELB181-19	MT456495
	JSTR00656_0101	R006560101	adult (♂)	France	Clapiers	43.658835/3.867831	16/04/2014	BCELB175-19	MT456489
	JSTR00666_0101	R006660101	adult (♂)	France	Arles	43.510278/4.781944	27/04/2014	BCELB176-19	MT456490
	JSTR00769_0101	R007690101	adult (♂)	France	Le-Bouchet-Saint-Nicolas	44.900330/3.748820	08/08/2014	BCELB177-19	MT456491
	JSTR01303_0101	R013030101	adult (♂)	France	La-Roche-Clermault	47.139333/0.228444	08/07/2005	BCELB178-19	MT456492
	JSTR04448_0101	R044480101	adult (♂)	Spain	Maçanet de la Selva	41.762700/2.758420	5/28/2017	BCELB182-19	MT456496
	JSTR04454_0101	R044540101	adult (♂)	Spain	Tordera	41.712840/2.686380	5/28/2017	BCELB183-19	MT456497
	JSTR04552_0101	R045520101	adult (♂)	Spain	Villafranca de Ebro	41.540200/–0.565540	6/8/2017	BCELB184-19	MT456498
Oulema obscura (Stephens, 1831)	JDCH00001_0102	–	adult (♂)	France	Berstett	48.682758/7.649488	07/07/2014	BCELB074-19	MT456542
	JDCH00001_0103	–	adult (♂)	France	Berstett	48.682758/7.649488	07/07/2014	BCELB075-19	MT456543
	JDCH00002_0102	–	adult (♂)	France	Mirecourt	48.286220/6.106403	25/03/2014	BCELB076-19	MT456544
	JDCH00002_0103	–	adult (♂)	France	Mirecourt	48.286220/6.106403	25/03/2014	BCELB077-19	MT456545
	JDCH00003_0102	–	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB078-19	MT456536
	JDCH00003_0103	–	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB079-19	MT456535
	JDCH00004_0102	–	adult (♂)	France	Houville-la-Branche	48.447991/1.625547	18/07/2014	BCELB080-19	MT456534
	JDCH00005_0102	–	adult (♂)	France	Treffendel	48.037544/–1.983165	06/06/2014	BCELB081-19	MT456533
	JDCH00005_0103	–	adult (♂)	France	Treffendel	48.037544/–1.983165	06/06/2014	BCELB082-19	MT456546
	JDCH00006_0102	–	adult (♂)	France	Saint-Aubin-du-Désert	48.322195/–0.203475	15/05/2014	BCELB083-19	MT456547
	JDCH00007_0102	–	adult (♂)	France	Cossé-le-Vivien	47.947795/–0.921883	15/05/2014	BCELB084-19	MT456548
	JDCH00009_0102	–	adult (♂)	France	Querrieu	49.945034/2.424406	25/07/2014	BCELB085-19	MT456549
	JDCH00009_0103	–	adult (♂)	France	Querrieu	49.945034/2.424406	25/07/2014	BCELB086-19	MT456550
	JDCH00010_0102	–	adult (♂)	France	Thiverval-Grignon	48.846643/1.951940	01/06/2014	BCELB087-19	MT456551
	JDCH00010_0103	–	adult (♂)	France	Thiverval-Grignon	48.846643/1.951940	01/06/2014	BCELB088-19	MT456552
	JDCH00012_0102	–	adult (♀)	France	Bruxerolles	46.611001/0.386810	18/05/2014	BCELB089-19	MT456553
	JDCH00020_0102	–	adult (♀)	France	Verchain-Maugré	50.266552/3.474370	19/04/2015	BCELB095-19	MT456559
	JDCH00024_0102	–	adult (♀)	France	Ladon	48.004543/2.533953	05/05/2015	BCELB096-19	MT456560
	JSTR01259_0102	–	adult (♂)	France	Lorgies	50.554260/2.793420	15/05/2014	BCELB090-19	MT456554
	JSTR01260_0101	–	adult (♂)	France	Neuville-Vitasse	50.255900/2.809390	15/05/2014	BCELB091-19	MT456555
	JSTR01265_0101	–	adult (♀)	France	Tilloy-lès-Mofflaines	50.283450/2.813050	15/05/2014	BCELB092-19	MT456556
	JSTR01265_0102	–	adult (♂)	France	Tilloy-lès-Mofflaines	50.283450/2.813050	15/05/2014	BCELB093-19	MT456557
	JSTR01272_0102	–	adult (♂)	France	Saint-Laurent-de-Lin	47.511404/0.271791	22/07/2014	BCELB094-19	MT456558
	JDCH00001_0101	H000010101	adult (♂)	France	Berstett	48.682758/7.649488	07/07/2014	BCELB097-19	MT456561
	JDCH00002_0101	H000020101	adult (♂)	France	Mirecourt	48.286220/6.106403	25/03/2014	BCELB098-19	MT456562
	JDCH00003_0101	H000030101	adult (♂)	France	Gannat	46.100167/3.199103	24/04/2014	BCELB099-19	MT456563
	JDCH00004_0101	H000040101	adult (♂)	France	Houville-la-Branche	48.447991/1.625547	18/07/2014	BCELB100-19	MT456564
	JDCH00005_0101	H000050101	adult (♂)	France	Treffendel	48.037544/–1.983165	06/06/2014	BCELB101-19	MT456565
	JDCH00006_0101	H000060101	adult (♂)	France	Saint-Aubin-du-Désert	48.322195/–0.203475	15/05/2014	BCELB102-19	MT456566
	JDCH00007_0101	H000070101	adult (♂)	France	Cossé-le-Vivien	47.947795/–0.921883	15/05/2014	BCELB103-19	MT456524
	JDCH00008_0102	H000080102	adult (♂)	France	Beugnâtre	50.129035/2.874760	14/05/2014	BCELB104-19	MT456525

Table 1 (continued).

Species	Specimens	Phylogenetic tree codes	Life stage (sex)	Country	Locality	Geographic coordinates Lon./Lat., decimal degr.	Sampling date	BOLD ID	GenBank Access no.
	JDCH00009_0101	H000090101	adult (♂)	France	Querrieu	49.945034/2.424406	25/07/2014	BCELB105-19	MT456526
	JDCH00010_0101	H000100101	adult (♂)	France	Thiverval-Grignon	48.846643/1.951940	01/06/2014	BCELB106-19	MT456527
	JDCH00011_0101	H000110101	adult (♂)	France	Tilloy-lès-Mofflaines	50.277301/2.806932	15/05/2014	BCELB107-19	MT456528
	JDCH00012_0101	H000120101	adult (♀)	France	Bruxerolles	46.611001/0.386810	18/05/2014	BCELB108-19	MT456529
	JDCH00020_0101	H000200101	adult (♀)	France	Verchain-Maugré	50.266552/3.474370	19/04/2015	BCELB114-19	MT456539
	JDCH00024_0101	H000240101	adult (♂)	France	Ladon	48.004543/2.533953	05/05/2015	BCELB115-19	MT456540
	JDCH00025_0101	H000250101	adult (♂)	France	Montargis	47.995572/2.730073	12/07/2015	BCELB116-19	MT456541
	JSTR01259_0101	R012590101	adult (♂)	France	Lorgies	50.554260/2.793420	15/05/2014	BCELB109-19	MT456530
	JSTR01260_0102	R012600102	adult (♀)	France	Neuville-Vitasse	50.255900/2.809390	15/05/2014	BCELB110-19	MT456531
	JSTR01262_0102	R012620102	adult (♀)	France	Tilloy-lès-Mofflaines	50.277510/2.799190	15/05/2014	BCELB111-19	MT456532
	JSTR01272_0101	R012720101	adult (♂)	France	Saint-Laurent-de-Lin	47.511404/0.271791	22/07/2014	BCELB112-19	MT456537
	JSTR01299_0101	R012990101	adult (♀)	France	Chambray-lès-Tour	47.339980/0.722099	16/05/2006	BCELB113-19	MT456538
"*Oulema* group *melanopus*"	EPIE00642_0103	–	larva	France	Beyssenac	45.411704/1.324250	07/06/2008	BCELB122-19	MT456426
	EPIE00642_0104	–	larva	France	Beyssenac	45.411704/1.324250	07/06/2008	BCELB123-19	MT456425
	EPIE00642_0105	–	larva	France	Beyssenac	45.411704/1.324250	07/06/2008	BCELB124-19	MT456424
	EPIE00642_0106	–	larva	France	Beyssenac	45.411704/1.324250	07/06/2008	BCELB125-19	MT456423
	JSTR00656_0103	–	adult (♀)	France	Clapiers	43.658835/3.867831	16/04/2014	BCELB117-19	MT456431
	JSTR00657_0101	–	adult (♀)	France	Clapiers	43.650652/3.873110	16/04/2014	BCELB118-19	MT456430
	JSTR00657_0103	–	adult (♀)	France	Clapiers	43.650652/3.873110	16/04/2014	BCELB119-19	MT456429
	JSTR01263_0101	–	adult (♀)	France	Tilloy-lès-Mofflaines	50.277510/2.799190	15/05/2014	BCELB120-19	MT456428
	JSTR01264_0101	–	adult (♀)	France	Tilloy-lès-Mofflaines	50.283450/2.813050	15/05/2014	BCELB121-19	MT456427
	JSTR03884_0101	–	adult (♀)	Italy	Cascina Litta	40.091173/18.167204	16/11/2016	BCELB126-19	MT456422
	CCOC02368_0101	C023680101	larva	France	Mer	47.721926/1.500711	15/05/2007	BCELB127-19	MT456421
	EPIE00642_0101	E006420101	larva	France	Beyssenac	45.411704/1.324250	07/06/2008	BCELB136-19	MT456412
	EPIE00642_0102	E006420102	larva	France	Beyssenac	45.411704/1.324250	07/06/2008	BCELB137-19	MT456411
	JSTR00656_0102	R006560102	adult (♀)	France	Clapiers	43.658835/3.867831	16/04/2014	BCELB129-19	MT456419
	JSTR00666_0103	R006660103	adult (♀)	France	Arles	43.510278/4.781944	27/04/2014	BCELB130-19	MT456418
	JSTR00669_0801	R006690801	adult (♀)	France	Combaillaux	43.666567/3.785286	18/05/2014	BCELB128-19	MT456420
	JSTR01258_0102	R012580102	adult (♀)	France	Lorgies	50.554260/2.793420	15/05/2014	BCELB131-19	MT456417
	JSTR01261_0102	R012610102	adult (♀)	France	Neuville-Vitasse	50.255900/2.809390	15/05/2014	BCELB132-19	MT456416
	JSTR01264_0102	R012640102	adult (♀)	France	Tilloy-lès-Mofflaines	50.283450/2.813050	15/05/2014	BCELB133-19	MT456415
	JSTR01297_0101	R012970101	adult (♀)	France	Sainte-Croix-de-Quintillargue	43.775419/3.907606	23/04/2006	BCELB134-19	MT456414
	JSTR01301_0101	R013010101	adult (♀)	france	Preuilly-sur-Claise	46.854377/0.928917	17/05/2006	BCELB135-19	MT456413
	JSTR03883_0101	R038830101	adult (♀)	Italy	Collemeto	40.203419/18.093920	16/11/2016	BCELB138-19	MT456410
	JSTR03885_0101	R038850101	adult (♀)	Italy	Galugnano	40.249590/18.229184	16/11/2016	BCELB139-19	MT456408
	JSTR03886_0101	R038860101	adult (♀)	Italy	Collemeto	40.203419/18.093920	11/16/2016	BCELB140-19	MT456409
Epitrix pubescens (Koch, 1803)	CCOC11818_0101	C118180101	adult	France	La Ville-aux-Dames	47.394035/0.770874	6/4/2011	BCELB185-19	MT456398

using Geneious v4.6.2 (Drummond et al., 2010). Sequences were aligned with default ClustalW parameters (1.81) (Thompson et al., 1997). Consensus sequences were translated to amino acids using MEGA 7 software (Kumar et al., 2016) to detect frameshift mutations and premature stop-codons, which may have indicated the presence of pseudogenes or contaminations. Voucher specimens and associated DNA are preserved in the INRAE collections of the CBGP (Centre de Biologie pour la Gestion des Populations, Montferrier-sur-Lez, France). All sequences were deposited in Barcode of Life Data System (BOLD; Ratnasingham & Hebert, 2007; www.boldsystems.org) and GenBank (NCBI). Our sequence dataset is available in BOLD (through the DOI dx.doi.org/10.5883/DS-BCOUL) and NCBI, accession numbers are provided in Table 1.

Pairwise nucleotide sequence divergences were calculated using the Kimura 2-parameter model of substitution (Kimura, 1980) in MEGA 7 software (Kumar et al., 2016) and the "pairwise-deletion" option. A preliminary phylogenetic tree of the genus *Oulema*, including all the specimens sequenced, was reconstructed based on the *COI* sequences. For a clearer view of the inferred tree, only the sequences of one or two specimens per locality are presented in Fig. 3 (specimens not highlighted in Table 1). We reconstructed a tree (Fig. 3) including the standard barcode *COI* solely for specimens reliably identified to species (only dissected males for the *melanopus/duftschmidi* complex) and the sequences published by Bezděk & Baselga (2015). Phylogenetic analyses were conducted using the Maximum Likelihood (ML) method and the SMS model (Smart Model Selection) (Lefort et al., 2017) in Phyml 3.0 software (Guindon et al., 2010). The aLRT approximation (approximate likelihood ratio test) was used to calculate the bootstrapping values for each node (Anisimova, 2006) (5000 replicates). The resulting phylogenetic trees were edited using iTOL software (Letunic & Bork, 2016).

RESULTS

Analysis of newly obtained sequences

The barcode fragment was successfully amplified for all specimens, regardless of sex or stage. In total, 185 specimens belonging to 12 species were sequenced (Table 1). The observed minimum interspecific genetic distance between species of Chrysomelidae in the neighbouring genera of *Oulema* based on *COI* ranged from 10.2 to 19.3% (Table 3). In the genus *Oulema*, *O. obscura* showed a minimum divergence of 18.5% with *Oulema melanopus* and *Oulema duftschmidi*. *O. melanopus* showed a minimum divergence of 3.1% with *O. duftschmidi*, its closest relative. Between genera of the Criocerinae, the minimum divergence varied from 16.5% between *Lema* and *Oulema* to 21.8% between *Crioceris* and *Oulema*. *Epitrix* had a minimum distance from the Criocerinae species tested ranging from 22.2 to 26.2%.

The intraspecific divergence levels ranged from 0 to 0.9% (mean 0.19% ± 0.06%) for *O. melanopus*, from 0 to

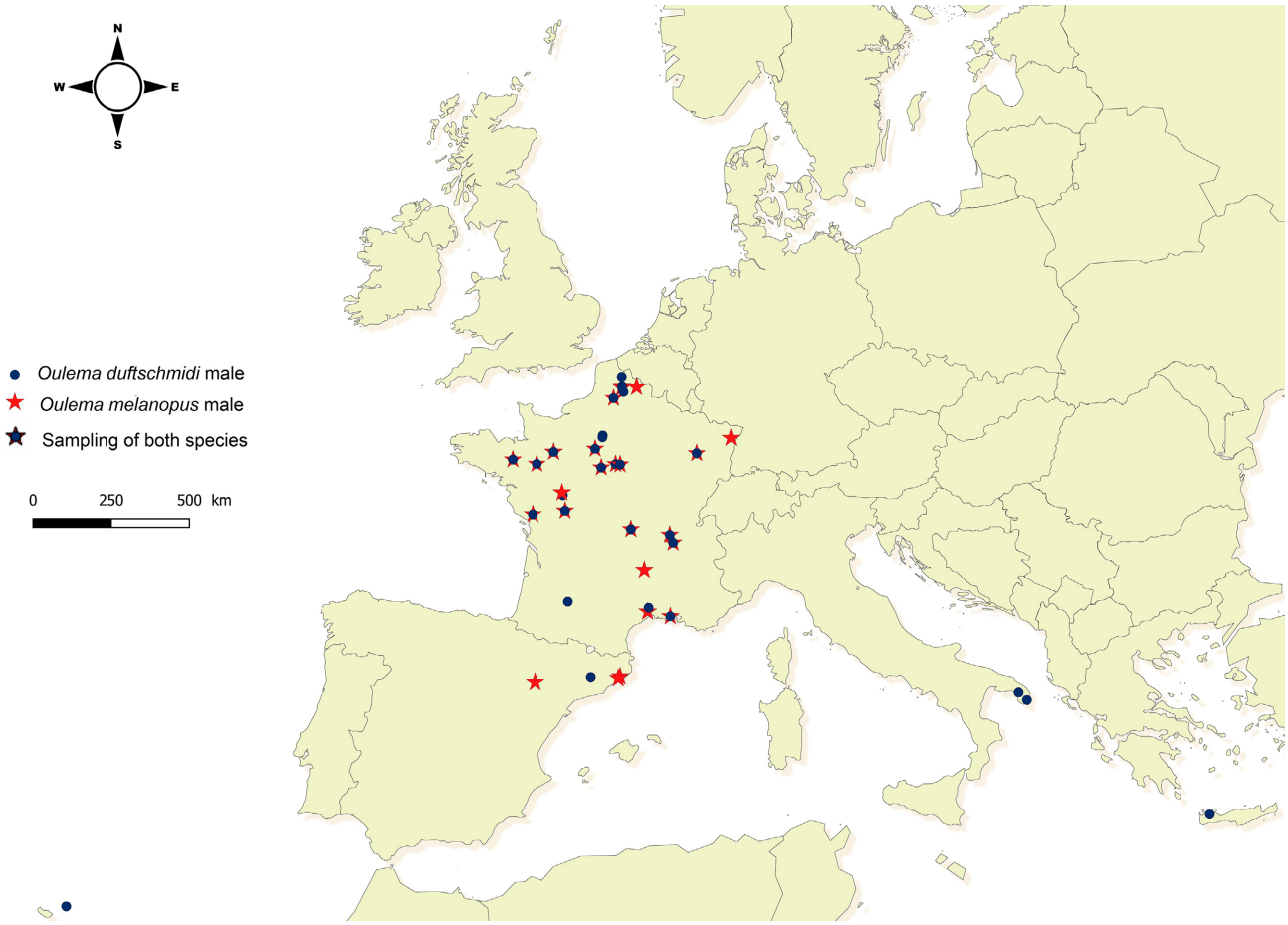

Fig. 1. Map showing the locations of the sites where males of *Oulema melanopus* (44 specimens) and *O. duftschmidi* (48 specimens) were collected. J. Leroy Design [QGIS Software version 2.18.12 (QGIS, 2016), Mapping Holdings Association: UE (Burke, 2012) and GREAT (UMS 2414 RIATE, 2018)].

Table 2. Mixture of PCR primers used in this study (based on Cruaud et al., 2010 and Germain et al., 2013). M13 tails (Ivanova et al., 2007) were used.

Name of the primer	Sequence 5'–3' of the primer
Forward	
LCO1490puc_t1	TGTAAAACGACGGCCAGTTTTCAACWAATCATAAAGATATTGG
LCO1490Hem1_t1	TGTAAAACGACGGCCAGTTTTCAACTAAYCATAARGATATYGG
Reverse	
HCO2198puc_t1	CAGGAAACAGCTATGACTAAACTTCWGGRTGWCCAAARAATCA
HCO2198Hem2_t1	CAGGAAACAGCTATGACTAAACYTCAGGATGACCAAAAAAYCA
HCO2198Hem1_t1	CAGGAAACAGCTATGACTAAACYTCDGGATGBCCAAARAATCA

1.7% (mean 0.57% ± 0.14%) for *O. duftschmidi* and from 0 to 0.5% (mean 0.09% ± 0.05%) for *O. obscura* (Table 4).

Phylogenetic reconstruction

The substitution models selected by PhyML 3.0 as the most appropriate were the GTR + G + I model for the tree in Fig. 3 (AIC = 10403,93488). That tree is based on both our sequences and those published by Bezděk & Baselga (2015). The genus *Oulema* is monophyletic (support 83.26%) with two sister clades. One (support 91.40%) contains *O. obscura* and *O. hoffmannseggii* from Spain (as in the phylogenetic tree of Bezděk & Baselga, 2015, in which, however, the latter species is paraphyletic); *O. obscura* contains two sister groups, one comprising specimens from the Iberian Peninsula and the other from France and the Czech Republic. The other clade containing the *O. melanopus* complex (including the 5 species recognized by Bezděk & Baselga, 2015) is highly supported (99.42%), but its internal relationships are problematic. *O. duftschmidi* forms a well-supported clade* (91.36%) which, however,

* Among the dissected males, only one mismatched specimen (JSTR02905_0101 from Alentejo, Portugal, morphologically clearly belonging to *O. duftschmidi*) was nested with specimens of *O. melanopus*. This could be a labelling error or contamination (see Discussion); therefore the specimen was removed from the final analyses, from the trees in Figs 3 and S1 and consequently from sequences deposited in Bold (Table 1).

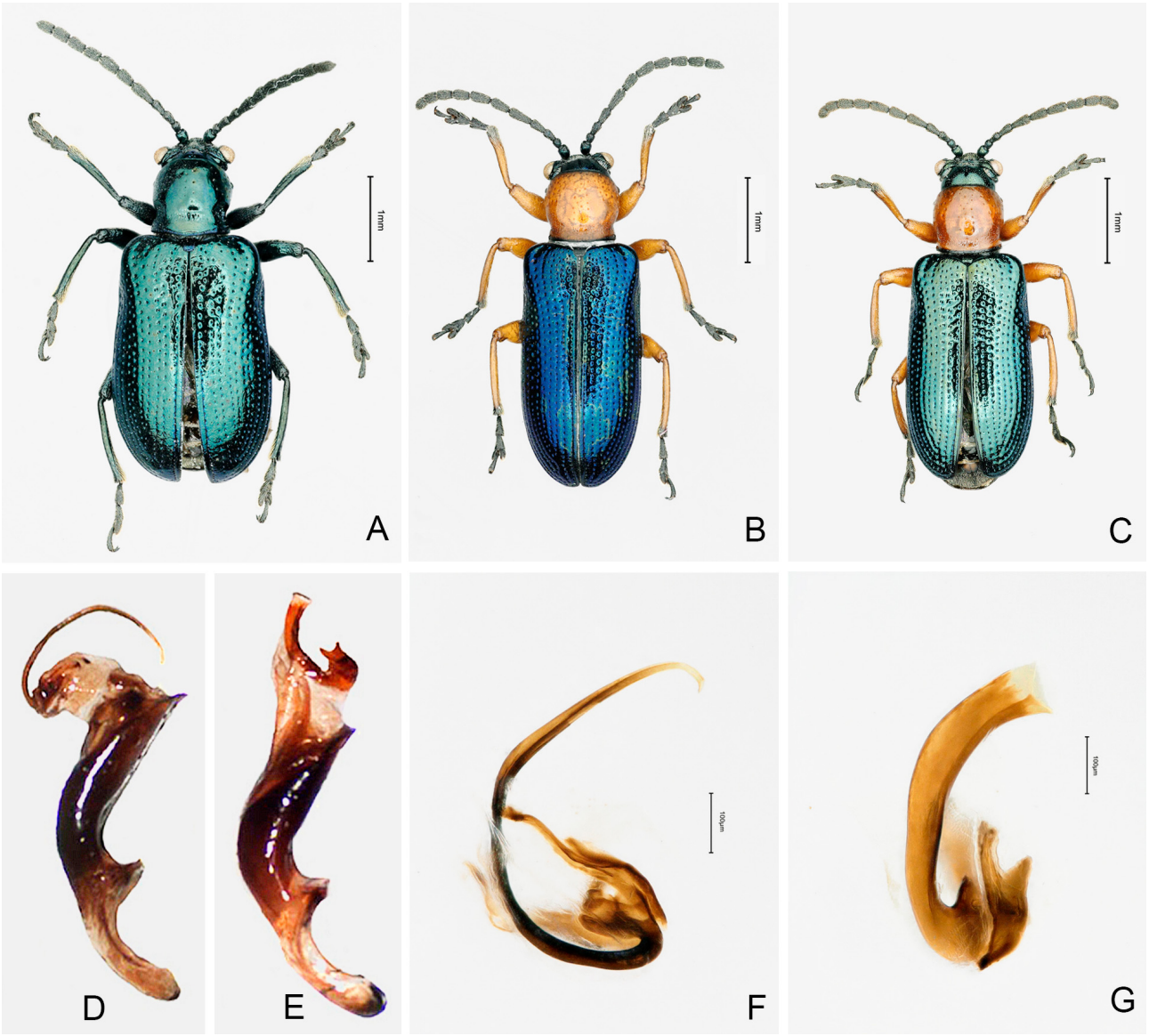

Fig. 2. Habitus and male genitalia of *Oulema* spp. A – *O. obscura* (specimen JSTR1259_0101). B – *O. duftschmidi* (specimen JSTR0666_0102). C – *O. melanopus* (specimen JSTR00769_0101). D – *O. duftschmidi* male aedeagus in lateral view with flagellum extracted. E – *O. melanopus*, idem. F – *O. duftschmidi* flagellum (specimen JSTR01302_0101). G – *O. melanopus,* idem (specimen CCOC11910_010, not sequenced). Photograhs: J.-C. Streito, except D and E, which are from Bukejs & Ferenca, 2010.

includes also the single available sequence of *O. mauroi* and one specimen from Morocco identified as *O. melanopus* (again similar to the tree in Bezděk & Baselga, 2015), whereas the other specimens (*O. rufocyanea*, *O. verae* and all remaining *O. melanopus*) form a markedly paraphyletic cluster and the latter two species (represented by more than one specimen) are both polyphyletic. In particular, five specimens from Galicia (NW Spain) identified as *O. melanopus* stand out and form a sister clade to *O. rufocyanea* (as in Bezděk & Baselga, 2015).

If we exclude the sequences of Bezděk & Baselga (2015), the resulting tree (not shown because it was essentially similar to the present Fig. S1) is congruent with morphological identifications and all species are monophyletic. The three cereal pest species had support of 100% for *O. obscura*, 65.94% for *O. melanopus* and 95.72% for *O. duftschmidi*.

When we included the unidentified specimens of the *O. melanopus* complex from France and Italy (see Table 1) in the above analysis, they were clearly placed in one or the other species (Fig. S1).

DISCUSSION

During this study, we sequenced 184 specimens of 11 species of Criocerinae in four genera (*Crioceris*, *Lema*, *Lilioceris* and *Oulema*) with a view to testing the possibility of routinely using the standard barcode *COI* for high-throughput and reliable identification of the three species of *Oulema* of agronomic interest, *O. obscura*, *O. melanopus* and *O. duftschmidi*. We included European specimens (*O. duftschmidi* was also available from Madeira) of 9 out of 28 species and subspecies of Criocerinae known to occur in Europe, and added specimens of 2 more *Lema* species from the Mascarenes. Combining our sequences with

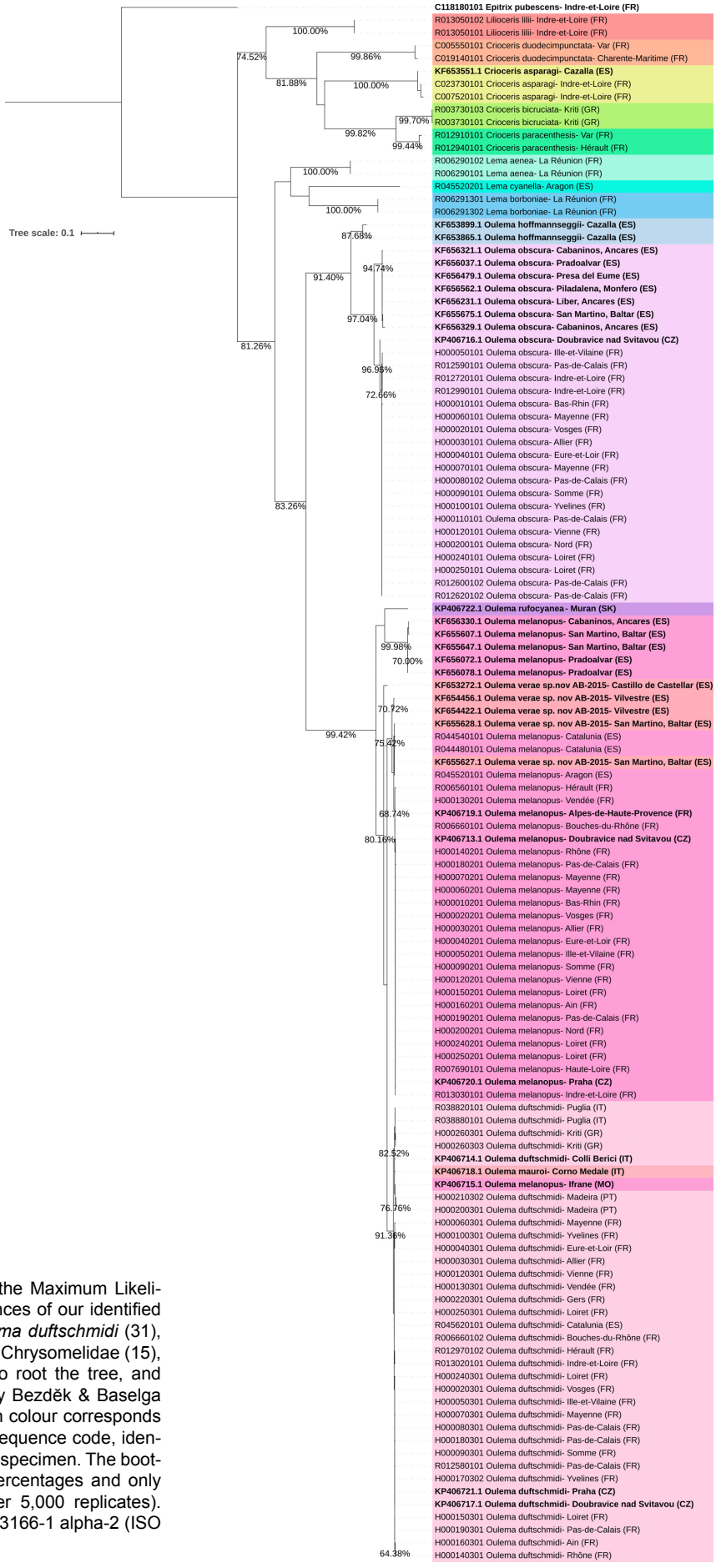

Fig. 3. Phylogenetic tree constructed using the Maximum Likelihood method (ML) and the COI gene sequences of our identified specimens of *Oulema melanopus* (25), *Oulema duftschmidi* (31), *Oulema obscura* (20) and 10 other species of Chrysomelidae (15), including *Epitrix pubescens* (in bold) used to root the tree, and the 30 sequences (also in bold) published by Bezděk & Baselga (2015), giving a total of 122 sequences. Each colour corresponds to a morphologically identified species. The sequence code, identity and geographical origin are given for each specimen. The bootstrap values located at branch nodes are percentages and only those greater than 64% are presented (over 5,000 replicates). The country name is coded according to ISO 3166-1 alpha-2 (ISO 3166, 2016).

Table 3. Kimura two-parameter pairwise distance values between species (interspecific divergence). In each box the first line gives the average value and the second the minimum value. The estimated standard errors (SE) are indicated in red above the diagonal. The blue boxes show the values of distances between genera and the yellow boxes the values within the genus. 161 sequences of *O. duftschmidi* (48) and *O. melanopus* (44) males and other species of Chrysomelidae (69) were included in this analysis. Sequences from Bezděk & Baselga (2015) are not included.

		A	B	C	D	E	F	G	H	I	J	K	L
A	*Lilioceris lilii*		0.020	0.019	0.018	0.021	0.019	0.019	0.020	0.020	0.020	0.021	0.020
B	*Crioceris asparagi*	0.218 [0,217]		0.018	0.018	0.017	0.019	0.020	0.019	0.021	0.021	0.021	0.023
C	*Crioceris bicruciata*	0.202 [0,202]	0.180 [0,176]		0.013	0.018	0.020	0.020	0.020	0.021	0.019	0.020	0.022
D	*Crioceris paracenthesis*	0.189 [0,186]	0.191 [0,186]	0.104 [0,102]		0.019	0.022	0.021	0.020	0.021	0.019	0.020	0.021
E	*Crioceris duodecimpunctata*	0.237 [0,224]	0.184 [0,179]	0.209 [0,184]	0.207 [0,193]		0.019	0.020	0.021	0.020	0.020	0.021	0.021
F	*Lema aenea*	0.204 [0,204]	0.197 [0,193]	0.218 [0,217]	0.242 [0,238]	0.204 [0,198]		0.015	0.018	0.017	0.018	0.018	0.021
G	*Lema borboniae*	0.208 [0,208]	0.226 [0,223]	0.219 [0,219]	0.230 [0,226]	0.229 [0,221]	0.136 [0,136]		0.017	0.018	0.019	0.019	0.020
H	*Lema cyanella*	0.214 [0,214]	0.211 [0,205]	0.231 [0,230]	0.227 [0,223]	0.228 [0,222]	0.169 [0,169]	0.159 [0,159]		0.019	0.020	0.021	0.020
I	*Oulema obscura*	0.206 [0,204]	0.240 [0,232]	0.242 [0,240]	0.229 [0,226]	0.222 [0,214]	0.165 [0,161]	0.166 [0,165]	0.190 [0,188]		0.020	0.019	0.022
J	*Oulema melanopus*	0.214 [0,208]	0.225 [0,217]	0.220 [0,215]	0.218 [0,211]	0.223 [0,212]	0.175 [0,170]	0.190 [0,186]	0.197 [0,192]	0.193 [0,185]		0.007	0.022
K	*Oulema duftschmidi*	0.222 [0,218]	0.232 [0,224]	0.226 [0,220]	0.235 [0,230]	0.237 [0,229]	0.176 [0,171]	0.191 [0,186]	0.206 [0,198]	0.192 [0,185]	0.037 [0,031]		0.021
L	*Epitrix pubescens* (Galerucinae) – outgroup	0.235 [0,235]	0.263 [0,262]	0.260 [0,260]	0.252 [0,250]	0.240 [0,206]	0.222 [0,222]	0.234 [0,234]	0.224 [0,224]	0.245 [0,243]	0.233 [0,230]	0.234 [0,228]	

those of Bezděk & Baselga (2015), 7 European species of *Oulema* (from the 11 known in Europe) were documented. Our results confirm those of Kubisz et al. (2012) and show that the standard DNA barcode can reliably differentiate between most European species of Criocerinae, but confirm also the results of Bezděk & Baselga (2015) that this marker cannot differentiate between all species of the genus *Oulema*. A complex of species occurs in the Mediterranean basin including at least *O. melanopus*, *O. verae*, *O. duftschmidi*, *O. mauroi* and possibly some undescribed cryptic species, which cannot be reliably distinguished by the standard barcode. In this study, sampling and marker selection were used to address agronomic questions. The lack of material coming from non-cultivated Mediterranean ecosystems prevented us addressing the problem of the species occurring around the Mediterranean basin where much more extensive sampling and the use of other markers and other methods would be needed to clarify the taxonomy of *Oulema*.

Table 4. Kimura two-parameter pairwise average distance values within the species studied (intraspecific divergence) (d – average; max – maximum). The estimated standard errors (SE) are given. 161 sequences of *O. duftschmidi* (48) and *O. melanopus* (44) males and other species of Chrysomelidae (69) were included in this analysis. Distances could not be estimated for species with only one individual (*Lema cyanella* and *Epitrix pubescens*). Sequences from Bezděk & Baselga (2015) are not included.

	d	SE	max
Oulema melanopus	0.0019	0.0006	0.009
Oulema duftschmidi	0.0057	0.0014	0.017
Oulema obscura	0.0009	0.0005	0.005
Crioceris duodecimpunctata	0.0183	0.0042	0.035
Crioceris asparagi	0.0037	0.0015	0.009
Crioceris bicruciata	0.0015	0.0015	0.002
Crioceris paracenthesis	0.0038	0.0016	0.008
Lema aenea	0	0	n/c
Lema borboniae	0	0	0
Lilioceris lilii	0	0	0

Distinguishing *Oulema obscura* from the *O. melanopus* complex

While adult specimens of *O. obscura* can be easily distinguished from those of the *melanopus* group on the basis of their general coloration (body entirely blue versus red pronotum and legs, respectively) (Fig. 2a versus 2b, c), the use of DNA barcodes for species identification can also be used to reliably identify the immature stages of these species. The sequence of the COI gene tested makes it possible to distinguish this species from the entire *melanopus* complex which includes the other two *Oulema* cereal pests (*O. melanopus* and *O. duftschmidi*). The minimum divergence of 18.5% between *O. obscura* and *O. melanopus*/*O. duftschmidi* is rather high and comparable to distances recorded between species in different genera in the same subfamily, such as *Crioceris* and *Lema* (Table 3).

Distinguishing between *O. melanopus* and *O. duftschmidi*

The minimum interspecific divergence of 3.1% between specimens of *O. melanopus* and *O. duftschmidi* (Table 3) means that these sibling species are more closely related than all the other species studied, which is consistent with their similar morphology and biology. In addition, such a value is congruent with what is reported for other sibling species in the family Chrysomelidae (Cognato, 2006). However, we recorded lower genetic distances between specimens of *O. duftschmidi* from very distant populations (North of France and the Italian province of Puglia) or isolated populations (such as those in Madeira and Crete), than between specimens of *O. melanopus* and *O.*

duftschmidi from France, despite being collected together, in the same place and at the same time (Tables 3 and 4).

Lastly, the interspecific percentage divergences (Table 3) were well above the maximum percentages of intraspecific divergence recorded (Table 4) and there was no overlap between the intra- and interspecific distances of *O. melanopus* and *O. duftschmidi*. Consequently, this argues in favour of a clear genetic differentiation of the sibling species *O. duftschmidi* and *O. melanopus*, which is supported by the phylogenetic trees (Figs 3 and S1). Of the 92 males studied, only one (JSTR02905_0101, a male specimen of *O. duftschmidi* from Alentejo, Portugal), was placed in a cluster that does not correspond with the species identification based on the morphology of its genitalia. A posteriori examination of the preserved adult and its dissected genitalia definitively excluded misidentification. However, we cannot exclude an error in tube labelling during handling or contamination. Wherever possible, we deliberately selected specimens of the two species for our dataset that were collected on the same day at the same location (see Table 1), to maximize the chances of recording potential hybridisation. Apart from this specimen, for which it was not possible to rule out a handling error, no other individual was incorrectly assigned in our data set. A second case of a mismatch between molecular and morphological identification was that of the Moroccan specimen (PK406715.1), which was genetically assigned to *O. duftschmidi*, whereas it was identified as *O. melanopus* by Bezděk & Baselga (2015). These authors (pers. com.) suspected that there was an undescribed cryptic species in Morocco to which this specimen belonged. Indeed, they noted differences between the genitalia of this specimen and typical specimens of *O. melanopus* with which it was tentatively identified. The method we used (extraction, amplification and sequencing of a gene) enabled correct assignment of males previously identified on the basis of dissected genitalia. The unidentified females and larvae that we tested were also unambiguously assigned to one of the two taxonomic groups. It would be interesting to test the method on a larger number of specimens in order to check whether introgression has occurred and assess its percentage of occurrence. Breeding tests would also be required to test this hypothesis.

Distinguishing other species in the *melanopus* complex and their intraspecific differences

The intraspecific diversity of specimens from the Mediterranean basin, especially those from the Iberian Peninsula, was greater than that of the French and Czech specimens (Fig. 3). This increase in genetic diversity with increase in the geographical coverage is documented (Bergsten et al., 2012) and due to the presence of Mediterranean glacial refugia and their associated biological diversity (Hewitt, 2001). Currently the data from the Mediterranean areas is limited and more extensive sampling could provide additional insights into the biological or biogeographical processes that resulted in the present diversity.

The other issue is distinguishing between the cereal pests and the rarer or more localized species that are described in the genus *Oulema.* Phylogenetic relationships between the five species of the *melanopus* group have been studied by Bezděk & Baselga (2015) based on the *COI* gene. They conclude that the relationships between the different species in this group were not well resolved on the basis of this gene. The results we obtained by combining their work with ours are slightly more optimistic at least in the possibility of distinguishing *O. duftschmidi* from any European specimens currently being morphologically identified as *O. melanopus* and support the idea that these two groups are genetically well separated. In Fig. 3, *O. mauroi* and the Moroccan specimen KF406717.1 presently identified as *O. melanopus* are nested within *O. duftschmidi*, and the sequences of *O. verae* are intermixed with the remaining specimens identified as *O. melanopus*. We did not undertake a morphological study of *O. mauroi* and *O. verae*, which are rare in collections, but according to Bezděk & Baselga (2015), the morphological differences between *O. verae*/*O. melanopus* and *O. mauroi*/*O. duftschmidi* are much more marked than the differences between *O. melanopus* and *O. duftschmidi*. *O. rufocyanea,* also a member of the *O. melanopus* complex, is clustered with a subgroup of Spanish specimens from Galicia (Bold BIN ACJ0414) morphologically identified as *O. melanopus*. The population from Morocco remains to be studied. It is possible that the standard *COI* barcode is not suitable for discriminating between species in the Mediterranean area. The possibility of introgressions having occurred will have to be explored along with increased sampling and use of more relevant molecular markers, especially nuclear markers.

Other European species of *Oulema*

Oulema obscura and *O. hoffmannseggii* form a sister group to the *O. melanopus* complex (as in Bezděk & Baselga, 2015). A more comprehensive sampling of the under-represented species and the addition of the 4 remaining species (*O. erichsonii*, *O. septentrionis*, *O. tristis* and *O. magistrettiorum*) should further improve our understanding of this genus. The fact that the specimens of *O. melanopus* from Galicia differed from the others also indicates that there may be additional cryptic diversity in the genus *Oulema*, warranting further studies using an integrated approach.

Oulema sequences available in BOLD and reliability of the barcoding identification of European species

BOLD system (Ratnasingham & Hebert, 2007) currently (November 2020) contains 436 *Oulema* sequences forming 15 Barcode Index Numbers (BINs). Three BINs contain most of the sequences:

AAK5928: 178 sequences of which 155 are identified as *O. melanopus*, two as *O. erichsonii*, and the remaining sequences are not identified to species level. This BIN includes 44 sequences from the present study identified as *O. melanopus*.

AAO0694: 107 sequences of which 83 are identified as *O. duftschmidi*, one as *O. mauroi* and two as *O. melanopus*. This BIN includes 48 sequences from the present study, all identified as *O. duftschmidi*.

AAN1559: 77 sequences of which 76 are identified as *O. obscura* (or its synonym *O. gallaeciana*) and one is unidentified.

Surprisingly, *O. verae* is not included in BOLD while *O. mauroi* from the same study (Bezděk & Baselga, 2015) is included.

The remaining 12 BINs are represented by a limited number of sequences (one to ten). Several species names are associated with several BINs: *O. erichsonii* (four different BINs among which is AAK5928); *O. duftschmidi* (AAO0694 and ADK1309 for one Indian sequence); *O. obscura* under the name *O. gallaeciana* (AAN1559 and ABW1444 for the 7 sequences from Spain, Galicia, see Fig. 3); *O. hoffmannseggii* (ABV0207 and ADU7791 for two sequences from Spain); *O. melanopus* (AAK5928 and four other BINs for sequences from different European and non-European countries, of which ABW1460 contains among other the 5 sequences from Spain, Galicia, sister to *O. rufocyanea* in our Fig. 3). That sequence of *O. rufocyanea* (KP406722.1) is associated with a unique BIN (ACJ0414). 44 sequences were not identified to species and 46 were not associated to a BIN due to their poor quality, insufficient length, etc.

BOLD provides the state-of-the-art barcoding information of the genus *Oulema* and highlights the need of clarification of the taxonomy in this group. The association of one species with several BINs and conversely several species within the same BIN may not be only due to misidentification. We cannot exclude some cryptic species such as the 7 specimens identified as *O. gallaeciana* from Spain that form a separate BIN. At the present state of knowledge, the use of the database for routine identification of *Oulema* can only be considered in a limited geographic context, keeping in mind possible misidentifications and the partly unresolved taxonomy. For that reason we chose to compare our results only to sequences resulting from a taxonomic study (Bezděk & Baselga, 2015).

CONCLUSION

Bergsten et al. (2012) highlighted that limited sampling, and thus a restricted set of sequences reflecting local biodiversity, improves the identification by barcoding. This is supported by our results. Depending on the geographical context and the agronomic versus natural context, the identification of *Oulema* species by barcoding may be more or less efficient.

This study showed that the standard *COI* barcode can be used to distinguish between some *Oulema* species, including *O. melanopus* and *O. duftschmidi*, but cannot distinguish some other species in the *melanopus* complex, suggesting that further analyses might be needed to validate their taxonomic status.

Very extensive sampling has been carried out recently in agricultural regions in France (Chapelin-Viscardi & Maillet-Mezeray, 2015; Leroy & Chapelin-Viscardi, 2018). Several thousand specimens were identified based on the shape of the male flagellum. Given the relatively clear morphological criteria that characterise *O. mauroi* and *O. verae* (Bezděk & Baselga, 2015), it is unlikely that they would have been confused with *O. melanopus* and *O. duftschmidi* in those studies. Identification based on male genitalia, and especially flagella, tested by specialists, was validated by our study, which also confirmed the quality of the morphological identifications. These surveys provide evidence that only three species of *Oulema* are present in cereal crops in France: *O. obscura, O. melanopus* and *O. duftschmidi*. In the absence of the other species of the *melanopus* complex, the results obtained show that DNA barcoding is a good method for differentiating between species of the genus *Oulema* in cereal crops in France, regardless of the developmental stage or sex of the specimens. In order to meet the needs of plant protection professionals, the method must provide both unambiguous and reliable results. To achieve this, it will be necessary first of all to associate the reference sequences present in the database (on which the identification of sequences will be carried out) to a given geographical area and context, in our case cereal fields in mainland France.

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Supplementary Fig. S1 follows on next page.

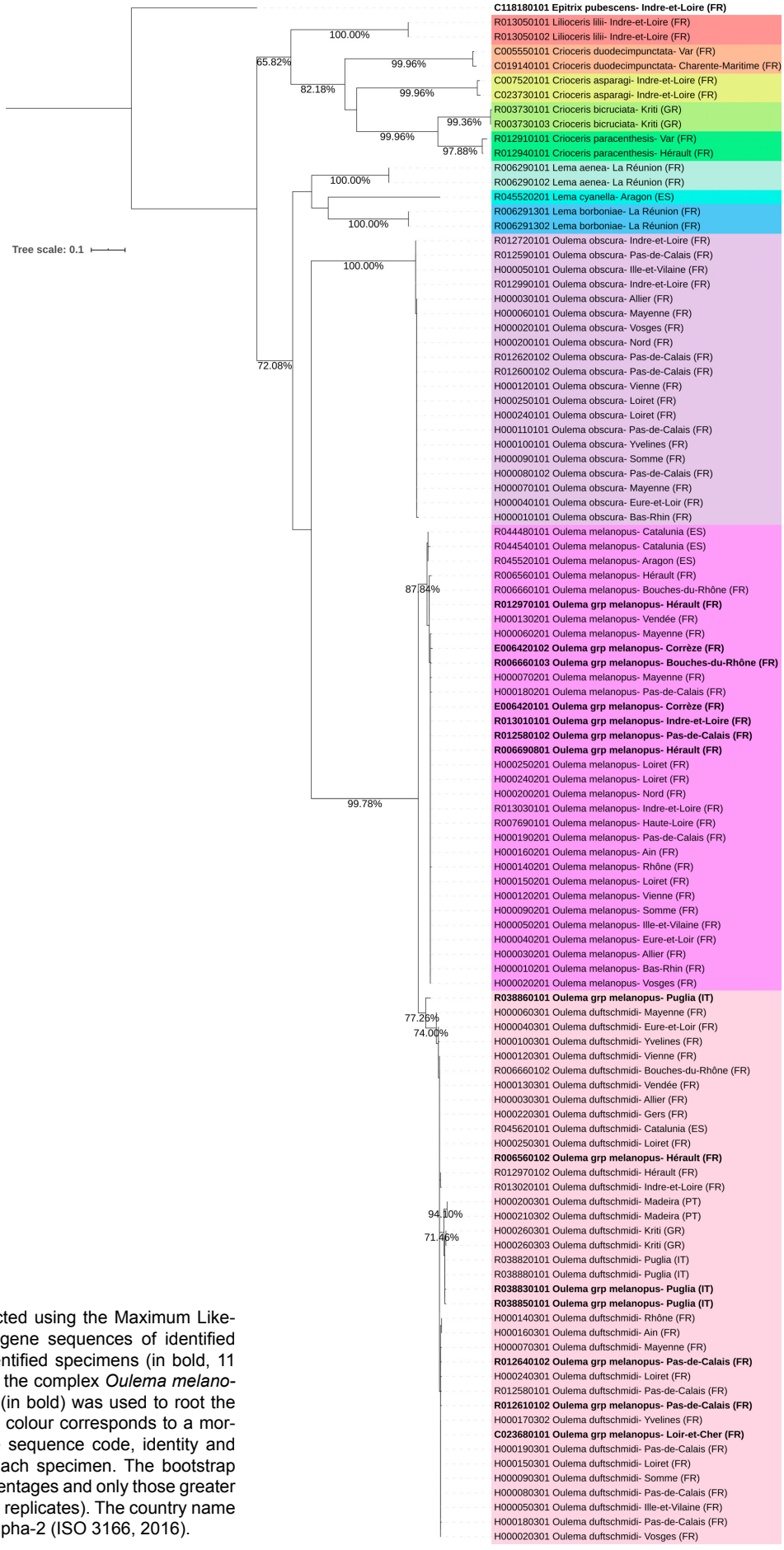

Fig. S1. Phylogenetic tree constructed using the Maximum Likelihood method (ML) and the COI gene sequences of identified specimens from Table 1 plus unidentified specimens (in bold, 11 females and 3 larvae), belonging to the complex *Oulema melanopus/duftschmidi*. *Epitrix pubescens* (in bold) was used to root the tree (106 sequences in total). Each colour corresponds to a morphologically identified species. The sequence code, identity and geographical origin are given for each specimen. The bootstrap values at the branch nodes are percentages and only those greater than 64% are presented (over 5,000 replicates). The country name is coded according to ISO 3166-1 alpha-2 (ISO 3166, 2016).