Diversity of introduced terrestrial flatworms in the Iberian Peninsula. A cautionary tale.

Many tropical terrestrial planarians (Platyhelminthes, Geoplanidae) have been introduced around the globe. One of these species is known to cause significant decline in earthworm populations, resulting in a reduction of ecological functions that earthworms provide. Flatworms, additionally, are a potential risk to other species that have the same dietary needs. Hence, the planarian invasion might cause significant economic losses in agriculture and damage to the ecosystem. In the Iberian Peninsula only *Bipalium kewense* Moseley, 1878 had been cited till 2007. From that year on, four more species have been cited, and several reports of the presence of these animals in particular gardens have been received. In the present study we have: (1) analyzed the animals sent by non-specialists and also the presence of terrestrial planarians in plant nurseries and garden centers; (2) identified their species through morphological and phylogenetic molecular analyses, including representatives of their areas of origin; (3) revised their dietary sources and (4) used Species Distribution Modeling (SDM) for one species to evaluate the risk of its introduction to natural areas. The results have shown the presence of at least ten species of alien terrestrial planarians, from all its phylogenetic range. International plant trade is the source of these animals, and many garden centers are acting as reservoirs. Also, landscape restoration to reintroduce autochthonous plants has facilitated their introduction close to natural forests and agricultural fields. In conclusion, there is a need to take measures on plant trade and to have special care in the treatment of restored habitats.

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20 Introduction

21   Most animal invasive species detected in Europe are terrestrial invertebrates (Roques et

22 al., 2009). Invading edaphic organisms can have dramatic effects on the environment, due to the

23 direct effects on native soil organisms, and through their interactions with the environment

24 aboveground. However, overall, their impact in human health and economy is greater than their

25 ecological impact (Vilà et al., 2010). Among these organisms, land planarians are becoming an

26 important and diversified group of introduced species in Europe.

27   Terrestrial planarians (Platyhelminthes, Geoplanidae) are divided into four subfamilies

28 (Bipaliinae, Microplaninae, Geoplaninae and Rhynchodeminae) with a cosmopolitan distribution

29 (Winsor, Johns & Yeates, 1998); however, most species are found in the southern hemisphere.

30 Bipaliinae (Fig. 1A) is absent from the American and European continents, Geoplaninae (Fig.

31 1B) have an exclusively Central and South American distribution, while Microplaninae (Fig. 2A)

32 and Rhynchodeminae (Fig. 2B) are the subfamilies with the most northerly distribution, including

33 Europe. Terrestrial planarians are the only free-living Platyhelminthes that do not live in an

34 aquatic habitat. However, they have not developed the capacity to prevent water loss and are thus

35 strongly dependent on environmental moisture level (Froehlich, 1956; McDonald & Jones, 2007).

36 They seem to withstand this limitation through behavioral strategies, such as hiding in damp

37 refuges during the day and becoming active during the night. Due to these characteristics, these

38 animals are considered to have a low capacity to disperse. In fact, in their areas of origin,

39 although a few species are well-adapted to open and human-transformed lands (Baptista & Leal-

40 Zanchet, 2010), most species are restricted to humid forest areas.

41   A total of 36 species of terrestrial planarians are known to have been introduced in

42 different countries around the globe. Most of these species have a big effect on terrestrial

43 ecosystem processes because they prey on soil invertebrates (see references in Winsor, Johns &

44 Barker, 2004). So far, five of these species are considered to be either invasive and cause

45 problems with local biodiversity (*Platydemus manokwari* De Beauchamp, 1963), or horticultural

46 pests (*Arthurdendyus triangulatus* (Dendy, 1894)) or earthworm farm pests (*Bipalium*

47 *adventitium* Hyman, 1943; *Bipalium kewense* Moseley, 1878; *Dolichoplana striata* Moseley,

48 1877, see Winsor, Johns & Barker, 2004).

49   In Europe there is evidence of the presence of at least 18 introduced terrestrial planarians

50 (Minelli, 1977; Ball & Reynoldson, 1981; Jones, 1988; 1998; Mateos, Giribet & Carranza, 1998;

51 Faubel, 2004; Jones et al., 2008 Vila-Farré et al., 2008; 2011; Justine et al., 2014). In the Iberian

52 Peninsula (IP) there are only a few published records of introduced terrestrial planarians, and the

53 only species cited are *Bipalium kewense* in Barcelona (Filella-Subirá, 1983), *Platydemus* sp. in

54 Málaga (Vila-Farré et al., 2011), *Obama* sp. in Asturias (Fernández et al., 2013) and

55 *Rhynchodemus* R02 and *Caenoplana coerulea* Moseley, 1877 in Girona (Mateos et al., 2013).

56 The last species has also been cited in Menorca (Breugelmans et al., 2012).

57 After receiving multiple reports from non-scientists on the presence of "large and

58 colored" terrestrial flatworms in several localities in the IP, and given their observed locations,

59 particularly in private gardens, we decided to analyze their presence in garden centers and plant

60 nurseries.

61 The aims of this work were to: (1) Estimate the number of terrestrial flatworm species

62 introduced in the IP, and find their region of origin; (2) check whether plant nurseries and garden

63 centers are acting as entrance gates and reservoirs; (3) estimate the invasive potential of some

64 introduced species by considering their diet and by using Species Distribution Modeling (SDM);

65 (4) propose measures to prevent their becoming invasive and to prevent further introductions and

66 spread.

67 Material and methods

68 Specimen collection

69 Specimens were sampled from four sources (Tables 1 and 2): (1) gardens, (2) nurseries

70 and plantations, (3) semi natural areas, and (4) from other countries (either the original area of

71 distribution or other invaded areas). Specimens from sources 1 and 2 were either sent by people

72 who knew our work through the information in social networks, or sampled by us (all the

73 localities reported by non-scientist collaborators correspond to gardens). Specimens from source

74 3 were sampled by us. Specimens from source 4 were sent by colleagues, specialists of the group,

75 to whom we requested material for comparison with the Iberian populations.

76 Data from a total of 13 domestic gardens, seven nurseries, two plantations (all confined,

77 humanized locations), and three semi-natural areas (humanized environments that are not

78 confined and in direct contact with agricultural and forest areas) have been analyzed (Table 1).

79 The three "semi natural areas", located in North-eastern Iberian Peninsula, were: (1) Cal Tet, Parc

80 Natural Delta del Llobregat, Barcelona (Fig. 3, Loc-code O); (2) Can Cabanyes, Granollers,

81 Barcelona (Fig. 3, Loc-code M); (3) Viaducte de Rubiò, Vall d'en Bas, Girona (Fig. 3, Loc-code

82 P). In all three places recent habitat restoration activities have been performed, including the

83 transplantation of autochthonous plant species from commercial nurseries.

84   Amateur collaborators photographed the animals alive and fixed them in absolute ethanol.

85   Specimens collected by us were also photographed and external morphological characters

86   recorded. Subsequently, animals were subjected to two different procedures to proceed to the

87   species identification: (1) specimens for molecular analyses were fixed in 100% ethanol and (2)

88   specimens for histological studies were killed with boiling water, fixed with 10% formalin for 24

89   hours, and then preserved in 70% ethanol.

90   Morphological studies

91   Preserved specimens were examined under a stereo microscope and notes of their

92   dimensions, appearance, color (though this is affected by preservation), eyes, any stripes or

93   pattern, the position of the pharyngeal aperture (mouth) and gonopore, if present, were taken.

94   Specimens with no visible gonopore were considered to be immature. It was possible to identify

95   some specimens, even immature ones, to species level without further examination. For

96   unrecognized specimens, or where identity was uncertain and required confirmation, a mature

97   specimen (evidenced by an open gonopore) was selected and divided into various portions, being

98   embedded in wax. The copulatory apparatus (gonopore) and a small anterior region were

99   sagittally and transversely sectioned at 10 or 15 μm, respectively, stained in Harris' haematoxylin

100   and eosin and mounted in Canada balsam.

101   DNA extraction, gene amplification and sequencing

102   A small piece of tissue fixed in absolute alcohol was digested with Wizard Genomic DNA

103   Purification lysis Buffer (Promega, Madison, WI, USA) and Proteinase K overnight at 37ºC,

104   following manufacturer's instructions. The rest of the tissue is kept as voucher in the Genetics

105   Department (Universitat de Barcelona).

106   We amplified an approximately 1 kb fragment of the mitochondrial cytochrome c oxidase

107   I (Cox1 gene) and a fragment of approximately 1,500 bp of the 28S rRNA gene (28S) by PCR

108   reaction. PCRs were carried out in a volume reaction mixture of 25 μl. For Cox1 we used

109   primers BarS (Álvarez-Presas et al., 2011) and COIR (Lázaro et al., 2009) and conditions were as

110   in Álvarez-Presas et al. (2011); 28S rDNA gene was amplified in two different overlapping

111   fragments using the primers 28S1F, 28S4R, 28S2F and 28S6R, and conditions as in Álvarez-

112   Presas et al. (2008). Amplification products were purified with a vacuum manifold

113   (Multiscreen_HTS Vacuum Manifold, Millipore Corporation, Billerica, MA 01821, USA). DNA

114   sequences were determined from both strands using Big-Dye Terminator (3.1, Applied

115 Biosystems, Foster City, CA, USA) and the reaction products were separated on the ABI Prism

116 3730 automated sequencer (Unitat de Genòmica dels Centres Científics i Tecnològics de la UB).

117   PCR products of the 28S gene for some individuals, that yielded double bands in the

118 direct sequences, were cloned using HTP TOPO TA Cloning Kit for Sequencing (Invitrogen) in

119 order to be sure that only one type of sequence was recovered (since the existence of a

120 duplication of the ribosomal cluster is known in terrestrial planarians, Carranza et al., 1996). The

121 sequences of the clones showed that these bands corresponded to polymorphisms of one of the

122 types. Seqman (v. 4.2.2, Gene Codes) was used to revise the chromatograms and obtain the

123 definitive sequences.

124 Molecular data analyses

125   Ribosomal sequences were aligned using MAFFT v. 7 (Katoh & Standley, 2013) with the

126 G-INS-i iterative refinement method and 1000 cycles. Mitochondrial coding DNA sequences

127 were translated into aminoacids and aligned manually in Bioedit v.7.0.9.0. (Hall, 1999). All

128 sequences were unambiguously aligned. We estimated the DNA sequence evolution model that

129 best fits the data for both molecules using jModelTest 2.1.4. (Darriba et al., 2012), applying the

130 Akaike information criterion (AIC). Phylogenetic relationships were estimated by Maximum

131 Likelihood (ML) using RAxML 7.0.0 software (Stamatakis, 2006) and Bayesian inference (BI)

132 using MrBayes v. 3.2. (Ronquist et al., 2012). Bootstrap support (BS) values were obtained for

133 ML trees from 10,000 replicates. In the BI analyses we ran four chains to allow heating and used

134 default priors, three million generations were run using the Markov Chain Monte Carlo (MCMC)

135 analysis in two independent runs. Sampling was every 1000 generations. The stationarity and

136 convergence of the runs were checked by plotting Log likelihood values vs number of

137 generations and inspecting when the standard deviation of split frequencies had reached < 0.01,

138 respectively.

139 Potential distribution modeling

140   Using data describing the known distribution of *C. coerulea* in Australia, we estimated the

141 potential distribution of this species in the Iberian Peninsula, as an exercise to find out whether

142 climatic variables could detect potentially at risk areas where the establishment of the introduced

143 species will be favored if only affected by climate. This could be a tool to help limit potential

144 activities in order to avoid the introduced animals becoming invasive in the most likely areas for

145 them to be successful.

146   For the SDM, a total of 179 Australian geographical coordinates of presence observations

147 extracted from the literature, internet sources and personal communications (L. Winsor, 2013)

148 were used for calibration of models (training dataset). To avoid over-parameterization and loss of

149 predictive power, we discarded the climatic variables that were highly correlated. To do this we

150 extracted environmental information from 10,000 randomly generated points and determined the

151 linear relationships among them using Spearman and Pearson correlations. Although all

152 correlations were significant they show low correlation coefficients ($r <= 0.12$). According to this

153 analysis we used the 9 bioclimatic variables from the WorldClim database v. 1.4.

154 (http://www.worldclim.org/, Hijmans et al., 2005) with less dependence, to form the present

155 climatic dataset at a scale of 30 arc seconds. Those variables were: annual mean temperature;

156 mean diurnal range; isothermality; maximum temperature of warmest month; minimum

157 temperature of coldest month; precipitation of wettest month; precipitation seasonality;

158 precipitation of wettest quarter; and precipitation of warmest quarter. The maximum entropy

159 model, a presence-only algorithm that requires known species occurrence points and

160 environmental variables (Maxent v.3.3.3k; Phillips, Anderson & Schapire, 2006), was applied.

161 We selected the software default values for the convergence threshold, regularization values, and

162 features. The maximum number of iterations was set to 1,000 and 1,000 bootstrap replicates were

163 used. All possible geographic locations were partitioned between training and test samples (75%

164 and 25%, respectively) in order to achieve higher predictive accuracy (Phillips & Dudík, 2008).

165 Once the models were trained, we projected the results using the IP climatic dataset, to study the

166 possible expansion of *C. coerulea* in the region. Model performance was evaluated using the

167 AUC test (area under the receiver operating characteristic curve (ROC)) and the binomial test of

168 the omission-dependent threshold was calculated by Maxent. Finally, binary maps of the outcome

169 of the models were overlapped in the geographic information system, ArcMap v.10 (ESRI 2011.

170 ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute).

171 Results
172 Morphological identification of the specimens

173   Based on the external appearance of the flatworms we initially grouped the specimens

174 into nine morphotypes. We classified four of them at the species level due to their characteristic

175 shapes or other external features, and the other five at genus or tribe level.

176 *Bipalium kewense* (Fig. 4) has been identified by the characteristic shape of the anterior

177 end and the pattern of stripes along the dorsal and ventral body surfaces. One specimen preserved

178 in 70% ethanol from Bordils locality (Loc code V in Table 1) has been deposited at the Natural

179 History Museum of the United Kingdom (NHMUK) with voucher number **NHMUK**

180 **2014.5.13.6.**

181 For *Caenoplana bicolor* (Graff, 1899) there is no published description of a sexually

182 mature specimen, hence the identification of the only specimen obtained, also an immature

183 individual, relied exclusively on its external appearance (Fig. 5). This specimen is deposited in

184 the tissue collection of the Genetics Department (Universitat de Barcelona).

185 Among the specimens with an external morphology initially ascribable to the *Caenoplana*

186 *coerulea* phenotype, we have found two morphotypes basing on their color pattern. Morphotype

187 Ca1 (Fig. 6) presents a dorsal coloration in dark blue with a yellow middle-dorsal stripe, and a

188 ventral light blue region (characteristic pattern of *Caenoplana coerulea*). The histological study

189 of one specimen from El Prat de Llobregat locality (Loc code O in Table 1) (**NHMUK**

190 **2014.5.13.14**) reveals that it may belong to *Caenoplana coerulea* species. Morphotype Ca2 (Fig.

191 7) presents a light brown dorsal region with a pale yellow middle-dorsal stripe, and a ventral light

192 blue-greenish region. The histological study of one specimen from Bordils locality (Loc code V

193 in Table 1) (**NHMUK 2014.5.13.12**) has revealed that its copulatory apparatus characters do not

194 fit any of the described *Caenoplana* species.

195 *Dolichoplana striata* (Fig. 8) could also be identified by its characteristic external

196 appearance. One specimen from Bordils locality (Loc code V in Table 1) has been deposited at

197 the NHMUK (**NHMUK 2014.5.13.7**).

198 *Kontikia ventrolineata* (Dendy, 1892) (Fig. 9) was externally identified, following NNSS

199 (2013). We assigned the specific name following Jones, Johns & Winsor (1998), who considered

200 *Parakontikia* Winsor, 1991 as junior synonym of *Kontikia* Froehlich, 1955. Three specimens

201 from Granollers locality (Loc code M in Table 1) are deposited at the NHMUK (**NHMUK**

202 **2014.5.13.3-5**).

203 We found one morphotype externally ascribable to the genus *Rhynchodemus*, but not to a

204 known species (Fig. 10). *Rhynchodemus* morph Rs1 has a dark brown pigmented body with two

205 black longitudinal stripes, and two large eyes situated a little distant from the anterior tip. One

206 specimen from Vall d'en Bas locality (Loc code P in Table 1) (**NHMUK 2014.5.13.9**) was

207 histologically studied but, unluckily, presented a copulatory apparatus not well developed,

208   preventing us from determining whether it could belong to *Rhynchodemus sylvaticus* (Leidy,

209   1851) to which it was extremely externally similar.

210   A morphotype externally ascribable to the tribe Rhynchodemini was found in

211   Benamargosa locality (Loc-code G), but its morphological features did not allow assigning it to

212   any genus. Rhynchodemini morph Ri1 presents a dark brown pigmented body with one dorsal

213   black line (no image available).

214   Specimens of *Obama* sp. (Fig. 11) have a characteristic leaf-shaped, broad, flattened body.

215   Externally, they are very similar to *Obama* sp. 6 *sensu* Carbayo et al., 2013 from Brazil

216   (Fernando Carbayo personal communication). One specimen from Bordils locality (Loc code V

217   in Table 1) is deposited at the NHMUK (**NHMUK 2014.5.13.8**).

218   Phylogenetic results

219   We inferred ML trees to check the diagnosis of the introduced specimens and to determine

220   their level of relatedness to the ones from the original areas of distribution. For this reason, the

221   datasets included, when possible, sequences belonging to morphologically diagnosed specimens

222   from the original area of distribution of the putative introduced species (obtained for this study or

223   coming from GenBank; Table 2).

224   We obtained 28S sequences for 15 individuals. One or two sequences from each

225   morphotype were aligned together with 19 GenBank ingroup sequences and 3 outgroup

226   sequences belonging to the *Dugesia* genus (terrestrial planarians sister group; Carranza et al.,

227   1998; Álvarez-Presas, Baguñá & Riutort, 2008). Cox1 sequences were obtained for all

228   individuals included in the study (Table 2). To obtain a more detailed picture of the situation

229   within the main clades, including introduced planarians found on the concatenated analysis, we

230   split the Cox1 sequences into four new datasets, one for each subfamily, tribe or genus:

231   Caenoplanini (56 ingroup + 4 outgroup), Geoplaninae (26 ingroup sequences + 2 outgroup),

232   Bipaliinae (9 ingroup + 3 outgroup) and Rhynchodemini (29 ingroup + 4 outgroup). For each

233   clade, its sister group was selected as the outgroup as shown on the concatenated analysis and/or

234   previous studies (Álvarez-Presas et al., 2008). The best-fit model of sequence evolution for the

235   28S was GTR+G and for Cox1 was GTR+I+G. We inferred a ML tree with partitions from a

236   concatenated dataset including 37 individuals for which both 28S and Cox1 sequences had been

237   obtained (Fig.12). The ML trees obtained from the Cox1 datasets are shown in Figs. 13-16.

238   For the concatenated dataset, the ML tree showed most introduced specimens constitute

239   monophyletic groups together with representatives of their species coming from the original

240 distribution area or other introduced localities. We have found introduced planarians in the IP for
241 all non-autochthonous terrestrial planarians subfamilies; in the case of the Rhynchodeminae there
242 are even representatives from two tribes (Rhynchodemini and Caenoplanini).

243   Within the Bipaliinae, *Bipalium* specimens found in the IP constitute a monophyletic
244 group together with *Bipalium* sequences from other species, *B. adventitium* being the closest
245 relative in the Cox1 tree (Figs. 12 and 13). The genetic diversity among the four *B. kewense*
246 sequences, coming from the IP and Açores Islands, was very small.

247   In the Geoplaninae clade (Figs. 12 and 14) the introduced specimens found in the IP
248 constitute a monophyletic group with a still not-described species from Brazil (*Obama* sp. 6 after
249 Carbayo et al., 2013, Fig. 14). In the Cox1 tree, specimens coming from the IP, United Kingdom
250 (both introduced) and Brazil (original area) constitute a highly-supported monophyletic group.
251 Within this group, the introduced individuals are divided in two quite differentiated clades
252 (*Obama* sp.A and *Obama* sp.B in Fig 14), also distinctly separated from the Brazilian individuals.
253 All the UK individuals fall within the clade *Obama* sp.A.

254   The Caenoplanini clade (Figs. 12 and 15) includes a high number of introduced
255 individuals and the broadest diversity of sequences. Even *Caenoplana coerulea* sequences, either
256 coming from GenBank, or from the individuals sent by our collaborator in Australia, are found in
257 very distinct genetic clades pointing to the existence of more than one species (see Discussion).
258 For this reason, we use the name *Caenoplana coerulea* s.l. to refer to all those specimens. In the
259 concatenated tree, the representative of *Caenoplana* morphotype Ca1 is closely related to
260 *Caenoplana coerulea* s.l. from Australia, while *Caenoplana* morphotype Ca2 is the sister group
261 of a clade constituted by *C. coerulea* s.l. and *C. bicolor*. The divergence among these three
262 lineages can be appreciated when compared to the other subfamilies present in the tree. In the
263 Cox1 tree (Fig. 15) genus, *Caenoplana* again shows high levels of genetic diversity, evidenced by
264 the long branches separating its subclades. Most *Caenoplana* morphotype Ca1 from the IP
265 constitute a low diversity clade including *C. coerulea* s.l. from its original area (Australia) and
266 also from UK and Menorca (also introduced). This clade is sister to another group including *C.*
267 *coerulea* s.l. originally from different localities in Australia (Sunnucks et al., 2006); however, the
268 differentiation among these two clades is extremely high. The other two *Caenoplana* morphotype
269 Ca1 individuals, coming from Townsville (Australia), constitute a highly differentiated clade that
270 also includes a GenBank sequence identified only to the genus level and one of the introduced
271 individuals. Finally, there is a clade including only introduced animals, one of them identified as

272   *C. bicolor* and the rest as morphotype Ca2. The genetic differentiation between the two lineages

273   within this clade is nonetheless extremely high.

274   In the Rhyncodemini clade (Figs. 12 and 16) we find representatives of three genera in the

275   IP. *Dolichoplana striata* sequences form a monophyletic clade in the Cox1 tree, including three

276   introduced animals in the IP and one coming from Brazil. The individuals assigned to

277   Rhynchodemini morphotype Ri1 collected in Málaga (Spain, Loc code G in Table 1; Vila-Farré et

278   al., 2011) cannot be assigned to any species, although they probably belong to *Dolichoplana*

279   given the relationships they show in the Cox1 tree. The four *K. ventrolineata* specimens analyzed

280   constitute a monophyletic group with a low variability, the French representative being the more

281   divergent. The genus *Rhynchodemus* is represented by at least three species in the Cox1 tree.

282   *Rhynchodemus sylvaticus* (considered an European autochthonous species), *Rhynchodemus*

283   morphotype Rs1, and a clade including two individuals from Panama that we had ascribed to the

284   Rhynchodemini by their external appearance, and they appear likely to belong to the genus

285   *Rhynchodemus*. It should be noted that the specific identification of all *R. sylvaticus* specimens

286   found in the IP (Boix and Sala, 2001; Mateos et al., 2009; Vila-Farré et al., 2008; 2011) have

287   been made based exclusively on external morphology (for this reason all these specimens have

288   been considered *Rhynchodemus* cf. *sylvaticus*). *Rhynchodemus* cf. *sylvaticus* clade, including

289   representatives from Spain, Portugal, UK and France, together with a specimen identified in a

290   previous study (*Rhynchodemus* cf. *sylvaticus* (Canyamars)), is sister group of a clade constituted

291   by *Rhynchodemus* morphotype Rs1 and one specimen of *R.* cf. *sylvaticus* (specimen 219).

292   Specimen distribution

293   Figure 3 and Tables 1 and 2 show the sampling localities of the animals analyzed in this

294   study. In all the plant nurseries, only one species of terrestrial planarian was found (*Bipalium*

295   *kewense,* Rhynchodemini Ri1 or *Obama* sp.), except in Bordils where six species were found

296   (Table 1, Loc-code V). The rest of the localities also contained a single species, with the

297   exception of Treto (a garden, Loc-code U) with two species, and the two ''semi natural areas''

298   situated in Vall de'n Bas (Loc-code P) and in Granollers (Loc-code M) also with two species

299   each. *Obama* sp. was the species most frequently found in plant nurseries, while *B. kewense*

300   predominated in private gardens. In the semi natural areas only the species *K. ventrolineata*, *C.*

301   *coerulea* s.l., and *Rhynchodemus* Rs1 (not found anywhere else) have been found.

302 Potential species distribution modelling

303   The result of projecting models for the potential distribution of *C. coerulea* s.l. in the IP

304 presents mean values of AUC beyond 0.9 (0.974) and significance for all tests of omission, which

305 indicates good performance of the models. Furthermore, predictions were significantly different

306 from random because binomial omission test thresholds were significant (p <0.01) in all 1,000

307 runs. A composite map showing the potential distribution models for *C. coerulea* s.l. species

308 projected on current climate layers is provided in Fig. 17.

309   The results of the potential distribution of the species in the IP, based on data from its

310 current distribution in their region of origin (Australia), show that the species can find extremely

311 suitable areas for its survival and expansion is the northern region, where the appropriate

312 temperature and humidity conditions occur.

313 Discussion

314 Species identification, or, how many species are out there?

315   External morphology (Figs. 4-14), analysis of histological sections, and phylogenetic

316 inference from molecular data (Figs. 12-16) have revealed the presence of five clearly

317 identifiable species of introduced exotic land planarians in the IP: *Bipalium kewense* (Bipaliinae),

318 *Caenoplana bicolor*, *Caenoplana coerulea* s.l. (Ca1), *Dolichoplana striata* (Rhynchodeminae,

319 Rhynchodemini), and *Kontikia ventrolineata* (Rhynchodeminae, Caenoplanini). However, the

320 phylogenetic trees obtained and the analysis of the external appearance of the specimens indicate

321 that probably at least five more species were present, including Rhynchodemini morph Ri1,

322 *Rhynchodemus* morph Rs1, *Obama* sp. and two more species within *Caenoplana*: *Caenoplana*

323 morph Ca2 and probably some individuals of *Caenoplana* morph Ca1 (see below).

324   The assignation of *Bipalium kewense* is based on its characteristic external morphology

325 (see Hill & Merickel, 2011; Jones, 1998). There are no published Cox1 gene sequences for this

326 species in Genbank, so those presented in this paper are the first available. Phylogenetic analysis

327 of these sequences point to an introduction from the same lineage. Surprisingly, all sequences

328 belonging to *Kontikia ventrolineata* (coming from Spain, France and UK) are situated within the

329 Rhynchodemini clade with high support in both trees. This situation contradicts the taxonomy of

330 Sluys et al. (2009) where the genus *Kontikia* belongs to tribe Caenoplanini.

331   The genetic differentiation observed within the group constituted by the genus

332 *Caenoplana*, monophyletic in the trees, leads us to predict that it includes more than one species.

333   In the Cox1 tree (Fig. 15), at least three monophyletic groups seem to be clearly defined and

334   probably represent different species. In fact, *C. coerulea* is considered by a specialist in this

335   group (L. Winsor, personal communication) as a complex of species, on the basis of internal

336   anatomical characters and stripe morphology. According to Winsor, there are at least three species

337   that are distinguishable morphologically; but there are probably more than three species in the

338   area of origin. One of the problems with the group is that the type of the species is non-sexually

339   mature. Hence, to clarify the situation and number of the species in this group, a broad sampling

340   in its original area of distribution is required, followed by a thorough morphological and

341   molecular study. Nonetheless, for the purpose of the present paper, the evidence is clear that at

342   least three different genetic lineages from Australia have been introduced in the IP, probably

343   independently.

344   In the case of *Rhynchodemus* Rs1, we cannot be sure if this is a distinct species or simply

345   a differentiated lineage of *R.* cf. *sylvaticus*. The latter has been generally regarded as an

346   introduced species in Europe from USA (Jones, 1988), but it is also considered as probable

347   species native to Europe (Jones, 1998; 2005) and introduced in the USA from Europe (Ogren &

348   Kawakatsu, 1998). The type locality of *R. sylvaticus* is Philadelphia, Pennsylvania, USA (1851).

349   This species has a wide distribution in the IP and two of the locations are plant nurseries, one in

350   Barcelona (Vila-Farré et al., 2008) and one in Málaga (Vila-Farré et al., 2011), while the other

351   localities can be considered natural habitats. In our molecular analysis there was no separation of

352   specimens according to their locality type (natural or artificial). Two distinct clades of European

353   *Rhynchodemus* were obtained (Fig. 16), suggesting the existence of two different species with

354   similar external morphology.

355   In the case of Rhyncodemini Ri1, this species probably belongs to the genus

356   *Dolichoplana*; however, we were only able to obtain three specimens and none of them were

357   sexually mature.

358   When specimens of *Obama* sp. were first found in the UK and the IP, they were identified

359   as *O. marmorata* (Schultze & Müller, 1857) due to their external appearance; however, molecular

360   data (unpublished) showed that the European specimens did not constitute a monophyletic group

361   with that species, indicating that they belonged to an unknown, still undescribed, Geoplaninae.

362   Sampling performed in Brazil since then has found another species (*Obama* sp.6), which is also

363   externally very similar. Molecular data show that it is closely related to the individuals found in

364   Europe (unpublished). As in the previous case, a morphological and molecular study should be

365   undertaken to clearly delimit and describe the new species. The two clades found in our Cox1

366   tree (Fig. 14), that may represent two different species, suggest that there have been two

367   independent introductions into the IP from different native sites in Brazil.

368   Overall, we have shown that at least ten introductions have occurred in the IP. These

369   introductions include species from all the non-European terrestrial planarian subfamilies from

370   native localities as far as South America and Australia. Since most of these species have

371   previously been reported to have been introduced in other countries, the introductions into the IP

372   have probably not been directly from the source countries, but were more likely to be indirect,

373   following plant trade routes. In most cases, all the individuals from the same species found in the

374   different localities are nearly identical, even when compared between Spain and the UK, which

375   can be interpreted as the result of a single introduction (or a single exportation from the place of

376   origin). In others, as in the case of *Caenoplana*, the observed diversity clearly indicates that the

377   introductions were from different lineages within this group and is likely to be the consequence

378   of more than one export from the native area.

379   What makes terrestrial planarians so successful as introduced species?

380   Temperature, humidity and food availability are the three basic factors determining the

381   geographical distribution of terrestrial planarians (Boag, Yeates & Johns, 1998). The feeding

382   habits of the introduced species in the IP indicate that all of them feed on invertebrate soil fauna

383   (Table 3). In plant nurseries and greenhouses microclimatic conditions are maintained artificially

384   (high humidity and stable temperature) and are likely to favor the presence of stable populations

385   of many species of terrestrial invertebrates. In nurseries visited by us, especially under

386   flowerpots, we have observed the presence of numerous specimens of snails, slugs, earthworms,

387   millipedes, isopods, beetles and various groups of microarthropods, including springtails.

388   Therefore, in this very suitable artificial microhabitat, there is likely to be a greater number of

389   species of terrestrial flatworms (as is the case of Bordils, Loc code V in Table 1, where six

390   species were detected in the same greenhouse).

391   Land planarians and their cocoons are very often associated with the soil of plants in pots

392   and certain types of fresh vegetables (Ogren, 1985; Mather & Christensen, 1992; Hogan &

393   Dundee, 1996). The transport of these pots and materials (which can occur over international and

394   intercontinental distances) may permit the transport of associated planarians and/or cocoons,

395   which is the primary means of introduction of exotic species of terrestrial planarians into

396   different contaminated countries (Winsor, Johns & Barker, 2004). The suitable conditions in the

397   plant nurseries and garden centers may explain their introduction success. In recent decades, the

398   adoption of free market policies and trade agreements have reduced barriers to plant trade among

399   different countries (Dehnen-Schmutz et al., 2010), but there has been insufficient attention given

400   to how such structural change in international trade can affect the risk of spread of invasive

401   species (Drew et al., 2010). Depending on the intricate network of commercial interactions

402   among European countries (see Dehnen-Schmutz et al., 2010), we expect a huge European

403   dispersal of exotic animal species associated with this trade.

404   Will planarians become invasive in the Iberian Peninsula as has occurred in other areas?

405   Exotic species present in an area could be categorized as introduced (detected in the area

406   but with unknown status), adventives or not established (they reproduce occasionally in the area

407   not constituting stable populations), naturalized or established (they form stable reproductive

408   populations in the area) and invasive (established and well spread in the area) (Richardson et al.,

409   2000; Simberlof et al., 2013). The "tens rule" (Williamson & Fitter, 1996; Williamson & Brown,

410   1986; Williamson, 1996) predicts that just one of hundreds of introduced species becomes

411   invasive (about 10% of the introduced species are established, and that 10% of those become

412   invasive). Based on the premise of the "tens rule", some researchers minimize the potential

413   impact of exotic species (National Research Council, 2002; Campbell & Gibson, 2001), while

414   others warn that this risk minimization is dangerous and, with respect to the possible impact of

415   introduced species, the adoption of the precautionary principle is crucial (Jaric & Cvijanovic,

416   2012), but unlikely! The problem with this sort of assumption or calculation is that, in most cases,

417   we simply have no knowledge of the unsuccessful introductions.

418   In the case of terrestrial planarians, some species are very tolerant of habitat modification

419   (Cannon et al., 1999; Carbayo, Leal-Zanchet & Vieira, 2002), facilitating their survival in

420   humanized environments. Many introduced species of terrestrial planarians are found confined to

421   these types of habitats (parks, private gardens, plant nurseries), but it is not known whether this

422   distribution is so restricted due to environmental constraints (planarians, coming from tropical

423   habitats cannot live outside these artificial habitats in the European environment) or to a low

424   velocity of dispersion to natural habitats (Ducey & Noce, 1998). In our case, most specimens

425   occurred in confined areas (gardens and nurseries). However, *Rhynchodemus* Rs1, *C. coerulea*

426   s.l. and *K. ventrolineata* have been also found in recently restored areas that were more or less

427 connected to natural and agricultural environments, which increases the danger of their becoming
428 naturalized or even invasive.

429   In the particular case of *C. coerulea* s.l., we performed a potential distribution study to
430 check whether the area around its present introduced localities in the IP may be suitable for its
431 expansion. The results show that the potential distribution of the species (Fig. 17) indeed includes
432 the countryside that was nearby to the localities of the IP where it is already present. The most
433 suitable area is the northern IP. This is not surprising when we consider that in this northern
434 region, the climatic conditions (temperature and humidity) are also more optimal for the presence
435 of native land planarians (Mateos et al., 2009; Álvarez-Presas et al., 2012). Thus, we show that
436 by having suitable climatic databases, it is possible to model the potential distribution of
437 introduced species, and thus predict their risk of becoming invasive. If we add to this information
438 the knowledge of some biological features of the terrestrial planarian species, such as their prey
439 preferences, we may be able to make an even more precise image of the sites where it is more
440 likely for the species to become invasive and thus concentrate prevention efforts in those areas.

441   Our results show that *C. coerulea* s.l. is apparently the most successful colonizer, since it
442 is the only species present in all three unconfined (semi-natural) areas sampled. This may be
443 because it feeds on several groups of arthropods that are abundant in areas where this species has
444 been detected (isopods, beetles, diplopods). The three species (*Rhynchodemus* Rs1, *C. coerulea*
445 s.l. and *K. ventrolineata*) we find in unconfined environments feed on arthropods, whereas the
446 other species (found only in confined environments) do not feed on arthropods, but instead on
447 other invertebrates that require extremely wet habitats. Hence, land planarian species that feed on
448 arthropods have their food "secured" in environments with a Mediterranean climate and, as a
449 consequence, have a higher likelihood of being successful and becoming established or even
450 invasive.

451 What consequences might the introduction of flatworms have on human economies and
452 biodiversity?

453   Another important question is: what are the negative effects of the spread of these
454 species? In literature, the primary problems reported are related to economic consequences for
455 agricultural activities (Boag & Neilson, 2006; Boag, Neilson & Jones, 2010). As predators of
456 earthworms, planarians can cause soil drainage and fertility to be severely compromised. The
457 ecological consequences of the presence of these predators depends on their propagation speed

458 and efficiency, but could have significant effects on processes mediated by earthworms in both

459 agroecosystems and forests (Lilleskov et al., 2010). Although there is still no direct impact study

460 of the presence of invasive planarians on agricultural production (Boag, Neilson & Jones, 2010),

461 data from farmers with infested farmland and from the scientific literature have suggested that it

462 could reduce grass yields significantly (Boag & Neilson, 2006).

463   No reference has been made to the effect of these species on autochthonous populations of

464 terrestrial planarians, probably because the knowledge of the autochthonous fauna is very scarce.

465 In the IP we have already performed some studies on the autochthonous terrestrial planarian

466 fauna and found that it is very diverse, including at least 15 species, of which some contain a

467 great deal of genetic diversity (Mateos et al., 2009; Álvarez-Presas et al., 2012). The potential

468 arrival of some of these introduced species in natural habitats, where the autochthonous ones are

469 localized (as predicted by the potential distribution studies), would have very negative

470 consequences. Since exotic planarians are, in general, bigger in size, more voracious, have more

471 aggressive behavior, and sometimes appear to have a generalist diet (pers. obs.), they may be

472 more resistant to extreme conditions than the native species.

473 A cautionary tale: Plant trading and landscape restoration

474   An important question raised by all these observations is whether governments in Europe

475 should be asked to propose new, more restrictive rules on the trade of plants coming from

476 outside, or alternatively, to establish better controls or protocols to avoid the introduction of

477 unwanted organisms together with the plants. However, it is probably now too late to have an

478 impact on the transport of species around the world. Nonetheless, we are still in time to avoid

479 invasions of terrestrial planarians. The restoration of degraded areas involves planting native

480 plant species. These plants are available from nurseries and transported to the restoration areas

481 accompanied by a certain amount of soil on the roots. If this land is not subject to any preventive

482 treatment, it may be contaminated with organisms that are also introduced in the area that is being

483 restored. Among these organisms may be unwanted species that, if given the right conditions, can

484 become invasive. It is important to warn agencies conducting such restorations of these dangers

485 and ask stakeholders to include in the protocols of landscape restoration the necessary steps to

486 avoid these unwanted introductions.

487   Some simple, easy-to-perform sanitizing procedures, such as heating the soil (EPPO,

488 2000a; 2000b; SEERAD, 2000; Sugiura, 2008) before transplanting the nursery plants to the

natural environment, may be sufficiently effective and reliable to ensure that there is no concomitant dispersal of flatworms. Such procedures, together with a periodic analysis of the introduced species present in garden centers and nurseries, and a study of the potential areas of flatworm distribution, would also help avoid the introduction of terrestrial planarians into areas where they are more likely to become invasive (DEFRA, 2005; DOVE, 2012).

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701 Winsor L, Johns PM, Barker GM. 2004. Terrestrial planarians (Platyhelminthes: Tricladida:
702   Terricola) predaceous on terrestrial gastropods. In: Barker GM, ed. *Natural Enemies of*
703   *Terrestrial Molluscs*. London: CAB International, 227-278.
704

Localities where introduced species have been found/recorded in the Iberian Peninsula.

Data organized chronologically. Sampling code: (fs) specimens from field surveys conducted by us in gardens, nurseries and semi-natural areas, (sbp) specimens sent by people who knew our work through the information in social networks, (bd) bibliographic data. Date in format yyyy/mm/dd. Collectors: AG, Alberto Gayoso; AL, Álvaro Leal; AT, Àngels Tudó; CC, Cristina Cabrera; CI, César de Inés; CS, Carmen Soler; EM, Eduardo Mateos; GG,, Georgina Gratacós;IV, Iván Salvia; JM, Jacobo Martín; MR, Marta Riutort; RS, Roberto Sáez; VS, Vicent Sancho; Montilivi-WEB, http,//www.iesmontilivi.net/WebProfes/jbarbara/web/Galeria/Imatges/Invertebrats/cuc.htm; XB, Xavier Béjar.

Sampling code	Loc code	Locality	Position	Habitat	Species	Date	Collector/Ref
bd	A	Caldes d'Estrac (Barcelona)	N41.569467 E2.526316	garden	*Bipalium kewense*	1983	Filella-Subirá 1983
fs	B	Barcelona (Barcelona)	N41.398539 E2.142162	garden	*Bipalium kewense*	1995	MR
sbp	C	Lourizan (Pontevedra)	N42.410111 W8.667716	nursery	*Bipalium kewense*	1990	AG
bd	D	Girona (Girona)	N41.964541 E2.827842	garden	*Bipalium kewense*	1994	Montilivi-WEB
sbp	E	Villamalea (Albacete)	N39.362159 W1.601281	nursery	*Bipalium kewense*	1998	VS
sbp	F	Bétera (Valencia)	N39.604153 W0.507864	garden	*Bipalium kewense*	1999	VS
bd	G	Benarmargosa (Málaga)	N36.8248 W4.1809	mango plantation	Rhynchodemini Ri1 [G]	2007/12/25	Vila-Farré et al. 2011 as *Platydemus* sp
sbp	H	Badalona (Barcelona)	N41.460177 E2.243985	garden	*Caenoplana* Ca1 [G]	2008	RS
bd	I	Menorca (Balearic Islands)	N39.95000 E3.850000	orchard	*Caenoplana coerulea*	2009/04	Breugelmans et al. 2012
fs	J	Torruella de Fluvià (Girona)	N42.17559 E3.03953	garden	*Obama sp.6* [G]	2010/04/04	MR
sbp	K	Òliva (Valencia)	N38.910550 W0.073200	garden	*Caenoplana* Ca1 [G]	2010/11/08	VS
sbp	L	Ames (A Coruña)	N42.857955 W8.653278	garden	*Caenoplana* Ca1	2010/12/10	AG
fs	M	Granollers (Barcelona)	N41.570240 E2.270532	semi-natural	*Caenoplana* Ca1 [G] *Kontikia ventrolineata* [G]	2011/02/28 2012/10/12	CS EM
sbp	N	Boadilla del Campo (Madrid)	N40.405270 W3.877014	garden	*Caenoplana* Ca1	2011/10/15	JM
fs	O	El Prat de Llobregat (Barcelona)	N41.309519 E2.120887	semi-natural	*Caenoplana* Ca1 [G M]	2011/11/05	EM & CC
fs	P	Vall d'en Bas (Girona)	N42.125939 E2.433678	semi-natural	*Caenoplana* Ca1 [G] *Rhynchodemus* Rs1 [G M]	2011/11/12 2011/11/26	EM & XB EM & MR

fs	Q	Gavà-1 (Barcelona)	N41.288100 E2.006233	nursery	*Obama sp*	2012/03/13	AT & MR
fs	R	Gavà-2 (Barcelona)	N41.293222 E2.017583	nursery	*Obama sp* [G]	2012/03/14	AT & MR
fs	S	Vilassar de Mar (Barcelona)	N41.497084 E2.376178	nursery	*Obama sp* [G]	2012/03/28	AT & MR
fs	T	Tortosa (Tarragona)	N40.767329 E0.556963	nursery	*Obama sp* [G]	2012/04/04	AT
sbp	U	Treto (Cantabria)	N43.392385 W3.470387	garden	*Obama sp* [G] *Bipalium kewense* [G]	2012/06/27	CI
fs	V	Bordils (Girona)	N42.034804 E2.898615	nursery	*Caenoplana* Ca1 [G] *Caenoplana* Ca2 [G M] *Caenoplana bicolor* [G] *Obama sp* [G] *Dolychoplana striata* [G] *Bipalium kewense*	2012/10/22	EM
sbp	W	Girona (Girona)	N42.009800 E2.825554	garden	*Caenoplana coerulea*	2013/09/11	GG
sbp	X	Polop (Alicante)	N38.622149 W0.126626	garden	*Caenoplana coerulea*	2014/02/01	AL
sbp	Y	Cártama (Málaga)	N36.748333 W4.586944	garden	*Obama sp*	2014/03/01	IV

[G] species with genetic sequences; [M] species sectioned for internal anatomy study (see Table 2).

Sequenced specimens.

To each new sequence a three digits numeric code was assigned. Sequences from the GenBank database do not have specimen code number, only when there are more specimens from the same species in the same locality a specimen code was assigned (three letters+one number). Loc code as in Table 1. Collector: DB, Dani Boix; EM, Eduardo Mateos; HJ, Hugh Jones; KA, Miquel Arnedo; LL, Laia Leria; LW, L.Winsor; MA, Marta Álvarez-Presas; SG, S.Graham; MV Miquel Vila.

Species/Morphotype	Code	Locality / Ref. or Loc code or collector and position	28S	COI
Family Geoplanidae				
Subfamily Bipaliinae				
Bipalium sp.		Japan / Baguñà *et al.* 2001 - Alvarez-Presas *et al.* 2008	DQ665959 [a]	AF178307[a,c]
B. adventitium		Leignston (USA) / Baguñà *et al.* 2001 - Alvarez-Presas *et al.* 2008	DQ665956 [a]	AF178306 [a,c]
B. kewense	894	Ponta Delgada, Illa São Miguel (Açores, Portugal) / DB N37.745196 W25.667408*	KJ599731 [a]	KJ659612* [a,c]
	621	Treto (Cantabria, Spain) / U*	KJ659703* [a]	KJ659609* [a,c]
	623			KJ659610* [a,c]
	666	Bordils (Girona, Spain) / V*		KJ659611* [a,c]
B. multilineatum		South Korea /GenBank Direct submission 2010-may-18		HM346600 [c]
B. nobile				HM346598 [c]
Novibipalium venosum		Japan / Alvarez-Presas *et al.* 2008		DQ666048 [c]
Subfamily Microplaninae				
Microplana nana		Vall d'en Bas (Girona, Spain) / Mateos *et al.* 2009	KJ599722 [a]	FJ969947 [a,c]
M. groga		Canyamars (Barcelona, Spain) / Mateos *et al.* 2009	KJ599721 [a]	FJ969964 [a,c]
M. terrestris		Pontedeume (A Coruña, Spain) / Mateos *et al.* 2009	KJ599724 [a]	FJ969952 [a,c]
Subfamily Rhynchodeminae				
Tribe Caenoplanini				
Arthurdendyus triangulatus		Wainui Barrys Bay (New Zealand) / Alvarez-Presas *et al.* 2008	DQ665953 [a]	DQ666027 [a,b,d]
Artioposthia sp.		Australia / Baguñà *et al.* 2001 - Alvarez-Presas *et al.* 2008	DQ665954 [a]	AF178325 [a,b,d]
A. testacea		Malborough (Australia) / Baguñà *et al.* 2001 - Alvarez-Presas *et al.* 2008	DQ665952 [a]	AF178305 [a,b]
Australoplana sp.		New Plymouth, Omata (New Zealand) / Alvarez-Presas *et al.* 2008	DQ665955 [a]	DQ666028 [a,b,d]
Caenoplana sp. 1		- / Alvarez-Presas *et al.* 2008	DQ665964 [a]	DQ666031 [a,b,d]
Caenoplana sp. 4		- / Alvarez-Presas *et al.* 2008		DQ666032 [a,b]
Caenoplana bicolor	654	Bordils (Girona, Spain) / V*	KJ659705* [a]	KJ659648* [a,b]
Caenoplana coerulea s.l.		New Plymouth, Omata (New Zealand) / Alvarez-Presas *et al.* 2008	DQ665961 [a]	DQ666030 [a,b]
		Menorca (Balearic islands, Spain) / Breugelmans *et al.* 2012		JQ514564 [a,b]
	Tal1	Tallaganda (Australia) / Sunnucks *et al.* 2006		DQ227621 [b]
	Tal2			DQ227625 [b]
	Tal3			DQ227627 [b]
	Tal4			DQ227629 [b]
	Tal5			DQ227631 [b]
	Tal6			DQ227632 [b]
	Tal7			DQ227633 [a,b]
	Tal8			DQ227635 [a,b]
	Vic1	Victoria (Australia) / Sunnucks *et al.* 2006		DQ465372 [a,b]
Caenoplana morph Ca1	399	El Prat de Llobregat (Barcelona, Spain) / O*		KJ659613* [a,b]
	400			KJ659614* [a,b]

402			KJ659615*[b]
403			KJ659616*[b]
404			KJ659617*[b]
415	Vall de'n Bas (Girona, Spain) / P*		KJ659618*[a,b]
416			KJ659619*[b]
417			KJ659620*[b]
418			KJ659621*[b]
419			KJ659622*[b]
420			KJ659623*[b]
421			KJ659624*[b]
422			KJ659625*[b]
423			KJ659626*[b]
424			KJ659627*[b]
443	Badalona (Barcelona, Spain) / H*	KJ659700*[a]	KJ659633*[a,b]
444			KJ659634*[a,b]
445			KJ659635*[b]
446			KJ659636*[b]
450			KJ659637*[b]
451			KJ659638*[b]
452			KJ659639*[b]
453			KJ659640*[b]
454	Òliva (València, Spain) / K*		KJ659641*[a,b]
601	Garden, Adelaide (Australia) / SG S34.988611 E138.599722*	KJ659702*[a]	KJ659642*[a,b]
603	Garden in Townsville (Palmetum, Australia) / LW S19.260277 E146.822222*		KJ659643*[a,b]
605			KJ659644*[a,b]
634	Nursery in Liverpool (UK) / HJ N53.3525 W2.902777*		KJ659645*[a,b]
649	Granollers (Barcelona, Spain) / M*		KJ659646*[a,b]
650			KJ659647*[a,b]
735	El Prat de Llobregat (Barcelona, Spain) / O*		KJ659651*[a,b]

Caenoplana morph Ca2

426	Bordils (Girona, Spain) / V*		KJ659628*[b]
427			KJ659629*[b]
428			KJ659630*[b]
430		KJ659699*[a]	KJ659631*[a,b]
431			KJ659632*[a,b]
657			KJ659649*[a,b]
658			KJ659650*[a,b]

Tribe Rhynchodemini
  Dolichoplana sp.

	- / Alvarez-Presas *et al.* 2008	DQ665971[a]	DQ666037[a,b,d]

Taxon	Code	Locality / Reference		
D. striata		Igreginha (Brazil) / Carbayo *et al.* 2013	KC608341 [a]	KC608226 [a,b,d]
	425	Bordils (Girona, Spain) / V*	KJ659698* [a]	KJ659679* [a,d]
	660			KJ659683* [a,d]
	661			KJ659684* [a,d]
Kontikia ventrolineata	638	Granollers (Barcelona, Spain) / M*		KJ659681* [a,d]
	639		KJ659704* [a]	KJ659682* [a,d]
	734	Nursery in Liverpool (UK) / HJ N53.3525 W2.902777*		KJ659687* [a,d]
	739	Saint Pée sur Nivelle (France) / MA N43.34235 W1.52650*	KJ599732 [a]	KJ659688* [a,d]
Platydemus manokwari		Townsville (Australia) / Baguñà *et al.* 2001 - Alvarez-Presas *et al.* 2008	DQ665986 [a]	AF178320 [a,b,d]
Rhynchodemus morph Rs1	411	Vall de'n Bas (Girona, Spain) / P*	KJ659697* [a]	KJ659676* [a,d]
	412			KJ659677* [a,d]
	414			KJ659678* [a,d]
	908	Luarca, Asturias (Spain) / LL 43°32'30.81"N 6°32'7.42"O*		KJ659696* [d]
Rhynchodemus cf. *sylvaticus*		Canyamars (Barcelona, Spain) / Mateos *et al.* 2009		FJ969946 [a,b,d]
	091	Canyamars (Barcelona, Spain) / EM N41.598317 E2.44302*		KJ659672* [d]
	219	Montjuich (Barcelona, Spain) / Vila-Farré *et al.* 2008 *		KJ659673* [d]
	569	Underbarrow (UK) / N54.31776 W2.80783*		KJ659680* [d]
	670	Pont en Royans (France) / N45.037875 E5.377033*		KJ659685* [d]
	675		KJ659706* [a]	KJ659686* [d]
	905	Benamargosa (Málaga, Spain) / Vila-Farré *et al.* 2011*		KJ659694* [d]
	900	Sueiro, Asturias (Spain) / LL N43.527130 W6.877329*		KJ659689* [d]
	901	Aljezur (Portugal) / LL N 37.316146 W8.803392*		KJ659690* [d]
	906	Benamargosa (Málaga, Spain) / Vila-Farré *et al.* 2011 *		KJ659695* [d]
Rhynchodemini morph Ri1	902	Benamargosa (Málaga, Spain) / Vila-Farré *et al.* 2011 *		KJ659691* [d]
	903	Benamargosa (Málaga, Spain) / Vila-Farré *et al.* 2011 *		KJ659692* [d]
	904	Benamargosa (Málaga, Spain) / Vila-Farré *et al.* 2011 *		KJ659693* [d]
Rhynchodemini sp2	262	Int. Park la Amistad, Pila (Panamá) / KA N8.524944 W82.618777*		KJ659674* [d]
Rhynchodemini sp3	264			KJ659675* [d]
Subfamily Geoplaninae				
Cratera crioula		São Paulo (Brazil) / Carbayo *et al.* 2013	KC608441 [a]	KC608324 [a,e]
C. tamoia		Teresópolis (Brazil) / Carbayo *et al.* 2013	KC608369 [a]	KC608254 [a,e]
Obama sp.6	Bra1	Paulo Lopes (Brazil) / Carbayo *et al.* 2013	KC608425 [a]	KC608308 [a,e]
	Bra2		KC608426 [a]	KC608309 [a,e]
Obama sp.	434	Bordils (Girona, Spain) / V*		KJ659652* [a,e]
	437			KJ659653* [a,e]
	438			KJ659654* [a,e]
	667			KJ659663* [a,e]
	668			KJ659664* [a,e]
	458	Gavà (Barcelona, Spain) / Q*		KJ659655* [a,e]

	Code	Locality / Reference		
	459			KJ659656* [a,e]
	593	Vilassar de Mar (Barcelona, Spain) / S*		KJ659657* [a,e]
	594			KJ659658* [a,e]
	595		KJ659701* [a]	KJ659659* [a,e]
	596	La Sínia (Tarragona, Spain) / T*		KJ659660* [a,e]
	610	Treto (Cantabria, Spain) / U*		KJ659661* [a,e]
	611			KJ659662* [a,e]
	895	Nursery in Liverpool (UK) / HJ N53.3525 W2.902777*		KJ659667* [a,e]
	896			KJ659668* [a,e]
	897			KJ659669* [a,e]
	898			KJ659670* [a,e]
	754	Torruella de Fluvià (Girona, Spain) / J*		KJ659665* [a,e]
	755			KJ659666* [a,e]
	899			KJ659671* [a,e]
O. burmeisteri		São Paulo (Brazil) / Álvarez-Presas *et al.* 2011 - Carbayo *et al.* 2013	KC608349 [a]	HQ542895 [a,e]
O. josefi		São Francisco de Paula (Brazil) / Carbayo *et al.* 2013	KC608435 [a]	KC608318 [a,e]
O. ladislavii sensu Froehlich, 1959	Bra1	Blumenau (Brazil) / Carbayo *et al.* 2013	KC608371 [a]	KC608256 [a,e]
O. ladislavii von Graff, 1899	Bra2		KC608373 [a]	KC608258 [a,e]
OUTGROUP: Family Dugesiidae				
Dugesia gonocephala		The Netherlands / Alvarez-Presas *et al.* 2008	DQ665965 [a]	DQ666033 [a]
D. ryukyuensis		- / Baguñà *et al.* 2001 - Alvarez-Presas *et al.* 2008	DQ665968 [a]	AF178311 [a]
D. subtentaculata		Spain / Alvarez-Presas *et al.* 2008	DQ665970 [a]	DQ666036 [a]

[a,b,c,d,e] a: Concatenated Dataset; b: Caenoplanini Dataset; c: Bipaliinae Dataset; d: Rhynchodemini Dataset; e: Geoplaninae Dataset

*Sequences obtained in this study

Feeding habits of the introduced terrestrial flatworm species in the Iberian Peninula.

Native region sensu Winsor et al 2004.

Species (native region)	Prey	Reference
Bipalium kewense (Vietnam to Kampuchea, possibly extending to Malaysia)	earthworms	See Winsor, Johns & Barker, 2004 for refs
Caenoplana coerulea (Eastern Australia)	gastropods, arthropods, earthworms isopods, diplopods, earwings *Ommatoiulus moreletii* (diplopod) beetles diptera larvae	See Winsor, Johns & Barker, 2004 for refs Olewine, 1972 Terrace & Baker, 1994 Mateos et al., 2013 Barnwell, 1978
Caenoplana bicolor (unknown)	isopods	Observations on captive specimen by H.D. Jones
Caenoplana C02 (unknown)	unknown	–
Dolichoplana striata (Indo-Malay region)	earthworms	See Winsor, Johns & Barker, 2004 for refs
Kontikia ventrolineata (Queensland, Australia)	gastropods, isopods snails, slugs, hawkmoth caterpillars isopods isopods earthworms	See Winsor, Johns & Barker, 2004 for refs NNSS 2013 Froehlich, 1956 Olewine, 1972 Present study
Obama (Brazil*)	mollusks, earthworms	Fernando Carbayo (Personal communication)
Rhynchodemini Ri1 (unknown)	unknown	-
Rhynchodemus Rs1 (unknown)	unknown for Rhynchodemus [genus]: springtails springtails springtails woodlice	 Wallner, 1937 Froehlich, 1956 Ogren, 1985 Jones, 2005

* sensu Carbayo et al. 2013

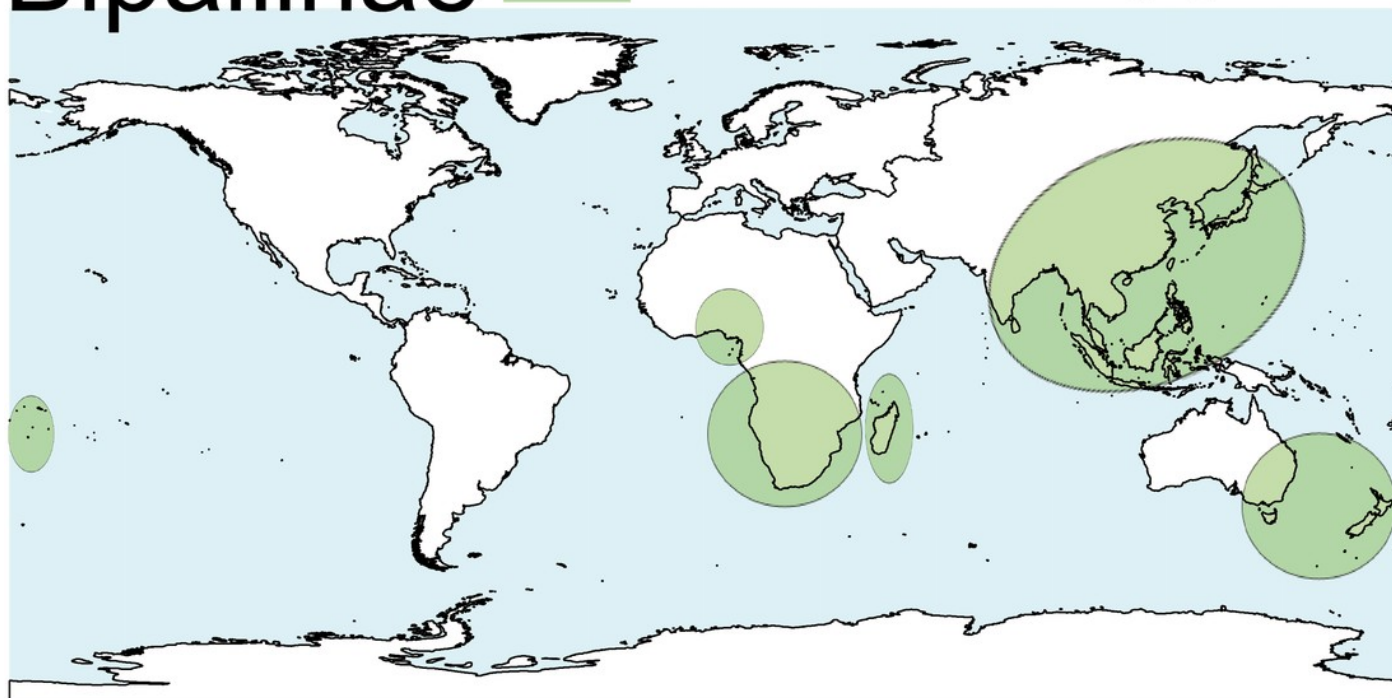
Figure 1

Distribution map of the terrestrial flatworms.

A, subfamily Bipaliinae. B, subfamily Geoplaninae. Information from

http://turbellaria.umaine.edu.

Bipaliinae

A

Geoplaninae

B

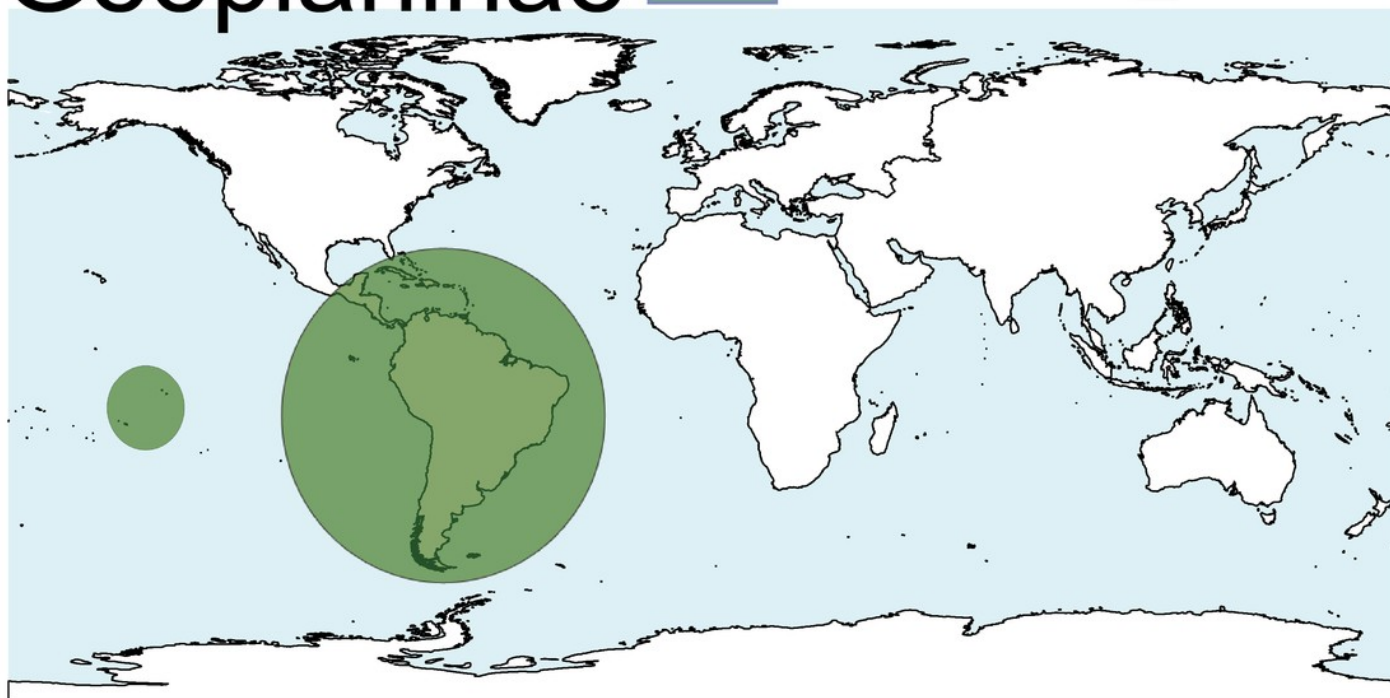
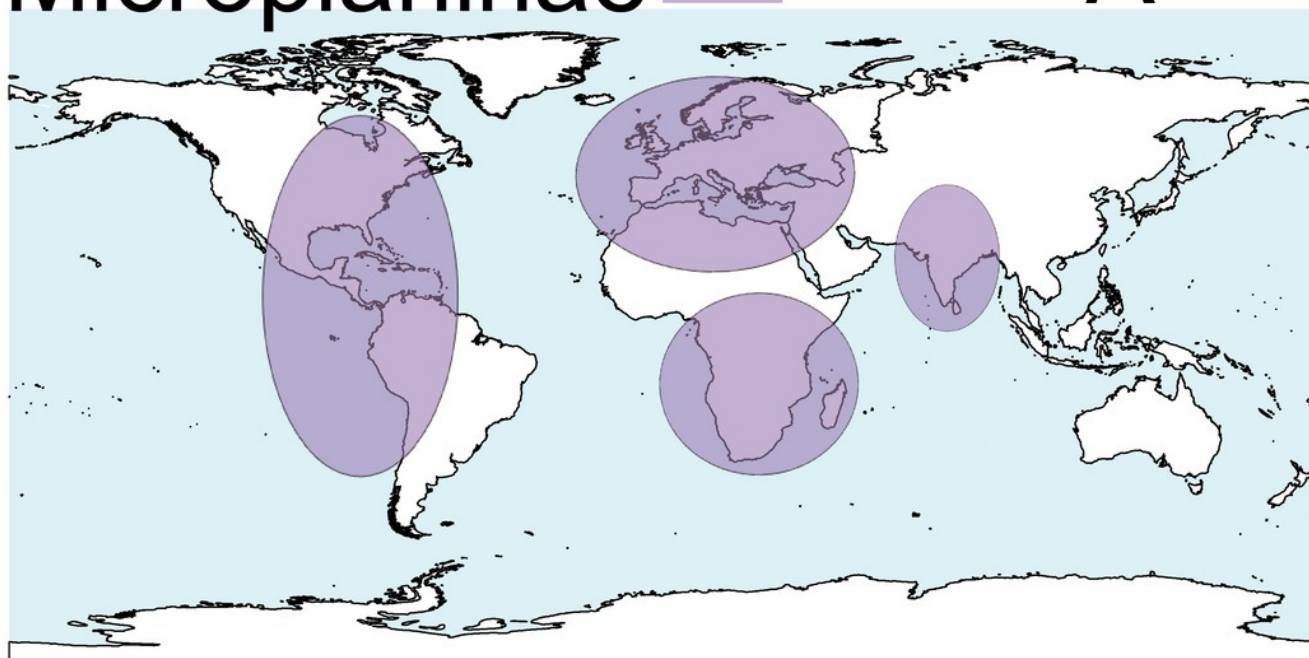



Figure 2

Distribution map of the terrestrial flatworms.

A, subfamily Microplaninae. B, subfamily Rhynchodeminae. Information from
http://turbellaria.umaine.edu.

Microplaninae

A

Rhynchodeminae

B

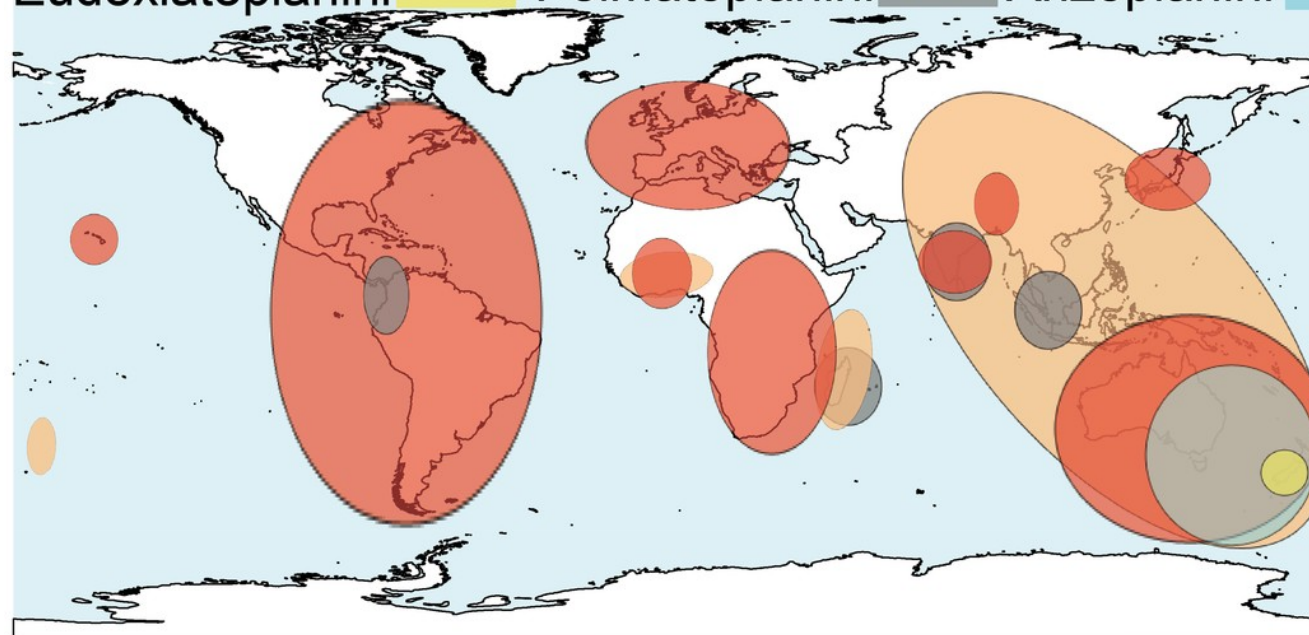
Eudoxiatoplanini | Pelmatoplanini | Anzoplanini

Rhynchodemini | Caenoplanini

Figure 3

Distribution of sampling localities of introduced terrestrial flatworms in the Iberian Peninsula.

Locality codes correspond to those in Table 1.

Figure 4

Bipalium kewense.

A, dorsal view. B, ventral view of median part. C, dorsal view of posterior end. D, dorsal view of anterior end. Scale bar 5 mm.

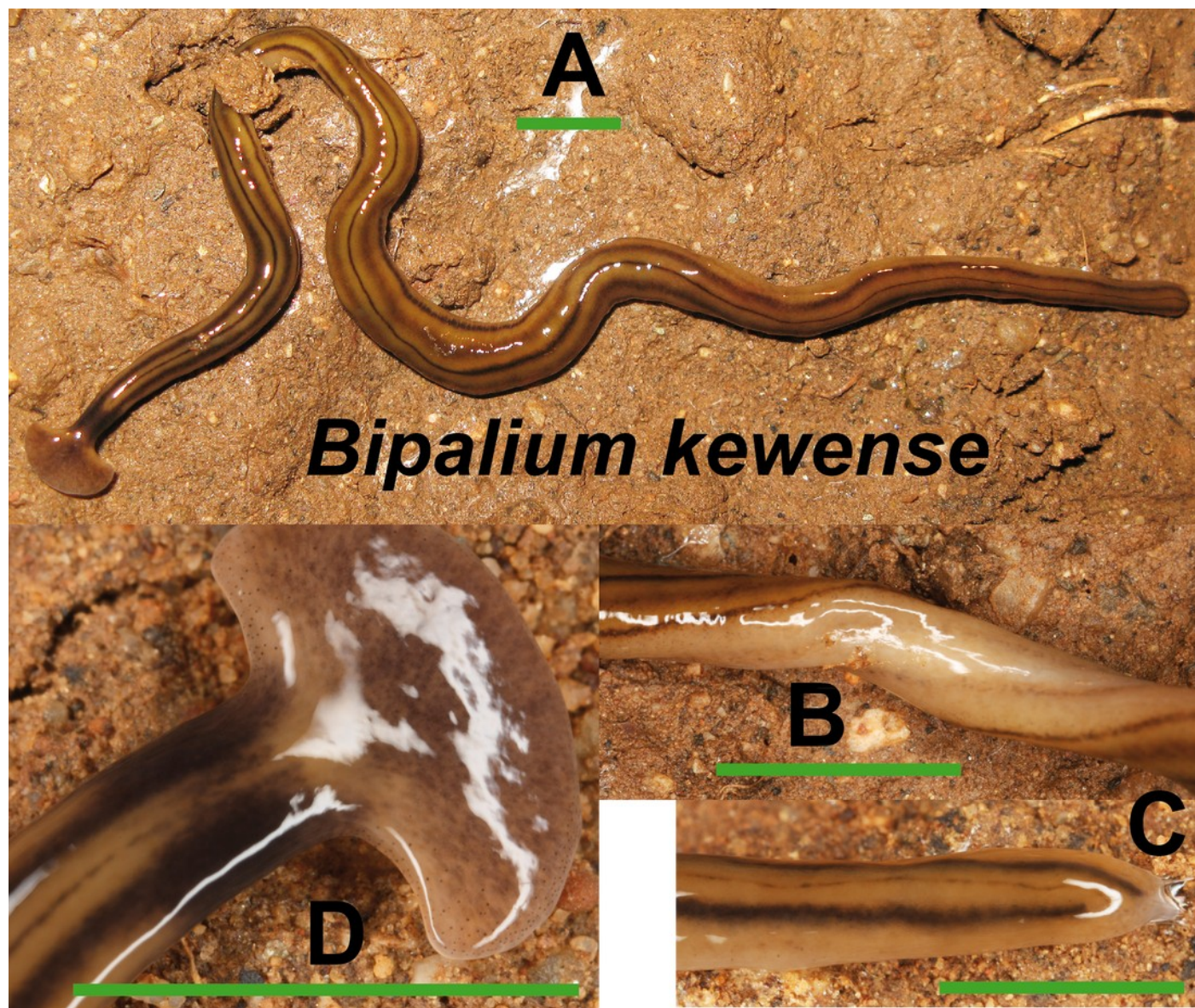



Figure 5

Caenoplana bicolor.

Dorsal view with partial ventral view in the center. The anterior end is not show (the specimen was damaged in this region). Scale bar 5 mm.

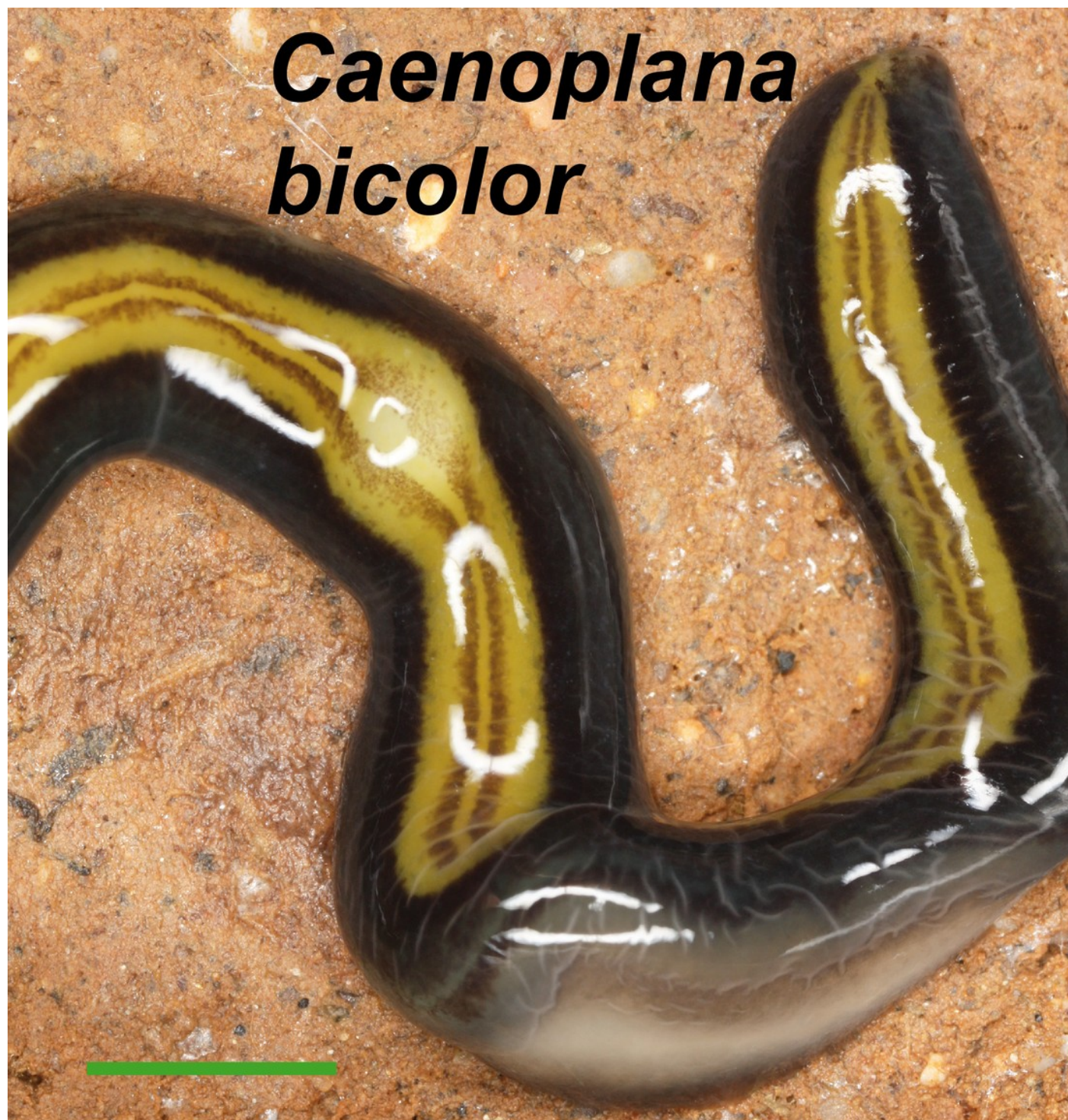



Figure 6

Caenoplana morph Ca1.

A, dorsal view. B, ventral view of median part. C, lateral view of anterior end showing line of eyes. Scale bar 5 mm.

A

Caenoplana Ca1

C

B

Figure 7

Caenoplana morph Ca2.

A, dorsal view. B, ventral view of median part and dorsal view of anterior end showing line of eyes. C, lateral view of anterior end showing line of eyes. Scale bar 5 mm.

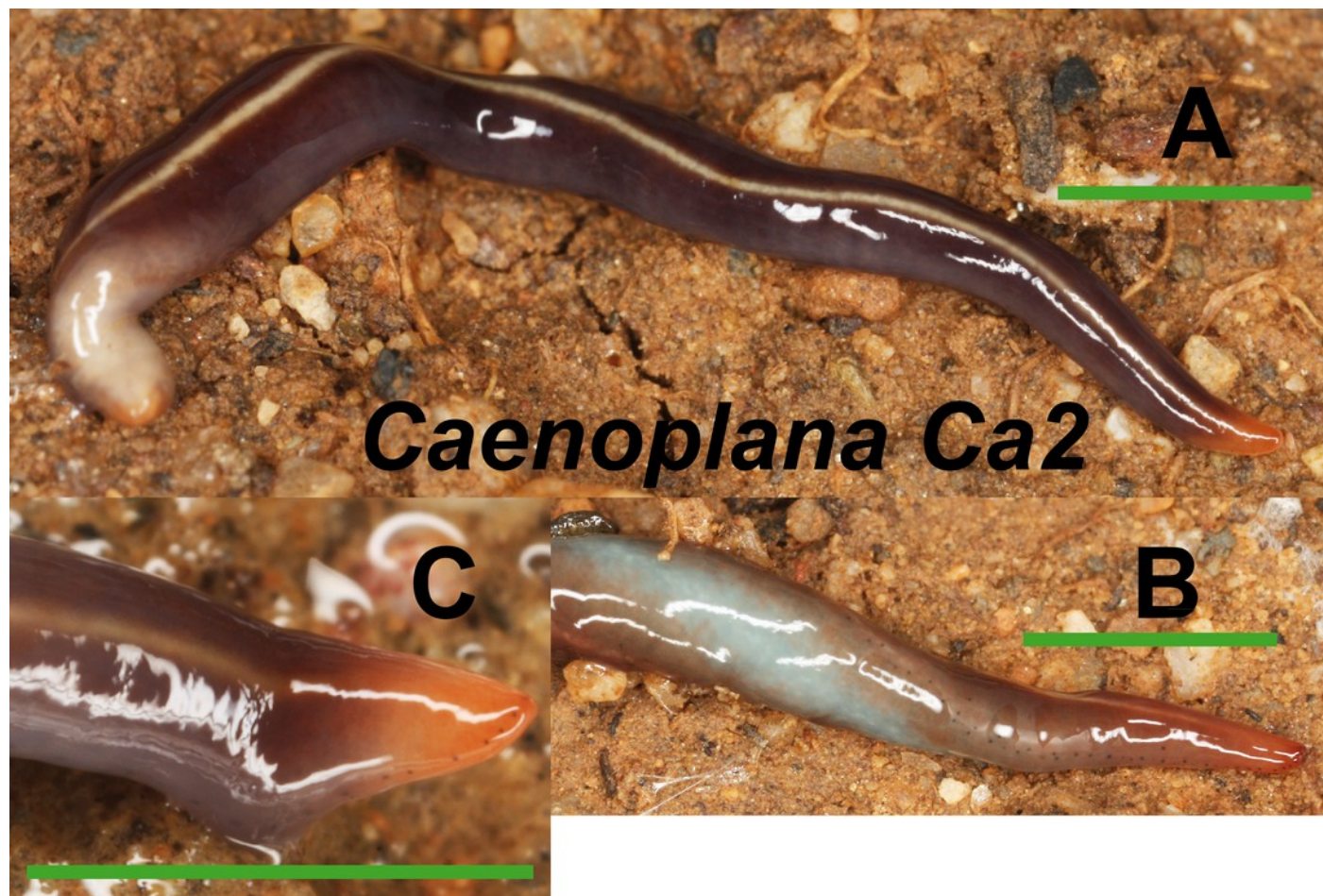



Figure 8

Dolichoplana striata.

A, dorsal view. B, ventral view of median part. C, lateral view of anterior end showing the eye spot. Scale bar 5 mm.

Dolichoplana striata

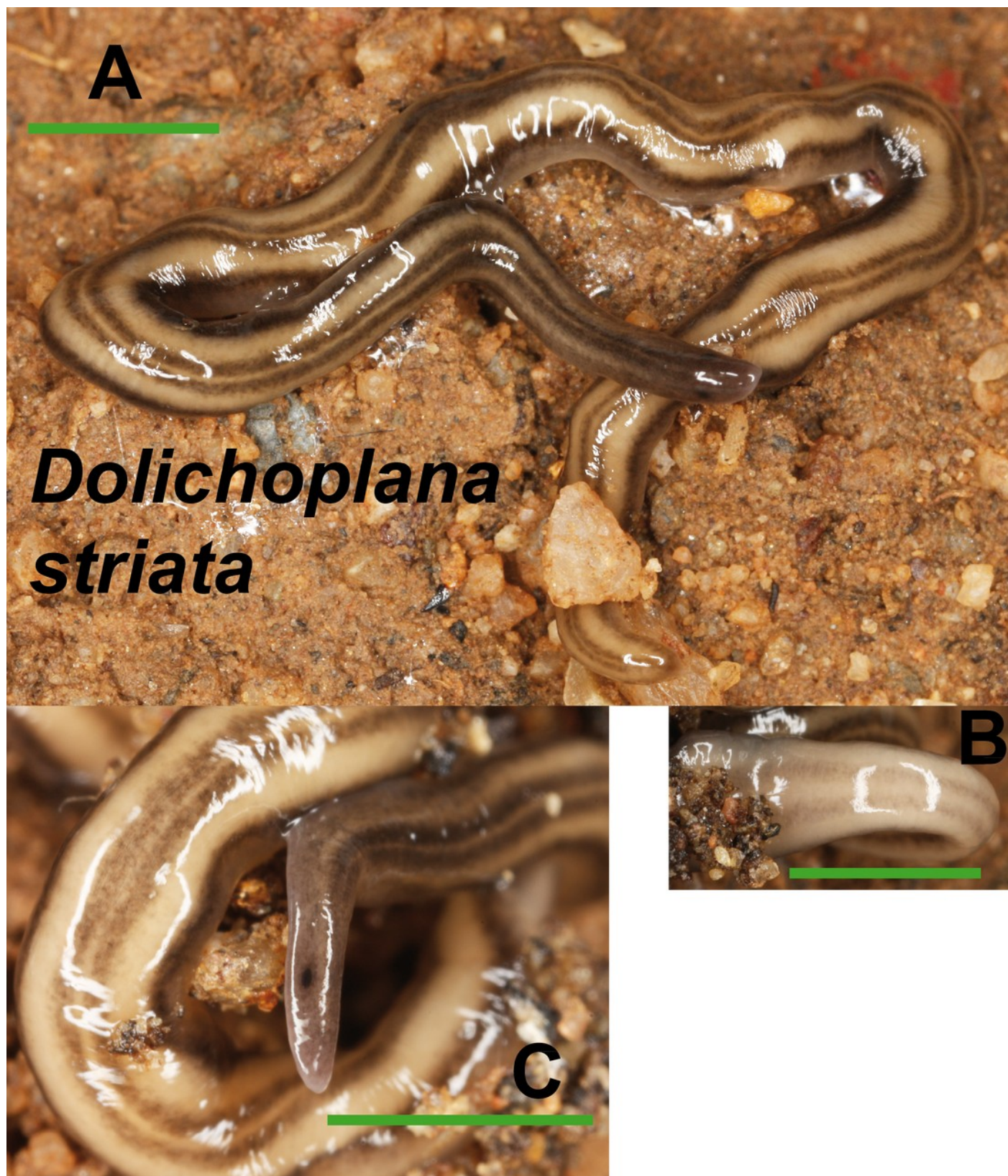



Figure 9

Kontikia ventrolineata.

A, dorsal view. B, ventral view of posterior end. C, lateral view of anterior end showing line of eyes. Scale bar 5 mm.

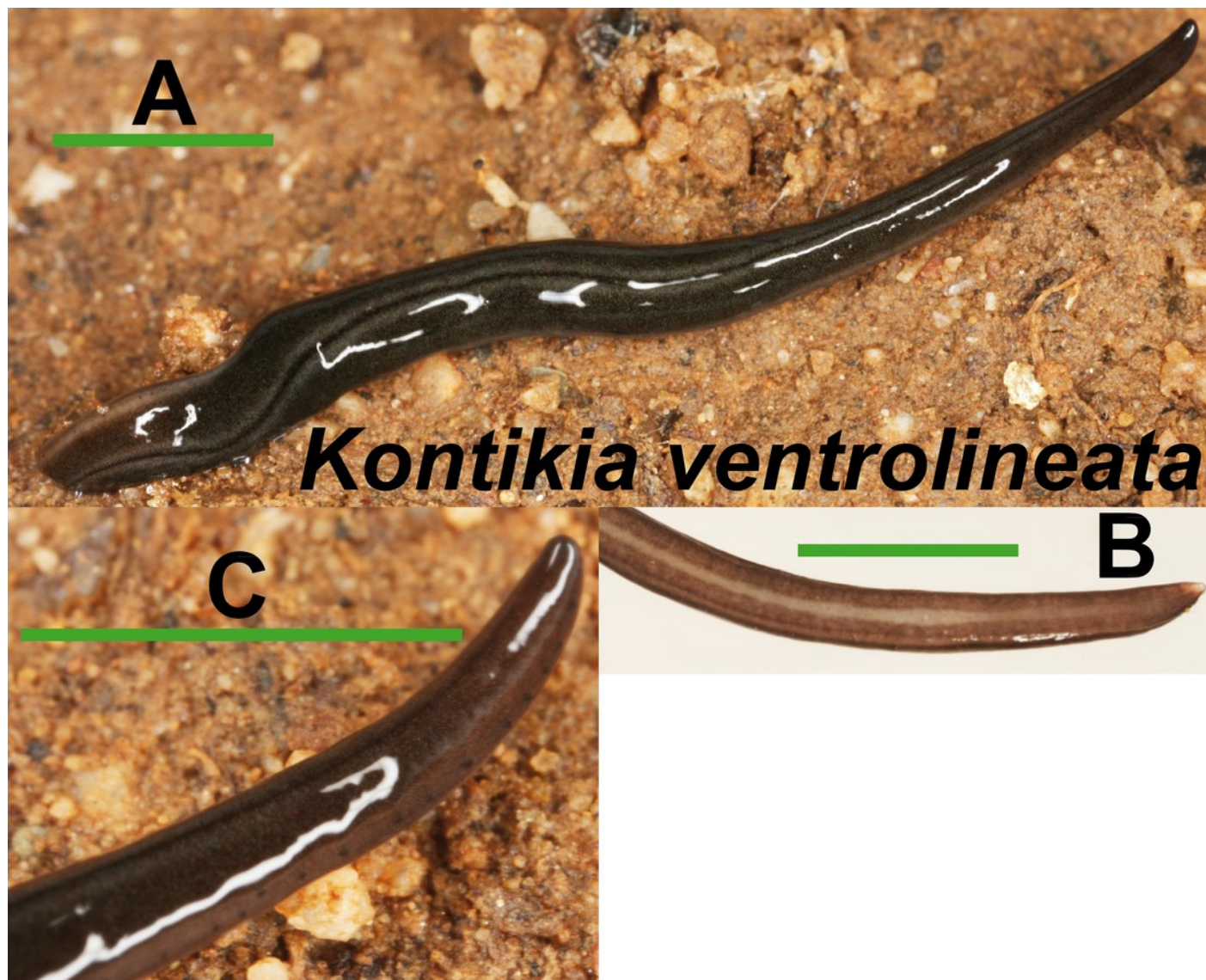



Figure 10

Rhynchodemus morph Rs1.

A, dorsal view, scale bar 5 mm. B, lateral view of anterior region, scale bar 2.5 mm.

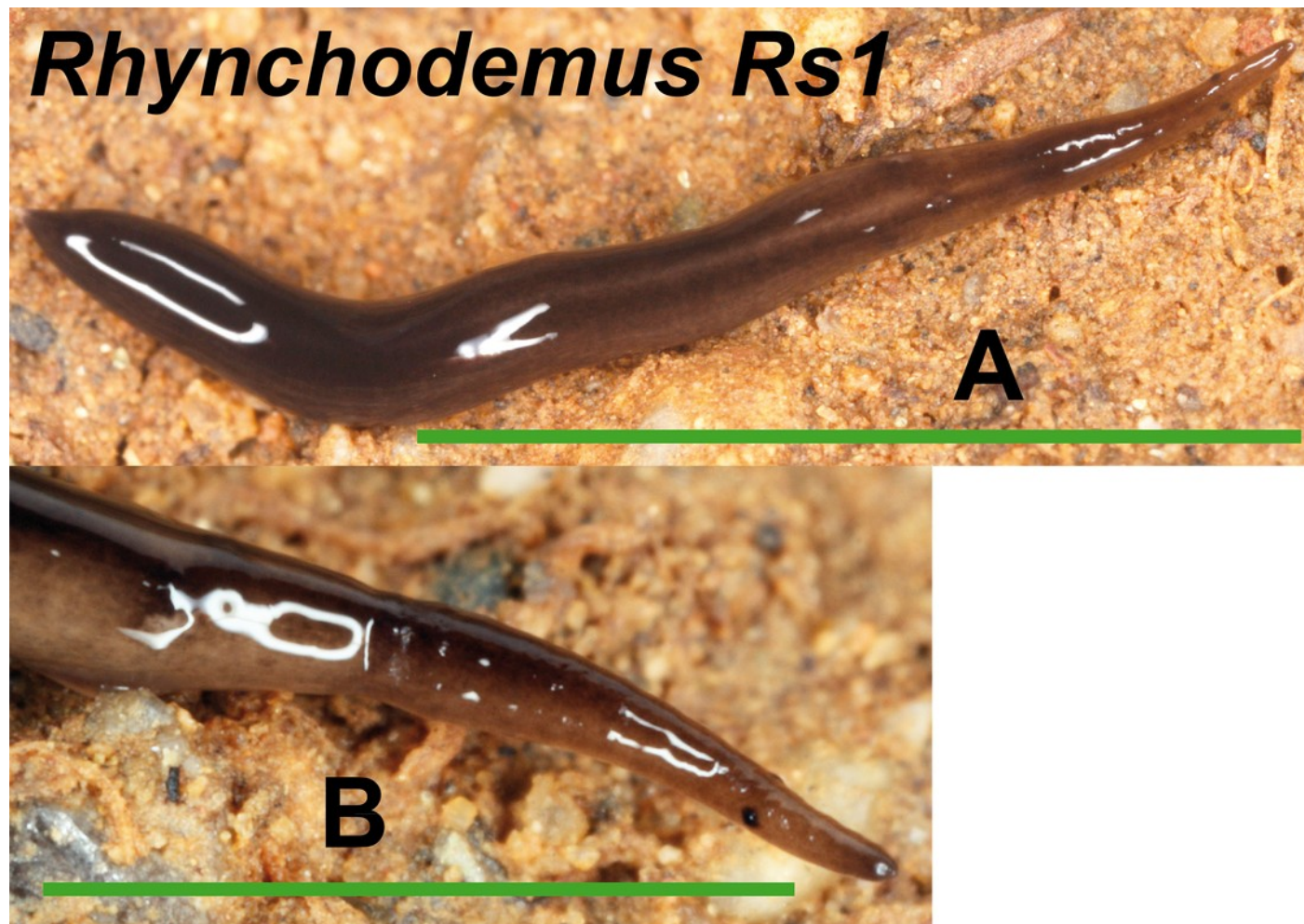



Figure 11

Obama sp.

A, dorsal view of two specimens (codes 434 and 610) from one *Obama* sp.A clade in the Geoplaninae Cox1 tree (Fig.14). B, dorsal view of two specimens (codes 437 and 594) from *Obama* sp.B clade in the Geoplaninae Cox1 tree (Fig.14). C, ventral view. D, lateral view of anterior end showing line of eyes. Scale bar 5 mm.

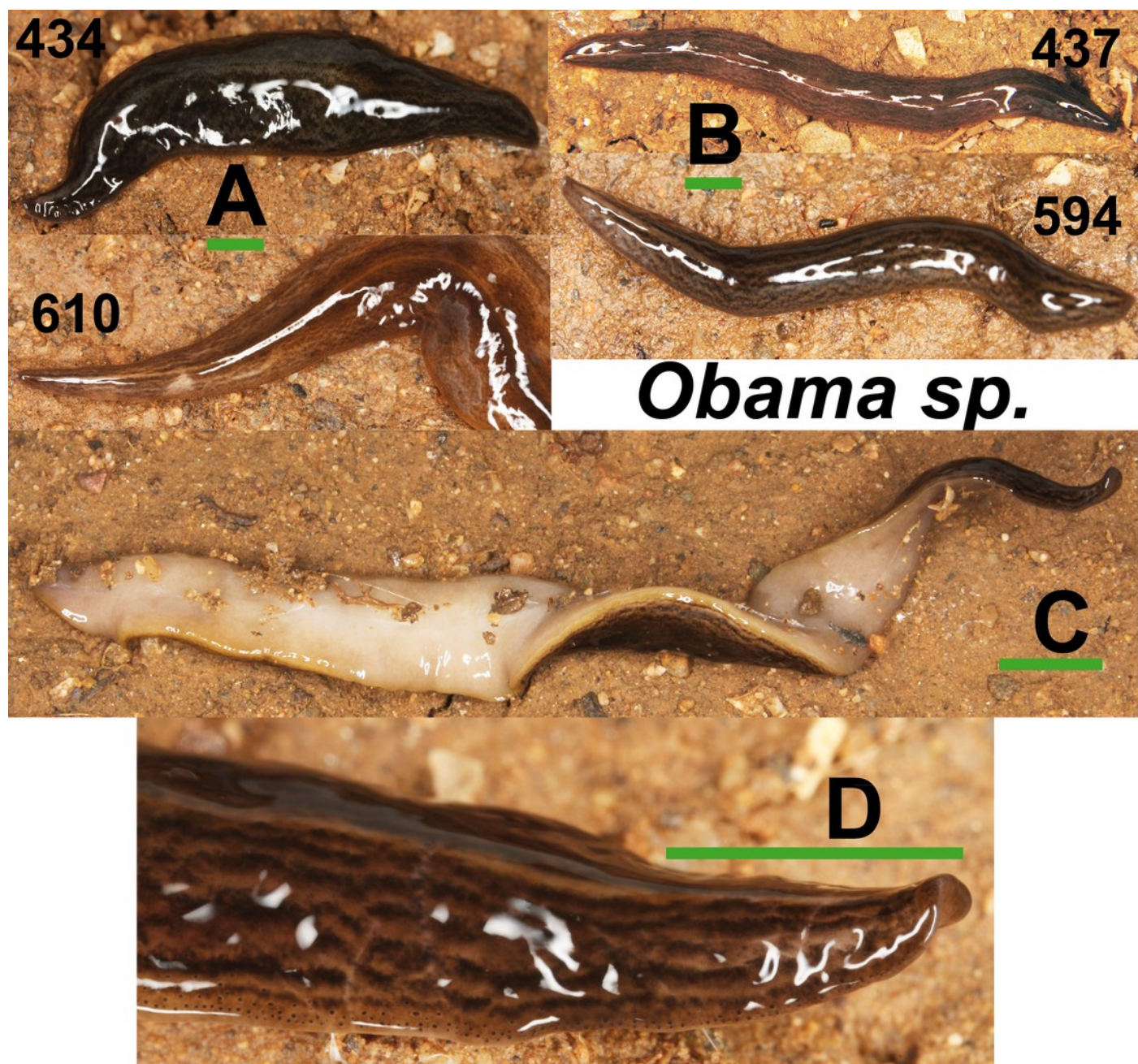



Figure 12

Maximum likelihood (ML) tree of the Geoplanidae subfamilies and tribes (Bipaliinae, Geoplaninae, Caenoplanini, and Rhynchodemini).

Tree inferred from the concatenated dataset (Cox1 and 28S genes). Three *Dugesia* species as outgroups. Values at nodes correspond to bootstrap (>75) for ML and posterior probability (PP) values from the Bayesian analysis (>0.95).

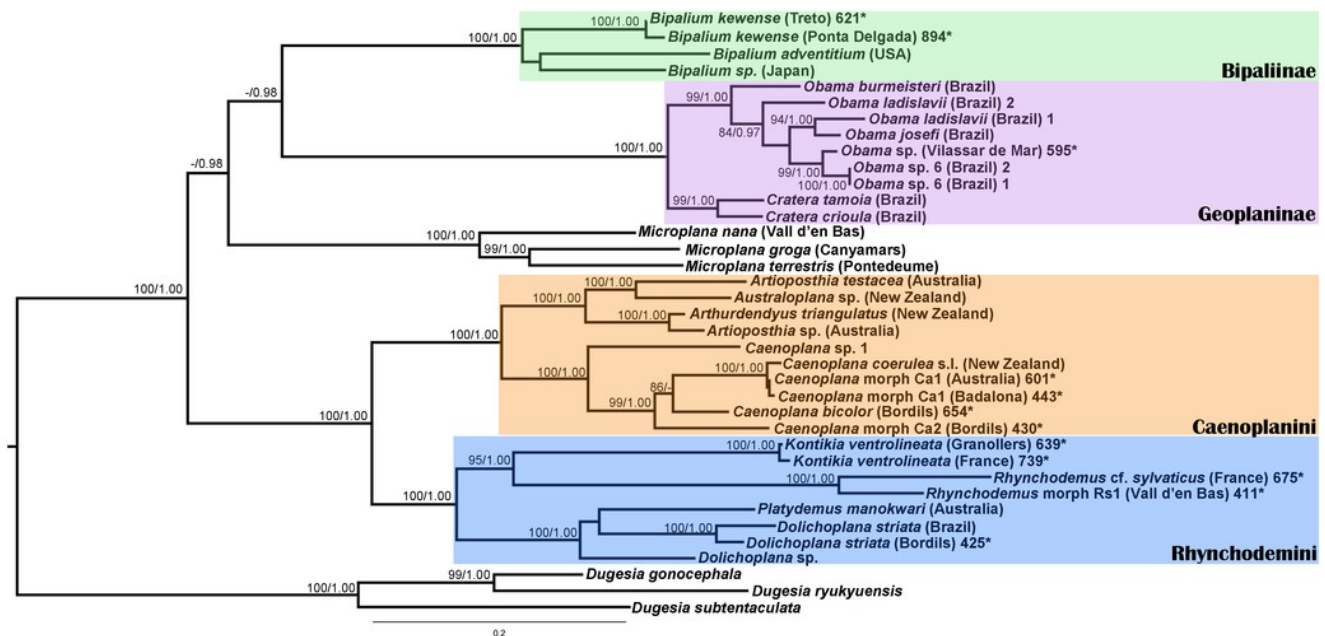



Figure 13

Bipaliinae dataset ML tree.

Tree inferred from the Cox1 gene. Three *Microplana* species as outgroups. Values at nodes correspond to bootstrap (>75) and PP (>0.95) values.

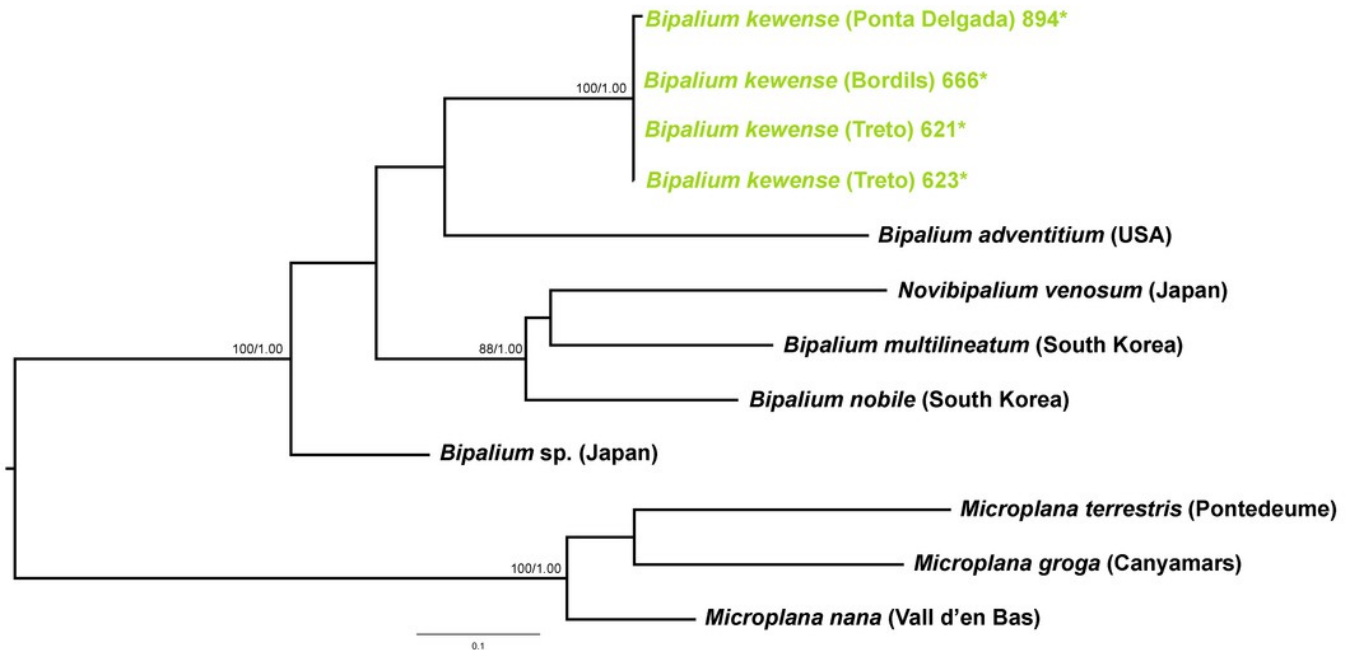



Figure 14

Geoplaninae dataset ML tree.

Tree inferred from the Cox1 gene. Two *Cratera* species as outgroups. Values at nodes correspond to bootstrap (>75) and PP (>0.95) values.

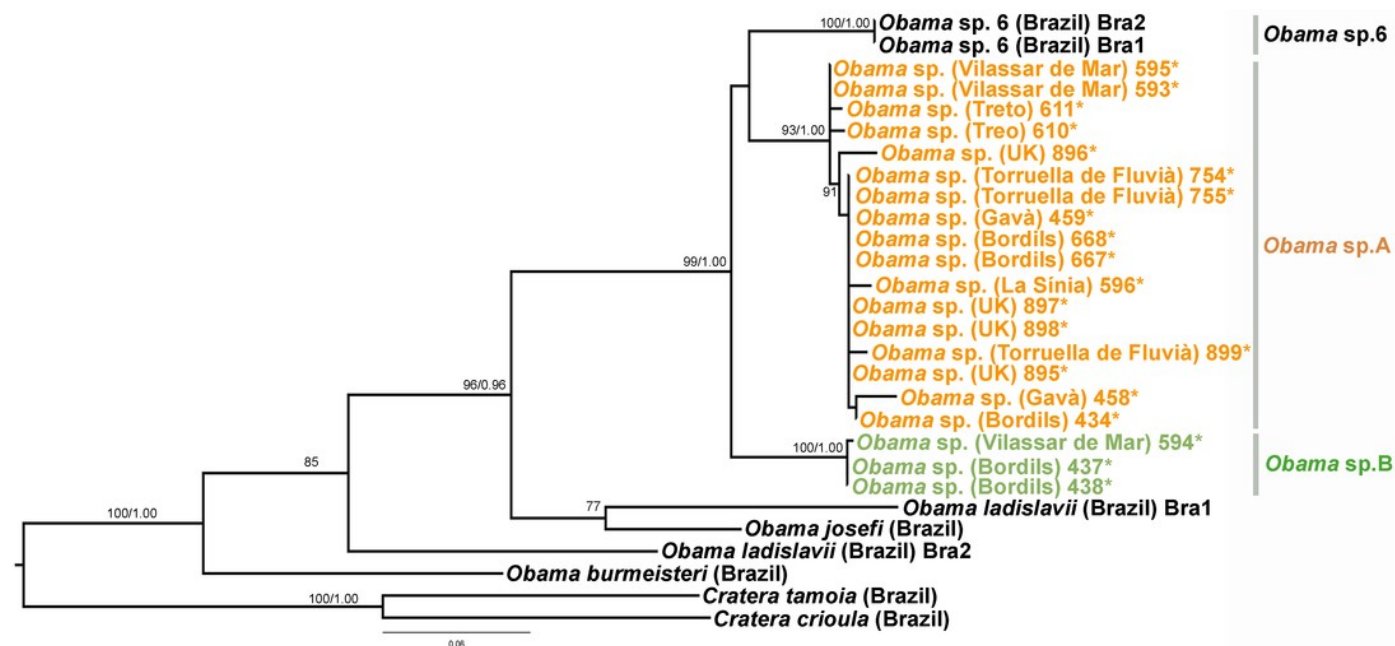



Figure 15

Caenoplanini dataset ML tree.

Tree inferred from the Cox1 gene. One *Rhynchodemus* species, one *Platydemus* species, and two *Dolichoplana* species as outgroups. Values at nodes correspond to bootstrap (>75) and PP (>0.95) values.

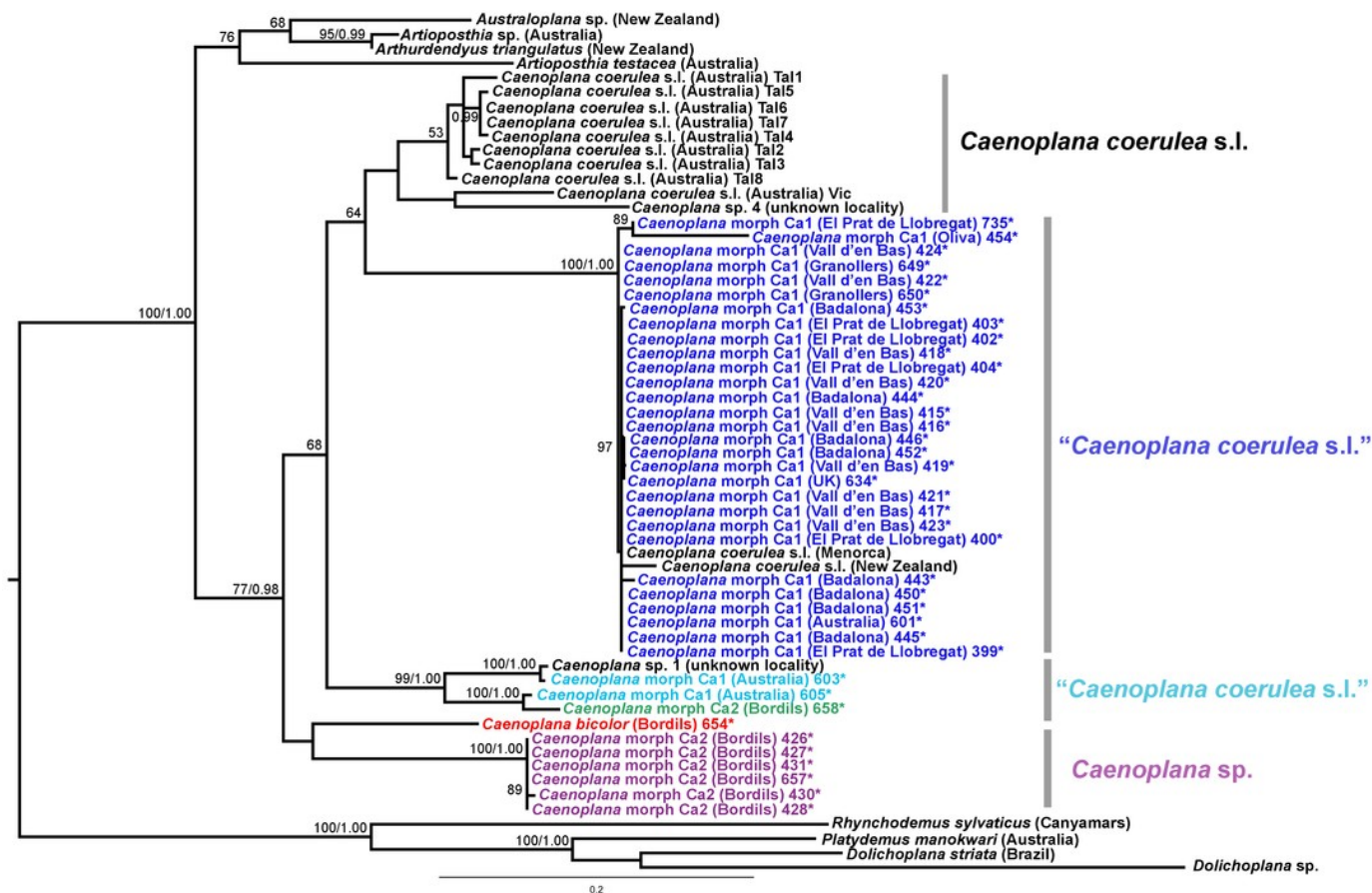



Figure 16

Rhynchodemini dataset ML tree.

Tree inferred from the Cox1 gene. One species of *genres Arthurdendyus*, *Artioposthia*, *Australoplana* and *Caenopolana* as outgroups. Values at nodes correspond to bootstrap (>75) and PP (>0.95) values.

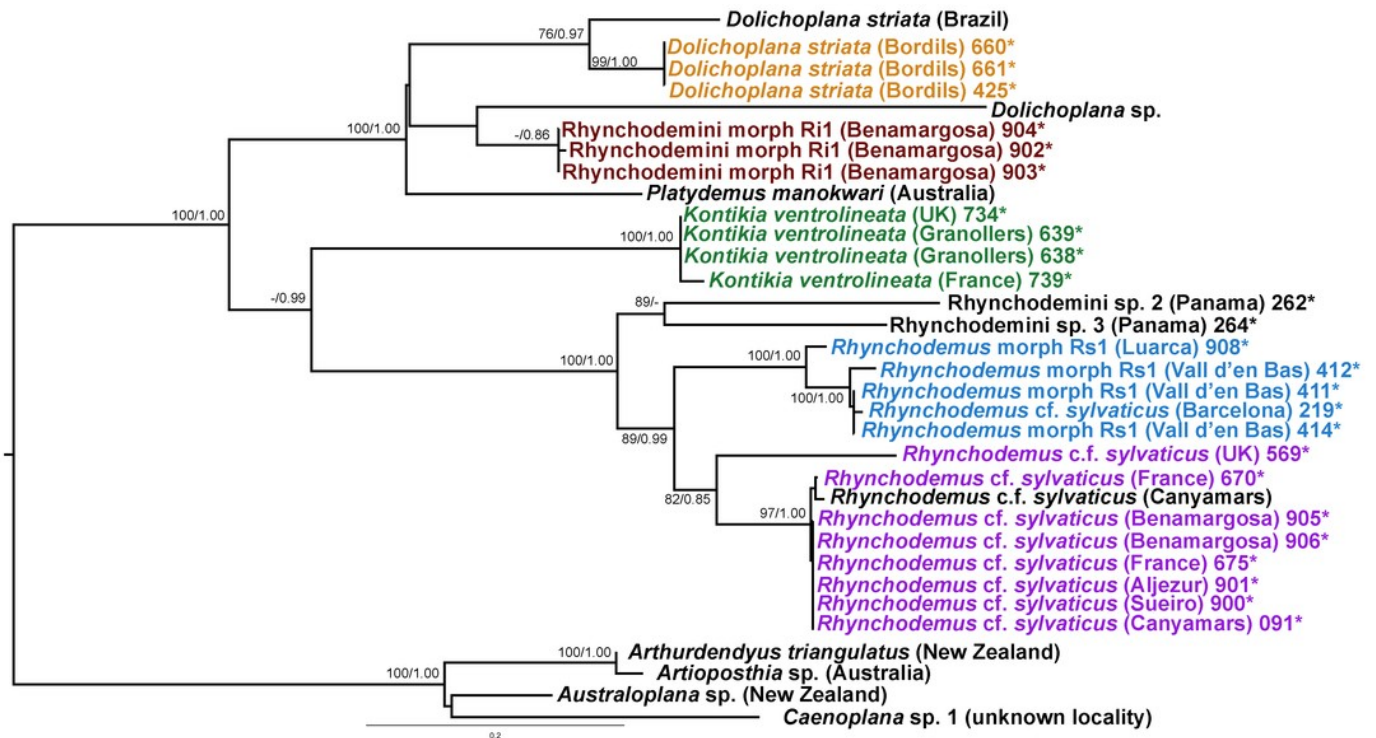



Figure 17

Potential distribution of Caenoplana coerulea species across the Iberian Peninsula.

The color gradient indicates the predicted likelihood that the environmental conditions suitable for the species based on the MaxEnt average output. Letters indicate localities where *C. coerulea* has been found, locality codes correspond to those in Table 1.