1   **DNA isolation in bark beetles: reliability of extraction methods and**

2   **application in downstream molecular procedures**

3   Leonel Stazione[1*], Victoria Lantschner[1], Juan Corley[1], Carolina Soliani[2]

4   [1] Grupo de Ecología de Poblaciones de Insectos, Instituto de Investigaciones Forestales

5   y Agropecuarias Bariloche, INTA EEA Bariloche - CONICET, Bariloche, Río Negro,

6   Argentina.

7   [2] Unidad de Genética Ecológica y Mejoramiento Forestal, Instituto de Investigaciones

8   Forestales y Agropecuarias Bariloche, INTA - CONICET, Bariloche, Argentina

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17   *Corresponding author: leonelstazione@hotmail.com

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19   **Abstract**

20   Molecular tools are increasingly used in entomology for several applications such as

21   taxonomy, genetics, ecology and evolution. For these studies, DNA extraction from

22   individual insects is a crucial step, since the yield and quality vary depending on the

23   methods used. Finding an ideal balance between quality and yield is particularly

24   difficult to reach and several constraints must be considered upon selecting the final

25   protocol. Bark beetles (Coleoptera: Curculionidae, Scolytinae) are a diverse group of

26   forest insects for which molecular studies at the individual level are important, given

27   that many species are invasive and may become pests. However, DNA extraction in

28   bark beetles is not easy, given their small size and complex molecules of diverse nature

29   conforming their exoskeleton and present in their digestive tract. Here, we carried out

30   a comparative analysis of DNA extraction performance in five bark beetle species:

31   *Hylurgus ligniperda, Hylastes ater, Orthotomicus erosus, Orthotomicus laricis* and

32   *Cyrtogenius luteus*. By assessing the efficiency of two different protocols, our aim was

33   to establish the best species-specific method for population-level studies. Our results

34   showed that a method's whole performance mainly depends on the species considered

35   and translates into DNA quantity and quality variance. We also noted that the

36   traditional method showed better PCR efficiency for the smallest species whereas the

37   commercial kit performed better for the larger beetles. Our comparative analysis

38   provides evidence that no single method for DNA isolation is best, and that each

39   particular species requires optimization.

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41   **Key words:** body size, DNA extraction, DNA quality, insects, PCR amplification.

42 **Introduction**

43 Molecular tools are increasingly used for a suite of applications in the field of

44 entomology including, taxonomy, genetics and evolution, studies of adaptation to

45 abiotic stress, or for tracking invasions (Borowiec et al. 2022; Lagisz et al. 2010; Miller

46 et al. 2009; Roques et al. 2009; Stevens et al. 2019). This type of molecular work often

47 requires DNA extraction from single individuals (Allen 2006; Dittrich-Schroder et al.

48 2012; Piper et al. 2019) which varies in yield amount and quality according to the

49 extraction methods deployed (Aboul-Maaty & Oraby 2019; Chen et al. 2010).

50 Traditional extraction methods work efficiently for DNA isolation from animal tissues

51 but use toxic chemicals and are time-consuming (Dairawan & Shetty 2020; Hajibabaei

52 et al. 2005). In turn, commercially available DNA extraction kits are becoming

53 increasingly popular because of their ease-of-use for a wide range of biological

54 samples, limited labor, and ability to consistently produce high-quality DNA (Nishiguchi

55 et al. 2002; Hajibabaei et al. 2005). However, some of the drawbacks of these kits

56 include their expense and the fact that prolonged incubation times cannot be avoided

57 in any case (Ball & Armstrong 2008). Therefore, an ideal balance between quality and

58 yield is desirable and, at the same time, methodological constraints should be

59 considered upon selecting the final protocol (e.g., the starting tissue, or the size of

60 specimens; Chen et al. 2010; Dittrich-Schroder et al. 2012; van der Loos & Nijland

61 2021).

62 Bark beetles (Coleoptera, Curculionidae, Scolytinae) are a highly diverse group of forest

63 insects that breed and feed on the phloem of trees (Kirkendall et al. 2015; Gomez et al.

64 2023). These insects are characterized by a small body size -most species range from 1

65   to 5 mm long-. Several species within this group are widely acknowledged as significant

66   agents of tree mortality in coniferous forests globally (Raffa et al. 2008; Biedermann et

67   al. 2019). Additionally, they are inadvertently transported via wood products and wood

68   packaging materials, and numerous species have effectively established worldwide,

69   constituting a notable group of invasive forest insects (Lantschner et al. 2020; Grégoire

70   et al. 2023).

71   Every extraction method has a percentage of DNA lost during procedure that should be

72   accounted for, to obtain a minimum amount of DNA for analysis. In the case of bark

73   beetles, the main constraints are related to their size and the presence of undesired

74   macromolecules. Their small size imposes a limitation to physical breakdown (milling)

75   that then translates into a lower efficiency of the cell lysis treatment and cell DNA

76   isolation. Working with small amounts of DNA also increases the risk of contamination

77   (van Oorschot et al. 2010). DNA contaminants can be introduced during sample

78   processing, consumables manipulation, laboratory reagents preparation (Ishak et al.

79   2011; Salter et al. 2014; Stinson et al. 2019), or inappropriate use of commercial DNA

80   extraction kits (Gefrides et al. 2010; Goodrich et al. 2014), in a proportion that may

81   exceed DNA, resulting in a useless extract.

82   Additionally, bark beetles have complex and large molecules of a diverse nature that

83   might be in part isolated with DNA during extraction procedures, affecting the

84   amplification process (Ferrara 2023; Hosseini 2010). These molecules are mainly

85   proteins and polysaccharides of their exoskeleton -as in most other insect species-, as

86   well as plant phenolics and tannins present in their digestive tract, associated to their

87   xylophagous diet. Macromolecules of this kind may act as inhibitors of PCR and are

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88   therefore undesired (Amini et al. 2021; Demeke & Jenkins 2010). For instance,

89   phenolics, tannins and other contaminants present in a DNA extraction solution can

90   inhibit enzymes such Taq polymerases, making extracted DNA unsuitable for PCR

91   amplification (Asghar et al. 2015). Moreover, the DNA extraction method could be

92   affected by different factors such as the storage conditions of the sample, and the cost

93   and duration of the extraction method (Psifidi et al. 2015).

94   We carried out a comparative study of two extraction methods for five different bark

95   beetle species: *Hylurgus ligniperda* (Fabricius 1787)*, Hylastes ater* (Paykull 1800)*,

96   Orthotomicus erosus* (Wollaston 1857)*, Orthotomicus laricis* (Fabricius 1792), and

97   *Cyrtogenius luteus* (Blandford 1894). The aim of our work was to understand the

98   relationship between the DNA concentration, yield and purity regarding to PCR

99   efficiency presented by each method, in each species. We provide information to

100   ascertain a suitable species-specific protocol that enables population-level studies.

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102   **Materials and Methods**

103   *Sample collection and preparation*

104   Bark beetles were collected from 17 pine plantation stands of southern South America

105   (Argentina, Chile and Uruguay). Insects were captured by placing traps (i.e., Lindgren

106   funnel traps, Cross vane panel traps) with generic bark beetle lures (i.e., ethanol, α-

107   pinene, and/or turpentine), and by manually collecting from cut pine logs available in

108   some sites (see Lantschner et al., 2024 for further details). All collected samples were

109   conserved in 70 - 95% ethanol and stored at -20°C. A total of 173 individuals of five

110   bark beetle species of different body sizes were obtained: 81 individuals of *Hylurgus*

111   ligniperda (4 to 6 mm), 30 individuals of *Hylastes ater* (3.5 to 4.5 mm), 22 individuals of

112   *Orthotomicus erosus* (3 to 3.8 mm), 20 individuals of *Orthotomicus laricis* (3 to 3.7 mm)

113   and 20 individuals of *Cyrtogenius luteus* (2.2 to 2.4 mm).

114   *DNA extraction*

115   Before extraction, the individuals were oven dried at 60°C for 15 minutes in sterile 1.5-

116   microcentrifuge tubes to ensure removing ethanol residues. We performed two DNA

117   extraction methods:

118   Method 1 (M1): This method was based on the protocol of Baruffi et al (1995) with

119   modifications. As a starting point, each sample was pre-washed with 50 μl of extraction

120   buffer (removed afterwards) and then grounded with a new aliquot of 100 μl of

121   extraction buffer (100 Mm NaCL, 200 mM sucrose, 50 mM EDTA, 100mM Tris-HC1 (pH

122   9.1), 0.5 % SDS) using a glass pestle. After this first step, 200 μl of extraction buffer and

123   2 μl of proteinase K enzyme (10 mg/ml) were added and incubated at 65 °C overnight.

124   Next, the homogenate was mixed with 56 μl of KAc 8M and incubated on ice for 30

125   minutes. The preparation was centrifuged at 14000 rpm for 15 min, twice

126   consecutively. The supernatant was recovered to then mix it with a solution of 400 μl

127   chloroform: isoamyl alcohol (24:1), following centrifugation at 14000 rpm for 10 min.

128   This step is crucial to ensure DNA isolation and the proper removal of other non-target

129   molecules. The aqueous phase was transferred to a new microcentrifuge tube and the

130   chloroform: isoamyl alcohol step repeated. The DNA was precipitated with 50 μl of

131   NaAc 3M and 400 μl of 100% ethanol. After centrifugation (14000 rpm for 20 min) the

132   resulting DNA pellet was washed twice with 500 μl 70% ethanol. Finally, the pellet was

133   air-dried and eluted in 50 μl TE (TRIS-EDTA 10:1) buffer.

134   Method 2 (M2): This method was based on the use of a commercial kit DNA Puriprep-T

135   kit (Inbio Highway®, Argentina). The entire body of the insects was grounded with a

136   glass pestle by adding 100 μl of the extraction buffer provided with the kit. Then 180 μl

137   of extraction buffer and 2 μl of proteinase K enzyme (10 mg/ml) were further

138   incorporated and samples were incubated at 56 °C overnight. The day after, the

139   homogenate was mixed with 200 μl of a lysis buffer (provided with the kit) and

140   incubated at 56°C for 15 minutes. This step is essential for proper lysis. Then, an aliquot

141   of 200 μl of 100% ethanol was added to the preparation. The entire volume was

142   transferred to a Colum Micro centrifuge tube and centrifuged at 13000 rpm for 2 min.

143   The DNA is retained in the column whereas the eluted volume was discarded. Column

144   membrane was washed twice with 500 μl of washing buffer (provided with the kit).

145   Finally, DNA was eluted from the membrane with 200 μl elution buffer (10 mM Tris-

146   HCl, 0.5 mM EDTA; pH: 9.0, at 70°C).

147   *Assessment of extraction success*

148   Extraction methods were evaluated using four variables: DNA concentration, yield,

149   260/280 ratio, and 260/230 ratio. DNA concentration of all samples was measured on

150   NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, USA) using 2 μl

151   of DNA extraction solution. Yield was reported as the mean ng DNA/mg tissue by

152   method. Purity of samples were reported as the ratio of the absorbance values at 260

153   and 280 nm (260/280 are the absorbance wave lengths of DNA and proteins,

154   respectively) and at 260 and 230 nm (260/230, are the absorbance wave lengths of

155   DNA, polysaccharides and other compounds like phenolics, respectively).

156   *PCR amplification*

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157 To assess amplification success, each DNA extraction sample was tested through

158 polymerase chain reaction (PCR). The mitochondrial gene cytochrome oxidase c

159 subunit I (COI) was used as the marker of choice - primer pair from Simon et al (1994)

160 (S1718 fw: 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′; A2411 rev: 5′-

161 GCTAATCATCTAAAAACTTTAATTCCWGTWG-3′). The PCR reaction consisted of 1 × buffer

162 (50 mm KCl, 10 mm Tris–HCl, pH 9), 1.5 Mm of MgCl2, 0.2 Mm of each dNTP, 0.16 µM

163 of each primer, 0.6 mg/ml of bovine serum albumin (BSA), 0.04 U of Taq polymerase

164 (Invitrogen®) and 20 ng of DNA, in a final volume of 15 µL. The temperature cycles for

165 the amplification were as follows: initial denaturation at 94 °C for 5 minutes; 40 cycles

166 of 94 °C for 1 minute, 58 °C for 45 seconds, 72 °C 45 seconds and a final extension of 10

167 minutes at 72 °C. The PCR products (~ 700 bp) were loaded into 1.5 % agarose

168 containing GelRed Nucleic Acid Gel Stain 10,000X (Biotium®) and electrophoresed in

169 0.5 × TBE buffer for 1 h at 3 V/cm, together with standard DNA ladders (1 Kb plus;

170 Promega Corp.) to confirm amplification. Samples were purified by using a

171 precipitation method based on sodium acetate solution and then sequenced with an

172 ABIPRISM 3700 capillary sequencer (Applied Biosystem).

173 *Data analysis.*

174 Differences in the extraction success variables (DNA concentration, yield and 260/280

175 and 260/230 purity ratios) between both extraction methods (M1 and M2) were tested

176 for each species individually. For that, a generalized linear model (GLM) was performed

177 using the logit link function and a Gaussian distribution (best fitted distribution of the

178 data). The extraction method was included as a fixed factor, and the replicate samples -

179 within the method- as a random factor. In addition, a Spearman correlation analysis

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180 was carried out to explore the relationship between DNA concentration, yield and

181 purity ratios. Finally, for each species, the proportion of samples for which successful

182 amplifications were obtained with each extraction method was estimated and a Chi-

183 Square (X^2) Goodness of Fit Test was performed to test for differences between

184 methods. All analyzes were performed using R software (version 4.2.2) for Windows (R

185 Core Team, 2023).

186

187 **Results**

188 The results of the GLM showed that the success of the two extraction methods varied

189 depending on the species (Fig 1). However, it is possible to identify some trending

190 patterns. Significant differences were revealed between the extraction methods for

191 some extraction success variables, with M1 exhibiting a lower average yield and higher

192 260/280 purity ratio than the M2, for *H. ligniperda* (z = 6.86, p < 0.001 for yield; z =

193 5.58, p< 0.001 for 260/280), *H. ater* (z = 2.77, p <0.01 for yield; z = 5.22, p < 0.001 for

194 260/280)*, O. laricis* (z = 5.82, p < 0.001 for yield, z = 6.97, p < 0.001 for 260/280) *and*

195 *O. erosus* (z = 2.10, p < 0.05 for yield) (Fig 1). DNA concentration showed variable

196 outcomes, with significantly higher values in M1 than M2 for *H. ater* (*z* = 2.44, p < 0.05;

197 and *C. luteus* (z = 2.67, p < 0.01) whereas the opposite occurred for *O. laricis* with M2

198 showing significant higher values of DNA concentrations than M1 (z = 6.22, p< 0.001;

199 Fig 1). No differences were found in 260/230 purity ratio between methods for any of

200 the five species (Fig 1).

201 The results of the Spearman correlations between variables of extraction success

202 showed significant positive correlations between DNA concentration and yield in all

203   species. Correlations between DNA concentration and 260/280 purity ratio as well as

204   between yield and 260/280 purity ratio were mostly negative and resulted significant in

205   *H. ligniperda*, *O. laricis* and *C. luteus*. Furthermore, the correlations between DNA

206   concentration and 260/230 purity ratio as well as between yield and 260/230 purity

207   ratio were mostly positive and resulted significant in *H. ligniperda*, *O. erosus* and *C.*

208   *luteus*. Finally, the correlations between 260/280 and 260/230 purity ratios were

209   significantly positive in *O. laricis* and significantly negative in *C. luteus* (Table 1, Fig S1,

210   S2, S3, S4, and S5).

211   A higher proportion of samples showing successful amplifications was obtained for M2

212   than for M1 in all of species except for *C. luteus*, where the pattern was opposite (X^2 =

213   9.41, p < 0.001 for *H. ligniperda*; X^2 = 86.7, p< 0.01 for *H. ater*; X^2 = 86.7, p < 0.001 for

214   *O. erosus*; X^2 =11.13, p< 0.001 for *O. laricis*; X^2 = 62.5, p < 0.001 for *C. luteus*; Fig 2).

215

216   **Discussion**

217   The efficiency of two DNA extraction methods, based on different isolation

218   approximations (phase separation and use of silica membranes) in each of five bark

219   beetle species was different. The DNA quantity and quality values showed a variance

220   that mainly depended on the species under consideration. In general terms, the results

221   indicated that the commercial method performed better in most species. However, in

222   *C. luteus* -the smallest species- the modified Baruffi method showed better PCR

223   efficiency. Therefore, we conclude that it is not possible to recommend a single

224   standard protocol for all species.

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225   We found that DNA concentration is not always predictive of higher PCR performance,

226   and that other properties of DNA quality may be particularly useful when considering

227   the likelihood of amplification success (Ballaré et al. 2019). It has often been assumed

228   that high levels of DNA concentration are necessary for high DNA quality (Andrews et

229   al. 2016; Puritz et al. 2014). However, in this study the method that returned the

230   highest DNA concentration as the most efficient in PCR amplifications was not

231   consistently observed.

232   When focusing on yield, better results were observed with the commercial kit

233   regardless of the beetle species. It is also important to note that yield is usually

234   influenced by many factors related to species' characteristics, as for example body

235   sclerotization, preservation method after field collection, or a particular and crucial

236   step during extraction procedure, (i.e., DNA precipitation; Lear et al. 2018). In our case,

237   and although all steps of material conservation, transportation and conditioning were

238   controlled, minor tissue damage could still have occurred that might have influenced

239   the yield and quality of extracted DNA (e.g., Chen et al. 2010), irrespective of the

240   species. These properties of template DNA could become a determining factor for PCR

241   efficiency (Schill 2007). In four out of five species, a higher PCR efficiency was observed

242   in samples that were extracted with the commercial kit. Conversely, in the smallest

243   species, *C. luteus,* a higher PCR success rate was observed for samples treated with M1,

244   in spite of minor yield, opposite to what was observed for the other species.

245   The 260/280 ratio, a proxy of purity, exhibited a negative relationship with DNA

246   concentration as well as yield in all species, regardless of their size. In addition, the

247   results showed that a higher 260/280 purity ratio did not guarantee PCR efficiency. This

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248   trend was consistent across most species, with the method identified as the best-

249   performing candidate (M2) showing a lower PCR success rate but significantly higher

250   260/280 purity ratio. In this sense, it is important to note that 83.8% of the samples

251   (145 out of 173 total) had a 260/280 purity ratio value higher than 1.8, indicating

252   "pure" DNA with little to no protein or other contaminants that absorb light at or near

253   280 nm (Simbolo et al. 2013).

254   In molecular studies that plan to extract DNA from a limited number of individuals, a

255   high PCR efficiency is particularly important. However, in cases where the initial

256   amount of tissue is small and/or the sample quality is low, the extracted DNA may

257   compete with DNA contaminants over the course of an experiment (Lusk 2014).

258   Implementing stricter laboratory precautions, such as routine UV irradiation of

259   reagents and tools and positive pressure laboratory ventilation systems, could help

260   minimize the risk of DNA contamination in the laboratory (Willerslev & Cooper 2005;

261   Champlot et al. 2010). While laboratory contamination is probable, previous studies

262   show that cross-contaminations were most likely introduced from the field (Goldberg

263   et al. 2013; Kranzfelder et al. 2016). The results seem to indicate that in bark beetles

264   the 260/280 purity ratio is not a variable that defines the efficiency of the PCR, but not

265   everything has been said when it comes to tiny specimens that seem exceptional.

266   The second indicative of DNA purity, the 260/230 ratio, showed a positive relationship

267   with DNA concentration as well as yield in most species. Due to the presence of

268   phenols, polysaccharide and other organic contaminants -that absorb light at 230 nm-

269   the 260/230 ratio could become relevant and tightly link to a particular bark beetle

270   species (Bilgin et al. 2009; Varma et al. 2007). We found opposite trends in purity ratio,

271   probably associated to the performance of each applied method of DNA isolation,

272   between *O. laricis* and *C. luteus*. As the number of PCR successful events were

273   contrasting in both species, we suggest that purity might have determined this

274   outcome relation. Achieving complete elimination of these contaminants is particularly

275   relevant in PCR amplification, given that contaminants cause inhibition in enzymes like

276   restriction endonucleases and Taq polymerases (Arif et al. 2010; Calderón-Cortés et al.

277   2010; Asghar et al. 2015). Considering the feeding interaction of bark beetles with

278   trees and their frequent exposure to plant products, we propose that there may be a

279   concomitant relationship between DNA concentration and organic contaminant

280   present in the wood during the extraction process. These contaminants might interfere

281   with the efficiency of PCR (Bilgin et al. 2009). Furthermore, this relationship is likely to

282   change depending on the insect size and species. It is expected that the amount of

283   genetic material extracted in smaller species would be less than that obtained in larger

284   species -using the same protocol- and in fact, the proportion of organic contaminants

285   will be greater as the smaller the insect. This could explain why M2 showed higher PCR

286   efficiency in larger species while it failed completely in *C. luteus* – the smallest species-

287   (Fig 4). In addition, the difference between protocols steps could be a key factor

288   determining the PCR efficiency. The Baruffi method includes steps to separate aqueous

289   phases and wash the extracted material with alcohols (see Materials and Methods),

290   which are carefully performed to reduce the losing pellet fractions, especially in small

291   species where the extracted material is even more limited. While in silica membrane-

292   based methods some loss of DNA is reported during the extraction (Rothe & Nagy

293   2016).

294   Considering the presence of possible organic wood residues that could interfere with

295   the PCR performance, future tests that include dissection of the digestive tract may be

296   the key to achieve the proper removal of these contaminants. Furthermore, the time

297   elapsed since the DNA extraction was carried out could be an important factor to the

298   PCR performance (Hazir 2019; Soto-Calderón et al. 2009) and should be considered.

299   The DNA isolated from xylophagous insects, which may contain polysaccharides, RNA

300   and phenolics, is not suitable for long storage periods and has a shorter storage life

301   (Horne et al. 2004; Lodhi et al. 1994; Rao & Doddamane 2022).

302   In bark beetles the presence of residual organic wood molecules and their small and

303   sclerotized bodies represent major constrains in the choice of a reliable extraction

304   protocol. Our results show different key factors to consider when choosing a suitable

305   method in these xylophagous insects. Although there is not a single extraction method

306   for all species, by assessing DNA concentration, yield and purity as estimators of PCR

307   success on several bark beetle species, we provide a novel approach to estimate the

308   applicability of widely used laboratory routines.

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495 Table 1. Spearman correlation analysis between pairs of extraction success variables: DNA concentration, yield and 260/280 and 260/230 purity
496 ratios. N: number of samples. CC: correlation coefficient. Asterisks indicate significant differences (* p < 0.05; ** p < 0.01; *** p < 0.001).

| Variable 1	Variable 2	*H. ligniperda*		*H. ater*		*O. laricis*		*O. erosus*		*C. luteus*	
		N	CC	N	CC	N	CC	N	CC	N	CC
DNA concentration	Yield	81	0.83***	30	0.55**	20	0.98***	22	0.79***	20	0.6**
DNA concentration	260/280	81	-0.36**	30	0.14	20	-0.77***	22	-0.2	20	-0.63**
DNA concentration	260/230	81	0.37***	30	0.28	20	-0.21	22	0.65**	20	0.54*
Yield	260/280	81	-0.65***	30	-0.56**	20	-0.81***	22	-0.19	20	-0.58*
Yield	260/230	81	0.28*	30	0.28	20	-0.27	22	0.81***	20	0.9***
260/280	260/230	81	0.13	30	-0.09	20	0.45*	22	-0.42	20	-0.49*
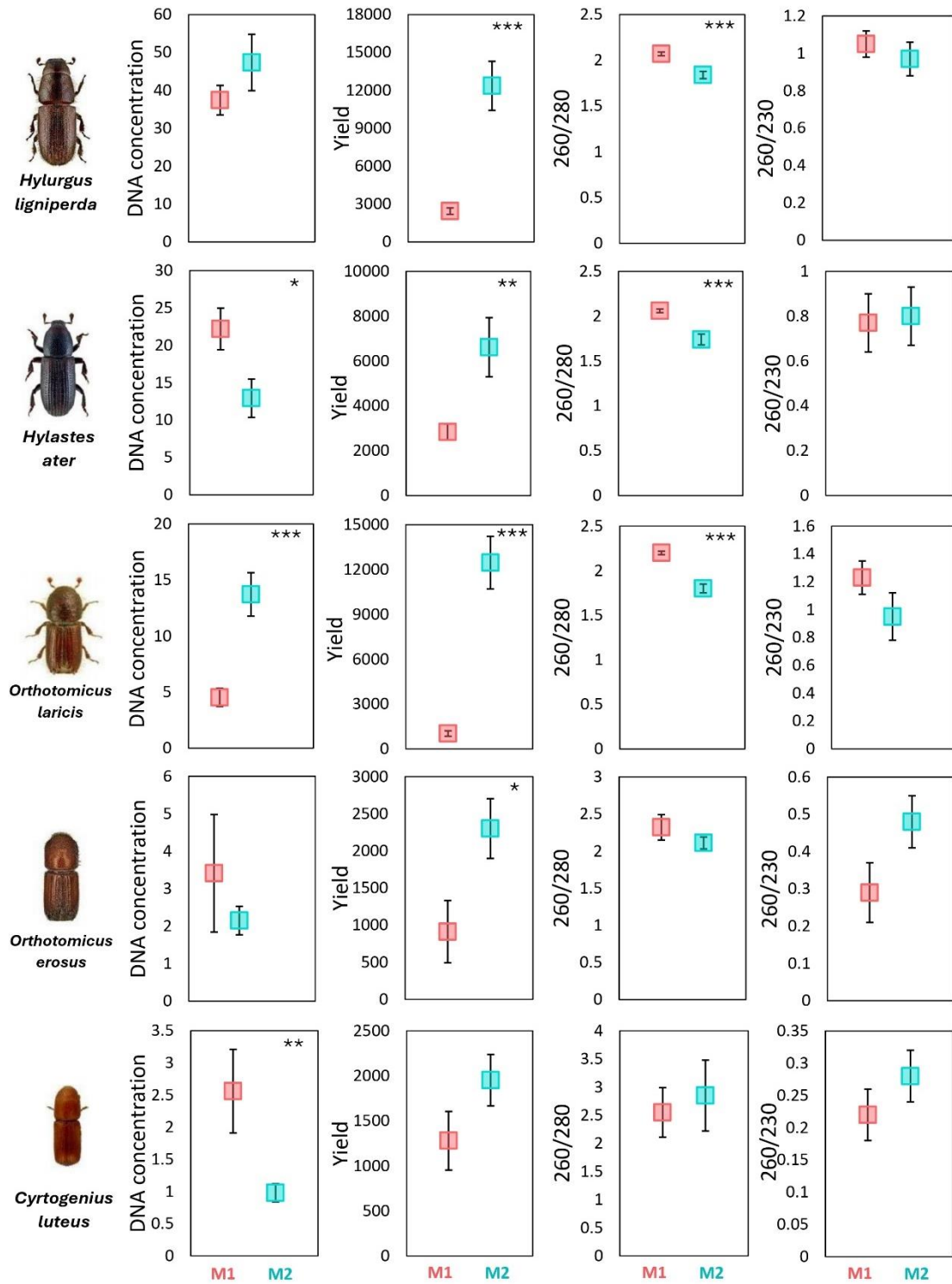
Figure 1. Mean values (±SE) of DNA concentration, yield, and 260/280 and 260/230 purity ratios for each extraction method, in each insect species separately. M1: Baruffi protocol, M2: commercial kit DNA Puriprep-T kit. Asterisks denote significant differences (*p < 0.05, **p < 0.01, ***p < 0.001). The proportionality in the size of the specimens for each species is in accordance with reality
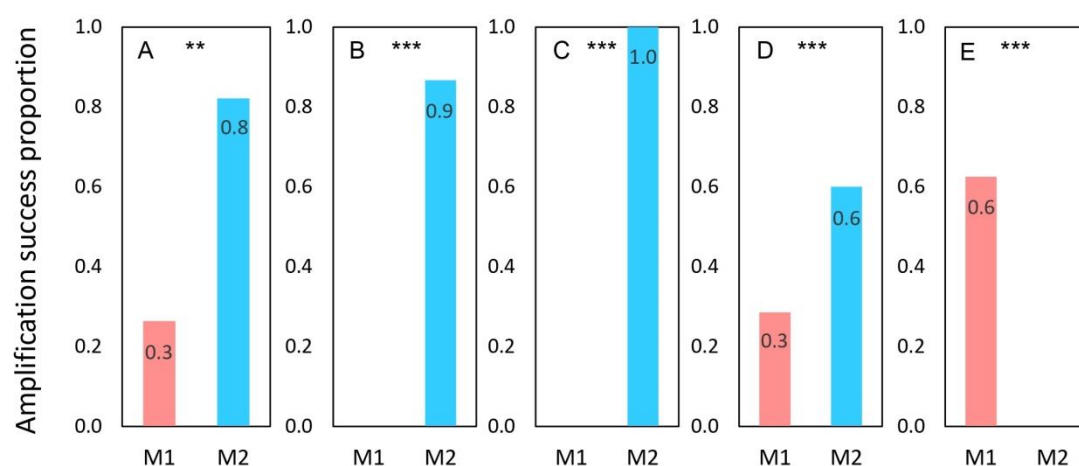
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Figure 2. Amplification success proportion for both extraction methods, in each species separately. A: *Hylurgus ligniperda,* B: *Hylastes ater*, C: *Orthotomicus laricis*, D: *Orthotomicus erosus*, E: *Cyrtogenius luteus*. M1: Baruffi protocol, M2: commercial kit DNA Puriprep-T kit. Asterisks denote significant differences (**p < 0.01, ***p < 0.001)