Pinpointing the level of isolation between two cryptic species sharing the same microhabitat: a case study with a scarabaeid species complex

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Understanding the processes of speciation is an important challenge in improving knowledge of the origin of biodiversity. One crucial point is to assess the causes of reproductive isolation, especially in the case of co-occurring species. Differences in microscale spatial distribution in small organisms may blur the issue. We explored biological processes underlying speciation within dung beetles belonging to the *O. vacca* species complex (Scarabaeidae: *Onthophagus*). The two taxa of this complex, *O. vacca* and *O. medius*, not only are known to have a large overlapping Palearctic distribution range but also share the same cowpat with no physical barriers and no observed specific aggregated patterns in the local distribution. The present study aimed at determining the level of isolation between the two taxa and discusses the most likely scenario of the speciation (sympatry vs. allopatry) based on the Coyne & Orr’s (2004) four criteria. We conducted a full study on populations sampled within the Mediterranean region integrating morphological analysis (digital image analysis of the elytral melanism pattern), two-gene phylogenies, population genetic analyses on populations sampled from an area where both species occur and another one with *O. vacca* only, as well as intra- and interspecific mating and crossing bioassays. The variation in the elytral melanism pattern clearly followed a bimodal distribution, with *O. medius* being more melanic than *O. vacca*, with a very limited overlapping area. The two taxa are reproductively isolated, with a strong postzygotic incompatibility despite the absence of sexual isolation. Sequence analysis of both nuclear and mitochondrial markers revealed a deep divergence between the two taxa dating back to 8.7 Mya. All findings concurred with some phenological observations and the conclusion that the most likely scenario for speciation in the *vacca* complex was an allopatric speciation followed by secondary contact.

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Introduction

Factors for gene-flow interruption during the speciation process in sexual organisms are recognized as numerous and various, although important debate is still ongoing about the relevance of the common distinction between allopatric and sympatric speciation modes (see Mayr 1942, 1963). Speciation is a continuous although irregular and reversible process driven by more or less contradictory evolutionary forces (Nosil 2008; e.g. migration vs. selection; Bolnick et al. 2009), depending on both organism’s intrinsic features and local constraints (dispersal ability and genetic isolation by distance, tolerance to acting selective pressures, interspecific competition…). Thus, those mechanisms involved may be extremely diverse and may succeed one another in various orders. Whilst some proposed a large-scale geographical framework of speciation modes (allopatric/sympatric, Mayr 1942, 1963; plus intermediate parapatric, Smith 1955), others, unsatisfied by such categorization, proposed to add a finer scale framework depending on specific ecological features of a variety of small organisms (e.g. allotopic/syntopic, Rivas 1964; allohospitalic/synhospitalic, Eichler 1966; ‘microallopatry’, Smith 1955). For Fitzpatrick et al. (2008), such categorization attempts have limited value with regard to the continuous nature of speciation, as opposed to studies evaluating the biological processes affecting divergence. Indeed, not only different species living in sympathy may in fact never come into contact due to different niche occupations, but also secondary contacts following allopatric speciation may explain sibling species co-occurrence.

In the former case, a spatial scale concern makes the definition of sympathy even more questionable; typically, ecological speciation is revealed to be consecutive to host switch in a diversity of plant-feeder insects (see e.g. Linn et al. 2004). In such cases, insect species which shift to a novel host plant might not be able to interplay with the congeners remaining on the ancestral host plant anymore, although plant hosts are still growing in the same geographical area. Indeed, the host plants or even plant organs constitute phytophagous insects’ life areas. Because the insects’ spatial scale does not match with the plants’ scale, the speciation process is initiated by an isolation in sympathy at the plant scale, but is completed in allopatry at the insect scale, that is in allotype sensu Rivas (1964). Plant-host switch, which was at first considered as the main speciation driver in plant feeders, appeared to be a less common speciation cause than expected (Nyman et al. 2010).

In the latter case, populations may have been physically isolated from each other for a long time, resulting in more or less markedly differentiated populations which later come into contact. If sexual incompatibility between the two populations is complete, the two species will simply co-occur, and the speciation process is considered to be complete. Such co-occurrence may persist or one species may displace the other one, depending on the extent of ecological similarity. If sexual incompatibility remains incomplete (incipient species), new gene flow will appear (speciation reversal, Seehausen et al. 2008).

In the case of effective ecological speciation of small organisms (in sympathy at a larger scale, but allopatry/allopatry at the considered organisms’ scale), investigating gene-flow interrupting factors during speciation (incipient species) at the organism- and/or population-level is often impossible or nearly impossible. This is mainly because it is difficult to distinguish currently present weak gene flow from very recently past important gene flow (e.g. Bourguet et al. 2014). In contrast, in the case of coexisting completely isolated species, biological processes involved in the gene-flow modulation and its history are more easily addressed (especially time since divergence, prezygotic and/or postzygotic factors of incompatibility).

To investigate such biological processes leading to differentiation between currently coexisting sibling species, we chose to study a biological model consisting of two congeners living mixed together, in strict sympathy and without, apparently, any local separation, which represents a good example of syntopy sensu Rivas (1964). *Onthophagus* (Coleoptera: Scarabaeoidea) with more than 2000 species described represents the largest genus of beetles, which originated 23–33 million years ago (Emlen et al. 2005). The evolution of this genus with the existence of many sibling species complexes and clear evidence of ongoing speciation is viewed as a very good example of rapid adaptive radiation (Pizzo et al. 2008, 2011; Macagno et al. 2011).

Two almost cryptic species *O. vacca* and *O. medius* having a wide overlapping distribution range and living in sympathy were recently compared and the latter described as a species by Rossner et al. (2010). The latter was long considered a form of *O. vacca*. Both species are known to have overlapping geographical distributions and commonly develop in the same individual cowpats (Lumaret & Stier-net 1994), thereby excluding possible gene-flow interruption due to physical isolation. In addition, the phylogenetic analysis published by Rossner et al. (2010) strongly suggests that both entities are sibling species. As a result, not only physical isolation between the two entities due to rough-grain scale could be ruled out, but also two of the four Coyne & Orr’s criteria (2004) required for a study case to be best explained by sympatric speciation are met: largely overlapping geographical range (first criterion), sister species according to the phylogenetic reconstruction (third criterion) based on mitochondrial DNA sequences.
In the present study, we decided to explore some of the biological processes possibly explaining the coexistence of two sibling species in the same microhabitat (a cowpat) which even has no evident pattern of spatial compartmentation (the two taxa are always spatially found together in cowpats in natura). We aimed at testing whether the second and fourth Coyne and Orr’s criteria are met for the vacca complex, that is:

- second criterion: speciation is completed (sexual incompatibility complete)
- fourth criterion: ‘biogeographical and evolutionary history of this vacca complex makes the existence of an allopatric phase very unlikely’.

As a first investigation of the factors explaining the maintenance of two sister dung beetle species in ‘syntopy’, this study should provide solid grounds for understanding speciation processes in general. The main questions are addressed: how deep is the divergence and how important is the recent/ongoing gene flow? Is gene flow between the two taxa still possible? To answer these questions, we used an integrative approach, involving analysis of the variation of the elytral melanism pattern using digital images and molecular (mitochondrial and nuclear DNA) phylogenies, as well as population genetics from populations sampled in both overlapping areas (South of France) and in a non-overlapping area (in Morocco, for O. vacca only), and bioassays for interspecific mating and assessment of hybrid fitness.

**Material and methods**

**Biological material**

Adult beetles were collected live in pitfall traps as well as by direct investigation of cowpats during the springs of 2012–2014 in several sites mainly located in France and in Morocco (see Appendix S1). All the beetles were used for phylogenetic analyses. For population genetic analyses, only samples collected at three sites were used: Salles-Curan, ‘Les Canabières’ (Altitude 900 metres) in the Aveyron Department (France), Saint Martin de Londres (Altitude 180 metres) in the Héraut Department (France) and Ifrane (Altitude 1400 metres) (Morocco). Insects dedicated for molecular analyses were preserved in absolute ethanol and kept frozen at −20 °C prior to analysis. Whole specimens, minus their 3rd leg (removed for DNA extraction), are housed as vouchers at the laboratory of CEFE (Montpellier). Insects dedicated for biological experiments were placed in pails with sandy soil as described below.

**Morphological differentiation and history of the elytral melanism pattern**

The melanism of the elytra is the major species-diagnostic character although it varies greatly within the two species (Rossner et al. 2010). However, reliable assignment to species based on visual inspection of this unique character is not straightforward mainly due to the subjective appraisal of the relative size of the melanic zones observed. However, the variability of this character is worth being used and analysed in order to complete our investigation of the level of sexual isolation within the vacca–medius complex. We expected the objective assessment of the melanism distribution in both O. vacca and O. medius to contribute to the characterization of inter- and intrataxa differentiation. For all these reasons, we chose to conduct a digital image analysis of this character.

Pictures of the right elytrum of each specimen to be sequenced were taken using a Nikon compact camera fixed onto a Leica binocular microscope (Leica Camera, Wetzlar, Germany). To calculate the ratio (R) of melanic areas over the total surface of the elytrum, the images were analysed under the freely available image-processing software Image J (Wayne Rasband, National Institute of Health, Bethesda, MD).

In order to assess to what extent this is a discriminant character, we measured the overlapping interval and the main parameters of the frequency distribution of R values in each. Additionally, to infer the evolutionary history of this continuous character between the two taxa, we performed some ancestral state reconstruction (ASR) (see below).

**Bioassays for assessing pre- and postzygotic compatibility between O. vacca and O. medius**

Cross-breeding experiments were carried out using laboratory-emerged progenies of O. vacca and O. medius females collected in natura from a field located in Salles-Curan (Aveyron, France). Beetles with dark colour elytra (O. medius) were distinguished from those with light colour elytra O. vacca and placed separately in pails filled with 5 L of an autoclaved moistened sandy soil. Each pail contained approximately 20 females for ovipositing. They were fed twice a week with good-quality dung which was kept frozen at −18 °C after collection during spring. After 1 month, the pails were sifted and the brood balls collected. They were placed into plastic boxes (30 broods/bag) filled with moist sandy soil and maintained in a climate chamber at 22 ± 1 °C, 60 ± 10% RH under a LD 14-h:10-h photoperiod. The newly emerging adults were sexed on a daily basis. Individuals were then separated by sex in a pail (30 individuals per pail of 5 L) and were fed with dung during 1 month at 22 °C. Then, pails were placed in growth chambers where the temperature was gradually lowered (5 °C per week) from 22 °C to 10 °C. Beetles were kept for 1 month at 10 °C. The temperature was then increased, following the same procedure as previ-
ously described, to reach 22 °C. The adults were then ready for cross-breeding.

**Interspecific hybridization experiments.** Interspecific hybridization experiments were carried out by crossing reciprocal pairs of *O. vacca* and *O. medius* to obtain F1 populations. Three males and three females were placed in one pail, and five replicates (pails) were performed per crossing. As controls, intraspecific crosses were also carried out (five replicates crossing). All pails were kept in growth chambers at 22 ± 1 °C, 60 ± 10% Rh under a LD 14-h:10-h photoperiod. Beetles were fed with dung twice a week and the experiment stopped when all females died. Once a month, each pail was sifted and brood balls collected were kept in new boxes for emergence. Adult emergence was checked weekly. After a period of 3 months, the unharvested broods were dissected to state whether it was empty (no egg laid inside) or the egg died subsequently (at egg, larval or nymphal stage).

Experiments of intraspecific–interspecific mate choice by females. Mate choice by females was conducted in laboratory conditions in order to determine to what extent females of one species would mate with males of the other species in the presence of conspecific males. For showing possible prezygotic isolation, the specific identity of the sperm found in the spermathecae of females was determined by analysing DNA.

Two females were placed in a pail in the presence of four males: two conspecific males and two males belonging to the other species. Ten pails representing 20 females were followed for each species ( *O. vacca* or *O. medius* ). Once a female started ovipositing resulting in 15 to 20 brood balls, the female was removed and her spermatheca extracted for further DNA analysis (see below). The DNA content of the spermatheca was systematically compared with the DNA content of one leg of the female.

**Molecular biology**

**DNA extraction, PCR amplification, sequencing and sequence alignments.** Genomic DNA of individual beetles was extracted from one of their 3rd legs (or from their dissected spermathecae) using QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Courtaboeuf, France) following the manufacturer’s protocol (DNA recovered in 35 µL of AE buffer), with one modification to improve yield and quality of DNA extracted: cell lysis was carried out overnight at 70 °C. A 710-bp fragment of the mitochondrial *CO1* barcode region was amplified using primers LCO_1490 and HCO_2198 (Folmer et al. 1994) and a 830-bp fragment of the nuclear ribosomal internal transcribed spacer 2 (ITS2) using primers ITS2f (Navajas et al. 1998) and RhiTS-R (De Rojas et al. 2002). PCR was performed with a 25 µL reaction mixture and 2 µL of diluted DNA (up to 20 ng) in a Perkin-Elmer PE 9700 Thermal cycler. The reagent concentrations were 1 × PCR buffer (QIAGEN), 0.04 U/µL Qiagen Taq Polymerase, 200 µM dNTPs and 0.3 µM of each primer, and 2.5 mM MgCl2 for *CO1* and 3.0 for ITS2. DNA amplifications were carried out as follows: initial denaturation at 94 °C for 5 min, followed by 5 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 48 °C ( *CO1* ) or 56 °C (ITS2), elongation for 1 min at 62 °C ( *CO1* ) or 72 °C (ITS2), 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 52 °C, elongation for 1 min at 62 °C ( *CO1* ) or 72 °C (ITS2), and final extension at 62 °C ( *CO1* ) or 72 °C (ITS2), for 10 min. Each PCR run included one negative control (water). Both strands of final PCR products were sequenced by capillary electrophoresis by MWG Eurofins Operon sequencing laboratory (Germany).

To estimate the divergence time between the two species, we took advantage of the most recent time-calibrated phylogeny of Scarabaeoidea published by Ahrens et al. (2014). Therefore, we also sequenced the region chosen by Ahrens et al. (2014), that is the 2183–3014 region lying downstream of the *CO1* (Simon et al. 1994) from 12 individuals belonging to either the *O. vacca* or the *O. medius* morphs, plus eight congener: *O. nuchicornis*, *O. taurus*, *O. similis*, *O. fracticornis*, *O. opacicollis*, *O. maki*, *O. raficipillus* and *O. verticicornis*, all sampled in the South of France. A 850-bp fragment of the mitochondrial *CO1* was amplified using primers C1-J-2183 (Jerry) and L2-N-3014 (Pat) (Simon et al. 1994) with the same reagent concentrations and PCR programs as described above. Sequences are available from GenBank under the accession numbers listed in Online-only material Appendix 1.

Both *CO1* and ITS2 sequence data sets were aligned using the MUSCLE option in SeaView version 4 (Gouy et al. 2010). All *CO1* sequences were translated into amino acids to check for the absence of any stop codon in this protein coding gene.

**Diagnostic PCR for bioassays.** To confirm that the F1 hybrids were indeed derived from reciprocal crosses and to assess their frequency, a PCR diagnostic assay was developed. Several species-diagnostic primer pairs were designed to amplify one target region within ITS2 and one within *CO1* for each taxa. These diagnostic PCRs were carried out with the same PCR mix and PCR programs as described above. Species-specific PCR amplicons were run in an (1%) agarose electrophoresis gel.

A total of 35 primers (sense or antisense, 17–25 bp of length), corresponding to 25 primer pairs, were designed from the aligned sequence matrix of ITS2 and *CO1* obtained
with Seaview v4 (see above). Several sites composed of few nucleotides which appeared to be polymorphic between *O. vacca* and *O. medius*, but monomorphic within each taxa were targeted for the design of species-specific primers. In some cases, we inserted additional mismatch at the adjacent base (penultimate) of a single nucleotide polymorphism (SNP) as recommended by Bui & Liu (2009) to increase the destabilizing effect of the target mismatch and so to optimize specificity of the primer. Species-specific primers were designed from the biparental nuclear ITS2 to evidence heterozygote hybrids. Species-specific primers were also designed from the uniparental CO1 to detect a small number of spermatozoa in spermathecae extracted in the mate choice experiments. Among 25 different primer pairs tested, four pairs were retained. The sensitivity of the most specific primer pairs generating one single amplicon with the target DNA was considered as a proof of an interspecific mating vent.

For the intraspecific–interspecific mate choice tests, DNAs extracted both from the spermathecae and from one leg of the female were tested by two different diagnostic PCRs (using the *vacca*-specific pair Diag2bv and the *medius*-specific pair Diag4bm) to (i) identify the female species from which spermatheca was extracted, (ii) evidence the absence or presence of DNA from the other species in the extracted spermathecae. In the case of presence, this was considered as a proof of an interspecific mating vent.

As for interspecific hybrids, ca. 75% of obtained F1 progenies were analysed using diagnostic PCR as follows: DNA extracted from one leg of each newly emerged individual (both female and male) was analysed by two distinct nuclear diagnostic PCRs (one with the *vacca*-specific pair ITSv and one with the *medius*-specific pair ITSm). Each PCR run included a PCR mix without DNA template as negative control and a PCR mix with a 50/50 (v/v) DNA template mix of the two species as positive control. When amplicons specific to the two species were generated from one individual, this was considered a true interspecific F1 hybrid.

**Genetic differentiation within and between taxa and demographic history**

*Nucleotide variation and F statistics.* The intra- and inter-taxa distributions of pairwise nucleotide variation (nucleotide differences π and nucleotide divergence D, resp.) were estimated using DnaSP 5.10 (Rozas & Rozas 1995; Librado & Rozas 2009). Average nucleotide diversity πxy between pairs of populations and the net number of nucleotide differences between populations (DA) were calculated using Arlequin 3.5.1.2. Their significance was tested with the permutation test (1000 permutations). Populations under test were samples with a suitable number of individuals (see Appendix S1).

**Bayesian clustering.** We used the Bayesian clustering method described by Pritchard et al. (2000) as implemented in STRUCTURE 2.3.4 on both the ITS2 genotype data set and the CO1 haplotype data set to identify genetically differentiated groups of individuals in the absence of preliminary information on group boundaries. The model assumes the existence of K clusters (the real number being unknown) and assigns individuals to clusters through a Markov chain Monte Carlo (MCMC) probabilistic approach so as to maximize the Hardy–Weinberg equilibrium within populations. To avoid violation of the HWE...
postulate, data sets to be analysed were restricted to one copy of each haplotype (COI) or genotype (ITS2). All analyses were based on 10 independent runs for each K value, with each individual run being based on a burn-in period of 100 000 iterations followed by 500 000 MCMC iterations without prior information on the taxonomy or the locality of origin of the individuals sampled. The admixture frequency model was run under the assumption of correlated allele frequencies to improve clustering of related lineages and identify possible hybridization patterns (Falush et al. 2003). The optimal number of clusters was verified using the ΔK statistical approach of Evanno et al. (2005).

**Haplotype networks and demographic history.** A median-joining network (Bandelt et al. 1999) of each DNA fragment was constructed using NETWORK v.4.5.1.6 (fluxus-engineering.com) with postprocessing parsimony analyses. The test for neutrality, Fu and Li’s F (Fu & Li 1993) was also assessed to get a rough overview of the demographic history using DNAsp v.5.10 software.

**Phylogenetic analyses**

**ML and MP phylogenies.** The evolutionary history was inferred using both the maximum likelihood (ML) and the maximum parsimony (MP) approaches with Mega version 6.06 (Tamura et al. 2013). ML trees were constructed using the Tamura 3-parameter model with a discrete Gamma distribution (T92 + G) to model evolutionary rate differences among sites (5 categories) for the COI sequence data set and the Kimura 2-parameter model with rate variation model allowing for some sites to be evolutionarily invariable (K2 + I) for the ITS2 data set, based on model test results using Mega 6.06 (BIC index). Initial tree(s) for the heuristic search was obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. MP trees were obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

**Divergence dating.** The data set comprised two sequences of *O. vacca* and *O. medius*, eight sequences of other congeners and 17 sequences of Scarabaeinae which were included in the most recent time-calibrated phylogeny of Scarabaeoidea (Ahrens et al. 2014). These include *Allogymnopleurus thalasi* (AY131898), *Canthidium guanacaste* (AY131867), *Canthon viridis* (AY131817), *Cheiorinitis boplosternus* (AY131940), *Circellium bacbus* (AF499750), *Copris lugubris* (AY131860), *Coptorina sp.* (AY131871), *Deltochilum gibbosum sublaeve* (AY131823), *Dicranocara desbodii* (EF656763), *Eucranium arachnides* (AF499752), *Euryternis angustulus* (AY131892), *Helicopris andersoni* (AY131878), *Kheper nigroaeneus* (AF499755), *Nessyphus fortitius* (AY131961), *Ontobophagus crinitis* (AY131924), *Phanaeus salli* (AY131951) and *Sisyphus fasciatus* (AY131964). One species, *Apbodius barbarus* (accession number AY132580) from the genus *Apbodius, which has been proposed a sister taxon of the Scarabaeinae (Monaghan et al. 2007) was used as an outgroup representative of the analysis. The 27 sequences were aligned using ClustalW executed in BioEdit v7.1.3.0 program (Hall 1999). The most appropriate model of sequence evolution was first computed in MODELTEST (Posada & Crandall 1998) implemented in PaupUP v1.0.3.1 (Calendini & Martin 2005) under the minimal AIC criterion (AICc-corrected Akaike information criterion). The program Beast V.1.8. (Drummond & Rambaut 2007), a Bayesian coalescent analysis with the MCMC procedure, was used to estimate time to most recent common ancestor (tMRCA). Lineage age was estimated under the lognormal uncorrelated model (relaxed molecular clock). Because the molecular clock model was rejected for COI (tested using the likelihood-ratio test of the clock hypothesis in Paup under the substitution model selected above: df = 25, \( \chi^2 = 48, 56, P = 0.003 \)), a relaxed-clock model (Drummond et al. 2006) was used in the Beast analysis. The Beast analysis was conducted, assuming the Yule speciation model, uncorrelated-relaxed molecular clock model, with all estimates utilizing the GTR + I + G model of substitution. Two independent Markov chains were run for 100 million iterations using a random starting tree, and all resulting effective sample size exceeded 100. The dating errors for the fossil (*Prionocephale deplanate*) used to calibrate the divergence time estimated into the Scarabaeinae place the fossil age in the upper Cretaceous between 92 and 83.5 Ma (Ahrens et al. 2014). To calibrate the root, we used the results of the first divergence estimate of the split between Scarabaeinae and Aphodinae around 108 Ma (Ahrens et al. 2014). We also used a second calibration time for the split between *Cheiorinitis boplosternus* and *Ontobophagus crinitis* that was given between 49 and 53.2 Ma by Ahrens et al. (2014). The program Tracer ver.1.6. (Rambaut & Drummond 2007) as implemented in the StarBeast package was used to assess the convergence between runs and posterior probabilities of the estimates. The results were summarized as maximum clade credibility (MCC) trees using Tree Annotator ver 1.8. Statistical uncertainty of divergence time estimates in the MCC trees was assessed by the 95% highest probability density (HPD) intervals (i.e. Bayesian equivalent of confidence interval).
History of the melanism character. To assess how the elytral melanism percentage has evolved in the *vaca* complex, an ancestral state reconstruction (ASR) of this continuous character was performed using the maximum parsimony method as implemented in Mesquite version 2.75 (Maddison & Maddison 2011).

Results
Melanism differentiation
The distribution of the ratio between the melanic areas and the total surface of the elytrum for *O. vacca* and *O. medius* is shown in Fig. 1. For both species, the percentage of melanism can be adjusted to a normal distribution (Kolmogorov–Smirnov test, \(D_{O. \text{ vacca}} = 0.107, p = 0.581; D_{O. \text{ medius}} = 0.072, p = 0.934; \alpha = 0.05\)). The mean value of this percentage was 12.9 and 33.5 for, respectively, *O. vacca* and *O. medius* (*N_{O. \text{ vacca}}(12.9;3.5); N_{O. \text{ medius}}(33.5;8.1)*). The distributions of *O. vacca* and *O. medius* were significantly different but slightly overlapping between each other, making an accurate assignment of individuals to specific identity difficult in the range 17–20% melanism.

Reproductive tests
Experiments of intraspecific–interspecific mate choice of females. Bioassays to estimate the ability of the two taxa to mate together resulted in an overall high frequency of interspecific matings, as estimated from DNA analyses of spermathecae excised from females under test: of the 16 *O. vacca* females tested, 12 were found to have mated with *O. medius* males (75% females had copulated with the opposite taxon in our laboratory conditions); of the 20 *O. medius* females tested, 8 were found to have mated with *O. vacca* males (40% interspecific mating in our laboratory conditions).

Interspecific hybridization experiments. All intra- and interspecific crossings produced viable offspring, although the number of offspring obtained was lower in interspecific crossings (<7 broods per female and <2 adults per female) than in intraspecific crossings (>19 broods per female and >7 adults per female) (Table 1). In intraspecific crossings, the number of broods and viable offspring were significantly higher in the *O. vacca* male: female crosses (\(F = 14.08\) and 43.01; \(P < 0.001\)). It was more than twice as high as the number obtained for the *O. medius* male: female crosses. No significant difference was found between the reciprocal male:female interspecies *O. vacca*-*O. medius* crossing for the number of broods produced and the number of adults that emerged from these broods (Table 1).

Obtained DNA sequences
A total of 45 ITS2 haplotypes (without considering indels) containing 105 parsimony informative sites were shown. A total of 63 haplotypes were recorded with indels considered (22 in *O. medius*, 41 in *O. vacca*) and a total of 13 haplotypes with indels as the only variation considered. It is worth noting that of 15 different sites with gaps in our dataset, 10 were discriminant indels (variable within taxa; 1–5 bp long). These were useful to design efficient diagnostic primers for prezygotic experiments. As for CO1, a total of 30 haplotypes were recorded (14 in *O. medius*, 16 in *O. vacca*), containing 44 parsimony informative sites. Accession numbers and sampling information are available in the Appendix S1.

Genetic structure and demographic history of the two taxa under scrutiny
The corrected average pairwise difference between populations *\(\pi_{xy}\)* when considering *O. vacca*-*O. medius* pairs was between 31.272 and 33.489 (with \(P < 0.001\) in all instances) in CO1 analyses and 68.448 and 70.435 (with \(P < 0.001\) in all instances) in ITS analyses (Appendix S2). One may

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**Table 1** *Onthophagus vacca* and *O. medius*, intra- and reciprocal interspecific cross-breeding

<table>
<thead>
<tr>
<th>Crossing</th>
<th>(\sigma \times \varphi)</th>
<th>(n)</th>
<th>Broods/(\varphi)</th>
<th>Adults/(\varphi)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. vacca</em> × <em>O. vacca</em></td>
<td>15</td>
<td>46.3 ± 7.9 a</td>
<td>14.4 ± 6.2 a</td>
<td></td>
</tr>
<tr>
<td><em>O. medius</em> × <em>O. medius</em></td>
<td>15</td>
<td>19.9 ± 6.5 b</td>
<td>7.9 ± 3.4 b</td>
<td></td>
</tr>
<tr>
<td><em>O. vacca</em> × <em>O. medius</em></td>
<td>20</td>
<td>6.6 ± 2.9 c</td>
<td>1.1 ± 0.9 c</td>
<td></td>
</tr>
<tr>
<td><em>O. medius</em> × <em>O. vacca</em></td>
<td>20</td>
<td>3.4 ± 2.1 c</td>
<td>0.7 ± 0.7 c</td>
<td></td>
</tr>
</tbody>
</table>

Values in the same row followed by the same letter are not significantly different (ANOVA and SNK tests, \(\alpha = 0.05\)).

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notice the important gap between inter- and intraspecific pairs in both genes: <2.240 within *O. vacca* and within *O. medius* in both genes; >31.270 and >68.440 between the two taxa in CO1 and ITS, respectively.

Bayesian clustering analyses on the whole data sets (i.e. including both *O. vacca* and *O. medius* CO1 haplotypes or ITS2 diploid genotypes) resulted in an optimal 2-cluster structure with both genes under study without any ambiguity (Appendix S3a,b). Almost no interindividual admixture (likelihood assignment values >0.9 in all cases) was found, and an exact correspondence between mitochondrial and nuclear clusters was recorded. When analyses were performed on single-taxon data sets, the retained optimal K value was without any ambiguity K = 1 in both genes for *O. medius* and in CO1 for *O. vacca*. The latter taxon resulted in an optimal K oscillating between 1 and 2 with ITS2 (Appendix S3c) and when K = 2 was considered, no geographical structure appeared, the two clusters being largely admixed with each other (no individual totally assigned to the less frequent).

Both phylogenetic CO1- and ITS2-based analyses resulted in two-clade topologies, with branch lengths much longer between the two taxa than within taxa (Fig. 2), in congruence with the CO1 and ITS2 haplotype median-joining networks (Appendix S4).

Moreover, both the CO1 and ITS2 networks highlighted a higher number of haplotypes separated by many mutational steps in *O. vacca* than in *O. medius*. This was expected given that *O. vacca* was sampled from very distant geographical locations (France and Morocco), whilst *O. medius* was sampled only in France. However, the observed pattern is also consistent with a recent demographic expansion in *O. medius*. This latter hypothesis is supported by significant negative values for Fu & Li’s F neutrality tests processed on Co1 sequences (Table 2).
Divergence between the two taxa and morphological character history

All retained phylogenetic trees (i) had all sequences of O. vacca grouped in one cluster and all sequences of O. medius grouped in another cluster, both clusters being strongly supported (Fig. 2), (ii) show the two groups as sister to each other and (iii) had much longer intertaxa than intrataxa branches. Additionally, whilst the divergence between the two taxa under test was congruent between mitochondrial and nuclear sequences, repeated incongruences occurred within both O. vacca and O. medius, which is consistent with the expected within-species reticulation.

Estimated divergence time (BEAST analysis) between O. vacca and O. medius lies between 5.28 and 12.18 million years ago (Fig. 3). This estimate of divergence dates the split to the late Miocene.

Table 2 Colour and genetic diversity, with neutrality estimates

<table>
<thead>
<tr>
<th>Gene under test</th>
<th>O. medius</th>
<th>O. vacca</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of sites</td>
<td>846</td>
<td>846</td>
</tr>
<tr>
<td>No of haplotypes, h</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Average number of nucleotide differences, k</td>
<td>1.346</td>
<td>1.295</td>
</tr>
<tr>
<td>Nucleotide diversity, π</td>
<td>0.00202</td>
<td>0.0021</td>
</tr>
<tr>
<td>Haplotype diversity, H(d) (±SD)</td>
<td>0.767 ± 0.034</td>
<td>0.488 ± 0.086</td>
</tr>
<tr>
<td>Fu and Li’s F</td>
<td>-0.781 (P &gt; 0.10)</td>
<td>-3.421 (P &lt; 0.02)</td>
</tr>
</tbody>
</table>

The maximum parsimony reconstruction of the melanism discrete character’s history (ASR) using observed states in terminal O. vacca and O. medius taxa on the Co1 topology resulted in a sharp and unambiguous separation of 11 classes of values, with the 3 lower ones (from 6 to 19.92%) restricted to the O. vacca clade and the 8 higher ones (up to 57.04%) to the O. medius clade (Fig. 4). The divergence was assumed to have occurred from common ancestral states comprised between 19.92 and 24.56%, with ancestral states <15% in the O. vacca clade and close to 30% in the O. medius clade. No clear grouping of extreme values was revealed, and the higher variance in O. medius was reflected by sparse darkening events in distal positions (three higher classes from 43.12 to 57.04%).

Fig. 3 Calibrated time tree of Scarabaeinae including O. vacca and O. medius and congeners obtained with beast for CO1. Divergence estimates correspond to the mean node ages in units of millions of years before present. Grey bars, 95% confidence intervals of the estimated ages for the nodes.
Discussion

Our study provides the first conclusive evidence for considering *O. vacca* and *O. medius* not only as phylogenetically distinct taxa, or as distinguishable genomic clusters in sympatry (Rossner et al. 2010), but also as two biological species in Mayr’s sense with clear postzygotic isolation.

Taken as a whole, our findings strongly support the following statements: (i) the population differentiation is much more important between the two taxa than within each taxon, with the genetic differentiation between the two taxa mirroring the melanic pattern of the elytra (only a very slight overlapping distribution ratios between taxa), (ii) the date of split between taxa appears to be ancient (ca. 8.7 Mya ago), and (iii) no gene flow is currently occurring between taxa. Although some interspecific F1 hybrids have been obtained in laboratory bioassays, no interspecific hybrids were evidenced from our sampling in natura. Besides, the much smaller number of F1 broods and viable offspring obtained in interspecific hybridization experiments compared with the values obtained in the intraspecific crosses, and the complete failure of F2 generation indicate that, whilst there is no prezygotic isolation between *O. vacca* and *O. medius*, breeding success is strongly reduced.

No biological prezygotic barrier was shown under laboratory conditions (recurrent interspecific mates in mate choice tests), contrary to evident signs of postzygotic incompatibility. Indeed, the fitness was very low for the F1 hybrids and null for the F2 (no living F2 were obtained). Results regarding the F2 should be taken with caution because of the few living F1 individuals available for the crossing. Yet, one of the prezygotic barriers which may occur during speciation is reinforcement by assortative mating, as a consequence of natural selection: ‘when two populations have diverged to such an extent that they produce unfit hybrids, […] characters increasing assortative mating will be favored until eventually two species may result’. (Butlin 1987). In the present study, no evidence for such reinforcement was shown.

Overall, the second Coyne & Orr (2004) criterion is validated: speciation is completed in *O. vacca* and in *O. medius*. As for the time since divergence, our results suggest that the speciation has occurred 8.7 Mya around
the Miocene–Pliocene boundary, and this speciation event could have followed major faunal turnover events including herbivores (Kohler et al. 1998). Species turnover events were over a limited time span close to the Miocene–Pliocene boundary resulting in a massive extinction of more than 60–70% of all Eurasian genera (Fortelius et al. 2006). Climate changes resulted in forest cover decline as well as a global increase in C4 biomass (Hsu et al. 1997; Fortelius et al. 2006). Open grassland habitats, which were exploited by an entirely new suite of mammals, replaced the earlier less seasonal woodland forest habitats. Thus, it is possible that the environmental changes associated with the Miocene–Pliocene boundary and the following emergence of new ecological niches such as open grasslands caused a new adaptive radiation of some Onthophagus. The diversification in the Cenozoic, most likely in the Oligocene (approximately 23–33 Mya) of the genus itself is also coincident with the expansion of grassland habitats and the radiation of mammals (Emlen et al. 2005). Intense competition for ephemeral food resources may have favoured specialization on novel dung sources (Villalba et al. 2002; Emlen et al. 2005). The comparison of our results with other taxa is limited as the dating of divergence events within tunnelling Onthophagus has not been published yet. Interestingly, the two species seemed to have originated earlier than the time frame of 3–4 Mya in the Pliocene, given to the split between O. taurus and O. illyricus (Pizzo et al. 2006), the Pliocene corresponding to an important frequency peak of speciation for several beetle taxa (Ribeira & Vogler 2004).

As for the fourth Coyne & Orr (2004) criterion, here it is to be asked whether ‘biogeographical and evolutionary history of the groups [makes] the existence of an allopatric phase very unlikely’. First, some phenological asynchrony was noticed between the two taxa based on field sampling and laboratory bioassays (OB, pers. obs., Rossner et al. 2010). In our experiments, overall, O. vacca emerged approximately 2 months earlier than O. medius in laboratory conditions. These observations do not preclude the possibility that reproductive activity overlaps between the two taxa in natura. Also, we observed that males of O. medius in most cases emerged before females. Owing to the fact that a higher mating success was obtained between O. vacca females and O. medius males, intertaxa hybridization event is shown possible, but intertaxa hybrids will likely be unfit relative to parents.

From an evolutionary point of view, it is not totally excluded that some shift in the life cycle could have played a part as a speciation driver between populations living in syntopy, resulting in a sharp sexual isolation, a process named allochronic speciation, that has been shown in some other insect species (e.g. Cooley et al. 2001; Santos et al. 2007). However, in such cases, the temporal shift should be accompanied by different selection pressures and by important gene-flow interruption to result in such sharp differentiation and reproductive incompatibility. Whilst above authors recorded several-year or 2-season time lags, the time lag in the O. vacca complex seems to be much less than a season and implies some overlapping period. This makes the allochronic speciation very unlikely.

Given that the two species develop in the same micro-habitat, with no reported local aggregation pattern, and that observed asynchrony is incomplete, the hypothesis of allopatric speciation followed by a secondary contact appears to be more probable. All the more, Coyne & Orr (1997) have shown that, in Drosophila, the postzygotic incompatibility dominates among allopatric species pairs, whereas prezygotic isolation predominates among sympatric species pairs. Under allopatric speciation hypothesis, the observed partial phenological asynchrony evidenced between the two taxa (see above) may explain why the elimination of one of the two co-occurring species has not occurred after the two taxa made contact.

The largely overlapping geographical range highlighted by Rosner et al. (2010) might be explained either by sympatric speciation or by the very ancient divergence time. The latter makes it possible that reproductive incompatibility was reached before large-scale spatial expansion with secondary contact occurred. One or the two species may have had time to widely spread and meet one other on the way after the incompatibility was complete. Yet, some apparent differences in the demographic history between present samples of O. vacca and O. medius suggest that O. vacca might have been installed in the sampled geographical areas (France, Morocco) for a longer time than O. medius, which might have entered the French part rather recently (thus showing signs of sudden expansion in CoI sequences). Such a spatial expansion could have been the cause of a secondary contact after speciation has occurred in allopatry. However, the very limited range of sampled areas in the present study and the current knowledge about the two species’ history is far from enough to be sure about this issue. The very large distribution areas for the two species as established by Rossner et al. (2010) apparently lies in a huge number of specimens mainly collected during the last century. It is not absolutely excluded that some sudden spatial expansion of O. medius has occurred before the before the twentieth century (i.e. ca. 120 generations ago) and that the imprint of it is detectable in current mitochondrial sequence batches, although not yet clearly in nuclear sequences (lower mutation rate expected). Nevertheless, nothing here allows confirmation, and present results only raise the question of a possible more or less recent colonization of some European areas by O. medius.
Conclusion
When considering the four criteria listed by Coyne & Orr (2004) required for a study case to be best explained by sympatric speciation, the first three criteria are fully met in case of the *O. vacca* complex: (i) the large overlapping geographical range was established in previous studies, (ii) the sister status of the two species as previously evidenced by Rossner *et al.* (2010) based on mitochondrial phylogenies was confirmed in the present study by mitochondrial and nuclear molecular phylogenetic reconstructions, and (iii) the speciation was clearly shown to be completed, with postzygotic complete incompatibility.

As for the fourth criteria (namely: ‘biogeographical and evolutionary history of the groups [makes] the existence of an allopatric phase very unlikely’), it is the most difficult to test with confidence and present data did not allow clearly deciding about it. A secondary contact subsequent to allopatric speciation could be the most likely scenario explaining the speciation in the *vacca* species complex. Indeed, the very ancient estimated divergence time (around the Miocene–Pliocene boundary), the ability of the two species to mate with each other (reinforcement absent) and the strong postzygotic incompatibility would most likely result from allopatric speciation. The partial asynchrony might explain the persistence of the two species in the same microhabitat. Some change in the spatial distribution of *O. medius*, as suggested by signs of sudden expansion in the mitochondrial sequence data of sampled populations, might be the cause of a secondary contact. However, this remains speculative.

To improve the assessment of the fourth criterion, it would be appropriate to perform some larger-scale investigation, involving larger beetle samples collected from across the whole distribution range of the two taxa and investigating the demographic history of these populations. This should allow the determination of whether *O. medius* more or less recently expanded its spatial range and what is the likely origin of this taxon. In addition, some further experiments dedicated to exploring the phenological traits of the two taxa and stating to which extent they are competitive to each other would be useful to improve our understanding of the functioning of this species complex.

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References


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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Information on genotyped beetle individuals with ENA (European Nucleotide Archive) accession numbers and geographic origin.

**Appendix S2.** Average number of pairwise differences between populations ($\pi_{XY}$), (a) using CO1 sequence data, (b) using ITS sequence data.

**Appendix S3.** Population structure of the *O. vacca* complex inferred with STRUCTURE (v2.3.4) software from the single genotype sequence datasets (admixture frequency model under the assumption of correlated allele frequencies, burn-in period of 100 000 iterations followed by 500 000 MCMC iterations without prior information on the taxonomy or the locality of origin of the individuals sampled).

**Appendix S4.** Haplotype MJN networks with postprocessing parsimony analysis (Network v4.5.1.6).