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Abstract:	<p>Morphologically cryptic lineages confound many estimates of global biodiversity and are often discovered in ecologically specialised taxa, subject to strong morphological constraint. Such a situation may apply in many extreme environments, including supralittoral rockpools, where dramatic fluctuations in water availability and salinity impose strong selection pressures on the inhabitants. Here we explore the genetic diversity and phylogeography of supralittoral rockpool <i>Ochthebius</i> beetles in the Eastern Atlantic and Western Mediterranean, using a combination of mitochondrial and nuclear markers and dense geographical sampling of the three recognised widespread species. Our results point to the existence of morphologically cryptic lineages within all currently named taxa and suggest that the distribution of these is linked to both historical and contemporary marine hydrogeography; a combination of ocean currents and winds apparently driving the spatial patterns observed. The main contemporary barrier to dispersal for <i>Ochthebius</i> is located around the Ibiza Channel, whilst the Messinian Salinity Crisis appears to have been the ultimate driver of lineage diversification in these insects. Our results show that oceanographic processes do not just shape the evolution of fully marine species, but also impact significantly on the terrestrially-derived inhabitants of the coastal zone.</p>

Abstract

Morphologically cryptic lineages confound many estimates of global biodiversity and are often discovered in ecologically specialised taxa, subject to strong morphological constraint. Such a situation may apply in many extreme environments, including supralittoral rockpools, where dramatic fluctuations in water availability and salinity impose strong selection pressures on the inhabitants. Here we explore the genetic diversity and phylogeography of supralittoral rockpool *Ochthebius* beetles in the Eastern Atlantic and Western Mediterranean, using a combination of mitochondrial and nuclear markers and dense geographical sampling of the three recognised widespread species. Our results point to the existence of morphologically cryptic lineages within all currently named taxa and suggest that the distribution of these is linked to both historical and contemporary marine hydrogeography; a combination of ocean currents and winds apparently driving the spatial patterns observed. The main contemporary barrier to dispersal for *Ochthebius* is located around the Ibiza Channel, whilst the Messinian Salinity Crisis appears to have been the ultimate driver of lineage diversification in these insects. Our results show that oceanographic processes do not just shape the evolution of fully marine species, but also impact significantly on the terrestrially-derived inhabitants of the coastal zone.

Keywords: cryptic species, rocky shores, dispersal, Mediterranean

1. Introduction

Despite much discussion of global biodiversity in recent decades, we still lack an accurate picture of the number of species on earth, but can be confident that most multicellular organisms are arthropods, in both terrestrial and aquatic habitats. Like most authors, Stork (2018) argued that described species represent a small fraction of those extant, estimating that there are over 7 million species of arthropods on earth, the majority of which are both undescribed and insects. One of the reasons behind our uncertainty is the existence of morphologically cryptic species, which have been shown to occur widely across taxa and biogeographical regions (Adams et al., 2014; Fiser et al., 2017; Pfenninger & Schwenk, 2007), particularly in the light of molecular genetic analyses (e.g., Aguilar et al., 2017; Blair & Bryson, 2017; Müller et al., 2018; Puillandre et al., 2012). Whilst a relative lack of morphological divergence may sometimes reflect recent evolutionary divergence (e.g., Zúñiga-Reinoso & Benítez, 2015), the morphological stasis of cryptic species can also persist for long periods when taxa are subject to strong ecological constraints (Cai et al., 2019; Cerca et al., 2020; Fiser et al., 2017; Pfingstl et al., 2019).

Morphological stasis may occur in harsh environments where stabilizing selection can lead to a reduced range of phenotypic traits (Bickford et al., 2007). This is the case with many organisms inhabiting extreme environments such as caves (Zhang & Li, 2014), hypersaline waters (Arribas et al., 2013), mountain summits (Adams et al., 2014) or the intertidal zone (Pfingstl et al., 2019; 2021). Ecomorphological constraints also operate on aquatic organisms more generally, however, particularly through their hydrodynamics, something which increases the likelihood of cryptic species complexes being present in such habitats (Céréginho et al., 2018; Domenici et al., 2007; Jami et al., 2021; Knowlton, 1993). Whilst lineage divergence without population isolation certainly does occur, particularly in marine

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4 scenarios (e.g., Bowen et al., 2013; Faria et al., 2021), speciation is thought much more likely
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6 to occur when there is an external barrier to gene flow, at least initially (Barraclough, 2019;
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8 Haak et al., 2019; Stock et al., 2019). Limitations to dispersal, and so gene flow, between
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10 populations may be rather cryptic themselves; marine systems providing good examples,
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12 including oceanic current systems which can be stable enough to act as geographical barriers
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14 for passively dispersing organisms. Examples of cryptic taxa apparently diverging as a result
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16 of such processes are varied, and include red algae (Billard et al., 2015), cave mysids
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18 (Rastorgueff et al., 2014) and chthamalid barnacles (Wares, 2020). In addition to obligately
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20 marine organisms, similar patterns have been demonstrated in some groups occupying the
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22 marine-terrestrial ecotone, both terrestrial and aquatic (Hurtado et al., 2014; Santamaria et al.,
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24 2016; 2017; Greenan et al., 2018; Mbongwa et al., 2019; González-Castellano et al., 2020),
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26 many of which also involve morphologically cryptic lineages.
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32 Supralittoral rockpools are complex and dynamic habitats, whose relatively simple
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34 communities reflect the challenges of living in these highly fluctuating environments
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36 (Vecchioni et al., 2021). Supralittoral pools undergo dramatic changes in water level, due to
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38 alternating cycles of rain and desiccation, and are also exposed to wide fluctuations in salinity
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40 as a result of such dilution and concentration events, coupled with occasional inundation by
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42 storms and spring tides (Powlik, 1999). Organisms permanently inhabiting such rockpools
43
44 have to cope with dramatic changes in temperature and salinity, which present considerable
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46 physiological challenges (Gaines & Denny, 2007; Powlik & Lewis, 1996). Whilst the harsh
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48 environment of supralittoral pools may select for morphological stability, as discussed above,
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50 these habitats have, until recently, not been considered to harbour significant cryptic diversity
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52 in their inhabitants, since the ephemeral and spatially patchy nature of pool availability would
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4 be expected to select for strong dispersal abilities (see Bilton et al., 2001; Ribera, 2008),
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6 resulting in high levels of gene flow.
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9 The depauperate fauna of supralittoral rockpools, coupled with their position between
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11 marine and terrestrial realms, means that these habitats have been relatively neglected in
12
13 ecological and evolutionary studies. It is clear, however, that the fauna of these habitats is
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15 relatively uniform in many parts of the world (e.g. Orchymont, 1932; Altermat et al., 2012),
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17 pools generally supporting a combination of microcrustacea, dipteran larvae (mostly
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19 Chironomidae) and in some regions, including the Western Palaearctic, adults and larvae of
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21 ochthebiine beetles (Coleoptera: Hydraenidae). Of these, rockpool *Ochthebius* Leach, 1815
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23 have received considerable attention in recent years, including research on their systematics,
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25 behaviour and physiology in various parts of the world (e.g., Bilton, 2021; Jäch & Delgado,
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27 2017; Mirón-Gatón et al., 2022; Ribera & Foster, 2018; Ribera & Hernando, 2019; Sabatelli
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29 et al., 2016; 2021; Porretta & Urbanelli, 2012; Urbanelli, 2002; Urbanelli & Porretta, 2008;
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31 Villastrigo et al., 2020). The genus includes more than 500 species, and members of a number
32
33 of lineages have shifted from inland waters to occupying supralittoral rockpools, including
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35 two in the western Palaearctic (Sabatelli et al., 2016; Villastrigo et al., 2019). Most
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37 *Ochthebius*, including rockpool taxa, are able to fly but their active dispersal capabilities may
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39 be limited by their small body size and weak flight (Urbanelli, 2002, unpublished
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41 observation), meaning that, like many small insects, they are likely to be largely subject to
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43 passive dispersal in air currents (see Bilton et al., 2001). Previous studies have argued for
44
45 possible sympatric speciation in rockpool *Ochthebius*, with only minute morphological
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47 differences in male genitalia but high genetic divergence based on mitochondrial markers
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49 (Antonini et al., 2010; Audisio et al., 2010). More recently, Ribera & Foster (2018) described
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51 *Ochthebius lanthanus* Ribera & Foster, 2018 from Gran Canaria, which is morphologically
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4 indistinguishable from its sister species *Ochthebius algicola* Wollaston, 1871 from Madeira,
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6 despite their estimated divergence time of ca. 14-15 MA (Villastrigo et al., 2020). Indeed,
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8 Sabatelli et al. (2016; 2021) and Villastrigo et al. (2019) have suggested that such cryptic
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10 species might be more common in these beetles, whose constrained morphology may hide a
11
12 multitude of independent evolutionary lineages. Whilst work to date suggests this may indeed
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14 be the case in rockpool *Ochthebius* (e.g., Antonini et al., 2010; Audisio et al., 2010; Urbanelli,
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16 2002; Urbanelli et al., 1996; Sabatelli et al., 2021), our understanding of the extent of this
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18 cryptic diversity and, crucially, what may drive it, remains very limited.
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23 Here we use dense geographical sampling to study supralittoral rockpool *Ochthebius*
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25 populations from the Eastern Atlantic and Western/Central Mediterranean coasts, exploring
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27 phylogeographical patterns and the distribution of major genetic lineages. We employ a
28
29 combination of mitochondrial and nuclear markers to determine the genetic diversity and
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31 phylogeographic structure of *Ochthebius*, and test whether historical and contemporary
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33 marine geographic barriers have driven genetic isolation between extant populations.
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35 Understanding the genetic diversity of these populations and their geographical subdivision
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37 sheds new light on the evolutionary biology of supralittoral rockpools and the influence the
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39 sea has on their inhabitants.
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45 **2. Material and Methods**

46 *2.1. Study area, study groups, and taxon sampling*

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48 Our study focussed on the *Ochthebius* beetles inhabiting rockpools of the Eastern Atlantic and
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50 Western/Central Mediterranean. We employed dense geographical sampling of populations in
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52 Iberia, in order to accurately characterise phylogeographic variation within *Ochthebius* as
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54 well as detect the distributional limits of genetic lineages. Members of two distinct groups of
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Ochthebius are found in rockpools in this region: the *quadricollis* species group of the subgenus *Ochthebius* s. str., the so-called ‘*Calobius*’ lineage, and the subgenus *Cobalius* (see Villastrigo et al., 2019). The former contains one species inhabiting the whole region, from the Eastern Atlantic to Central Mediterranean, *O. quadricollis* Mulsant, 1844, which overlaps with the frequently microsympatric, morphologically cryptic, *O. urbanelliae* Audisio, Trizzino & De Biase, 2010 on Italian Tyrrhenian coasts. *Cobalius* currently includes three species in the study area, *O. subinteger* Mulsant & Rey, 1861 and *O. lejolisii* Mulsant & Rey, 1861, considered endemic to Mediterranean and Atlantic coast, respectively, as well as the recently described *O. evae* Villastrigo, Hernando, Millán & Ribera, 2020. *O. evae* belongs to a newly recognised lineage within the subgenus *Cobalius* (the *biltoni* species group) whose flightless members inhabit rock crevices (Villastrigo et al., 2020) rather than pools. *Ochthebius evae* is known only from a very limited number of specimens and localities, in southern Iberia and Morocco, and as a consequence we do not consider this species further here. All known localities of the three species studied, including data gathered for this work, are mapped in Figs. S1-S3.

To understand current phylogeographical patterns and their evolutionary history, we compiled two datasets, examining different temporal and evolutionary scales. Dataset A included all currently recognised lineages within ‘*Calobius*’ and *Cobalius*, all major lineages of the tribe Ochthebiini-~~tribe~~, plus a selection of other Hydraenidae (genera *Limnebius* Leach, 1815; *Laeliaena* Sahlberg, 1900 and *Hydraena* Kugelann, 1794) and Ptiliidae taxa as outgroups. This dataset was constructed using sequences from the GenBank database (Sayers et al., 2021), across five commonly used gene markers: two fragments of cytochrome *c* oxidase subunit I, 16S RNA plus tRNA-Leu and the 5’ end of NADH dehydrogenase subunit I, an internal fragment of the large ribosomal subunit (28S), and an internal fragment of the

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4 small ribosomal unit (18S). Dataset B mostly comprised samples of the three putative species
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6 inhabiting the study area, *O. quadricollis*, *O. lejolisii* and *O. subinteger*, to which we added
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8 existing sequences from GenBank. Beetles were collected mainly during extensive field
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10 campaigns from 2016-2019 from the Atlantic and Mediterranean coasts of the Iberian
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12 Peninsula and the Balearic Islands, plus samples taken in 2016 and 2017 from Atlantic and
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14 Mediterranean coasts of Morocco. All samples were collected by hand in the field using
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16 brushes and preserved in absolute ethanol. In total, the dataset A comprised 79 individuals,
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18 whilst dataset B included 171 individuals from 57 different localities (see Table S1).
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22 Geographical information of localities is available in Table 1 and Fig. 1.
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26 27 2.2. DNA extraction and sequencing

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30 We randomly selected three specimens of each species per population to generate the novel
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32 sequences used in dataset B. In total, DNA from 160 specimens was extracted, using a non-
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34 destructive protocol following the instructions of Qiagen DNeasy Blood & Tissue Kit
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36 (Hildesheim, Germany). Specimens were subsequently dried and kept in the collection of the
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38 Aquatic Ecology research group at the University of Murcia. We amplified and sequenced
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40 two genes: i) the 3' end of mitochondrial gene cytochrome *c* oxidase subunit I (primers Pat
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42 and Jerry, Simon et al., 1994), and ii) a fragment of the nuclear gene wingless gene (primers
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44 Wg550F and WgAbRZ, Wild & Maddison, 2008). Sequences were assembled and edited
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46 using Geneious v10.1 (Kearse et al. 2012) and were deposited in the ENA database (see Table
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48 S1 for a complete list of specimens, localities and accession numbers).
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54 55 2.3. Phylogenetic analyses

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4 Sequences in dataset A were aligned using the G-INS-I algorithm implemented in MAFFT
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6 7.407 (Kato & Standley, 2013) and concatenated. PartitionFinder v2.1.1 (Lanfear et al.,
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8 2016) was used to select the optimal partition scheme and the evolutionary models based on
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10 AIC selection criteria, considering initially one partition per gene fragment. A phylogenetic
11
12 tree was reconstructed using Bayesian Inference in BEAST v1.10.4 (Suchard et al. 2018),
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14 using the partition scheme and evolutionary models selected by PartitionFinder, plus one
15
16 strict clock per partition and Yule speciation process as priors. Four different calibration
17
18 points were used in the phylogenetic analysis: i) the estimated split of Ptiliidae and
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20 Hydraenidae at ca. 165 Ma (95% confidence interval 110-195 Ma) by McKenna et al. (2019),
21
22 which is compatible with prior analyses (Hunt et al. 2007; McKenna et al. 2015); ii) the
23
24 Burmese amber fossil *Archaeodraena cretacea* Jäch & Yamamoto, 2017, likely to be a
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26 member of the crown Hydraenidae (Yamamoto et al., 2017), with a hard minimum bound of
27
28 98.79 Ma (see Shi et al. (2012)) and the split between Macaronesian endemic species setting
29
30 hard maximum bounds in uniform priors as in Villastrigo et al. (2020); iii) the separation
31
32 between the species of the Canaries and Madeira (14.3 Ma, Machado et al., 2017) and iv) the
33
34 separation between the species of the islands of Sal and Santiago in the Cabo Verde
35
36 archipelago (10.3 Ma, Cunha et al. 2017). The analysis was run for 100 million generations,
37
38 and convergence checked to measure the burn-in fraction using Tracer v1.7.1 (Rambaut et al.,
39
40 2018). The maximum clade credibility tree was reconstructed using TreeAnnotator v1.10.4
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42 included in Beast v1.10.4.

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45 Dataset B was divided into three subsets, based on each of the putative species, *O.*
46
47 *quadricollis*, *O. lejolisii* and *O. subinteger*. Owing to possible nucleotide ambiguities due to
48
49 the use of Sanger sequences in diploid organisms, haplotypes were inferred using PHASE
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51 (Stephens et al., 2001) for 1000 iterations, applying a burn-in of 100 and thinning parameter
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4 5. To interpret the biogeographical structure of rockpool *Ochthebius*, we performed a spatial
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6 analysis of molecular variation (SAMOVA; Dupanloup et al., 2002), which automatically
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8 defines clusters of populations that are homogeneous both geographically and genetically,
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10 maximizing differences amongst clusters, using SAMOVA 2.0 (available at
11
12 <http://cmpg.unibe.ch/software/samova2/>). We used a variable number of possible groups (k ,
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14 ranging from 2 to 8) per gene. We selected the homogeneous number of clusters (k) that
15
16 supported the highest increase of the FCT parameter, relative to the FCT value for $k-1$ clusters
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18 (see Dupanloup et al., 2002). Haplotype networks were estimated using TCS v1.21 (Clement
19
20 et al., 2000) with gaps as missing data and a fixed connection limit of 95%. Enhanced
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22 visualization of TCS networks was performed using tcsBU (Múrias dos Santos et al., 2016),
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24 using defined geographical areas based on features known to have driven speciation in other
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26 animal groups: the Gibraltar Strait (Lowe et al., 2012), the Almeria-Oran Front (González-
27
28 Castellano et al., 2020) and the Ibiza Channel (García-Mechán et al., 2012). In addition, we
29
30 incorporated additional putative boundaries that may promote genetic differentiation: i) the
31
32 Alboran Sea was divided into two different regions, east and west (Booth-Rea et al., 2018);
33
34 and ii) the Balearic Islands were divided into two entities corresponding with their extent
35
36 during the Last Glacial Maximum (LGM), the Pityusic and Gymnesian Islands, respectively
37
38 (Mora et al., 2017). All these barriers affect not only the connectivity of terrestrial taxa, but
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40 also drive the isolation of marine organisms due to the dynamics of oceanic currents (e.g., the
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42 Ibiza Channel separates the Iberian Peninsula and the Balearic Islands, where details of
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44 oceanic geology result in separate Northern and Balearic Currents, which in turn shape coastal
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46 wind patterns, see Pinot et al., 2002). In addition to haplotype networks, gene trees (see Figs.
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48 S4 and S5 for COI and wingless genes respectively) were constructed using the raw
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4 sequences in IQTree v2.0 (Minh et al., 2020), including bootstrap and SH-ALRT metrics
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6 (both 1000 replicates) under the preferred model selected by IQTree (Chernomor et al., 2016).
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10 11 **3. Results**

12 13 *3.1. Calibrated phylogenetic tree for rockpool Ochthebius*

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16 The selected partition scheme included one partition per mitochondrial fragment, plus an
17 additional partition for pooled nuclear genes. Three different evolutionary models were
18 preferred: i) GTR+G+I for the 3' end of cytochrome *c* oxidase subunit I and the pooled
19 nuclear genes, ii) HKY+G+I for the 5' end of cytochrome *c* oxidase subunit I, and iii)
20 TN93+G+I for 16S RNA. The resulting phylogenetic tree (Fig. 2) revealed a similar topology
21 to that in a recent revision of Ochthebiini (Villastrigo et al., 2019), but with different
22 estimated ages for the study groups. *Cobalius* and 'Calobius' were estimated as originating
23 ca. 30 Ma and ca. 25 Ma respectively, whilst Villastrigo et al. (2019) estimated older ages (ca.
24 60 and ca. 55 Ma respectively). As expected, we verified that *O. subinteger* and *O. lejolisii*
25 are sister species that diverged ca. 14 Ma from a common ancestor. Each of the putative
26 species studied was divided into distinct, divergent lineages, of considerable apparent age: 1)
27 *O. quadricollis* originated ca. 19 Ma, with three topological splits detected at ca. 17, 7 and 2
28 Ma respectively; 2) *O. subinteger* originated at 14 Ma, with a single topological split at ca. 6
29 Ma; and 3) *O. lejolisii* originated 14 Ma, with two relatively recent splits at ca. 2 and 1 Ma
30 respectively.
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51 52 *3.2. Intraspecific genetic variability*

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55 We detected moderate genetic variability within each of the studied lineages (Table 2); >6.5%
56 for the 3' end of cytochrome *c* oxidase subunit I in two out of three studied whilst *O. lejolisii*
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4 displays variability >3%. Variations in the nuclear wingless gene were moderate, in line with
5 expected lower divergence of nuclear genes, reaching 2.47% in *O. quadricollis*, 1.27% in *O.*
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9 *subinteger* and 1.29% in *O. lejolisii*. We observed high haplotype diversity for both COI and
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11 wingless genes (greater than 0.7 - see Table 2), together with relatively low nucleotide
12
13 diversity (especially for wingless - see Table 2).
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16 In SAMOVA (Table 3; see Tables S2-S4 for details on *O. quadricollis*, *O. subinteger* and
17
18 *O. lejolisii* respectively), we found a considerable increase in FCT values when 3 clusters
19
20 were defined for *O. quadricollis* (Fig. S6) and *O. lejolisii* (Fig. S7) for both mitochondrial and
21
22 nuclear genes, and a more complex pattern in *O. subinteger*, with 4 homogeneous clusters
23
24 with COI but only 3 for wingless (Fig. S8). Detailed results of analyses for each species are
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26 now presented in turn.
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32 3.3. *Ochthebius quadricollis* geographic structure and haplotype networks

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34 The best fitted geographical clusters based on SAMOVA (Fig. S6) revealed a common
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36 scenario for both genes, with a geographical barrier roughly corresponding with the Ibiza
37
38 Channel and a second barrier where the Alboran Sea is divided into western and eastern
39
40 basins, based on anticyclonic oceanic gyres. These barriers match the two oldest topological
41
42 splits within *O. quadricollis*, separating three different lineages located on the Atlantic coast
43
44 and the western Alboran Sea, the eastern Alboran Sea up to the Ibiza Channel, including the
45
46 Balearic Islands, and on coasts north of the Ibiza Channel, including France, Italy and Malta.
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48 These same clusters can be seen in the haplotype network for the wingless gene (Fig. 3). In
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50 contrast, the mitochondrial network comprises four disjointed clusters, in which populations
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52 from southeastern Iberia are grouped either with those from the eastern Alboran Sea or with
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54 those from the Balearic Islands. In all the analyses, samples from the Balearic Islands were
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4 associated with populations located south of the Ibiza Channel. Detected geographical barriers
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6 did not include either the Gibraltar Strait or the Almeria-Oran front, with either approach.
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10 11 3.4. *Ochthebius subinteger* geographic structure and haplotype networks 12

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14 *O. subinteger* was found in coastal rockpools in the Central Mediterranean basin plus the
15 eastern coast of Spain up to Cartagena. The restricted geographical distribution of this species
16 in the study region (Fig. S2) limited the number of samples, and so the estimated number of
17 SAMOVA clusters auto-generated using the wingless gene. As with *O. quadricollis*,
18 populations north and south of the Ibiza Channel were separated in the best-fitted scenarios
19 generated by SAMOVA (clusters with $k = 4$ for COI and $k = 3$ for wingless; Fig. S7). This
20 split is also reflected in the haplotype networks (Fig. 4), which also exhibit a notable but
21 unexpected geographical structure, with a split between different populations on the Balearic
22 Islands. Beetles from the Pityusic islands - the ones closest to the Iberian Peninsula - are
23 associated with populations from south-eastern Spain, whilst samples from the Gymnesian
24 islands (i.e., Menorca) are distributed in both networks. The COI of one Menorcan specimen
25 clustered with sequences from north of the Ibiza Channel, whilst the wingless sequence was
26 identical to some from specimens south of this channel.
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46 3.5. *Ochthebius lejolisii* geographic structure and haplotype network 47

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49 The putative species with the broadest geographical distribution is also the one with the most
50 complex genetic structure. Traditionally believed to be an endemic Atlantic taxon, *O. lejolisii*
51 was detected here for the first time in the Mediterranean, in multiple localities throughout
52 south Spain up to the Ibiza Channel. SAMOVA results (Fig. S8) were consistent with 3
53 clusters in both mitochondrial and nuclear genes, but with no consistent pattern, except the
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4 separation of European Atlantic populations from the rest, with a mixture of Mediterranean
5 and African populations in the case of the mitochondrial marker, whilst the nuclear wingless
6 gene separates European and African populations at Gibraltar Strait. Haplotype networks
7 (Fig. 5) also suggest a complex history, but with COI also revealing a separation of samples
8 from the European Atlantic coast. In the case of wingless, samples from the Atlantic coast of
9 Morocco are separated from those in the Mediterranean, whilst with COI Moroccan samples
10 closest to the Iberian Peninsula (Larache, Asilah and Cap Spartel) are more similar to those in
11 the Mediterranean than others in the Atlantic.
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25 **4. Discussion**

26
27 Our study reveals the presence of cryptic lineages, often with high genetic and haplotype
28 divergence and marked phylogeographic structure in coastal *Ochthebius* populations in the
29 Atlanto-Mediterranean region. Whilst previous studies have also suggested that these
30 rockpool beetles harbour considerable cryptic diversity (e.g., Sabatelli et al., 2016, Villastrigo
31 et al., 2019; 2020), ours is the first investigation to employ detailed enough sampling to
32 identify the drivers of such diversity. In all three species, a combination of historical and
33 contemporary marine hydrogeography appears to shape the distribution of lineages in these
34 small and flying insects, whose diversification has occurred over a range of spatial and
35 temporal scales.
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51 *4.1. Calibrated phylogenetic tree*

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53 Our calibrated molecular phylogeny reflects the most up-to-date information on the origin and
54 evolution of ‘*Calobius*’ and *Cobalius*. This phylogeny is highly congruent in both topology
55 and timing with previous phylogenetic studies of *Cobalius* (Villastrigo et al., 2020), but
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4 suggests slightly older divergence times than those proposed by Sabatelli et al. (2021). Our
5 analyses also suggest a very different age estimate for ‘*Calobius*’ than previous studies (17
6 Ma - Sabatelli et al., 2016; 55 Ma - Villastrigo et al., 2019). These differences likely result
7 from the previous use of less precise calibrations (Sabatelli et al., 2016, 2021; Villastrigo et
8 al., 2019), limited taxon sampling (Sabatelli et al., 2016) and the absence of comprehensive
9 phylogenies to contextualise the calibration (Sabatelli et al., 2021). Whilst Sabatelli et al.
10 (2016; 2021) employed sequence divergence rates estimated for a closely related family and
11 Villastrigo et al. (2019) used a secondary calibration point, we opted for an integrative
12 approach including all major lineages within the genus *Ochthebius*, and not only secondary
13 calibration points but also one fossil and two biogeographic events to set hard age constraints
14 (e.g. the geological origin of some Macaronesian islands, see Machado et al. 2017; Cunha et
15 al. 2017), all approaches likely to result in more accurate phylogenetic dating.
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34 4.2. *Phylogeographic structure and geographical patterns*

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37 We consistently detected a geographical barrier in the region of the Ibiza Channel for species
38 inhabiting both sides (*O. quadricollis* and *O. subinteger*). We also found that *O. lejolisii*
39 reaches, but does not pass the Ibiza Channel, the first time this species, long considered an
40 Atlantic endemic, has been detected in the Mediterranean. The location of the Ibiza Channel
41 barrier coincides with the area where the Northern Current and the Balearic Front are
42 responsible for most of the surface-flow characteristics of the Balearic Sea. In all cases, the
43 cluster of populations from south of the Ibiza Channel also includes those from Denia and
44 Jávea, two localities situated in the Ibiza Channel itself. Oceanic currents in the Channel
45 region are known to influence the phylogeography of a number of fully marine taxa (Schunter
46 et al., 2011; García-Mechán et al., 2012). In the Ibiza Channel, northwestern Mediterranean
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4 water is redirected northwards, following the Balearic coast, with just a small fraction of the
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6 current actually crossing the channel itself (El-Geziry & Bryden, 2010). The Ibiza Channel
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8 may shape *Ochthebius* lineage structure through associated coastal wind currents determining
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10 their passive dispersal (Arribas et al., 2012) and/or dispersal of individuals of these salinity-
11
12 tolerant beetles in marine currents themselves (see e.g., Niikura et al., 2015). The degree to
13
14 which the Ibiza Channel could serve as a geographical barrier in this way will vary
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16 seasonally; whilst the Northern Current is affected by an anticyclonic gyre for almost the
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18 whole year, limited flow does occur from north to south during winter (Pinot et al, 2002). An
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20 additional explanation for the genetic structuring detected around this region may involve the
21
22 scarcity of suitable habitat on the Iberian coast north of the Ibiza Channel, where rocky
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24 coastline gives way to a sandy coast for around 250 km up to the Ebro Delta. The Ibiza
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26 Channel may reduce or completely block gene flow, depending on the season, and the
27
28 absence of suitable habitats in adjacent mainland Iberia enhance the isolation of lineages.
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30 Previous studies, whilst much less extensive than ours, have also suggested genetic
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32 differences in *O. quadricollis* populations in this region. Sabatelli et al. (2016) found that
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34 populations from Calpe (southern coast of the Ibiza Channel) represent a different molecular
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36 entity to those from Italy. Villastrigo et al. (2019; 2020) also detected considerable genetic
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38 variation in *O. quadricollis*, across specimens from the Atlantic coast and Mediterranean
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40 coasts of Spain, France and Italy.
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48 The estimated timescale of lineage divergence in *O. subinteger* suggests a role for the
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50 Messinian Salinity Crisis (MSC) (e.g., García-Castellanos et al., 2009; Krijgsman et al., 1999)
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52 in establishing present genetic diversity patterns. As well as separating the Mediterranean and
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54 Atlantic basins, the MSC also connected the Balearic Islands with the Iberian Peninsula (Mas
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56 et al., 2018), preventing gene flow between mainland Iberian populations spanning this
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4 region, but favouring it between the Iberian Peninsula and the present-day Balearic Islands.
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6 This situation ended in the Zanclean Flood ca. 5.33 MA, when Balearic populations were
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8 once again isolated from the mainland. This pattern of connectivity and isolation is reflected
9
10 in endemic Balearic species whose closest relatives inhabit the Iberian Peninsula, including
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12 some *Ochthebius* species (Jäch, 2000) and other animal groups as diverse as spiders (Mora et
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14 al., 2017) and mammals (Torres-Roig et al., 2019).
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18 Evidence for the impact of a second geographical barrier was detected only in *O.*
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20 *quadricollis*, where populations from the Atlantic coast and western Alboran Sea were
21
22 separated from the rest. The Alboran Sea is divided by two surface water gyres, whose
23
24 interface may operate as a barrier. Atlantic Ocean waters enter the Mediterranean through the
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26 Gibraltar Strait, where their surface component becomes trapped in the permanent western
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28 Alboran gyre (García-Ladona, 2017; Maggi and González-Wangüemert, 2015). This barrier
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30 coincides with a possible long-term marine refuge for Mediterranean species that provided
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32 suitable habitat during the MSC (Booth-Rea et al., 2018), reconnecting with the Atlantic coast
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34 after the opening of the Gibraltar Strait ca. 5.3 MA (Andújar et al., 2012). The evolutionary
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36 history of *O. quadricollis* s. lat. spans approximately 20 million years, with two topological
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38 splits at ca. 17 and ca. 7 MA, respectively. The first split involves isolation of central
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40 Mediterranean basin populations from western ones. The second, more recent, split may be
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42 related to the MSC, during which populations in the Alboran Sea and the rest of the Iberian
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44 Peninsula would have been isolated in various “patches” or sub-basins within the
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46 Mediterranean. Such isolation would have driven genetic differentiation, with the subsequent
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48 refilling of the Mediterranean contribution to the current phylogeographic structure through
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50 recolonization of coastlines.
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4 This study revealed the presence of *O. lejolisii* in the Mediterranean for the first time,
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6 this supposed Atlantic species reaching the Ibiza Channel. The timing of colonization of the
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8 Mediterranean by this species is unclear, but recent invasion has been suggested for some
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10 coastal taxa such as the gastropod *Nassarius reticulatus* Linnaeus, 1758 (Albaina et al., 2012).
11
12 *O. lejolisii* and *O. subinteger*, were previously thought to be allopatric, separated by some
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14 barrier in southern Spain (e.g., the Gibraltar Strait). Our results instead demonstrate that the
15
16 two species overlap in part of their distributional range and may indeed occur in true
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18 sympatry in southeastern Spain. Both species were found at one of our study sites (Cala
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20 Reona, Murcia), but, interestingly, in different years (*O. subinteger* in 2016 and *O. lejolisii* in
21
22 2019). These two species are estimated to have diverged ca.15 MYA, but despite their
23
24 considerable genetic divergence we were not able to detect any unambiguous morphological
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26 characters by which they could be distinguished. Jäch (1989) in his morphological revision of
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28 *Cobalius* separated *O. lejolisii* and *O. subinteger* on pronotal punctuation. These differences
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30 separate specimens from Atlantic versus Mediterranean rockpools but not specimens of the
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32 two lineages collected in the Mediterranean.
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39 As previously highlighted, a key trait underlying the genetic structure of coastal
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41 *Ochthebius* populations is their dispersal capabilities. Our results suggest that passive
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43 dispersal through coastal winds and/or marine currents may be the main way in which these
44
45 insects disperse over medium to large spatial scales. Long-distance dispersal by wind currents
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47 is recognised as an important factor in the colonization dynamics of small arthropods
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49 (Gillespie et al., 2012, Beltrán Epele et al., 2021), including many aquatic taxa (Bilton et al.,
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51 2001). In coastal *Ochthebius*, another factor that may promote gene flow amongst populations
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53 is the action of cyclones (Lionello et al., 2006). In such conditions, supratidal environments
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55 are directly exposed to tidal inundation, and resistant stages, including eggs, can be dispersed
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4 in ocean currents, as has been reported in springtails (McGaughran et al., 2010) and mites
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6 (Pfungst, 2013). Authors' unpublished observations have revealed that all life history stages of
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8 *O. lejolisii* are able to survive for weeks across a wide range of salinities, including that of
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10 oceanic water.
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13 Overall, our results demonstrate that surface ocean currents, and their associated
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15 winds, may fundamentally shape the evolutionary history of non-strictly marine inhabitants of
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17 the coastal zone. We establish the presence of strongly divergent cryptic lineages in two of
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19 our study species, moderate divergent cryptic lineages in the third, and show that the
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21 distribution of these lineages appears to have been shaped by a combination of historical and
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23 contemporary changes to the coastal seascapes of the Atlantic Ocean and the Mediterranean
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25 Sea. Whilst we refrain from formal species descriptions at present, it seems highly likely that
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27 the lineages we detect correspond to cryptic species, whose distribution should be considered
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29 in biodiversity assessments of the coastal zone.
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38 **Data Accessibility**

39 DNA sequences generated during this study are uploaded to the ENA and GenBank
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41 databases. Accession numbers are available in Table S1.
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31 **Figure captions**

32
33 Figure 1. Distribution of sampling localities (major figure) and main surface marine currents
34 and potential geographic barriers to dispersal (top-right corner).
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36
37 Figure 2. Calibrated phylogenetic tree obtained with BEAST v1.10.4 of *Ochthebius* with
38 focus in *Cobalius* subgenus and *quadricollis* species group (former ‘Calobius’ subgenus).
39 Numbers in nodes represent posterior probabilities. Calibrations points used in analysis are
40 specified by grey dots.
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43 Figure 3. Haplotype network for *Ochthebius (Ochthebius) quadricollis*. Colours represent the
44 main geographic areas as indicated in the legend.
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47 Figure 4. Haplotype network for *Ochthebius (Cobalius) subinteger*. Colours represent the
48 main geographic areas as indicated in the legend.
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51 Figure 5. Haplotype network for *Ochthebius (Cobalius) lejolisii*. Colours represent the main
52 geographic areas as indicated in the legend.

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54 Figure S1. Currently known localities in which the presence of *Ochthebius (Ochthebius)*
55 *quadricollis* is confirmed.

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57 Figure S2. Currently known localities in which the presence of *Ochthebius (Cobalius)*
58 *subinteger* is confirmed.
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4 Figure S3. Currently known localities in which the presence of *Ochthebius (Cobalius) lejolisii*
5 is confirmed.
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8 Figure S4. Phylogenetic tree for the COI gene obtained with IQTree for the studied samples.
9 Numbers in nodes represent support measures (ultrafast bootstrap/SH-like aLRT support).
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12 Figure S5. Phylogenetic tree for the wingless gene obtained with IQTree for the studied
13 samples. Numbers in nodes represent support measures (ultrafast bootstrap/SH-like aLRT
14 support).
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17 Figure S6. Best fitted geographic clusters based on SAMOVA for *Ochthebius (Ochthebius)*
18 *quadricollis* for the COI and wingless genes.

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20 Figure S7. Best fitted geographic clusters based on SAMOVA for *Ochthebius (Cobalius)*
21 *subinteger* for the COI and wingless genes.
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24 Figure S8. Best fitted geographic clusters based on SAMOVA for *Ochthebius (Cobalius)*
25 *lejolisii* for the COI and wingless genes.
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29 **Table captions**

30
31 Table 1. Detailed information on sampling localities, including the number of specimens used
32 for each lineage.

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34 Table 2. General genetic statistics (N = number of samples, h = number of haplotypes, Hd =
35 haplotype diversity, π = nucleotide diversity, Max. Div. = maximum sequence divergence)
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38 Table 3. Distribution of genetic diversity as inferred by analysis of molecular variance (K =
39 best-fitted number of homogeneous groups defined by SAMOVA2)

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41 Table S1. List of material used for molecular analyses, including details of vouchers,
42 localities, collectors, dataset in which they were used and accession numbers. In bold,
43 sequences newly generated for this work.
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46 Table S2. SAMOVA2 detailed information for *Ochthebius (Ochthebius) quadricollis*,
47 including information on the number of homogeneous groups (K), the genetic variability
48 among populations within groups (FSC), among populations (FST), among groups (FCT), and
49 the increase of FCT relative to the FCT value for k-1 clusters (Δ FCT).

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51 Table S3. SAMOVA2 detailed information for *Ochthebius (Cobalius) subinteger*, including
52 information on the number of homogeneous groups (K), the genetic variability among
53 populations within groups (FSC), among populations (FST), among groups (FCT), and the
54 increase of FCT relative to the FCT value for k-1 clusters (Δ FCT).
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57 Table S4. SAMOVA2 detailed information for *Ochthebius (Cobalius) lejolisii*, including
58 information on the number of homogeneous groups (K), the genetic variability among
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4 populations within groups (FSC), among populations (FST), among groups (FCT), and the
5 increase of FCT relative to the FCT value for k-1 clusters (Δ FCT).
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For Review Only

Table 1. Detailed information of sampling localities, including the number of specimens used for each lineage.

#	Location	Latitude	Longitude	COI			Wingless		
				<i>quadricollis</i>	<i>subinteger</i>	<i>lejolisia</i>	<i>quadricollis</i>	<i>subinteger</i>	<i>lejolisia</i>
1	Saint Jean de Luz, France	43.4033	-1.6582			1			
2	Ibarranguelua, Spain	43.4107	-2.6547			1			
3	Rinlo, Spain	43.5592	-7.1055			1			
4	Nigrán, Spain	42.1419	-8.8063	1		1			
5	Luz, Portugal	37.0756	-8.7502	1					
6	Isla de Tarifa, Spain	36.0019	-5.6124	3		3	3		3
7	Cala Milla de Plata, Spain	36.3065	-5.2602	3		3	3		3
8	Nerja, Spain	36.7409	-3.8841	3		3	3		3
9	Cala de Maro, Spain	36.7529	-3.8465			1			
10	Velilla, Spain	36.7449	-3.6616	3		3	3		3
11	Cala Rijana, Spain	36.7092	-3.3923	3		3	3		3
12	El Playazo, Spain	36.8628	-2.0036	3		3	3		3
13	Cala de las Conchas, Spain	37.2841	-1.7310	3		3	3		3
14	Pozo del Esparto, Spain	37.3190	-1.7001	3		2	2		1
15	Cabo Cope, Spain	37.4372	-1.4840	3		3	2		2
16	Cala de las Pulgas, Spain	37.4678	-1.4712	3		3	3		3
17	Percheles, Spain	37.5293	-1.3795	3		3	3		3
18	Cala Reona, Spain	37.6173	-0.7130	4	3	3	3	2	3
19	Punta del Cocedor, Spain	37.7504	-0.7281	4		3	3		3
20	Cabo de Santa Pola, Spain	38.1948	-0.5173	3		3	3		3
21	La Illeta, El Campello, Spain	38.4316	-0.3807	3		3	3		3
22	Calpe, Spain	38.6399	0.0679	1					
23	Moraira, Spain	38.6849	0.1252	3		3	3		3
24	Denia, Spain	38.8335	0.1385	3	3		3	3	
25	Sant Carles de la Rapita, Spain	40.5816	0.5564	3	3		3	3	
26	Cala de Sant Francesc, Spain	41.6793	2.8084	3			3		
27	Sant Feliu de Guixols, Spain	41.7711	3.0300		1				
28	L'Escala, Spain	42.1181	3.1536		3			3	
29	Plateau de Malmousque, Marseille, France	43.2804	5.3479	1	1				

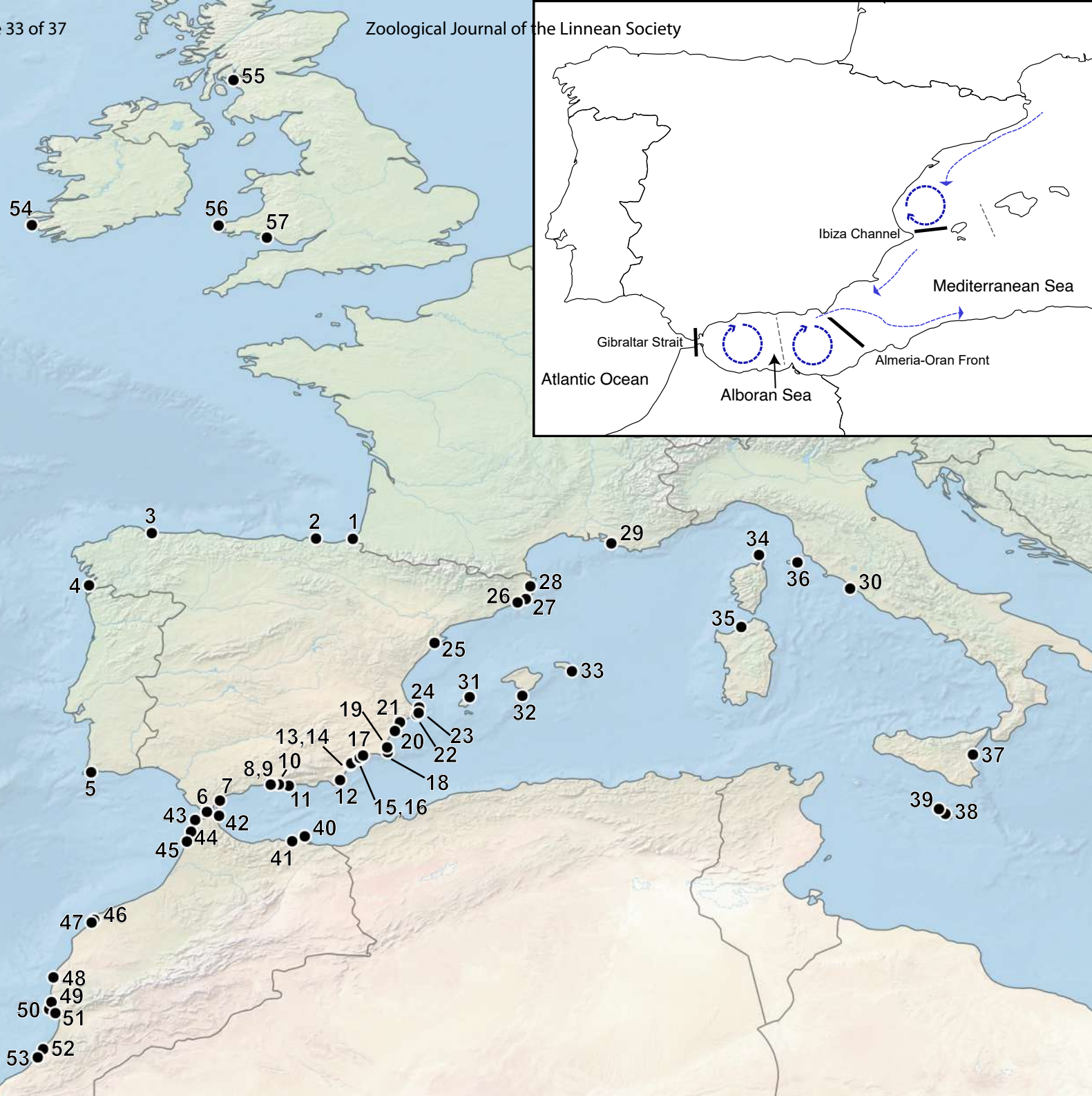
Table 2. General genetic statistics (N = number of samples, h = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, Max. Div. = maximum sequence divergence)

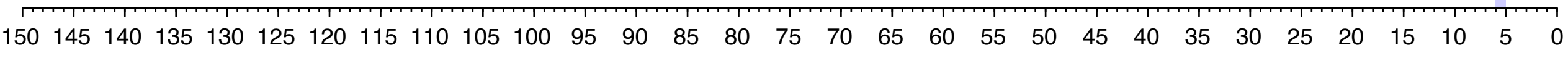
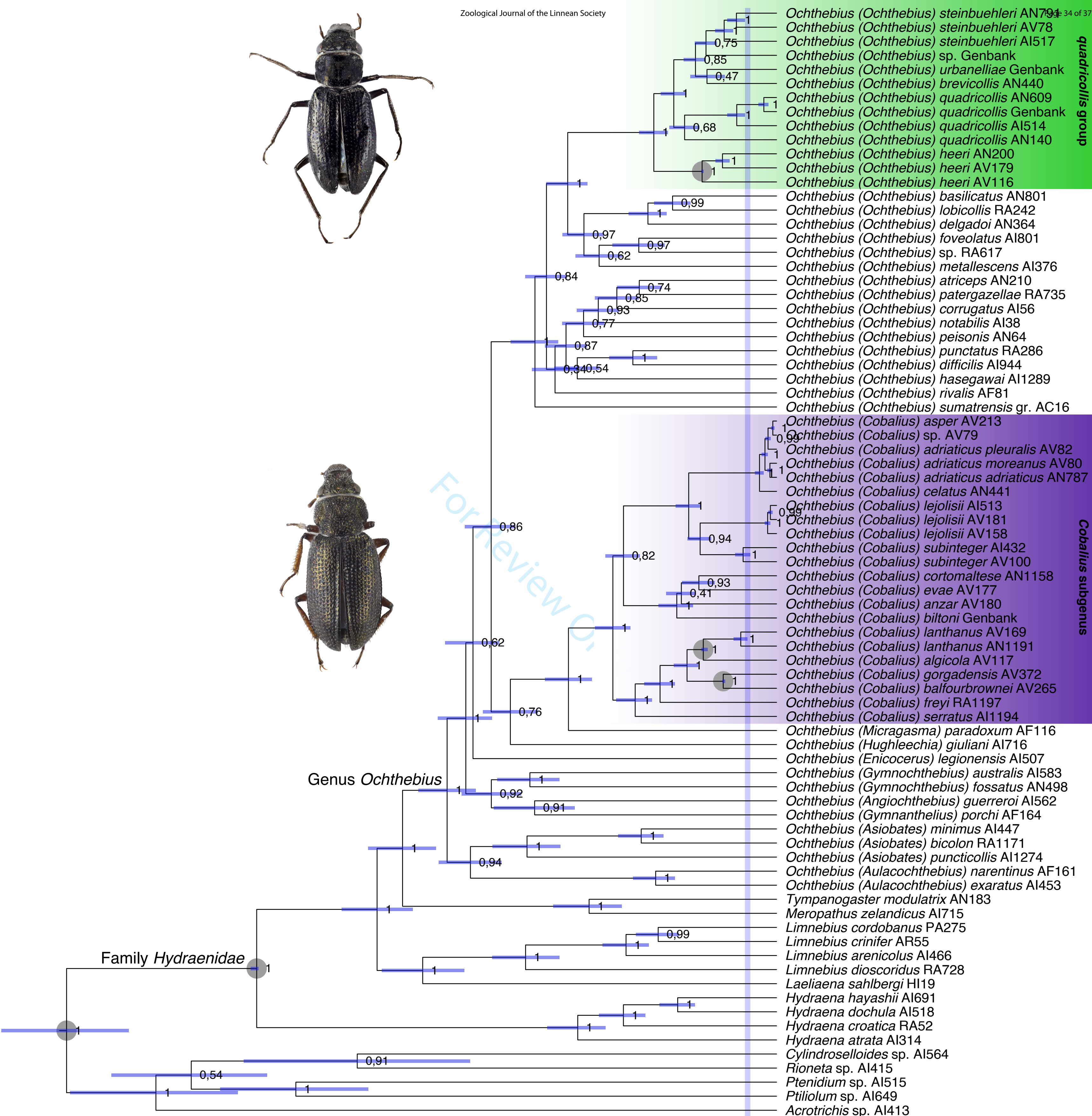
	N	Localities	COI				Wingless			
			h	Hd	π	Max. Div.	h	Hd	π	Max. Div.
<i>O. quadricollis</i>	84	41	46	0.964	0.04431	8.20%	17	0.875	0.00852	2.47%
<i>O. lejolisii</i>	65	34	29	0.887	0.00891	3.26%	14	0.727	0.00334	1.29%
<i>O. subinteger</i>	22	10	17	0.974	0.03543	6.64%	7	0.808	0.00453	1.27%

Table 3. Distribution of genetic diversity as inferred by analysis of molecular variance (K = best-fitted number of homogeneous groups defined by SAMOVA2)

Species	Gene	K	Among groups (FCT)	Among populations within groups (FSC)	Within populations (FST)
<i>O. quadricollis</i>	COI	3	0.80803	0.78152	0.95806
	Wingless	3	0.75466	0.62258	0.90740
<i>O. lejolisii</i>	COI	3	0.85110	0.79014	0.96875
	Wingless	3	0.67995	0.40891	0.81082
<i>O. subinteger</i>	COI	4	0.83428	0.10928	0.85239
	Wingless	3	0.79792	0.27489	0.85347

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