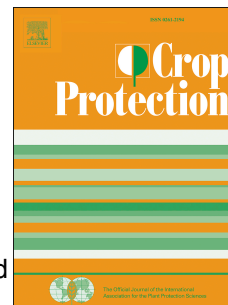


# Journal Pre-proof

Bats as natural samplers: First record of the invasive pest rice water weevil  
*Lissorhoptus oryzaophilus* in the Iberian Peninsula

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1     **Bats as natural samplers: First record of the invasive pest rice water weevil *Lissorhoptus***  
2     ***oryzophilus* in the Iberian Peninsula**

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19

20 **Abstract:** We face an increasing global food security challenge as the human population  
21 continues to grow across the globe. As agricultural production rises to keep up with food  
22 demand, so too does the expansion of crop detrimental pest species. Early detection can be  
23 crucial to control their damage and relies on the use of accurate and dependable techniques. We  
24 report the first record of rice water weevil (*Lissorhoptrus oryzophilus*) in the Iberian Peninsula.  
25 The distribution range expansion of this invasive rice pest weevil is confirmed through  
26 traditional sampling and through natural sampling by bats combined with DNA metabarcoding,  
27 a non-invasive technique with strong potential for early detection of pests in agricultural areas.  
28 Presence of rice water weevil was confirmed genetically from bat faecal samples collected from  
29 a *Pipistrellus pygmaeus* bat roost in the Ebro Delta (Catalonia, Spain). The known distribution  
30 of this rice pest, native to North America, now includes four of the top five rice-producing  
31 European countries. To date, the economic damages caused by this pest in Europe are minimal  
32 and concentrated in small areas, but chemical and agronomic controls are implemented to  
33 control their populations. The nature of this record highlights the potential of insectivorous bats  
34 as natural samplers to detect (and possibly control) pest species in crops. Further studies are  
35 required to determine the full extent of the rice water weevil's spread, and to investigate  
36 integrated pest management systems, including biological control to suppress their populations.

37 **Keywords:** *Pipistrellus pygmaeus*, invasive species, pest control, metabarcoding, ecosystem  
38 services, biological control

39

40 **INTRODUCTION**

41 Agricultural food production continues to increase to keep up with the food demand of the  
42 world's growing human population (Molotoks et al., 2018). As croplands expand and intensify,  
43 detrimental crop pest species also spread and thrive, causing huge losses in yield and quality of  
44 agricultural production (Savary et al., 2019). In order to control their damage and avoid higher  
45 economic losses, early detection of these pests is key. Therefore, exploring new methods to  
46 provide rapid and accurate pest detection can help in deploying effective management  
47 strategies.

48 Pest species have infested many food crops, including rice (*Oryza sativa*) crops that are a staple  
49 food of more than half of the world's population (Muthayya et al., 2014). One crucial rice pest  
50 is the rice water weevil, *Lissorhoptrus oryzophilus* Kuschel, 1952 (Coleoptera: Eirirhinidae).  
51 This species is indigenous to North America, where it causes annual losses of up to 25% of rice  
52 crops (Aghaee and Godfrey, 2014). This highly invasive pest quickly spread its range in the  
53 1950s, infesting rice fields across North America (CABI, 2020; Lange and Grigarick, 1959).  
54 The accidental introduction of this species in Asia led to a rapid spread through China, Japan,  
55 South Korea, North Korea and India (Lupi et al., 2015). This led to significant losses of rice  
56 production in newly infested areas, for instance causing initial losses of up to 80% of infested  
57 rice crop areas in China (Chen et al., 2005). The first record of the rice water weevil in Europe  
58 was in Italy in 2004 (Caldara et al., 2004), and its presence continues to expand with confirmed  
59 reports in France (Ferrand, 2017) and Greece (Giantsis et al., 2017). This expansion has been  
60 detected through visual observations, insect sweep nets, rice root searches, root/soil core  
61 sampling, and scouting for leaf scars (Cave, 1983; Cherry et al., 2016; Lupi et al., 2010). The  
62 first records of this pest in Italy and Greece were both incidental detections (Caldara et al.,  
63 2004; Giantsis et al., 2017).

64 This invasive pest is ecologically suited to infest rice crops, exacerbated by its parthenogenic  
65 reproduction in non-native areas, where only females can be found (Chen et al., 2005; Huang et  
66 al., 2017). Adults of the rice water weevil are brown to black, small (2.8 mm long, 1.2-1.8 mm

67 wide), sexually dimorphic, semi-aquatic, and nocturnal fliers (CABI, 2020; Saito et al., 2005).  
68 The female lays its eggs in submerged rice leaf sheaths, and the hatched larvae crawl down the  
69 plant to the roots where they spend about a month before enclosing themselves in a mud cocoon  
70 attached to the root to pupate (Saito et al., 2005). After 5 to 7 days, the adults emerge from the  
71 cocoons and move to the aerial part of the plant. The rice water weevil can complete multiple  
72 generations per year, associated with the presence of water and young rice plants. The number  
73 of generations is therefore linked to the number of rice crop cycles per year, and the  
74 implementation of staggered planting approaches (CABI, 2020; Chen et al., 2005; Saito et al.,  
75 2005). Many of the areas in which this crop has spread, including those in Europe, are single  
76 cropped areas, and therefore the rice water weevil completes a single generation (CABI, 2020;  
77 Lupi et al., 2015). Rice plants are vulnerable to damage from rice water weevil from their early  
78 vegetative stage to the reproductive stage, and can be damaged by both larval and adult weevils  
79 (Aghaee and Godfrey, 2014).

80 With a continuous expansion of *L. oryzaophilus* and other pest species, genetic confirmation of  
81 new records is particularly valuable to rapidly and accurately identify invasive species in new  
82 areas, providing the confidence needed to act on early warning detections (Comtet et al., 2015).  
83 Our study highlights a promising technique for early detection of pest insects - combining the  
84 natural sampling capacity of insectivorous bats with DNA metabarcoding. The concept of  
85 natural sampling involves assessing the biodiversity present in an area by using DNA extracted  
86 from predatory or scavenging organisms (e.g. Siegenthaler et al., 2018). Natural samplers have  
87 potential in providing a cost-efficient way to obtain large amounts of highly informative  
88 biodiversity samples from a variety of ecosystems (e.g. Mariani et al., 2019). In the case of  
89 insectivorous bats, their potential as broad natural samplers is enhanced by their huge insect  
90 consumption and ecological diversity with diverse species specialised in different foraging  
91 techniques and targeting a huge variety of invertebrate prey (Altringham, 2011).

92 Overall, this article aims to: i) report the first records of *L. oryzaophilus* in the Iberian Peninsula  
93 through both traditional sampling methods and DNA metabarcoding of bat faeces; and, ii)

94 exemplify the potential of bats as natural samplers of invertebrates, and more specifically,  
95 detrimental crop pest species.

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96 **MATERIALS AND METHODS**

97 In August 2018, weevil samples were collected from rice crops in the towns of L'Ampolla,  
98 Amposta and Sant Carles de la Ràpita during entomological field studies in the Ebro Delta in  
99 Catalonia (Northeastern Iberian Peninsula). In August 2019, faecal samples were collected from  
100 12 roosts of *Pipistrellus pygmaeus* bats located across the Ebro Delta as part of a bat research  
101 study. For each of the roosts, two replicate samples (of ~20 droppings each) were collected from  
102 underneath the roosts following the protocol for bat dropping collection for DNA analysis  
103 described in Collins (2016). All samples were collected using clean tweezers that were  
104 disinfected between sites to avoid cross contamination. Samples were stored dry in Eppendorf  
105 plastic tubes. In accordance with the protocol, fresh and damp samples were air dried on a clean  
106 sheet of paper at room temperature to help preserve the DNA.

107 DNA was extracted from the samples using a Stool DNA isolation kit (Norgen Biotek).  
108 Sequences from each sample were obtained using a massive-parallel barcoding strategy  
109 (Ershova et al., 2019). The polymerase chain reaction (PCR) was conducted using the universal  
110 Leray-XT primer set (Wangensteen et al., 2018) to amplify a 313 base pair (bp) fragment of the  
111 mitochondrial cytochrome c oxidase subunit I (COI) gene, as in Kemp et al. (2019). We used  
112 forward primer mlCOIint-XT (5'-GGWACWRGWTGRACWITITAYCCYCC-3') and reverse  
113 primer jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA-3'). Each primer was attached  
114 to a unique 8-bp sample-tag and a 1-step PCR protocol was followed for the amplification. The  
115 PCR mix comprised 10µl of ThermoFisher's Amplitaq Gold 360 master mix, 3 µg bovine serum  
116 albumin, 1 µl of 5 µM tagged forward primer, 1 µl of 5 µM tagged reverse primer and 2 µl of  
117 extracted DNA. The PCR programme started with a 10 min cycle at 95°C to denature the  
118 blocking antibody of Taq polymerase, followed by 35 cycles of: 1 min at 94°C, 1 min at 45°C,  
119 and 1 min at 72°C, and ending with an extension time of 5 min at 72°C. An extraction blank and  
120 a PCR blank with molecular-biology grade water were amplified along with the samples and  
121 added to the multiplexed pool. The amplification was checked by gel electrophoresis and the  
122 tagged PCR products were pooled together into a library and purified using MinElute columns

123 (QIAGEN) to remove fragments below 70 bp. The DNA concentration in the final pool was  
124 measured using a Qubit fluorometer (Invitrogen). A PCR-free ligation protocol was followed to  
125 attach the Illumina adaptors to the pooled amplicons, using the NEXTflex PCR-Free DNA  
126 Sequencing Kit (BIOO Scientific). A 1% PhiX library was mixed in with our libraries as a  
127 sequencing positive control. Sample denaturation was completed following the manufacturer  
128 protocol and total concentration was adjusted to 12 pM to be loaded into a MiSeq™ Reagent  
129 Nano Kit v2 (2x250 cycles) for the sequencing.

130 A metabarcoding pipeline based on OBITools (Boyer et al., 2016) was used to analyse the  
131 metabarcoding sequences. We retained aligned paired-end sequences with alignment scores of  
132 >40 and lengths between 301 and 319bp (after primer removal) and reads with ambiguous bases  
133 or missing forward or reverse tag sequences were removed. Dereplication of sequences was  
134 done using the ‘obiuniq’ command to determine unique sequences and calculate the number of  
135 reads in each sample. Taxonomic assignment was done for the ten most abundant unique  
136 sequences detected from each sample, using the OBITools ecotag algorithm against a reference  
137 database of COI Leray sequences, downloaded from the NCBI database and the BOLD database  
138 (NCBI, 2020; Ratnasingham and Hebert, 2007).

139



140 **RESULTS**

141 The weevil samples collected in August 2018 were confirmed taxonomically as *Lissorhoptrus*  
 142 *oryzophilus*, photographed (Fig. 1) and reported to the Spanish Ministry of Agriculture,  
 143 Fisheries and Food on 26 September 2018.



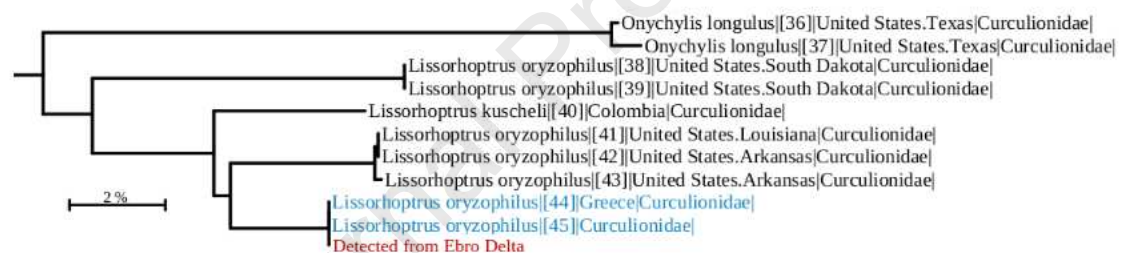
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145 **Fig. 1** Parthenogenic females of *Lissorhoptrus oryophilus* sampled in the Ebro Delta in 2018,  
 146 showing the same specimen in (a) dorsal view, (b) lateral view, (c) ventral view (scale bar 2  
 147 mm); (d) four specimens showing diverse elytral color morphs (scale bar 2 mm); and, (e) a  
 148 closeup of head and prothorax, with rostrum and antennae (scale bar 1 mm)

149

150 From the 24 bat faecal samples collected in 2019, both samples collected at one roost at the  
 151 edge of the urban area of Riumar in the Ebro Delta (0°50'18.90"E, 40°43'24.55"N) contained  
 152 the pest species *L. oryzaophilus*. In the two faecal samples, 2.22% and 2.78% of the  
 153 metabarcoding reads corresponded to *L. oryzaophilus*. No arthropod sequences were detected  
 154 from any of the blank samples. Identification was confirmed with 100% sequence similarity  
 155 with three reference sequences in the Genbank and BOLD databases (accession numbers:  
 156 KY212031, KC510131 and GU478339), sampled from Greece, South Korea and China. The  
 157 high taxonomic resolution of this marker, which is able to detect intra-species genetic diversity  
 158 (Turon et al., 2020), together with the comparison to barcoded sequences of close relatives,  
 159 allows confirmation of the identity of the species beyond any reasonable doubt (Fig. 2).

160



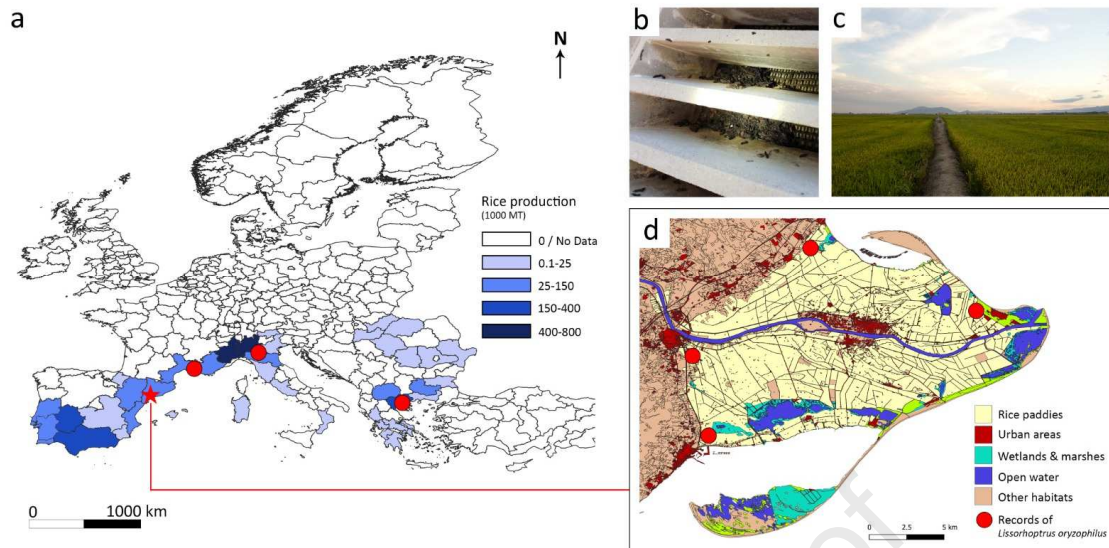
161

162 **Fig. 2** Detail of the neighbor-joining tree (K2P distance, automatically generated by BOLD-  
 163 systems) showing the 100% identity of the sequence detected from bat faeces in the Ebro Delta  
 164 with a previously sequenced *Lissorhoptrus oryzaophilus* haplotype

165

166 The four *L. oryzaophilus* records we confirm are spread out in the Ebro Delta, which has a land  
 167 cover of 70% of rice paddies (Fig. 3). Moreover, our results expand the current known European  
 168 distribution of this rice pest into a fourth country of the top five rice-producing European  
 169 countries (Fig. 3).

170



171

172 **Fig. 3** Distribution of the rice pest *Lissorhoptrus oryzophilus* in Europe, showing (a) the first  
 173 record confirmed in each country and harvested rice production (2010-2014 average) across the  
 174 continent; (b) the bat roost in the Ebro Delta from which genetic metabarcoding of the bat  
 175 faeces confirmed predation of *L. oryzophilus*; (c) a typical rice paddy field of the Ebro Delta;  
 176 and, (d) the first records of rice water weevil confirmed in the Ebro Delta. The map of rice  
 177 production has been modified from data provided online by the Foreign Agriculture Service of  
 178 the United States Department of Agriculture (<https://ipad.fas.usda.gov/>). Habitat layers for the  
 179 Ebro Delta map were reclassified from cartographic data from Catalonia  
 180 (<http://territori.gencat.cat/> and <http://www.icc.cat/>)

181

182 **DISCUSSION**

183 Our results represent the first record of *L. oryzoophilus* in the Iberian Peninsula. The Ebro Delta  
184 has a land cover of 70% of rice paddies, contributing significantly to the national rice crop  
185 production of Spain - the second largest producer nation in Europe (Kraehmer et al., 2017).  
186 With this finding, the current known distribution of this rice pest includes four of the top five  
187 rice-producing European countries (Fig. 3). In Italy, the distribution of *L. oryzoophilus* has  
188 expanded vastly and covers 86% of the country's rice crop areas (Lupi et al., 2010). Expansion  
189 has been driven by adult insect dispersal, accidental human transportation, and the absence of  
190 known natural predators (Lupi et al., 2010). However, despite the increase of its distribution  
191 range, the economic damages caused by the pest are minimal and concentrated in small areas  
192 (Lupi et al., 2015). This is partly because the pest is unable to complete a second generation  
193 during the year, due to unsuitable winter temperatures and the limitation of a single annual  
194 harvest cycle of rice (Lupi et al., 2015). Nonetheless, Italy and other countries implement  
195 control measures of rice water weevil populations using agronomic and chemical treatments  
196 (CABI, 2020; Lupi et al., 2015).

197 Integrated Pest Management (IPM) is a sustainable ecosystem-based approach to pest control  
198 that combines different management strategies to minimise the use of chemical pesticides and  
199 grow healthier crops (FAO, 2020). One pest control strategy often implemented in IPM is the  
200 use of natural biological controls by promoting natural enemies that can help suppress pest  
201 populations. Few known natural predators are reported for *L. oryzoophilus*, including a few  
202 species of frogs, insects and birds (CABI, 2020; Cave, 1983; Mercer, 2015). Our data report the  
203 first evidence of a bat foraging on rice water weevil. Bats are known predators of other rice  
204 pests similar to *L. oryzoophilus*, as shown in Kemp et al. (2019), Puig-Montserrat et al. (2015)  
205 and Wanger et al. (2014). Furthermore, the *Pipistrellus pygmaeus* bat confirmed to predate on  
206 the rice water weevil in this study have previously been shown to reduce pest populations of the  
207 Asiatic rice borer, the crambid moth *Chilo suppressalis* (Puig-Montserrat et al., 2015). Our

208 research therefore highlights bat predation as a focal point for further research on biological  
209 control of this pest species.

210 The significance of bats as natural enemies of insect pests in agricultural systems is only  
211 beginning to be uncovered and there is huge potential to implement more metabarcoding  
212 techniques to better quantify the potential of bats as natural samplers. Beyond rice crops, bats  
213 have been demonstrated to prey on pests and therefore reduce yield losses in cotton fields  
214 (Cleveland et al., 2006; Lopez-Hoffman et al., 2014), coffee plantations (Karp and Daily, 2014),  
215 cacao farms (Gras et al., 2016; Maas et al., 2013), corn crops (Maine and Boyles, 2015),  
216 macadamia orchards (Taylor et al., 2018) and vineyards (Rodríguez-San Pedro et al., 2020). To  
217 build on this growing body of evidence, implementing metabarcoding analysis of faecal samples  
218 of insectivorous bats has huge potential to confidently confirm the presence of pest species in  
219 new areas, providing early detection warnings that could be vital to control pests in their early  
220 stages of spread.

221 Insectivorous bats comprise the majority of the 1400+ described bat species (Simmons and  
222 Cirranello, 2020), occupying every type of environment on Earth except for the most extreme  
223 poles. These bats consume insects throughout the night, and their increased mobility through  
224 flight enables them to cover large geographical areas in a single night (Altringham, 2011),  
225 enhancing their power as natural samplers. Consequently, in this method, bats do most of the  
226 sampling work – naturally sampling insects through consumption – and the subsequent  
227 collection of faeces from roosts for analysis is quick, straightforward and non-invasive. Bats can  
228 be great natural samplers to provide not only new records of insect distribution, but also to  
229 discover and describe new insect species altogether (e. g. Corley et al., 2019). Importantly,  
230 careful consideration must be applied to avoid possible contamination sources during field  
231 sample collection and laboratory work. As metabarcoding techniques become increasingly  
232 affordable, genetic dietary analysis of bats can provide a comprehensive tool for naturally  
233 sampling insects and a powerful tool for early detection of pest species in agricultural lands  
234 where bats forage.

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241

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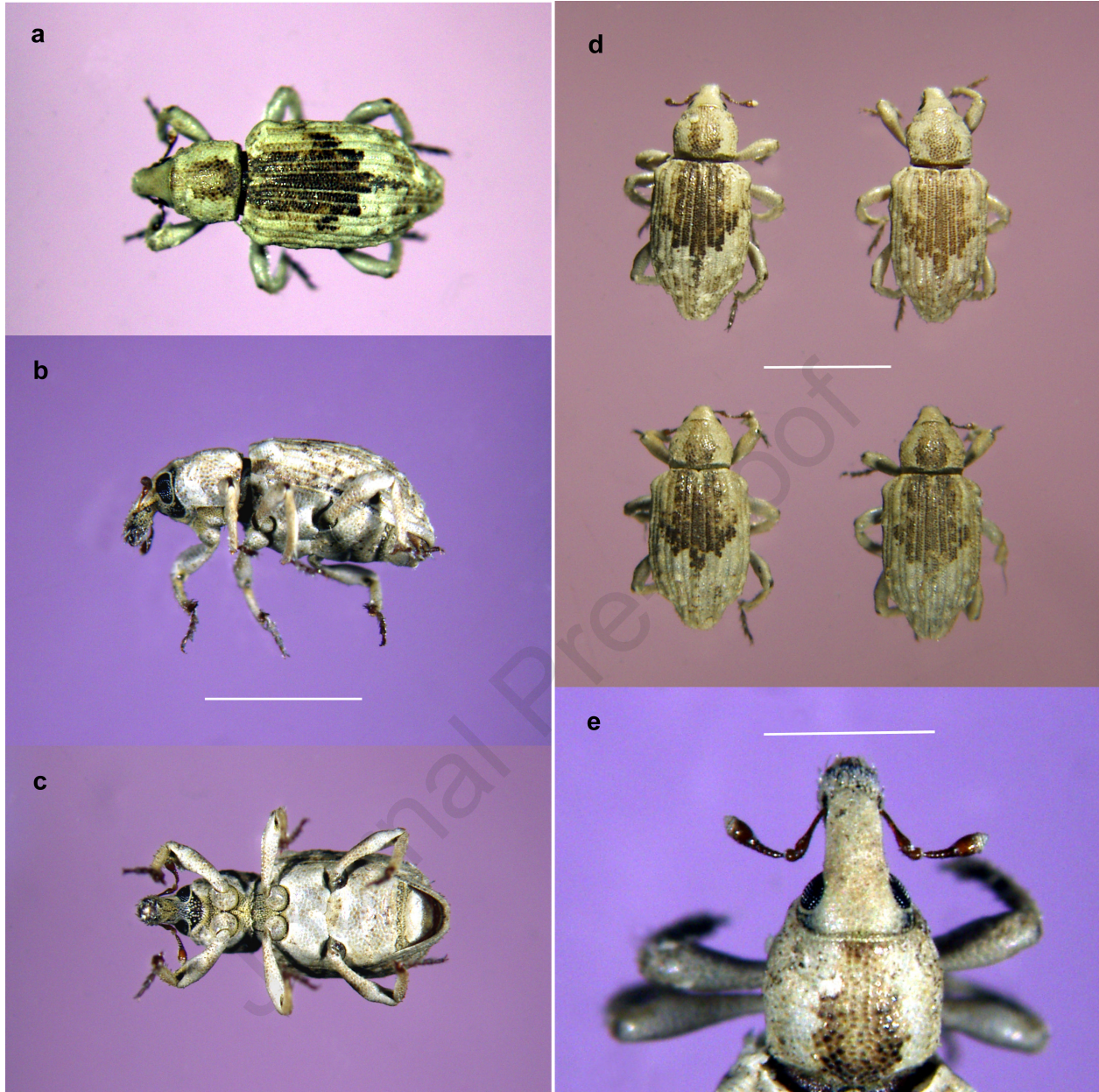


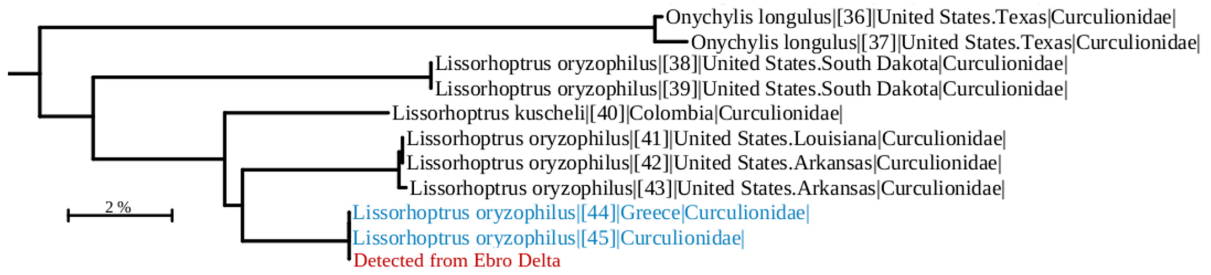
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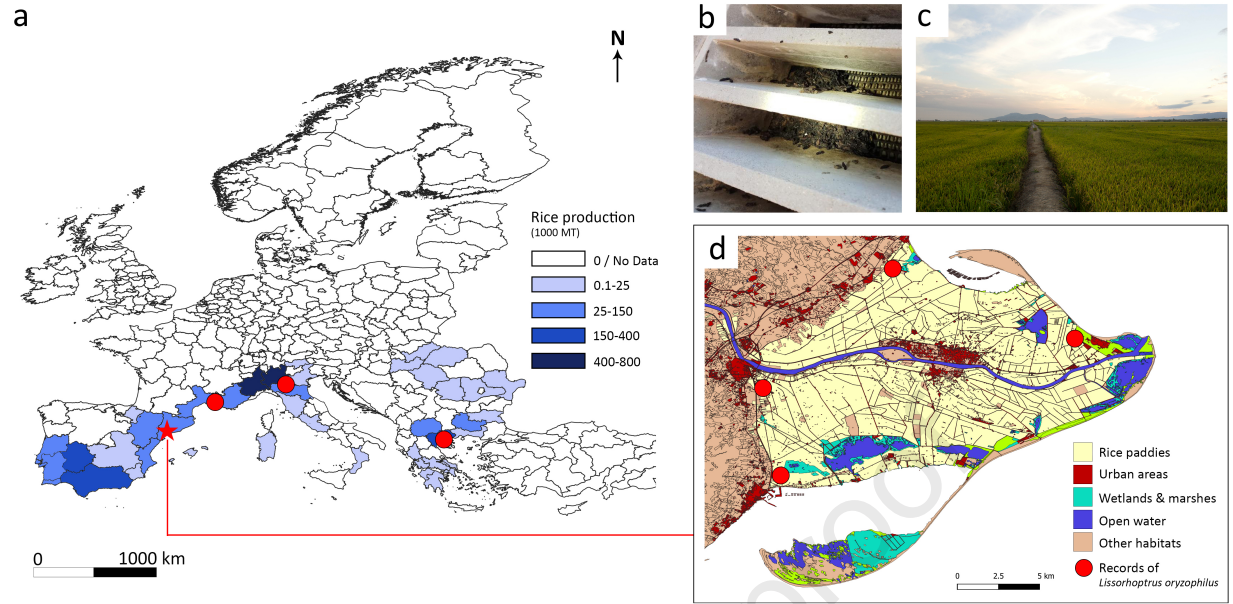
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## Highlights

- Insectivorous bats consume a large diversity of detrimental crop pests
- DNA metabarcoding of bat faeces provides a powerful natural sampling tool
- This non-invasive technique can provide important early detection warnings of pests
- With this method, we confirm the first record of the rice water weevil pest in Spain
- This invasive weevil is now in four of the top five rice producing European countries

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**Cecilia Montauban:** Writing - Original Draft; Conceptualization; Methodology; Investigation;  
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**David Gisbert Fornós:** Validation; Writing - Review & Editing

**Xavi Ferré Mola:** Validation; Writing - Review & Editing

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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