Integrative taxonomy and conservation of cryptic beetles in the Mediterranean region (Hydrophilidae)

Paula Arribas, Carmelo Andújar, David Sánchez-Fernández, Pedro Abellán & Andrés Millán

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Because biodiversity inventory forms the basis for the effective conservation of species and habitats, there is an imperative need to discover and describe new species. A significant part of presently unknown biodiversity is constituted by cryptic species complexes, where traditional taxonomy usually fails due to a lack of clear taxonomic characters in the external structures of specimens. Integrative taxonomy offers a powerful tool to shed light on this part of encrypted biodiversity, combining multiple operational criteria in an evolutionary context in order to delineate species boundaries. The present study used an integrative approach to explore the species boundaries in a water beetle complex (*Enochrus falcarius* species complex) inhabiting saline streams, a rare and threatened habitat across the Mediterranean region. First, hypotheses about the candidate species on the basis of phylogenetic analyses and biogeographical information were proposed. Second, lineage divergence was evaluated between candidate species using (i) molecular cluster delimitation, (ii) morphometry (both linear body morphometrics and pronotum outlines) and (iii) ecological niche similarity estimates. We found divergence between candidate species on the basis of molecular, biogeographical and niche information, and consequently, four species were delimited within the *E. falcarius* complex (i.e. *Enochrus jesusarribasi* sp. n., *Enochrus blazquezae* sp. n., *Enochrus risii* sp. n. and *Enochrus falcarius*), despite the fact that they showed high morphological similarity. *Enochrus falcarius*, as previously considered, had not been proposed to be of conservational concern, because until now, it had been regarded as a single broadly distributed species in the Mediterranean region. However, the four entities here delimited within this species complex displayed characteristics that categorised them as vulnerable taxa. Hence, these results show how applying integrative taxonomy approaches and rapid vulnerability assessments to lineages from threatened habitats with the potential to comprise cryptic diversity could become a fundamental tool for biodiversity conservation, driving the discovery of cryptic species and consequently the modification of previous, inadequately evaluated vulnerability categorisations.

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Introduction

The species is one of the fundamental units of biology (Mayr 1982; De Queiroz 2005a), and its conceptualisation and delimitation are central for the development of systematic biology, but also primary for analysis in biogeography, ecology and evolution and especially relevant for disciplines like conservation biology and biodiversity research (Caldecott *et al.* 1996; Agapow *et al.* 2004; Padial

& De la Riva 2006). Although evolutionary biologists mostly agree that species are separately evolving metapopulation lineages (sensu Wiley 1978), they tend to disagree in adopting different properties acquired by lineages during the course of divergence (Mayden 1997; De Queiroz 1998; Naomi 2011). De Queiroz (1998, 2005b, 2007) attempted to resolve this controversy by dividing both the theoretical species definition and secondary properties used in the species delimitation process. He proposed that evolutionary independence is a species' only necessary attribute, and considered the other attributes as different lines of evidence (operational criteria) relevant in assessing lineage delimitation. This perspective has promoted the emergence of an integrative taxonomy that brings together new theories and methods to delimit species using an objective and rigorous framework (Dayrat 2005; Padial et al. 2010).

Morphology, DNA sequences, geographical distributions and ecological niches are recognised as some of the most relevant and useful operational criteria used to assess lineage divergence (e.g. DeSalle et al. 2005; Padial et al. 2010; Schlick-Steiner et al. 2010). For practical reasons, morphology has been historically considered as a primary operational criterion to delimit species (Dayrat 2005). The majority of species delimitation studies rely entirely on traditional comparative morphology, but other techniques, such as morphometrics and image analyses, have arisen to provide statistical rigour, also allowing to detect hidden divergence among groups (Becerra & Valdecasas 2004; Mutanen & Pretorius 2007). Nevertheless, applying these techniques into species delimitations has been limited until recently, particularly regarding the integrative taxonomy approaches (however see Leaché et al. 2009; Gebiola et al. 2012).

On the other hand, the increasing availability of DNA sequence data has given biologists a new tool for detecting and differentiating morphologically similar species. DNA sequences provide a large number of characters that are readily comparable among entities to assess the evolutionary independence of putative new taxa (Vogler & Monaghan 2007; Cardoso et al. 2009). However, recent studies have revealed the shortcomings of exclusively using molecular taxonomy (e.g. Wheeler 2004; Bond & Stockman 2008; Sauer & Hausdorf 2011), and as a result, it would be preferable to combine DNA sequence data with additional criteria for species delimitation.

The degree of ecological interchangeability is also considered as a decisive taxonomic character for species delimitation, as it represents an important stage of the lineage separation process (Templeton 2001; Wiens et al. 2010). Collecting life history data is usually problematic and time-consuming, which limits the use of ecological niche information on species delimitation (Hawlitschek et al. 2011). Ecological niche modelling and niche divergence tests offer a novel way to visualise and statistically analyse ecological divergence between organisms (Wiens & Graham 2005; Warren et al. 2008), and so, the potential of such analyses on systematic biology research is broadly recognised (e.g. Raxworthy et al. 2007; Rissler & Apodaca 2007).

The lack of knowledge concerning how many and what kinds of species exist has been denominated the *Linnaean shortfall* (Brown & Lomolino 1998; Whittaker et al. 2005). Because a vast part of the Earth's biodiversity has been undisclosed until now, there is an imperative need to discover and describe species (Mace 2004; Padial et al. 2010), primarily for groups like insects that suffer an enormous *taxonomic deficit* (i.e. the ratio of expected taxa vs. named taxa; Lambshead 1993). Additionally, a significant part of this unknown biodiversity is constituted by cryptic species complexes, where comparative morphology usually fails because no clear taxonomic characters are found in the specimens' external structures (Bickford et al. 2007). In this sense, integrative taxonomy has become a powerful tool to shed light on this important portion of encrypted biodiversity, combining multiple operational criteria in an evolutionary context to delineate species boundaries (Schlick-Steiner et al. 2010).

The identification and description of cryptic species have important implications for biodiversity conservation. For instance, the existence of cryptic taxa in particularly endangered regions and habitats could result in significant biodiversity losses, because this undetected diversity is lacking a proper vulnerability evaluation and protection (Bickford et al. 2007; Trontelj & Fiser 2009). In accordance with this and stressed by accelerated extinction rates, the prioritised application of integrative taxonomy approaches to lineages from threatened habitats with potential to comprise cryptic diversity could help to preserve a high number of endangered species (e.g. Witt et al. 2006; Condon et al. 2008; Vieites et al. 2009).

The present study uses an integrative approach that explores species boundaries in a complex of habitat-specialist water beetles (*Enochrus falcarius* species complex) from the Mediterranean region. *Enochrus falcarius* Hebauer 1991 has been traditionally viewed as a single species occurring in saline streams across the western Mediterranean (Schödl 1998; Hansen 1999). In this region, saline and hypersaline streams are uncommon, broadly threatened habitats that contain communities particularly rich in rare or endemic species, especially aquatic Coleoptera (Sánchez-Fernández et al. 2008; Millán et al. 2011). While several other species of water beetles occurring in these habitats have been identified as globally endangered (see

e.g. Abellán *et al.* 2005; Verdú & Galante 2006; Sánchez-Fernández *et al.* 2008; Bennas *et al.* 2009; Verdú *et al.* 2011), *E. falcarius* was not believed to be of conservational concern because it had been regarded as a single broadly distributed Mediterranean species. However, a recent study has revealed that *E. falcarius* (as currently understood) comprises four lineages, each with restricted and disjunct geographical distributions (Arribas *et al.* 2012b).

In the present study, we first propose hypotheses about the candidate species included in the *E. falcarius* species complex on the basis of phylogenetic and biogeographical information. Second, lineage diversification of the proposed entities is tested using DNA grouping, morphometrics and niche similarity measurements. Finally, the degree of vulnerability of each of the delimited species within *E. falcarius* complex is assessed to know whether they have enough risk features to be considered as endangered taxa. In this sense, the case of the *E. falcarius* species complex could be paradigmatic in terms of how the failure of traditional morphology-based taxonomy could lead to important biodiversity losses and how integrative taxonomy together with rapid vulnerability assessments could be a window on the conservation of endangered cryptic species.

Methodology

Two main steps were taken in the process of species delimitation: first, basal lineages were identified and hypotheses about the candidate species were proposed, and second, divergences in operational criteria were assessed between these lineages delineated as candidate species (Bond & Stockman 2008; Schlick-Steiner *et al.* 2010).

Delimitation of the candidate species

Sequences of three mitochondrial (3′ end of cytochrome *c* oxidase subunit I, *cox1*; an internal fragment of cytochrome *b*, *cob*; 3′ end of the large ribosomal unit plus Leucine transferase and the 5′ end of NADH dehydrogenase subunit 1, *rrn*L + *trn*L + *nd1*) and one nuclear gene (an internal fragment of the large ribosomal unit, *LSU*) for specimens of *E. falcarius* and related species were obtained from two previous studies (Arribas *et al.* 2012a,b; see Tables S1 and S2). The samples comprised 55 specimens from 43 localities in the western Mediterranean and covered the entire known range of *E. falcarius*, including populations from the Iberian Peninsula, Sicily, Tunisia and Morocco (Fig. 1, Table S1). Specimens of the other species within the same species group in the region (i.e. *Enochrus bicolor* Fabricius and *Enochrus segmentinotatus* Kuwert; Schödl 1998) and several species of *Enochrus* in the same subgenus (*Lumetus* Zaitzev) were also included. *Enochrus natalensis* (Gemminger & Harold) from the subgenus *Methydrus* Rey was used as outgroup.

Bayesian (BI) and maximum-likelihood (ML) analyses were conducted on the combined sequence matrix using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and RAxML 7.0.3 (Stamatakis 2006), following the same methodology and parameters as in Arribas *et al.* (2012b).

Topology tests were performed to assess the support of the paraphyletic nature of *E. falcarius*. The statistical significance of the likelihood difference between the best previously obtained topology and the best topology with a monophyletic *E. falcarius* grouping as estimated using same parameters and constraining such monophyly in RAxML was tested using SH and AU tests in the program Consel (Shimodaira & Hasegawa 2001).

Finally, we also used the uncorrected p-distances of *cox1* data set to study the genetic divergences and perform fast distance-based clustering of the data (for an example with water beetles see Hendrich *et al.* 2010). We checked clustering of sequences at different preset thresholds using SpeciesIdentifier module of TaxonDNA software v.1.6.2 (Meier *et al.* 2006). SpeciesIdentifier recognises *a priori* delineated species from the sequence name and summarises the number of clusters found under each threshold, the number of clusters that contain only one species name (perfect clusters i.e. monophyletic *a priori* species), the number of split clusters (one species split into more than one cluster, i.e. paraphyletic *a priori* species) and lumped clusters (more than one species name in a cluster; for details, see Meier *et al.* 2006). Therefore, this test was first used as an additional support for the delimitation of candidate species and second as a first step of the candidate species evaluation, once it was repeated including the proposed candidate species.

DNA grouping based on the coalescent theory

Additional BI analyses were conducted in Beast v.1.6.1 (Drummond & Rambaut 2007) to obtain ultrametric trees. A relative root age of 100 (Normal distribution, Mean = 100, SD = 0.1) and an uncorrelated lognormal clock were set. Four independent runs of 25 million generations were performed, sampling every 1000 generations, partitioning by gene and using the best-fitting evolution model for each partition, with ten gamma categories when required. Posterior probabilities of nodes were used as support values. Beast analyses were computed on CIPRES portal (Miller *et al.* 2010).

The generalised mixed Yule coalescent (GMYC) method (Pons *et al.* 2006) was applied to delimit clusters on the basis of phylogenetic DNA information (i.e. ML entities, hereafter ML entities). This tree-based method identifies the threshold for the transition from a neutral coalescent model of population differentiation (intraspecific scope) to species diversification under a Yule pure birth model
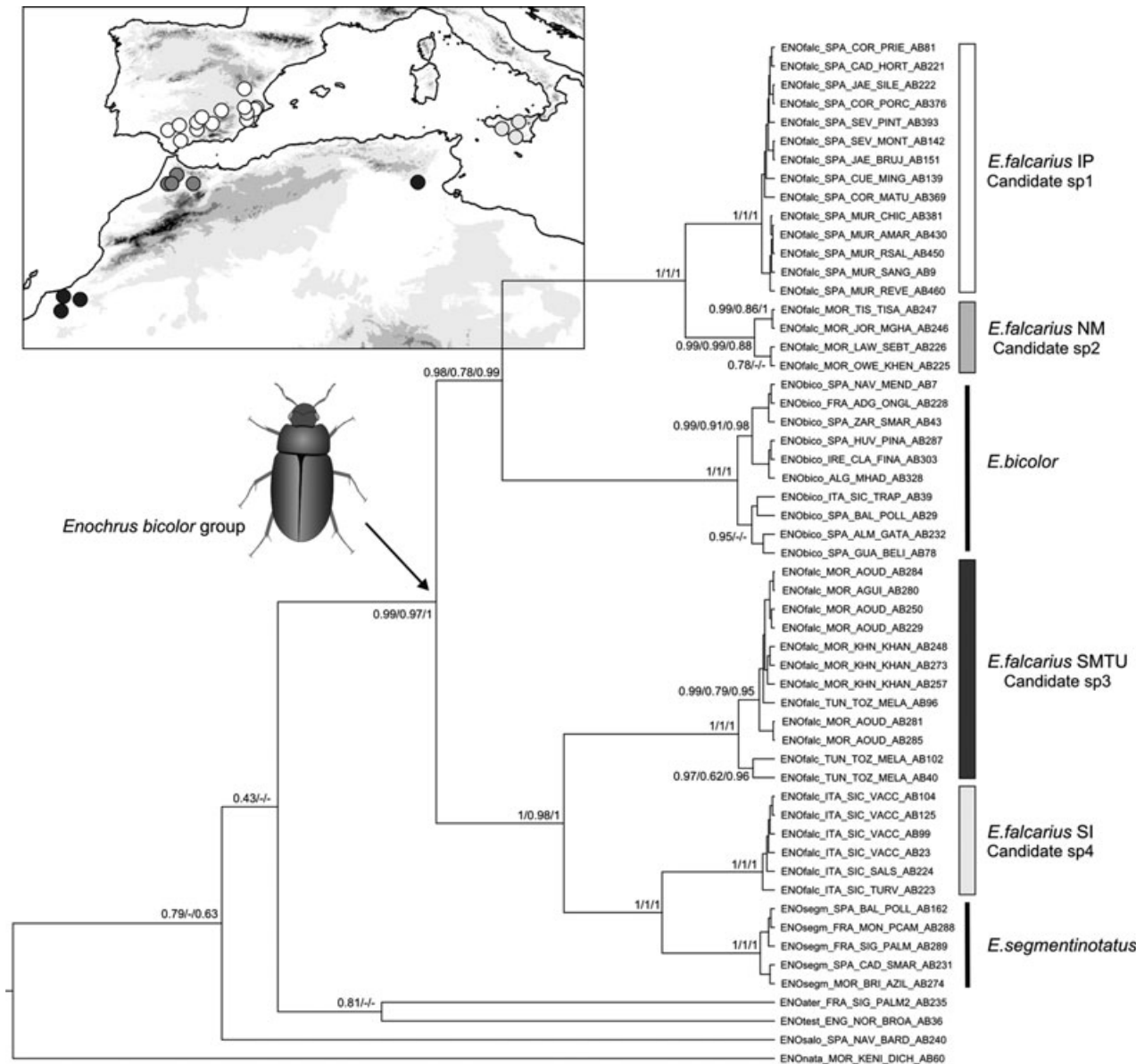
Fig. 1 Phylogenetic reconstruction of the *cox1*, *cob*, *rrn*L + *trn*L + *nd1* and *LSU* haplotypes of the *Enochrus falcarius* species complex and related species, obtained with Bayesian analysis (BEAST). Numbers above the main nodes are Bayesian posterior probabilities (MRBAYES)/maximum-likelihood bootstrap values ($\times 10^{-2}$; RAxML)/Bayesian posterior probabilities (BEAST). Only support values of the main nodes are shown. Codes correspond to morphological species, vouchers and localities (see Tables S1 and S2 in Supporting information). Map shows sampling sites in the western Mediterranean for the *E. falcarius* species complex, with colours referring to four main lineages. IP, Iberian Peninsula; NM, northern Morocco; SI, Sicily; SMTU, southern Morocco and Tunisia.

(interspecific scope) and has been used to define species boundaries in many taxonomic groups (e.g. Pons *et al.* 2006; Monaghan *et al.* 2009). The GMYC test was performed for the previously estimated ultrametric tree using the R package 'splits' (Ezard *et al.* 2009) with default options for single and multiple threshold models. The set of best models as determined by AIC scores were used to

construct a pairwise probability graphic showing the probabilities that two individuals belong to the same cluster. For each model in the set, each pair of specimens was scored as being predicted to belong, or not, to a common cluster. The probability that each pair of specimens belongs to a common cluster was then calculated as the Akaike weighted sum of these scores (for details see Powell 2012).

Morphometric evaluation of the candidate species

Linear morphometrics. Twenty-six linear quantitative measurements of principal exoskeletal pieces were collected from 84 specimens (50 males and 34 females) from the *E. falcarius* species complex, including most of the specimens used in phylogenetic analyses and some additional material from the same populations (Table S2). Measurements were chosen to best characterise shape and size, not systematic characters, and included major linear dimensions of main exoskeletal pieces (for details see Table S3). To minimise the measurement errors owing to differences in body position, specimens were dissected, and exoskeletal pieces were mounted with a 50% dimethyl hydantoin formaldehyde (DMHF) solution onto entomological cardboard labels. Exoskeletal pieces were photographed under a Zeiss Stemi 2000C Trinocular Zoom Stereomicroscope (Zeiss, Thornwood, NY, USA) and measured using a Spot Insight Firewire digital camera (Spot Imaging Solutions, Sterling Heights, MI, USA) and associated software.

Body length (BL) was estimated as the sum length of the left elytra and pronotum and was used as a proxy of body size because both will scale in a very similar manner across such morphologically similar species (Benke *et al.* 1999). Specimen body size was compared between the four candidate species using ANOVA and post hoc tests with Tukey correction.

To estimate size-independent measurements of the specimens' shapes, residuals of the linear regressions of each individual's log-transformed measurement combined with their log-transformed BL were considered as new variables (Ribera *et al.* 1999). The new variables were summarised using a principal component analysis (PCA), and main axes coordinates were used to test differences among candidate species using a multivariate analysis of variance (MANOVA). ANOVAs and post hoc tests with Tukey correction for each individual axis were performed. All statistical analyses were conducted using R (R Development Core Team 2011).

Outline morphometrics: pronotum comparisons. Morphometric elliptic Fourier analyses of the pronotum outlines were conducted for the 84 specimens used in linear morphometrics. The dorsal surface of the dissected pronotums was pasted onto cardboard labels, and digital images were obtained as previously indicated for linear measurements. Information describing the ventral outlines of the pronotums was automatically extracted and used for calculating the normalised elliptic Fourier descriptors (EFDs) for 60 Fourier harmonic ellipses on CHAINCODER and CHC2NEF programs from the SHAPE 1.3 package (Iwata & Ukai 2002). EFDs were normalised based on the first harmonic ellipse and used to perform a PCA and inverse Fourier reconstructions to visualise the shape changes associated with each PCA axe, both using the PRINCOMP program from the same package.

A MANOVA using coordinates from the main PCA axes was performed to test for differences between the four candidate species. ANOVAs and post hoc tests with Tukey correction for each individual axis were performed. All statistical analyses were conducted using R (R Development Core Team 2011).

Ecological niche similarity between candidate species

Ecological niche modelling (ENM) based on large-scale climatic data sets and known occurrence points was used to characterise each candidate species' environmental niche and to test for niche divergence among them. Climatic data were obtained at a spatial resolution of 2.5 arc-minutes from WORLDCLIM, version 1.3 (19 bioclimatic variables from http://www.worldclim.org; Hijmans *et al.* 2005). Spearman correlations ($R \geq 0.9$) were used to reduce the number of bioclimatic variables for the western Mediterranean region in order to avoid problems due to multicolinearity of predictive variables in the modelling process. As records of species occurrences, we employed all known localities for the *E. falcarius* species complex, most of them used on molecular and morphological analyses (Table S1).

Ecological niche models were generated using the widely used MAXENT niche modelling software (Phillips *et al.* 2006). Then, Schoener's D metric (Schoener 1968) as a measure of niche similarity between each pair of proposed entities was calculated using ENMTOOLS (Warren *et al.* 2010). These values were calculated by comparing the climatic suitability of each grid cell in the study area obtained with MAXENT. Because niche differences may be simply a result of the spatial autocorrelation of the explanatory environmental variables (Warren *et al.* 2008), we conducted a *background similarity test*, also implemented in ENMTOOLS. This test generates a null distribution of 1000 niche similarity values obtained by comparing the model suitability values of one candidate species to those generated from random cells drawn from the background area of the other candidate species. Then, the observed *D* value of niche similarity between them was compared with the null distribution generated for each entity. The background area of a species should be adjusted to the habitat available for each studied entity and have a biological justification (Warren *et al.* 2010). Despite previous studies that pointed to the limited dispersal capacity of some entities within the *E. falcarius* species complex (i.e. Arribas *et al.* 2012a,b), little is known about its specific dispersal rate. So, we defined the background area for each candidate species as the environmentally suitable places in which a species could occur according to the values of the

environmental variables in the observed localities using a multidimensional envelope procedure (MDE; Chefaoui & Lobo 2008; Jiménez-Valverde *et al.* 2008). Details of this methodology and its application for similar purposes can be found in Aragón *et al.* (2010) and Sánchez-Fernández *et al.* (2011), respectively.

Assessment of vulnerability for the delimited species

The vulnerability degree of each delimited species was evaluated using the methodology proposed by Abellán *et al.* (2005) with slight modifications to adapt to a broader scale (see Table S4 for details). This methodology assesses the vulnerability of each species on the basis of six criteria: general distribution, endemicity, rarity, persistence, habitat rarity and habitat loss (see Abellán *et al.* 2005 for methodological details and Sánchez-Fernández *et al.* 2008; Bennas *et al.* 2009 for previous applications of this methodology to species from the Iberian Peninsula and Morocco). Finally, the conservation status of each delimited species was assessed following IUCN Red List criteria (IUCN 2011).

Results

Delimitation of the candidate species

The aligned sequence data matrix had 2595 characters, of which 506 were variable (97% of them located in the mitochondrial markers). For MrBayes analyses, the standard deviation of the split frequencies between the two runs reached a value lower than *c.* 0.005 at 10 million generations, and the half compact consensus tree was calculated by removing 10% of the initial trees as a 'burn-in'. The four independent runs of the Beast analyses resulted in similar likelihood scores, with ESS >200, as checked using Tracer 1.5.4 (Rambaut & Drummond 2007). Then, trees from the four runs were pooled by removing 10% of the samples as an initial burn-in, and the Bayesian consensus tree was obtained using median values for branch lengths with TreeAnnotator v1.5.4 (Drummond & Rambaut 2007).

Phylogenetic trees were congruent with those from a previous study on this group's phylogeny (Arribas *et al.* 2012b). The topology was similar for the three reconstruction methods, BI (MrBayes and Beast) and ML, with high support values for the main nodes (Fig. 1). As reported in Arribas *et al.* (2012b), putative species from the *E. bicolor* group (i.e. *E. falcarius*, *E. bicolor* and *E. segmentinotatus*) formed a clade with strong support that excluded the remaining *Enochrus* species. Two main, well-supported clades could be recognised within this group: one clade that encompasses all populations of *E. bicolor* and the Iberian and northern Moroccan populations of *E. falcarius*, which in turn, constitutes two independent well-supported clades, IP and NM clade respectively. The other main clade includes all populations of *E. segmentinotatus*,

together with Sicilian, Tunisian and southern Moroccan *E. falcarius*. Within this second main clade, *E. segmentinotatus* is sister to the Sicilian *E. falcarius* (SI clade), and these two together are sisters to a clade that includes the populations of *E. falcarius* from southern Morocco and Tunisia (SMTU clade; Fig. 1).

Topology test results showed that the likelihood of topology with a monophyletic *E. falcarius* grouping was significantly lower than topology without such constraint for SH and AU tests ($P \leq 0.001$ for both tests).

Similarly, distance-based clustering of the data using the three putative species from the *E. bicolor* group (i.e. *E. falcarius*, *E. bicolor* and *E. segmentinotatus*) as *a priori* entities showed that across the threshold range of 1.3–2.9% (which matches values displaying a good performance for species delimitation; e.g. Hendrich *et al.* 2010), *E. bicolor* and *E. segmentinotatus* were recovered as perfect clusters (monophyletic), but *E. falcarius* resulted as a paraphyletic entity, splitting into four clusters which correspond with Iberian, northern Moroccan, southern Moroccan-Tunisian and Sicilian populations (Table S5).

In accordance with these results, four candidate species were proposed within the *E. falcarius* species complex: IP clade: candidate species 1, NM clade: candidate species 2, SMTU clade: candidate species 3 and SI clade: candidate species 4.

Distance-based clustering and genetic divergences between candidate species

Using candidate species as *a priori* entities, distance-based clustering displayed a perfect cluster matching within the threshold range from 1.3 to 2.9% (Table S6). Ideally, within-species genetic divergences should be smaller than divergences from sister species, with no overlap between both (the so-called *barcoding gap*; Hebert *et al.* 2003). Considering candidate species, mean interspecific distances within the *E. bicolor* group were clearly higher (9.3%) than the mean intraspecific distances (0.4%). Both interspecific and intraspecific distances showed no overlap, with a *barcoding gap* from 2.2 to 2.9% (see Fig. S1).

DNA grouping based on the coalescent theory

The likelihood of the single and multiple threshold GMYC models was significantly higher than the likelihood of the null model ($P < 0.001$ for both). The single threshold GMYC analysis was the best solution as determined by AIC scores and delimited six ML entities within the *E. bicolor* group: the recognised species *E. bicolor* and *E. segmentinotatus*, and the four purposed candidate species within the *E. falcarius* species complex. Pairwise probability graphic also reported such entities, all of them over a probability of 0.70 (Fig. 2).
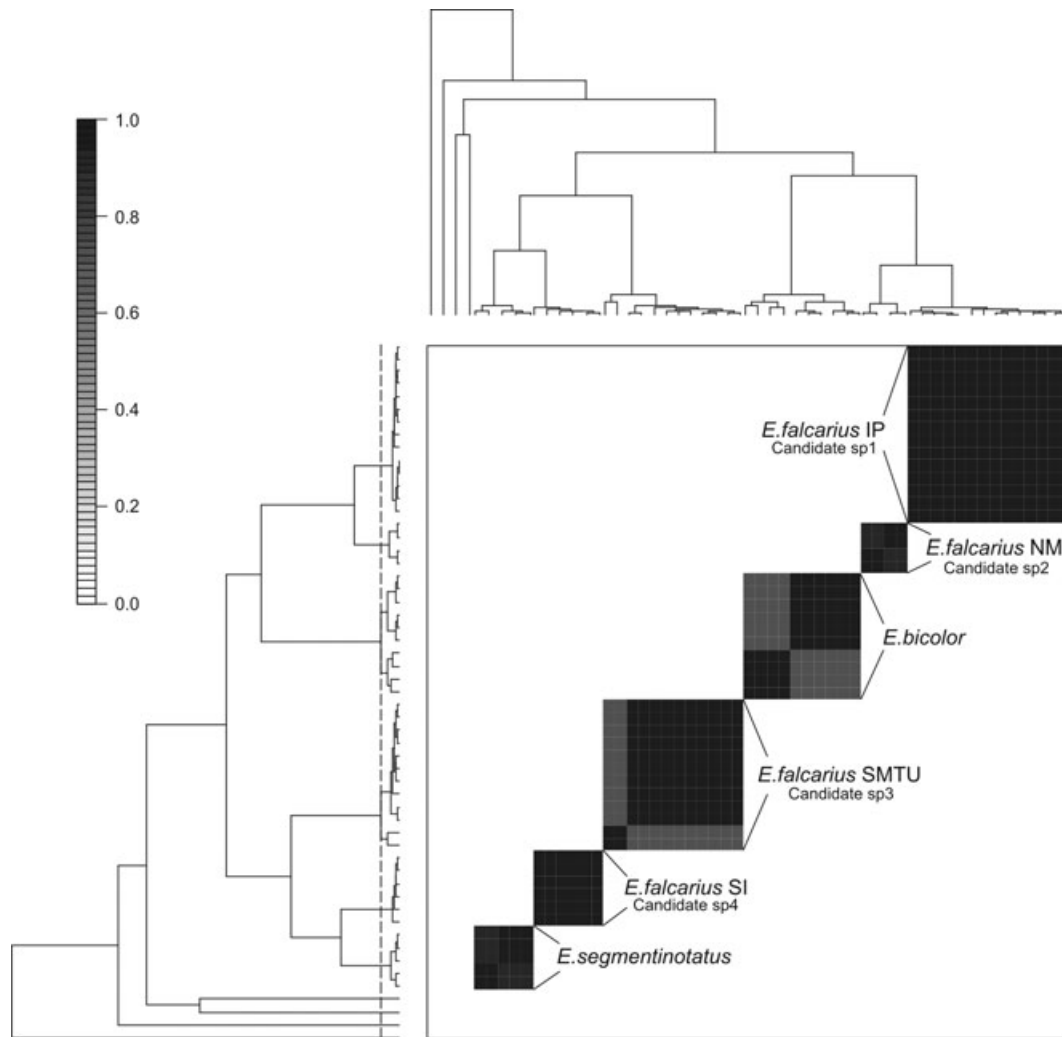
Fig. 2 Generalised mixed Yule coalescent (GMYC) analysis results for the *Enochrus falcarius* species complex and related species. Discontinue line is the best species delimitation solution as determined by AIC scores. Pairwise probability graphic shows model-averaged GMYC predictions of pairwise probabilities that two individuals of the *E. falcarius* species complex and related species belong to the same cluster. Probabilities are shown on upper left grey scale.

Morphometric evaluation of the candidate species

Linear morphometrics. All candidate species showed significant differences in BL with the exception of the IP and SMTU clades (ANOVA taxa, d.f. = 3, $F = 48.1$, $P < 0.001$). The highest body size corresponded with SI populations, and the lowest, with the IP and SMTU populations, and NM populations presented an intermediate body size.

The first ten axes of the PCA on the specimens' shape accounted for more than 75% of the total variance, and subsequent axes did not significantly contribute to the explained cumulative variance. In the ordination scatter plots using pairs of these first ten axes, no clear aggregations were observed, and thus, specimens of the four candidate species appeared mixed in the point cloud (Fig. S2).

No significant differences among the four candidate species were found in the MANOVA analysis including the first ten PCA axes coordinates as variables (MANOVA taxa, d.f. = 3, *Pillai* = 0.4, $P = 0.465$, Table S7A). Similarly, independent ANOVAs for each axis showed no significant differences among candidate entities, with the exception of axis six, which presented a P value slightly lower than the established threshold (ANOVA taxa, d.f. = 3, $F = 2.9$, $P = 0.049$; Table S7A). However, no significant differences between candidate species were detected for axis six coordinates in the corresponding *post hoc* analysis.

Outline morphometrics: pronotum comparisons. The first five axes of the PCA accounted for more than 85% of total

variance of the pronotum outlines and subsequent axes did not significantly contribute to the explained cumulative variance. In the ordination scatter plots for these five axes, the SI clade (candidate species 4) specimens were clearly

separated from the other candidate species in the second axis (Fig. 3A).

Significant differences among the candidate species were found in the MANOVA that included the first five PCA axes
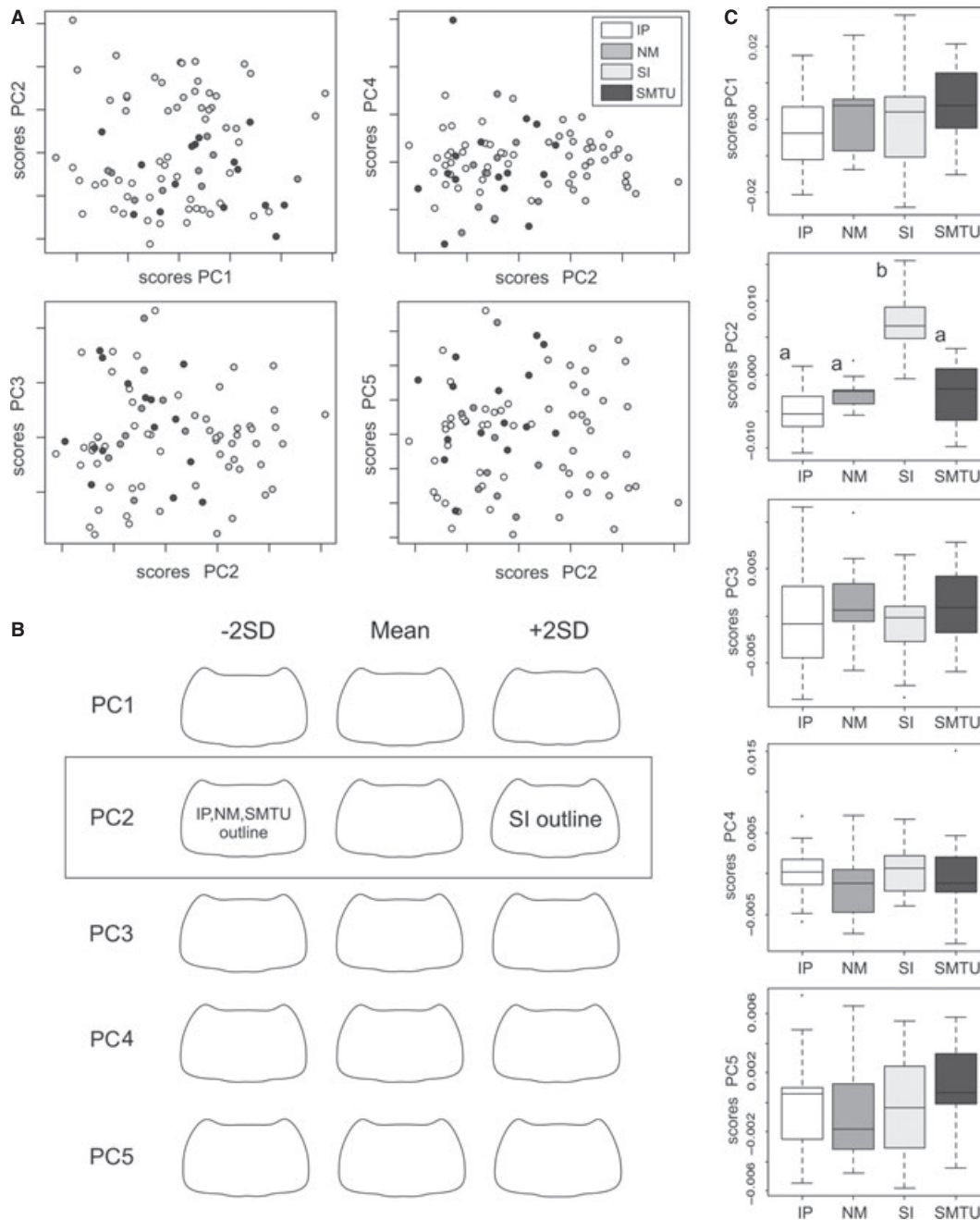
Fig. 3 Pronotum outline morphometrics for candidate species within *Enochrus falcarius* complex. —A. Scatter plots for the five main axes of pronotum outlines ordination. —B. Inverse reconstructions of pronotum outlines for the same five ordination axes. —C. Ordination coordinate differences between the four candidate species for the same five axes. Box plots represent median and quartiles. Significantly different means (*P* < 0.05) between species are indicated by different letters (according to Tukey correction).

coordinates as variables (MANOVA taxa, d.f. = 3, *Pillai* = 0.9, *P* < 0.001; Table S7B). Independent analysis of each variable only showed significant differences for axis two, where the SI clade differed from the other candidate species (ANOVA taxa, d.f. = 3, *F* = 79.6, *P* < 0.001; Fig. 3C, Table S7B).

Inverse Fourier reconstructions based on the PCA axis two showed that the main differences between the SI clade specimens and the rest of candidate species were due to the basal width of the pronotum, which was markedly larger than apical width in SI clade specimens (trapezoidal ventral outline) and moderately larger in the other candidate species (rectangular ventral outline; Fig. 3B).

Ecological niche similarity between candidate species
Potential distributions from MAXENT for each candidate species presented a low spatial overlap. Similarly, the degree of niche overlap estimated by Schoener's D statistic showed values lower than 0.37 for all pairwise comparisons between the four candidate species, thus pointing that niche characteristics differ among them (Fig. 4, Table S8).

Background similarity test results demonstrated that observed overlap values (Schoener's D) between species were significantly lower than null model estimations (i.e. differences were greater than the background environmental divergence), with the exception of IP and SI clades (candidate species 1 and 4), where despite the low *D* value (0.29), it was not significantly different than expected by chance (Fig. 4).

Taxonomic treatment and vulnerability assessment for the delimited species
The integrative comparison of operational criteria between the candidate species suggested the presence of four separately evolving meta-population lineages and so four species within *E. falcarius* (Fig. 5). Lineages from the IP, NM and SM-TU (i.e. candidate species 1, 2 and 3) were identified as three new species, hereafter referred to as *Enochrus jesusarribasi* sp. n., *Enochrus blazquezae* sp. n. and *Enochrus risii* sp. n., respectively. The specific name *Enochrus falcarius* was conserved for the Sicilian lineage (candidate species 4), where it was originally described (Hebauer 1991). After
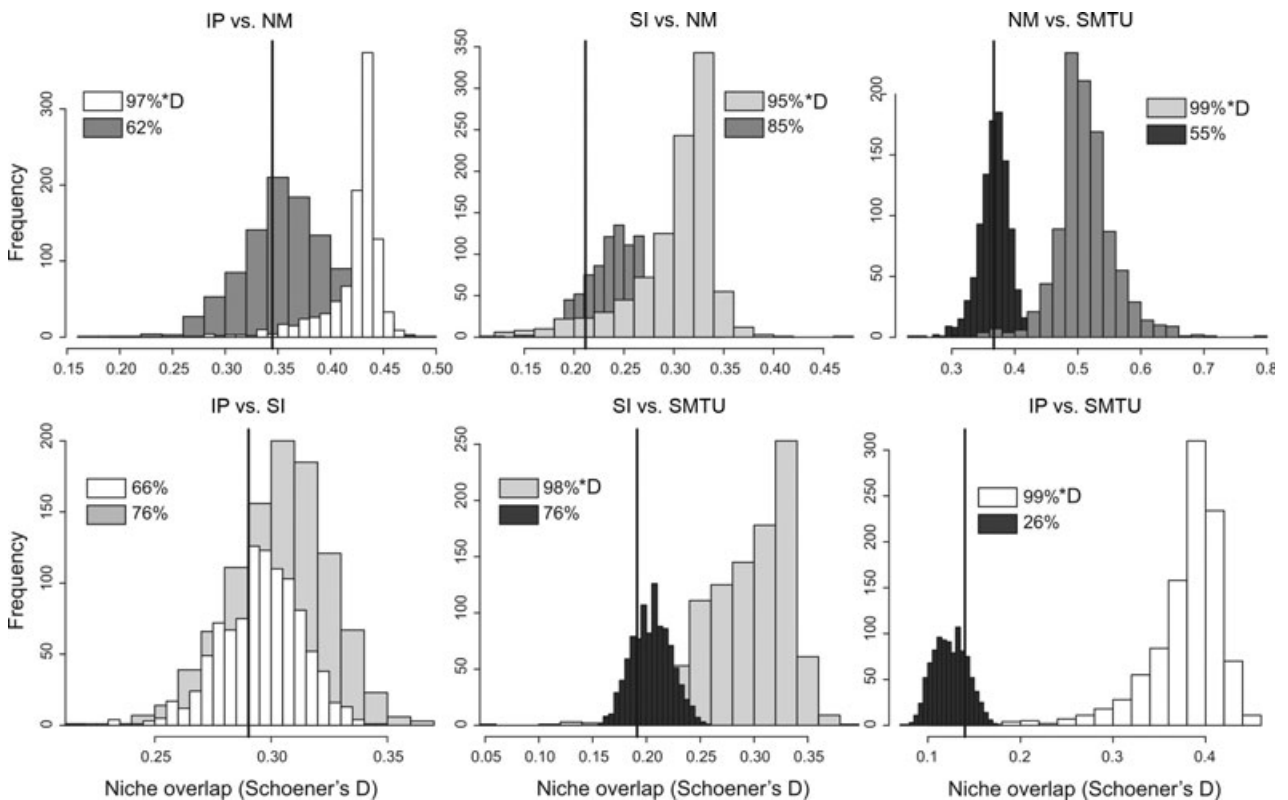
Fig. 4 Background similarity test results for candidate species within the *Enochrus falcarius* complex. Observed niche overlap values (vertical lines) were compared with null distributions (1000 replicates) generated by comparing model suitability values of one candidate species to those generated from random cells drawn from the background area of the other candidate species. Percentages represent the proportion of null distribution over observed niche overlap values. When percentage is higher than 95% (*P* < 0.05), *D* indicates niche divergence between candidate species.
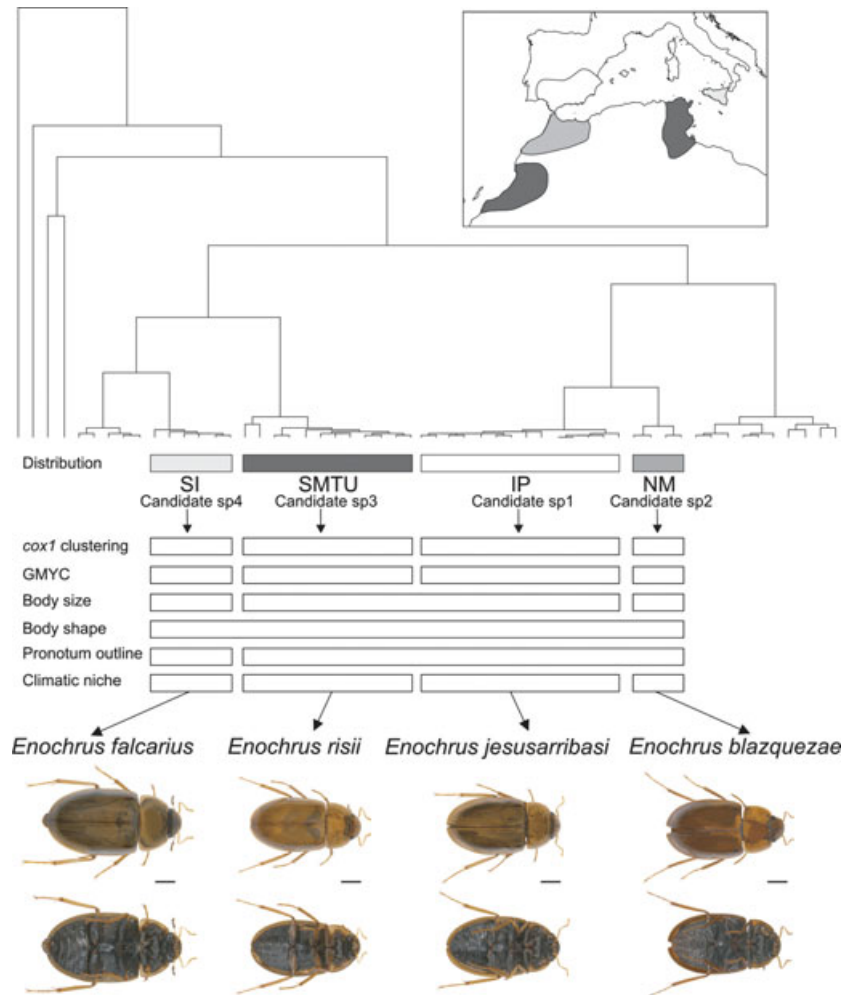
Fig. 5 Synthetic representation of the integrative taxonomy approach and resulting four species delimited within the *Enochrus falcarius* species complex. Scale bars correspond to 1 mm.

that, the detailed comparative microscopic study of the four species allowed some species identification characters to be recognised (see Fig. S3), which were used in the identification key provided (Fig. S4).

Vulnerability assessment of the delimited species following Abellán *et al.* (2005) showed that *E. jesusarribasi* sp. n. and *E. falcarius* Hebauer present features to be included in the *very high vulnerability* category and *E. blazquezae* sp. n. and *E. risii* sp. n. in the *high vulnerability* category (see Table S4 for detailed scores). Finally, following IUCN (2011), all delimited species could be categorised as *Vulnerable* (VU B2abiii).

Descriptions of species
Genus *Enochrus* Thomson, 1859

Enochrus jesusarribasi sp. n. Arribas & Millán (Fig. S3A)

Holotype. 1M, SPAIN, Siles, Arroyo Salado, lon/lat −2.547°/38.408°, Andújar & Arribas, 24/10/2009, (Naturhistorisches Museum, Vienna, Austria).

Paratypes. 1F, same data as for holotype, voucher IBE-AB222; 1M, 2F, SPAIN, El Bosque, Salinas de Hortales, arroyo, −5.537°/36.738°, Andújar & Arribas, 29/01/2010, voucher IBE-AB221; 1M, 1F, SPAIN, Baena, Salinas de la Maturra, arroyo, −4.355°/37.676°, Ribera, 28/07/1998, voucher IBE-AB369; 2M, SPAIN, Priego de Córdoba, Río Salado, −4.18°/37.407°, Ribera & Cieslak, 02/06/2002; 1M, 2F, SPAIN, Minglanilla, Arroyo Salado, −1.563°/39.548°, Arribas & Arribas, 22/11/2009; 2M, SPAIN, Brujuelo, Salinas de Brujuelo, arroyo, −3.669°/37.888°, Millán & col., 21/07/2006, voucher IBE-AB151; 3M, SPAIN, Valenzuela, Salinas de Porcuna, arroyo, −4.218°/37.789° Millán & col., 12/10/2003, Ribera, 28/07/1998; 1M, 1F, SPAIN, Cieza, Rambla de Agua Amarga, −1.503°/38.307°, Andújar & Arribas, 27/11/2010, voucher IBE-AB430; 1M, 1F, SPAIN, Abanilla, Río Chícamo, −1.052°/38.216°, Millán & col., 21/09/2007; 2M, SPAIN, Mazarrón, Rambla del Reventón, −1.367°/37.643°, Andújar & Arribas, 27/11/2010; 2M, SPAIN, Fortuna, Rambla

Salada, −1.135°/38.135°, Arribas & Millán, 07/07/2009; 1M, 1F, SPAIN, Murcia, Salinas de Sangonera, arroyo, −1.289°/37.953°, Millán & col., 22/01/2008; 1M, 2F, SPAIN, Montellano, Arroyo Montero, −5.693°/36.976°, Millán & col., 21/07/2006, voucher IBE-AB142. All paratypes were dissected, mounted and deposited in the Departamento de Ecología e Hidrología, Universidad de Murcia, Spain.

Etymology. The specific epithet is a Latinised eponym, genitive case, based on the name and surname of Jesús Arribas, first author's father.

Diagnosis. Body length (elytra length plus pronotum length): 4.42–5.93 mm. Amber colour in dorsal view. Black femur with a well-defined yellow spot on apical extreme (Fig. S4B). Medially flat labrum–clipeus suture (Fig. S4C). Apical extreme of the parameres elongated, moderately arched on the internal edge, the external edge enlarged steadily towards the basis and tips slightly dilated (Fig. S4F). Ventral outline of the pronotum type rectangular (Fig. S4I).

Description. Body length (elytra length plus pronotum length): 4.42–5.93 mm. Males (4.42–5.45) are generally smaller than females (5.32–5.93). Body outline elongatedly oval, markedly interrupted between pronotum and elytra. Amber colour in dorsal view and very dark in ventral view.

Head. Length: 0.91–1.21, Width: 0.89–1.20. Colouration is variable, but in general, amber with darkened frons, frons–clypeus suture, labrum and central clypeus. Densely punctated, also in labrum. Medially flat clipeus–labrum suture (Fig. S4C). Yellow palps without any spot, second segment convex and longer than the third. Antennae brown, short, and pilose.

Pronotum. Length: 1.10–1.54, Apical width: 1.18–1.60, Basal width: 1.81–2.43. Colouration is variable, but in general, amber and slightly darked in pronotal disc. Densely punctated. Underside dark brown. Somewhat rectangular ventral outline, with basal width moderately longer than apical width (Fig. S4I). Corners rounded.

Elytra. Length: 3.27–4.48. Basal width: 0.80–1.12. Amber and partly transparent. Densely but weakly punctated. Ten punctuation series and suture very patent.

Ventral meso- and metathorax. Length: 1.36–1.79. Width: 1.44–2.01. Black, punctated and pilose. Prosternum medially simple, without pronounced keel and variable size and shape. Mesosternal lamella pointed posteriorly and variable in size.

Abdomen. Black, punctated and densely pilose. Last sternite without notch.

Legs. 1st femur length: 0.89–1.18. 2nd femur length: 0.80–1.08. 3rd femur length: 0.76–0.99. 1st tibia length: 1.29–

1.68. 2nd tibia length: 0.99–1.21. 3rd tibia length: 0.82–1.09. Black and pilose femur with a well-defined yellow spot on the apical extreme (Fig. S4B), especially in the forelegs. Dark yellow tarsus and tibia, the latter with abundant thick pilosity. Extremely elongated pretarsal claws both in males and females. The male foreleg claws are more robust and elongated and inner claw more pronounced than in females.

Aedeagus. Length: 1.19–1.34. Maximum width: 0.32–0.39. Apical extreme of the parameres elongated, moderately arched on the internal edge, the external edge enlarged steadily towards the basis and tips slightly dilated (Fig. S4F). The tip of the median lobe not reaching apex of parameres and slightly dilated. Basal piece constricted basally.

Distribution and habitat. Enochrus jesusarribasi sp.n. is distributed across the south eastern IP, in the Segura, Júcar and Guadalquivir river basins. This species is found in meso- and hypersaline streams (7–85 g/L), where it is one of the most abundant and specialised macroinvertebrates able to inhabit these extreme ecosystems.

Proposed IUCN conservation status. Vulnerable (VU B2abiii)

Genus *Enochrus* Thomson, 1859
Enochrus blazquezae sp. n. Arribas & Millán (Fig. S3B)
Holotype. 1M, MOROCCO, Oued Khendek, lon/lat −5.35°/34.69°, Millán & col. 18/04/2006, (Naturhistorisches Museum, Vienna, Austria).

Paratypes. 2M, 3F, same data as for holotype, voucher IBE-AB225; 1M, MOROCCO, Oued Mghassem, −5.502°/34.388°, Millán & col. 18/04/2006, voucher IBE-AB246; 1F, MOROCCO, Oued Sebt Lawdaya, −5.5°/34.39°, Millán & col. 18/04/2006, voucher IBE-AB226; 1M, MOROCCO, Salines de Tissa, −4.68°/34.29°, Millán & col. 19/04/2006, voucher IBE-AB247. All paratypes were dissected, mounted and deposited in the Departamento de Ecología e Hidrología, Universidad de Murcia, Spain.

Etymology. The specific epithet is a Latinised eponym, genitive case, based on the surname of Llanos Blázquez, the first author's mother.

Diagnosis. Body length (elytra length plus pronotum length): 5.20–5.85 mm. Amber colour in dorsal view. Black femur with a well-defined yellow spot on apical extreme (Fig. S4B). Medially flat labrum–clipeus suture (Fig. S4C). Apical extreme of the parameres slightly shortened, moderately arched on the internal edge, the external edge abruptly enlarged towards the basis and tips slightly dilated (Fig. S4G). Ventral outline of the pronotum type rectangular (Fig. S4I).

Description. Body length (elytra length plus pronotum length): 5.20–5.85 mm. Males (5.20–5.69) are generally

smaller than females (5.71–5.85). Body outline elongatedly oval, markedly interrupted between pronotum and elytra. Amber colour in dorsal view and very dark in ventral view.

Head. Length: 1.01–1.16. Width: 1.05–1.18. Similar to what is described above for *E. jesusarribasi* sp. n.

Pronotum. Length: 1.31–1.56. Apical width: 1.43–1.61. Basal width: 2.14–2.50. Similar to what is described above for *E. jesusarribasi* sp. n.

Elytra. Length: 3.85–4.34. Basal width: 0.92–1.10. Similar to what is described above for *E. jesusarribasi* sp. n.

Ventral meso- and metathorax. Length: 1.60–1.90. Width: 1.75–2.04. Similar to what is described above for *E. jesusarribasi* sp. n.

Abdomen. Similar to what is described above for *E. jesusarribasi* sp. n.

Legs. 1st femur length: 1.07–1.26. 2nd femur length: 0.98–1.11. 3rd femur length: 0.84–0.95. 1st tibia length: 1.56–1.71. 2nd tibia length: 1.12–1.30. 3rd tibia length: 0.97–1.10. Similar to what is described above for *E. jesusarribasi* sp. n.

Aedeagus. Length: 1.29–1.43. Maximum width: 0.36–0.38. Apical extreme of the parameres slightly shortened, moderately arched on the internal edge, the external edge abruptly enlarged towards the basis and tips slightly dilated (Fig. S4G). Its other features are similar to those described above for *E. jesusarribasi* sp. n.

Distribution and habitat. Enochrus blazquezae sp. n. is distributed in Morocco across the northern region of the Middle Atlas. This species is found in meso- and hypersaline streams (10–90 g/L), where it is one of the most abundant and specialised macroinvertebrates able to inhabit these extreme ecosystems.

Proposed IUCN conservation status. Vulnerable (VU B2abiii)

Genus *Enochrus* Thomson, 1859
Enochrus risii sp. n. Arribas & Millán (Fig. S3C)
Holotype. 1M, MOROCCO, Oued Aoudrei, lon/lat −12.18°/27.95°, Millán & col., 05/04/2007, (Naturhistorisches Museum, Vienna, Austria).

Paratypes. 4M, 3F, same data as for holotype, voucher IBE-AB284, IBE-AB250, IBE-AB229, IBE-AB281, IBE-AB285; 2M, MOROCCO, Oued El-Aguig, −11.63°/28.25°, Millán & col., 03/04/2007, voucher IBE-AB280; 1M, 2F, MOROCCO, Oued Khanoi Naâm, −12.22°/27.68°, Millán & col., 04/04/2007, voucher IBE-AB248, IBE-AB273, IBE-AB257; 2F, TUNISIA, Oued El Melah, 8.576°/34.339°, Ribera & Cieslak, 26/10/2001, voucher IBE-AB102, IBE-AB40. All paratypes were dissected, mounted and deposited in the Departamento de Ecología e Hidrología, Universidad de Murcia, Spain.

Etymology. The specific epithet is a Latinised eponym, genitive case, based on the surname of Jesús María Arribas 'Risi', the first author's brother.

Diagnosis. Body length (elytra length plus pronotum length): 4.63–5.85 mm. Pale amber colour in dorsal view. Brown femur without a well-defined yellow spot on apical extreme (Fig. S4A). Medially flat labrum–clipeus suture (Fig. S4C). Apical extreme of the parameres elongated, markedly arched on the internal edge, the external edge enlarged steadily towards the basis and tips noticeably dilated (Fig. S4H). Ventral outline of the pronotum type rectangular (Fig. S4I).

Description. Body length (elytra length plus pronotum length): 4.63–5.85 mm. Males (4.63–5.23) are generally smaller than females (4.77–5.85). Body outline elongatedly oval, markedly interrupted between pronotum and elytra. Pale amber colour in dorsal view and very dark in ventral view.

Head. Length: 0.94–1.12. Width: 0.97–1.19. Slightly darker than the pronotum and elytra. The other features are similar to those described above for *E. jesusarribasi* sp. n.

Pronotum. Length: 1.18–1.55. Apical width: 1.23–1.61. Basal width: 1.95–2.47. Pale amber colour. The other features are similar to those described above for *E. jesusarribasi* sp. n.

Elytra. Length: 3.41–4.30. Basal width: 0.83–1.00. Pale amber. Densely and coarsely punctated. The other features are similar to those described above for *E. jesusarribasi* sp. n.

Ventral meso- and metathorax. Length: 1.45–1.84. Width: 1.52–2.03. Similar to what is described above for *E. jesusarribasi* sp. n.

Abdomen. Similar to what is described above for *E. jesusarribasi* sp. n.

Legs. 1st femur length: 0.89–1.24. 2nd femur length: 0.80–1.09. 3rd femur length: 0.71–0.90. 1st tibia length: 1.30–1.70. 2nd tibia length: 0.94–1.25. 3rd tibia length: 0.82–1.04. Brown and pilose femur without a well-defined yellow spot on apical extreme (Fig. S4A), just slightly darkened on borders. Bright yellow tarsus and tibia. The other features are similar to those described above for *E. jesusarribasi* sp. n.

Aedeagus. Length: 1.19–1.30. Maximum width: 0.33–0.39. Apical extreme of the parameres elongated, markedly arched on the internal edge, the external edge enlarged steadily towards the basis and tips noticeably dilated (Fig. S4H). The other features are similar to those described above for *E. jesusarribasi* sp. n.

Distribution and habitat. Enochrus risii sp. n. is distributed across the southern Morocco, in the southern region of the Anti-Atlas range and the northern Tunisia. This species is found in meso- and hypersaline streams (15–70 g/L), where it is one of the most abundant and specialised macroinvertebrates able to inhabit these extreme ecosystems.

Proposed IUCN conservation status. Vulnerable (VUB2abiii)

Genus *Enochrus* Thomson, 1859
Enochrus falcarius Hebauer 1991 (Fig. S3D)
Studied specimens. Holotype and paratypes were not examined, although specimens from the type localities were studied (i.e. Torrente Vaccarizzo, Torrente Mandre and Fiume Salso) and some additional material from other localities in Sicily: 4M, 2F, ITALY, Sicily, Torrente Mandre, lon/lat 14.32°/37.711°, Abellán & Picazo, 12/06/2007; 1M, ITALY, Sicily, Fiume Salito, 13.863°/37.51°, Abellán & Picazo, 15/06/2007; 5M, 5F, ITALY, Sicily, Afluente mesosalino del Fiume Salso, 14.188°/37.639°, Gutiérrez-Cánovas, 27/07/2009, voucher IBE-AB224; 7M, 2F, ITALY, Sicily, Fiume Tùrvoli, 13.452°/37.497°, Gutiérrez-Cánovas, 26/07/2009, voucher IBE-AB223; 1M, 3F, ITALY, Sicily, Castello, Torrente Vaccarizzo, 14.109°/37.611°, Abellán & Picazo, 12/06/2007, voucher IBE-AB125, IBE-AB99. All of this material was dissected, mounted and deposited in the Departamento de Ecología e Hidrología, Universidad de Murcia, Spain.

Diagnosis. Body length (elytra length plus pronotum length): 5.44–6.92 mm. Dark amber colour in dorsal view. Black femur with a well-defined yellow spot on apical extreme (Fig. S4B). Medially pointed labrum–clipeal suture (Fig. S4D). Apical extreme of the parameres elongated, moderately arched on the internal edge, the external edge enlarged steadily towards the basis and tips slightly dilated (Fig. S4F). Ventral outline of the pronotum type trapezoidal (Fig. S4J).

Description. Body length (elytra length plus pronotum length): 5.44–6.92 mm. Males (5.44–6.44) are generally smaller than females (5.57–6.92). Body outline elongatedly oval, markedly interrupted between pronotum and elytra. Dark amber colour in dorsal view and very dark in ventral view.
Head. Length: 1.04–1.28. Width: 1.09–1.35. Dark amber colour. Labrum completely black. Medially pointed clipeus–labrum suture (Fig. S4D). The other features are similar to those described above for *E. jesusarribasi* sp. n.
Pronotum. Length: 1.41–1.89. Apical width: 1.44–1.82. Basal width: 2.38–2.86. Dark amber colour. Trapezoidal ventral outline, with basal width markedly longer than apical width (Fig. S4J). The other features are similar to those described above for *E. jesusarribasi* sp. n.
Elytra. Length: 3.97–5.12. Basal width: 0.93–1.34. Dark amber colour. Densely but coarsely punctated. The other features are similar to those described above for *E. jesusarribasi* sp. n.

Ventral meso- and metathorax. Length: 1.69–2.16. Width: 1.79–2.39. Similar to what is described above for *E. jesusarribasi* sp. n.
Abdomen. Similar to what is described above for *E. jesusarribasi* sp. n.
Legs. 1st femur length: 1.10–1.45. 2nd femur length: 0.99–1.27. 3rd femur length: 0.87–1.11. 1st tibia length: 1.63–1.99. 2nd tibia length: 1.19–1.39. 3rd tibia length: 1.01–1.20. Bright yellow tarsus and tibia. The other features are similar to those described above for *E. jesusarribasi* sp. n.
Aedeagus. Length: 1.28–1.57. Maximum width: 0.32–0.45. Similar to what is described above for *E. jesusarribasi* sp. n. In the aedeagus figure of *E. falcarius* description (i.e. Hebauer 1991), the parameres are more open than in a natural reposed position.

Distribution and habitat. Enochrus falcarius is distributed across the most arid regions of Sicily. This species is found in meso- and hypersaline streams (10–95 g/L), where it is one of the most abundant and specialised macroinvertebrates able to inhabit these extreme ecosystems.
Proposed IUCN conservation status. Vulnerable (VU B2abiii)

Discussion

Species delimitation within the E. falcarius species complex
Consistency between molecular analyses and geographical distributions allowed the clear delimitation of four candidate species within *E. falcarius* that were all distinguishable at the molecular (mainly mitochondrial information) and geographical level (i.e. all monophyletic and allopatric entities; Fig. 1). The four candidate species, together with the recognised species *E. bicolor* and *E. segmentinotatus*, were perfectly clustered across a broad range of genetic distance thresholds (1.3–2.9% for *cox1* p-distances), which matches values proposed as having a good performance to delimit species (see Hendrich *et al.* 2010 for a study using water beetles). Similarly, such entities were recovered as different ML entities in GMYC analysis. Long-term extrinsic barriers defining allopatric lineages tended to generate prominent genetic differentiation due to the deep limitation of gene exchange (Avise 2000). Because saline streams are restricted to circum-Mediterranean areas, habitat gaps among them (e.g. resulting from the sea or mountain ranges) could be acting as effective barriers to gene flow between species within the *E. falcarius* complex, which is in agreement with patterns found for other water beetles living in similar environments in this region (Abellán *et al.* 2009). Despite the fact that they are winged and able to fly, dispersal ability for beetles in this species complex seems to be limited (e.g. Abellán *et al.* 2012; Arribas *et al.* 2012b), which could be promoting allopatric speciation.

Morphometry did not allow unambiguous discrimination between the candidate species within the *E. falcarius* species complex. Regarding the linear morphometric analyses, a high morphological similarity was found between candidate species, and only significant variations in body size were found. Although the size differences among the candidate species pointed to lineage diversification, they should be considered carefully, especially in groups with high body size plasticity such as aquatic insects (e.g. Barahona *et al.* 2005), as they could be the result of phenotypic adaption to local conditions (Peters 1983). On the other hand, the only significant differentiation detected in the pronotum outline of one candidate species was subtle and difficult to perceive visually; indeed, such differences were not detected by linear morphometrics. These results agree with previous studies (e.g. Leaché *et al.* 2009), showing that linear measurements have a low resolution for capturing slight variations between complex shapes. In contrast, outline analyses provide a high-resolution technique for analysing shapes in multivariate space and could be used as a complement for the study of potentially informative anatomical features (Garnier *et al.* 2005).

High morphological similarity between candidate species was also reported after detailed microscope revision of the dissected specimens. However, some microscopic differences were detected in few exoskeletal pieces after comparative exploration of candidate species (Fig. S3). Such unapparent differences could offer additional evidence for the diversification between the *E. falcarius* complex entities. Additionally, these slight variations are useful for identifying specimens and act as a bridge that connects integrative taxonomy results with classical species descriptions. Linking the delimitation process of integrative taxonomy with the naming and visual description of traditional taxonomic practice (i.e. the incorporation of visual descriptors as identifiers) improves access to the biological knowledge tied to names (Dayrat 2005; Valdecasas *et al.* 2008; Schlick-Steiner *et al.* 2010), thus fomenting the 'integrative' advance in taxonomy and biodiversity cataloguing.

The high morphological similarity found among candidate species despite high levels of molecular divergence (which is congruent with the previous consideration of *E. falcarius* as a single species) agrees with the results found for other species living in Mediterranean saline aquatic habitats (e.g. Gómez *et al.* 2002; Abellán *et al.* 2007). Extreme habitat conditions could promote morphological stasis between divergent lineages because they undergo a strong natural selection on behavioural, physiological or morphological characters (Schonrogge *et al.* 2002; Bickford *et al.* 2007), which could explain the high similarity founded in the morphometric analyses. Additionally, evolving under severe habitat conditions (e.g. under high osmotic stress, as in saline streams) can also promote morphological convergence resulting from similar selection pressures (Trontelj & Fiser 2009; Trontelj *et al.* 2009). Saline aquatic ecosystems are very stressful habitats where extreme thermal fluctuations and high salinity levels act as colonisation barriers for many aquatic organisms (Millán *et al.* 2011). Thus, species inhabiting saline water habitats are highly specialised and present thermal tolerances and a variety of physiological mechanisms that palliate salt's effects (Bradley 2008; Sánchez-Fernández *et al.* 2010; Arribas *et al.* 2012b). Along these lines, the morphological similarity observed in species within the *E. falcarius* complex could be considered as a clear example of how the high degree of habitat specialisation promoted by extreme conditions could result in cryptic diversification.

With respect to ecological niche differentiation, our results provide strong evidence for niche divergence between the candidate species. Correlation between niche conservatism and allopatric distributions has been reported for different groups, suggesting that maintaining ancestral ecological adaption along an evolutionary lineage promotes allopatric divergence in fragmented habitats (Peterson *et al.* 1999; Kozak & Wiens 2006). Additionally, highly, geographically structured lineages emerging from allopatric speciation may reduce diversification in many operational criteria, because reinforcement or cohesion mechanisms acting after speciation are not promoted (Harrison 1991; Lehtinen *et al.* 2011). In this sense, both morphological and ecological differentiation could be limited for allopatric entities. Nevertheless, the present study found significant niche divergence among the four disjunct candidate species, which seems to be the result of local adaption to climatic conditions across each independent geographical entity (Losos & Glor 2003; Coyne & Orr 2004). Such ecological divergence could have promoted reproductive isolation between delimited entities, avoiding contact and any gene flow between them (Templeton 2001; Wiens & Graham 2005; Rissler & Apodaca 2007). The four species occur in arid regions of the Mediterranean basin where annual precipitations range from 30 to 600 mm, and hot summers are punctuated by severe drought. *Enochrus risii* sp. n. inhabits the most arid habitats of the four species that combine acute xeric and hot conditions (arising to 1–11 mm and 29.7–33.8 °C in the warmest months). The other African species, *E. blazquezae* sp. n., occupies the second most arid habitats where also experiences severe droughts (12–23 mm) during the hottest season. Finally, *E. jesusarribasi* sp. n. and *E. falcarius* occur in less arid sites (19–56 mm and 20.5–26.4 °C in the warmest months), the latter appearing in the areas with the most moderate dry and warm conditions of the four species.

To integrate the results reported for the different operational criteria, a congruence framework was followed that considered that concordant divergence patterns among several taxonomic characters indicated that full lineage separation, as it was highly improbable that a coherent concordance pattern would emerge by chance (DeSalle *et al.* 2005; Padial *et al.* 2010). Full concordance was found between molecular, biogeographical and climatic niche information, pointing to the existence of four separately evolving meta-population lineages, with additional slight, difficult-to-appreciate external differences that support the independent diversification between them. So, we hypothesised that the *E. falcarius* complex comprises four species: *E. jesusarribasi* sp. n., *E. blazquezae* sp. n., *E. risii* sp. n. and *E. falcarius* Hebauer (Fig. 5). The present study includes classical species descriptions that provide traditional identification tools for such delimited species (Fig. S4). While some authors (e.g. Bond & Stockman 2008) have advocated that the presence of a significant geographic/habitat break between candidate allopatric entities is enough to validate them as two distinct species because their disjunct distributions point to a low probability of genetic exchange (i.e. low gene flow), others (e.g. Padial *et al.* 2010) have claimed that evidence for ecological divergence and/or reproductive isolation is also required to delimit such entities as species. Despite insufficient mating experiment data and the fact that aedeagal differences are likely not enough to be considered as a reproductive barrier, the disjunct distribution of the proposed species, their reduced dispersal capacity (Arribas *et al.* 2012a,b) and their climatic niche divergences point to very low probability of genetic exchange between them, as exhibited by the clear molecular differentiation. Such evidences allowed the four entities within the *E. falcarius* complex to be delimited as species, also following the last most conservative criterion. Given the high reported morphological similarity, these new entities can be considered as cryptic or pseudo-cryptic species (Saez & Lozano 2005; Bickford *et al.* 2007) with only slight, difficult-to-appreciate external differences. Hence, the case of the *E. falcarius* species complex exemplifies how species delimitation approaches exclusively focussed on visual assessment may have grossly oversimplified and underestimated diversity (Mutanen & Pretorius 2007).

In summary, because these entities are monophyletic, clearly divergent on the basis of mtDNA, disjunct, with low dispersal capacities, reduced morphological divergence and show differentiated environmental niches, it is hypothesised that these species would emerge via allopatric speciation, and adaptation to different climatic regimes could have arisen after they split into separately evolving meta-population lineages. Further studies will be needed to explore the temporal framework of the speciation processes acting within the *E. falcarius* species complex.

Integrative taxonomy and biodiversity conservation

Discovering diversity is a potentially important factor influencing future conservation decisions (Witt *et al.* 2006; Condon *et al.* 2008) and, in the case of cryptic diversity, highly dependent upon the application of integrative taxonomy approaches (Bickford *et al.* 2007; Trontelj & Fiser 2009). Therefore, identifying geographical and habitat-related patterns in the distribution of cryptic species could promote conservation of covert endangered taxa, but also discover as yet unknown pockets of endemism and diversity that might warrant reconsideration for particular habitats or conservation sites (Bickford *et al.* 2007). In accordance with this, prioritising integrative taxonomy in the extreme habitats of highly diverse regions could be fundamental for an accelerated biodiversity inventory and effective biodiversity protection (Dayrat 2005; Schlick-Steiner *et al.* 2010; Nair *et al.* 2012). This seems to be the case for saline and hypersaline environments in the Mediterranean region, which are increasingly being recognised as holding cryptic diversity usually associated with morphological stasis (Gómez *et al.* 2002; Abellán *et al.* 2007, 2009; Ortego *et al.* 2009, 2010; Sánchez-Fernández *et al.* 2011). Furthermore, considering the dramatic decline and loss of saline habitats as a consequence of the rapid changes in land uses taking place in some Mediterranean areas (Millán *et al.* 2011; Gutiérrez-Cánovas *et al.* 2012), recognising and evaluating such cryptic diversity and subsequently applying appropriate conservation measures are of immediate importance. The biodiversity of saline streams often includes endemic species displaying high habitat specificity, restricted geographical ranges and often occurring as highly isolated populations (Millán *et al.* 2011). These factors together with the vulnerability of Mediterranean saline habitats have promoted the recognition of several endemic beetles as highly vulnerable species (Abellán *et al.* 2005; Sánchez-Fernández *et al.* 2008; Bennas *et al.* 2009; Verdú *et al.* 2011). However, *E. falcarius* was previously not considered to be of conservational concern because of its incorrect classification as a single broadly distributed species. The present integrative taxonomic study delimited four species within this species complex, which should be recognised as vulnerable and which shared very similar biogeographical, ecological and habitat-related attributes (i.e. habitat rarity and habitat loss) and also had fewer populations and smaller distributional ranges than the currently accepted single entity. Regarding strategies for the protection of these species, a recent study focussed on the effect of climate change on saline water species (Arribas *et al.* 2012a) has shown that

the persistence capacity of *E. jesusarribasi* sp. n. in its current localities is high, and as a result, *in situ* management centred on maintaining these populations and minimising impacts in these habitats could be the most efficient and practical conservation strategies for the *E. falcarius* species complex. Our results show how applying integrative taxonomy approaches to lineages from threatened habitats with potential to comprise cryptic diversity could be a fundamental tool for biodiversity conservation, driving the discovery, naming and evaluation of cryptic species and the modification of previous, inadequately assessed vulnerability categorisations.

Acknowledgements

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Supporting Information

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

Fig. S1. Intra and interspecific *cox1* uncorrected p-distances for the *Enochrus bicolor* group considering the four delimited candidate species within the *Enochrus falcarius* species complex.

Fig. S2. Linear body morphometrics for candidate species within the *Enochrus falcarius* complex.

Fig. S3. Detailed microscope photographs of characters for the *Enochrus falcarius* complex species identification.

Fig. S4. Key for identification of the *Enochrus falcarius* complex species.

Table S1. Localities sampled for the species delimitation integrative approach.

Table S2. List of studied specimens in the species delimitation integrative approach.

Table S3. Linear measurements used in the body morphometrics study of the *Enochrus falcarius* species complex.

Table S4. Detailed species vulnerability assessment scores following Abellán *et al.* (2005) for the species within the *Enochrus falcarius* species complex.

Table S5. Distance-based clustering of the *cox1* data for different divergence thresholds and matching with a putative species within the *Enochrus bicolor* species group.

Table S6. Distance-based clustering of the *cox1* data for different divergence thresholds and matching with candidate species within the *Enochrus bicolor* species group.

Table S7. Morphometric evaluation of the candidate species within the *Enochrus falcarius* species complex.

Table S8. Observed niche overlap values for species pairs within the *Enochrus falcarius* species complex.