A population genetic study of the egg parasitoid Baryscapus servadeii reveals large scale automictic parthenogenesis and almost fixed homozygosity

Mauro Simonato, Marco Pilati, Emmanuelle Magnoux, Claudine Courtin, Laure Saune, Jérôme Rousselet, Andrea Battisti, Marie-Anne Auger-Rozenberg, Carole Kerdelhue

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30 **Highlights**
31 • *B. servadeii* is a specialist egg parasitoid of the pine processionary moth
32 • Microsatellite markers were developed to track its possible range expansion
33 • Very high levels of homozygosity were discovered, with only 2 main MLG
34 • *Rickettsia*, not *Wolbachia*, is likely the symbiont inducing parthenogenesis
35 • The markers show variability in the cryptic *B. transversalis* hyperparasitoid
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Abstract

The pine processionary moth *Thaumetopoea pityocampa - T. wilkinsoni* is one of the main defoliators of pine and cedar forests in the Mediterranean area. Its distribution is currently expanding northward and to higher altitudes due to climate warming. This species is associated with a rich community of predators and parasitoids. The specialist egg parasitoid *Baryscapus servadeii* is among the most abundant, and is thought to significantly affect the population dynamics of its host. To determine if the parasitoid tracks the range expansion of its host and shows similar population genetic patterns, we developed eight de novo microsatellite markers using SSR enrichment and 454 pyrosequencing. Despite a large-scale sampling design, ranging from Northern Africa to Western Europe, Crete and Cyprus, genotyping revealed an unexpectedly high level of homozygosity and reduced genetic diversity, with 90% of studied individuals being characterized by only 2 multi-locus genotypes. We then tested if *B. servadeii* is associated with parthenogenesis-inducing endobacteria, and found that most individuals were associated with a *Rickettsia* symbiont, not *Wolbachia*. Interestingly, genetic diversity and an absence of symbionts were documented in some individuals of the congeneric hyperparasitoid *B. transversalis*, which were found in the same samples. Altogether, the results suggest that microsatellite nuclear markers will not be useful for deciphering the demography and range expansion routes of this parasitoid. However, *B. servadeii* can be considered an interesting and novel example of *Rickettsia* inducing thelytoky in chalcid parasitoids.

1. Introduction

Climatic and land use changes affect both species distribution and life history traits. Poleward and upward shifts due to climate warming have been observed in many temperate species limited by cold (Lenoir and Svenning 2015), and climate warming tends to advance the development of poikilothermic organisms (Bartomeus et al. 2011). Still, if the impacts of current environmental changes on individual populations or species are increasingly well-understood, less attention has been paid to the fate of biotic interactions (Thompson et al. 2013). Climate change and landscape heterogeneity are major disruptors of ecological relationships, because species may evolve along different "adaptation routes" in front of similar environmental changes and selective pressures. In multi-trophic systems facing environmental changes, sustainability of interspecific interactions relies on the maintenance of both a fit between phenotype and environment at each trophic level, and phenological and spatial overlaps of interacting species (Rafferty et al. 2013). In the case of pest - natural enemies relationships, disruptions of the interaction lead to weakened biological control services and increased risks of pest outbreaks (Thomson et al. 2010), even though opposite situations are predicted in some cases (Berggren et al. 2009). Moreover when natural enemies fail to efficiently track their expanding host in newly colonized areas, the subsequent lowered levels of mortality in the pest tend to increase its population dynamics and hence its expansion rate (Liu and Stiling 2006; Colautti et al. 2004). Inferring routes of expansion and characterizing dispersal of natural enemies is an essential step to develop efficient methods to manage invasive or expanding species. Population genetics and phylogeographic studies on natural enemies are necessary tools to understand the dynamics of host tracking of these species in the areas recently colonized by their hosts (Gebiola et al. 2014).

The pine processionary moth (PPM, *Thaumetopoea pityocampa* (Den. & Schiff.) and *T. wilkinsoni* Tams, Lepidoptera, Notodontidae) is one of the main pine and cedar forest pests in the Mediterranean basin, as it causes heavy economic damage due to tree defoliation and represents a threat for human health due to the release of urticating setae during the late larval stages (Battisti et al. 2017). In the last decades, as a result of the increased average winter temperatures, this moth has been expanding its range in Europe to both higher latitudes and elevations (Battisti et al. 2005; Robinet et al. 2014). Long-distance, accidental man-aided migration has been suggested to play a role in this rapid expansion (Robinet et al. 2012). In its native range, this pest species is associated to a rich community of predators and parasitoids that play an important role in its control (Battisti et al. 2015). In particular, two main species of egg parasitoids, namely a specialist *Baryscapus servadeii* (Domenichini) (Hymenoptera, Eulophidae) and a generalist *Ooencyrtus pityocampae* (Mercet) (Hymenoptera, Encyrtidae), can cause an egg mortality up to 40% in southern Europe and

99   could efficiently reduce the damage caused by the PPM (Auger-Rozenberg et al. 2015a). Both

100   species occur across the Mediterranean Basin and are present all over the distribution of their main

101   host; they both mostly reproduce through thelytokous parthenogenesis (Battisti et al. 1990). In this

102   mode of reproduction, males are extremely rare and diploid females are produced from the

103   development of unfertilized eggs. Diploidy can be restored by diverse mechanisms leading to

104   different genetic outcomes (Rabeling and Kronauer 2013). Depending on the mechanism at play,

105   the offspring can be clonally identical to the mother and heterozygozity is maintained; or they can

106   be fully homozygous. Thelytoky can either have an intrinsic genetic basis, or it can be induced by a

107   microbial infection (Ma and Schwander 2017). Three genera of parthenogenetic-inducing bacteria

108   are known to occur in Hymenoptera, namely *Wolbachia*, *Rickettsia,* and *Cardinium*. The first one is

109   by far the most frequently insect-associated symbiont inducing parthenogenesis, in particular in

110   chalcid wasps (Rabeling and Kronauer 2013; Ma and Schwander 2017).

111   In a recent study, Auger-Rozenberg et al. (2015b) investigated the phylogeographical structures

112   of *B. servadeii* and *O. pityocampae* over the Mediterranean Basin using mitochondrial and nuclear

113   sequences. They showed contrasting levels of diversity in both species, the specialist *B. servadeii*

114   having 87 haplotypes whereas only 16 were found in *O. pityocampae* over the same spatial scale.

115   The two species were found to have drastically different Quaternary evolutionary histories.

116   Interestingly, *B. servadeii* showed a strong geographical structure, with 3 main clades partially

117   mirroring the genetic structure found in its host insect (Kerdelhué et al. 2009); a fourth, strongly

118   divergent mitochondrial clade, linked to a similarly strong differentiation of the nuclear marker, was

119   suggested to correspond to a congeneric hyperparasitoid, *Baryscapus transversalis* Graham. This

120   species is difficult to differentiate based on morphological characters. It occurs in the Balkans,

121   Spain and Turkey (Auger-Rozenberg et al. 2015b; Boyadzhiev et al. 2015). Contrary to *B.*

122   *servadeii*, males are frequent and probably involved in sexual reproduction. Presence of males is

123   actually one of the cues used to suggest occurrence of *B. transversalis*. Yet, complementary data

124   using polymorphic nuclear loci are still needed to confirm this result.

125   At a finer geographical scale, studies carried on latitudinal and altitudinal gradients along the

126   expanding front of the PPM suggested that the egg parasitoids tend to track the expansion of their

127   PPM host, yet with a time lag (Auger-Rozenberg et al. 2015a; Robinet et al. 2012; Zovi et al. 2006).

128   Using mitochondrial markers, a strong genetic structure was found along these gradients for *B.*

129   *servadeii* (Auger-Rozenberg et al. 2015a), with a higher variability in the core areas compared to

130   the expansion areas, as it is expected in case of diffusive expansion. On the other hand, a study

131   carried on a few populations in northeastern Italy, using variable genetic markers (AFLP),

132   underlined the lack of geographical patterns at a finer geographical scale, which could be due to

133 higher dispersion of *B. servadeii* compared to *O. pityocampae* (Simonato et al. 2012).

134 Characterizing population structure and dispersal modes using polymorphic and codominant

135 nuclear markers is now necessary to compare host and parasitoid spatial expansions, in particular in

136 the case of the specialist species, which strictly depends on its insect host.

137   Obtaining a set of variable microsatellite loci is thus a crucial step for future studies on the

138 expanding patterns of *B. servadeii* at a finer geographical scale in the PPM expanding areas, as well

139 as to characterize the divergence between *B. servadeii* and *B. transversalis*. Here we report the

140 development of a set of 8 polymorphic microsatellite markers, retrieved by high throughput

141 genomic sequencing. Our primary goal was to use these markers to characterize genetic diversity

142 and structure in populations chosen to represent south – north gradients in Western Europe, and to

143 include sites in the Balkans where *B. transversalis* is expected to occur. Yet, given the very low

144 genetic diversity found in *B. servadeii*, we completed the sampling design by adding sites from

145 North Africa, Cyprus and Crete, where the *B. servadeii* mitochondrial clades II and III occur

146 (Auger-Rozenberg et al. 2015b). A further goal was to determine if both the parasitoid and the

147 hyperparasitoid were associated with parthenogenesis-inducing bacterial symbionts that could

148 explain the different reproduction type.

149

150 **2. Materials and Methods**

151 *2.1. Sampling*

152 Samples were collected from egg masses of the pine processionary moths *Thaumetopoea*

153 *pityocampa* and *T. wilkinsoni* following the procedure described in Auger-Rozenberg et al. (2015b),

154 and adults were morphologically identified as *Baryscapus* spp. as discrimination between *B.*

155 *servadeii* and the hyperparasitoid *B. transversalis* is difficult. Three successive sampling were used

156 during the course of the project (hereafter named Sets-a, -b and -c, see Table 1):

157 Set-a: a pool of individuals chosen from Auger-Rozenberg et al. (2015b) to represent the diversity

158 of *B. servadeii* in Europe was used for microsatellite library construction. This pool was constituted

159 from five localities from France, Spain and Portugal;

160 Set-b: 28 individuals sampled in 14 localities chosen all over the distribution range of *B. servadeii*

161 and belonging to the 4 main mitochondrial clades identified in this species were used to test the

162 microsatellite primer pairs designed during marker development;

163 Set-c: Ten populations (9 to 31 individuals each) were finally genotyped using the validated

164 microsatellite loci. The initial objective was to use Western European samples along north-south

165 gradients to test for signs of geographic expansion and host tracking. The sampling was enlarged to

166 Eastern Europe and North Africa in a second step, when the first results suggested that this species

167 has an extremely low genetic diversity.

168

169 *2.2. DNA extraction and mitochondrial characterization*

170 For each population except Longarone (Italy), we used the DNA of individuals from Auger-

171 Rozenberg et al. (2015b) and completed the sampling using other individuals from the same

172 localities, emerged from different egg batches to avoid siblings. Individuals from Longarone

173 originated from the study of Simonato et al. (2012). Genomic DNA was extracted using

174 NucleoSpin® Tissue XS kit (Macherey-Nagel, Germany), according to the manufacturer's protocol.

175 Quantification and quality check of the extracted DNA were assessed by a

176 Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Within each

177 sampling site, a sub-sample of 1 to 12 individuals was further sequenced using the same

178 mitochondrial marker as in Auger-Rozenberg et al. (2015b) to ascertain identification and define

179 their mitochondrial clades (CI to CIV, see Auger-Rozenberg et al. 2015b). In particular, all

180 individuals belonging to the G1 group defined from microsatellite analyses (see below) were

181 sequenced.

182

183 *2.3. Microsatellite isolation and primer development*

184 The microsatellite library was constructed following Malausa et al. (2011) as detailed in Retamal et

185 al. (2016). Library enrichment and sequencing were carried out by GenoScreen (Lille, France) on a

186 Roche 454 GsFLX Titanium system (Brandford, USA) using 1 µg of the pooled DNAs from Set-a

187 individuals. Possible primer pairs were identified using the QDD software (Meglécz et al. 2010)

188 and chosen using the following parameters (modified from Retamal et al. 2016): target PCR product

189 size between 80 and 300 bp, perfect and various microsatellite motifs with at least 4 repetitions,

190 primer annealing temperature close to 60°C. In total, 42 primer pairs were selected and first tested

191 using Set-b individuals with non-fluorescent primers to determine if amplification was successful

192 for all individuals before proceeding with microsatellite development. The PCR program consisted

193 in an initial step of denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at

194 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 min, and a final extension at

195 72°C for 10 min. PCR products were then run on a 3% agarose gel stained with ethidium bromide,

196 and DNA bands visualized on a UV transluminator. The loci for which the amplification produced

197 bands in the expected size range for all individuals of Set-b and showed some variability (at least

198 between *B. servadeii* and *B. transversalis*) were then selected for a further amplification with

199 forward primers labeled with the fluorescent dyes 6-FAM, PET, NED or VIC (Applied

6

200 Biosystems). The PCR products were visualized using an ABI 3500 Genetic Analyzer (Applied

201 Biosystems) with the 600 LIZ^{TM} GeneScan^{TM} size standard and scored with the GENEMAPPER^{TM}

202 4.1 software. The loci showing unambiguous genotype patterns for the individuals of Set-b were

203 then organized in 2 multiplex and used to genotype individuals from Set-c. Primer sequences and

204 multiplex compositions are given in Table 2.

205

206 *2.4. Genetic data analyses*

207 Number of alleles and observed/expected heterozygosities were estimated using Arlequin 3.5

208 (Excoffier et al. 2005; Excoffier and Lischer 2010). The number and frequency of the different

209 multilocus genotypes (MLG) found in each population were estimated using the package 'poppr'

210 (Kamvar et al. 2014) of the R software (R Core Team 2018).

211 Using genotyping data obtained for Sets-b and -c, we performed a Principal Component Analysis

212 (PCA) to explore population genetic structure using the R package adegenet 1.4-2 (Jombart 2008).

213 Individuals from Set-c were further assigned to genetic clusters using the Bayesian clustering

214 method implemented in Structure 2.3.4 (Pritchard et al. 2000) under the model with admixture. We

215 set *K*, the number of clusters, varying from 1 to 6, and a burn-in of 100,000 steps followed by

216 100,000 iterations of the Markov Chain. To assess the consistency of results, we performed 10

217 independent runs for each value of *K* and compared the obtained individual Q-values. The number

218 of clusters *K* that best explained the data was chosen by examining the curve of Log P(X|K) as well

219 as using the ΔK method described in Evanno et al. (2005) and implemented in Structure Harvester

220 (Earl and vonHoldt 2012). Pairwise F_{ST} values were estimated using Arlequin. Cavalli-Sforza and

221 Edwards chord distances were calculated using Populations 1.2.30 (Olivier Langella,

222 http://bioinformatics.org/Btryphon/populations/).

223

224 *2.4. Screening of the* Baryscapus spp. – Rickettsia *and* Baryscapus spp. – Wolbachia *associations*

225 Given the extremely low genetic diversity found in *B. servadeii*, all individuals of Set-c were tested

226 for the presence of *Wolbachia* using the same primers as in Boivin et al. (2014), amplifying the

227 surface protein, namely wsp81F TGGTCCAATAAGTGATGAAGAAAC and wsp691R

228 AAAAATTAAACGCTACTCCA. They were also tested for *Rickettsia* using the primers Rct1-F

229 CCGCGTCAGATTAGGTAGTT and Rct1-R TCAGTTGTAGCCCAGATGAC that specifically

230 amplify the 16S region of this endosymbiont (Simonato 2010). PCR cycles were set up as follows:

231 initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C (1 min),

232 annealing at 55°C (1 min) and elongation at 72°C (1 min). PCR products were then run on a 1.5%

233 agarose gel stained with ethidium bromide and visualized by a UV transluminator. A subset of 19

234   *Rickettsia* PCR products (1-3 from each population) were then purified and Sanger sequenced

235   following the same protocol as in Auger-Rozenberg et al. (2015b). Three *Rickettsia* sequences

236   obtained from the insect hosts *Neochrysocharis formosa* (Westwood), *Pnigalio soemius* (Walker)

237   and *Bembidion articulatum* (Panzer) were retrieved from GenBank (accession numbers

238   AB231472.1, JN182552.1 and MK078278.1), aligned to the sequences obtained from *Baryscapus*

239   *servadeii*, and p-distances were calculated using MEGA3 (Kumar et al. 2004).

240

241

242   **3. Results**

243   *3.1. Microsatellite library and marker development*

244   Using QDD, we identified 1101 microsatellite loci for which satisfactory primer pairs could be

245   designed, and we further selected 42 candidates using the criteria described above. After testing

246   these primer pairs on Set-b, 17 loci were monomorphic and 15 could not be successfully genotyped

247   for some individuals. Hence, 10 loci were selected and further amplified using fluorescent primers.

248   After genotyping of Set-c individuals, 8 loci were finally selected as resulting in 100% genotyping

249   success and were organized in 2 multiplex (Table 2). Number of alleles ranged from 2 to 15 across

250   loci.

251

252   *3.2. Genotyping and population genetics results*

253   All 241 individuals (Set-b and Set-c) were successfully genotyped, without any missing data. The

254   PCA clearly showed that individuals were grouped in 2 clusters separated along axis 1 (Fig. 1). One

255   group (hereafter G1) had negative coordinates along that axis and gathered 11 individuals (9 from

256   Pukë, Albania; 1 from Venzone, Italy; 1 from Lilyanovo, Bulgaria) distributed along axis 2, while

257   the other group (G2) contained all 230 remaining individuals, all located very close to the origin of

258   the PCA. Structure results suggested that K = 3 was the best solution (Fig. 2). One cluster

259   corresponded to the 11 individuals from G1, while the two other clusters corresponded respectively

260   to (i) all individuals from Algeria, Morocco, Orléans (France) as well as 1 individual from Chania

261   (Crete) and (ii) all remaining individuals from G2. Sequencing of COI haplotypes of all individuals

262   from G1 and a subset of individuals from G2 showed that all G1 individuals belonged to Clade IV

263   sensu Auger-Rozenberg et al. (2015b), while the individuals from G2 belonged to the other 3

264   clades. These results consistently suggested that all individuals belonging to G1 corresponded to the

265   species *B. transversalis*. We thus proceeded with population analyses of Set-c after splitting the

266   individuals from Pukë in two groups corresponding to *B. servadeii* (10 individuals) and *B.*

267   *transversalis* (9 individuals), and excluding the *B. transversalis* individual found in Venzone, Italy.

268   Observed heterozygosity (Ho) was null in almost all populations and loci in *B. servadeii*, and it

269   reached 0.22 at most in Senalba (Algeria). In the only population of *B. transversalis*, it ranged

270   between 0 (Bary19) and 0.89 (Bary13). The genetic differentiation between *B. servadeii* and *B.*

271   *transversalis* populations was high and significant (pairwise F_{ST} spanning between 0.61 and 0.80,

272   Cavalli-Sforza and Edwards chord distance between 0.74 and 0.76). Among *B. servadeii*

273   populations, high and significant differentiation was found between populations from Algeria and

274   Orléans vs. all other populations (pairwise F_{ST} spanning between 0.73 and 0.96 and Cavalli-Sforza

275   and Edwards chord distance between 0.13 and 0.19, see Table 3). Interestingly, all 9 *B.*

276   *transversalis* individuals from Pukë had different genotypes (9 MLG for 9 individuals), while 90%

277   of *B. servadeii* individuals (183/203) belonged to 2 MLG, one mostly found in Algeria (Moudjbara

278   and Senalba) and in Orléans (France), and the second in all the other populations. The remaining 16

279   MLG were rare and each was found in 1 or 2 individuals only. Note that most MLG found in *B.*

280   *servadeii* only slightly differed from each other, and one single allele was fixed for most loci; for

281   instance the major MLG23 and MLG26 only differed by a different allele fixed for locus Bary-13

282   (allele 88 vs. allele 90), the very same alleles being present in both MLG for all other loci. Detailed

283   distributions and genotypes of the 27 MLG are given in Table 4. The populations characterized by

284   the occurrence of MLG23 actually corresponded to cluster 2 in Structure, while the populations

285   characterized by MLG26 corresponded to cluster 3.

286

287   *3.3. Patterns of* Baryscapus – Rickettsia *and* Baryscapus – Wolbachia *associations*

288   The PCR screening performed to detect the presence of *Wolbachia* returned only negative results in

289   *Baryscapus* spp., while a positive control taken from Boivin et al. (2014) was successfully

290   amplified. The screening performed to detect *Rickettsia* showed that this bacterium was absent from

291   all *B. transversalis* individuals whereas it was present in all *B. servadeii* populations and in 89% of

292   the studied individuals for that species. The rates of association were generally very high (100% in

293   Motril (30/30 individuals), Evrychou (20/20) and Longarone (19/19); 97% in St Guilhem (30/31);

294   90% in Moudjbara (9/10) and Pukë (9/10); 89% in Senalba (8/9); 86% in Orléans (25/29)) but were

295   somewhat lower in Razanac and in Venzone (67% in both sites). We obtained 426 bp long

296   sequences from the obtained amplicons, corresponding to 2 alleles differing by only 1 mutation

297   (G/A at position 94) for *B. servadeii*. One allele was found in Algeria whereas the other was found

298   in all other sequenced individuals. The *Rickettsia* sequences obtained from *B. servadeii* were

299   slightly different from the ones retrieved from GenBank for different hosts (p-distance between 0.9

300   and 1.4%). Sequences of the two alleles found in this study were deposited in GenBank (accession

301   numbers MN400060 and MN400061).

302

4. Discussion

The primary goal of our paper was to develop microsatellite markers for the focal species *B. servadeii*, and to study the patterns of genetic diversity along north – south gradients to confirm the signals of expansion found previously using mitochondrial markers. As expected in Hymenoptera, we did identify a high number of candidate loci after the sequencing of DNA libraries enriched with SSR motifs (Arthofer et al. 2007). Yet, the full procedure of marker development, followed by genotyping of 10 populations sampled over the whole distribution range of the species, highlighted an unexpectedly low genetic variation over a large geographical scale, and a very high homozygosity.

This result contrasts with the high mitochondrial haplotype diversity found during previous works at different spatial scales (Auger-Rozenberg et al. 2015a; 2015b), and suggests that peculiar genomic mechanisms lead to high levels of homozygosity and low levels of variability. Indeed, using 8 microsatellite markers, we showed that 90% of *B. servadeii* individuals fell in 2 fully homozygous multi-locus genotypes (MLG) differing only by the fixed allele for locus Bary-13, while all other MLG differed from those 2 main ones by one or two alleles at most.

Males are extremely rare in this species, which is thought to reproduce mostly through thelytokous parthenogenesis. Schematically, parthenogenesis is apomictic when it is due to mitotic mechanisms and does not involve any meiotic event. In that case, the offspring is clonally identical to the mother (Lorenzo-Carballa and Cordero-Rivera 2009; Tsutsui et al. 2014), heterozygosity is maintained and mutations tend to accumulate independently. These features are not consistent with the results found here for *B. servadeii*. On the other hand, automictic parthenogenesis involves a meiotic reduction followed by diploidy restoration either through the fusion of central or polar nuclei, or through gamete duplication. Automixy with central fusion leads to the maintenance of heterozygosity and is difficult to distinguish from apomixy, while automixy with terminal fusion or gamete duplication both lead to full homozygosity in the offspring (Rabeling and Kronauer 2013). If the first mechanism is only known from a small number of solitary Hymenoptera, the second was documented in all cases of parthenogenesis induced by *Wolbachia* infection studied so far (Ma and Schwander 2017; Rabeling and Kronauer 2013).

In the case of *B. servadeii*, rare heterozygous loci in some of the MLG confirmed that females are actually diploid, even if homozygosity is very high. More, we showed that all populations were infected by a *Rickettsia* symbiont, even if the PCR diagnostic test was negative for some individuals, which could be either due to technical issues (low bacterial DNA obtained after extraction) or actual loss of the symbiont in some individuals. On the contrary, *Wolbachia* was not

336   involved in the studied system. We thus suggest that thelytokous parthenogenesis in *B. servadeii* is

337   induced by *Rickettsia* and probably corresponds to a mode of automictic parthenogenesis involving

338   gamete duplication, which leads to full homozygosity in the progeny. This hypothesis should now

339   be confirmed by detailed cytological studies.

340   *Wolbachia* frequently occurs in a wide range of hymenoptera families, particularly within the

341   Chalcidoidea superfamily, and was expected to be the main parthenogenesis-inducing bacterium in

342   the case studied here (Rabeling and Kronauer 2013). Moreover, even if *Rickettsia* bacteria are

343   described from chalcid wasps, they are much rarer and the only case in which the cytological

344   mechanisms were described concluded that *Rickettsia* induces heterozygous offspring identical to

345   the mother through a single equational division during meiosis (Adachi-Hagimori et al. 2008). The

346   case of *B. servadeii* thus raised original results, both concerning the type of endosymbiont identified

347   and the cytological mechanisms suggested, which is very similar to *Wolbachia*-induced thelytoky.

348   Mutation is then the single strength potentially inducing some diversity, and would explain the

349   occurrence of the rare MLG documented in this study.

350   The very strong reduction of diversity and large-scale homozygosity also suggest that thelytoky is

351   by far the most frequent reproduction mode in this species. In a recent review, Ma & Schwander

352   (2017) showed that endosymbiont-induced parthenogenesis tends to facilitate the maintenance of

353   both sexual and parthenogenetic strains. Yet, our work suggests that *B. servadeii* has a

354   parthenogenetic reproduction at a large geographical scale, encompassing all the mitochondrial

355   lineages identified in this species (clades I, II and III from Auger-Rozenberg et al. 2015b). This

356   hypothesis is supported by the contrasting results we obtained for the sexually-reproducing *B.

357   transversalis* (Boyadzhiev et al. 2015), for which no association with *Rickettsia* was found and

358   genetic diversity and heterozygosity were documented using the same microsatellite markers.

359   Microsatellite genotypes and mitochondrial sequences both confirmed the occurrence of *B.

360   transversalis* in the Balkans (Albania and Bulgaria), but also in Italy where this species was not

361   mentioned so far but was suspected to occur based on the high number of males (unpublished

362   results). Indeed, both *B. servadeii* and *B. transversalis* can be found in PPM eggs, but males are

363   known mostly from the second species, which is likely to reproduce sexually rather than through

364   thelytoky.

365   The genetic pattern found may have some profound implications in the regulation of the PPM

366   populations across the range. *B. servadeii* is tracking the host in the expansion range in a

367   homogenous way, ensuring a relatively quick density-dependent control and bringing the initially

368   explosive population growth observed at range edge to the levels observed in the core range

369   (Auger-Rozenberg et al. 2015a). Yet, lack of genetic diversity in the nuclear genome suggests

370 limited adaptation capacities. This could negatively affect the ability of *B. servadeii* to adapt to the

371 new environments colonized by the PPM, such as urban areas, northern regions or new host trees,

372 and the efficiency of biological control. The lack of genetic diversity may also hamper the response

373 to the current global changes (climate warming, increased risks of extreme climatic events, land-use

374 changes...). On the other hand, the fact that *B. servadeii* is associated to a *Rickettsia* symbiont and

375 has a pure thelytokous reproduction can ease colonization or recolonization of certain

376 environments, as a single female can produce many offspring. Successful expansion can thus

377 originate from a limited number of dispersing individuals, as was already evidenced in an invasive

378 chalcid wasp (Auger-Rozenberg et al. 2012).

379 The hyperparasitoid *B. transversalis* does not seem to track the egg parasitoid as it could be

380 expected, occurring sporadically and in limited number. This lack of specialization can be explained

381 by the generalist trait of the hyperparasitoid, known to be associated also with other egg parasitoids

382 of the PPM, such as *O. pityocampae* (Tsankov et al. 1996). In addition, *B. transversalis* is

383 characterized by multivoltinism whereas *B. servadeii* is generally univoltine, matching perfectly the

384 annual appearance of the PPM eggs.

385 Altogether the results found here are striking, and suggest that microsatellite nuclear markers will

386 not be useful in future studies to decipher the demography and expansion routes of *B. servadeii*,

387 while they could be used for the hyperparasitoid *B. transversalis*. Interestingly, mutations seem to

388 accumulate in the mitochondrial DNA that is directly inherited from mother to daughters, and this

389 organelle DNA could be sufficiently informative to study spatial patterns of expansion in Europe,

390 even though it will not allow to test contrasting scenarios as it can be done with Approximate

391 Bayesian Computation approaches using nuclear markers (Estoup et al. 2018). High-throughput

392 sequencing and SNP genotyping technologies will be necessary to confirm that the high

393 homozygosity documented here is a genome-wide characteristic, and to use a larger portion of the

394 mitochondrial genome in future population studies. Finally, *B. servadeii* should now be seen as an

395 interesting example to study the mechanisms inducing thelytoky in an original *Rickettsia* – chalcid

396 parasitoid association.

397

401

402 **6. References**

403 Adachi-Hagimori T., Miura K. & Stouthamer R. 2008. A new cytogenetic mechanism for
404   bacterial endosymbiont-induced parthenogenesis in Hymenoptera. *Proceedings of the*
405   *Royal Society of London B-Biological Sciences* 275(1652): 2667-2673
406   http://dx.doi.org/10.1098/rspb.2008.0792
407 Arthofer W., Schlick-Steiner B., Steiner F., Avtzis D., Crozier R. & Stauffer C. 2007. Lessons from
408   a beetle and an ant: Coping with taxon-dependent differences in microsatellite
409   development success. *Journal of Molecular Evolution* 65(3): 304-307
410   http://dx.doi.org/10.1007/s00239-007-9012-1
411 Auger-Rozenberg M.-A., Barbaro L., Battisti A., Blache S., Charbonnier Y., Denux O., Garcia J.,
412   Goussard F., Imbert C.-E., Kerdelhué C., Roques A., Torres-Leguizamon M. & Vétillard F.
413   (2015a) Ecological responses of parasitoids, predators and associated insect
414   communities to the climate-driven expansion of the pine processionary moth. In:
415   Roques A (ed) *Processionary moths and climate change : an update.* Springer
416   Netherlands, pp 311-357 http://dx.doi.org/10.1007/978-94-017-9340-7_7
417 Auger-Rozenberg M.-A., Boivin T., Magnoux E., Courtin C., Roques A. & Kerdelhué C. 2012.
418   Inferences on population history of a seed chalcid wasp: invasion success despite a
419   severe founder effect from an unexpected source population. *Molecular Ecology*
420   21(24): 6086-6103 http://dx.doi.org/10.1111/mec.12077
421 Auger-Rozenberg M.-A., Torres-Leguizamon M., Courtin C., Rossi J.-P. & Kerdelhué C. 2015b.
422   Incongruent evolutionary histories of two parasitoids in the Mediterranean Basin:
423   influence of host specialization and ecological characteristics. *Journal of Biogeography*
424   42(6): 1040-1051 http://dx.doi.org/10.1111/jbi.12495
425 Bartomeus I., Ascher J.S., Wagner D., Danforth B.N., Colla S., Kornbluth S. & Winfree R. 2011.
426   Climate-associated phenological advances in bee pollinators and bee-pollinated plants.
427   *Proceedings of the National Academy of Sciences USA* 108(51): 20645-20649
428   http://dx.doi.org/10.1073/pnas.1115559108
429 Battisti A., Avcı M., Avtzis D.N., Ben Jamaa M.L., Berardi L., Berretima W., Branco M., Chakali G.,
430   El Alaoui El Fels M.A., Frérot B., Hódar J.A., Ionescu-Mălăncuş I., İpekdal K., Larsson S.,
431   Manole T., Mendel Z., Meurisse N., Mirchev P., Nemer N., Paiva M.-R., Pino J., Protasov
432   A., Rahim N., Rousselet J., Santos H., Sauvard D., Schopf A., Simonato M., Yart A. &
433   Zamoum M. (2015) Natural history of the processionary moths (*Thaumetopoea* spp.):
434   new insights in relation to climate change. In: Roques A (ed) *Processionary moths and*

435   *climate change: an update*. Springer / Quae Editions, pp 15-80 http://dx.doi.org/
436   10.1007/978-94-017-9340-7_2

437 Battisti A., Isantoanne P., Milani N. & Zanata M. 1990. Preliminary accounts on the rearing of
438   *Ooencyrtus pityocampae* (Mercet) (Hym., Encyrtidae). *Journal of Applied Entomology*
439   110(1-5): 121-127 http://dx.doi.org/10.1111/j.1439-0418.1990.tb00105.x

440 Battisti A., Larsson S. & Roques A. 2017. Processionary moths and associated urtication risk:
441   global change–driven effects. *Annual Review of Entomology* 62(1): 323-342
442   http://dx.doi.org/10.1146/annurev-ento-031616-034918

443 Battisti A., Stastny M., Netherer S., Robinet C., Schopf A., Roques A. & Larsson S. 2005.
444   Expansion of geographic range in the pine processionary moth caused by increased
445   winter temperatures. *Ecological Applications* 15(6): 2084-2096
446   http://dx.doi.org/10.1890/04-1903

447 Berggren Å., Björkman C., Bylund H. & Ayres M.P. 2009. The distribution and abundance of
448   animal populations in a climate of uncertainty. *Oikos* 118: 1121–1126
449   https://doi.org/10.1111/j.1600-0706.2009.17558.x

450 Boivin T., Henri H., Vavre F., Gidoin C., Veber P., Candau J.-N., Magnoux E., Roques A. & Auger-
451   Rozenberg M.-A. 2014. Epidemiology of asexuality induced by the endosymbiotic
452   *Wolbachia* across phytophagous wasp species: host plant specialization matters.
453   *Molecular Ecology* 23(9): 2362–2375 https://doi.org/10.1111/mec.12737

454 Boyadzhiev P., Dautbasic M., Mujezinovic O., Mirchev P., Georgiev G. & Georgieva M. 2015.
455   *Baryscapus transversalis* Graham (Hymenoptera: Eulophidae) - A new species for the
456   fauna of Bosnia and Herzegovina. *Sumarski List* 139(1-2): 69-71

457 Colautti R.I., Ricciardi A., Grigorovich I.A. & MacIsaac H.J. 2004. Is invasion success explained
458   by the enemy release hypothesis? *Ecology Letters* 7(8): 721-733
459   https://doi.org/10.1111/j.1461-0248.2004.00616.x

460 Earl D.A. & vonHoldt B.M. 2012. STRUCTURE HARVESTER: a website and program for
461   visualizing STRUCTURE output and implementing the Evanno method. *Conservation*
462   *Genetics Resources* 4(2): 359-361 http://dx.doi.org/doi:10.1007/s12686-011-9548-7

463 Estoup A., Raynal L., Verdu P. & Marin J.-M. 2018. Model choice using Approximate Bayesian
464   Computation and Random Forests: analyses based on model grouping to make
465   inferences about the genetic history of Pygmy human populations. *Journal de la Société*
466   *Française de Statistiques* 159(3): 167-190

467   Evanno G., Regnaut S. & Goudet J. 2005. Detecting the number of clusters of individuals using
468   the software STRUCTURE: a simulation study. *Molecular Ecology* 14: 2611-2620
469   https://doi.org/10.1111/j.1365-294X.2005.02553.x
470   Excoffier L., Laval G. & Schneider S. 2005. Arlequin (version 3.0): an integrated software
471   package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47-
472   50
473   Excoffier L. & Lischer H.E.L. 2010. Arlequin suite ver 3.5: A new series of programs to perform
474   population genetics analyses under Linux and Windows. *Molecular Ecology Resources*
475   https://doi.org/10: 564-567 10.1111/j.1755-0998.2010.02847.x
476   Gebiola M., Lopez-Vaamonde C., Nappo A.G. & Bernardo U. 2014. Did the parasitoid *Pnigalio*
477   *mediterraneus* (Hymenoptera: Eulophidae) track the invasion of the horse chestnut
478   leafminer? *Biological Invasions* 16(4): 843-857 https://doi.org/10.1007/s10530-013-
479   0542-8
480   Jombart T. 2008. Adegenet: a R package for the multivariate analysis of genetic markers.
481   *Bioinformatics* 24: 1403-1405 https://doi.org/10.1093/bioinformatics/btn129
482   Kamvar Z.N., Tabima J.F. & Grunwald N.J. 2014. Poppr: an R package for genetic analysis of
483   populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* 2: e281
484   http://dx.doi.org/10.7717/peerj.281
485   Kerdelhué C., Zane L., Simonato M., Salvato P., Rousselet J., Roques A. & Battisti A. 2009.
486   Quaternary history and contemporary patterns in a currently expanding species. *BMC*
487   *Evolutionary Biology* 9: 220 http://dx.doi.org/10.1186/1471-2148-9-220
488   Kumar S., Tamura K. & Nei M. 2004. MEGA3: Integrated software for Molecular Evolutionary
489   Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5(2): 150-163
490   http://dx.doi.org/10.1093/bib/5.2.150
491   Lenoir J. & Svenning J.C. 2015. Climate-related range shifts – a global multidimensional
492   synthesis and new research directions. *Ecography* 38(1): 15-28
493   http://dx.doi.org/10.1111/ecog.00967
494   Liu H. & Stiling P. 2006. Testing the enemy release hypothesis: a review and meta-analysis.
495   *Biological Invasions* 8(7): 1535-1545 http://dx.doi.org/10.1007/s10530-005-5845-y
496   Lorenzo-Carballa M.O. & Cordero-Rivera A. 2009. Thelytokous parthenogenesis in the
497   damselfly *Ischnura hastata* (Odonata, Coenagrionidae): genetic mechanisms and lack of
498   bacterial infection. *Heredity* 103(5): 377-384 http://dx.doi.org/10.1038/hdy.2009.65

499   Ma W.-J. & Schwander T. 2017. Patterns and mechanisms in instances of endosymbiont-
500   induced parthenogenesis. *Journal of Evolutionary Biology* 30(5): 868-888
501   http://dx.doi.org/10.1111/jeb.13069

502   Malausa T., Gilles A., Meglécz E., Blanquart H., Duthoy S., Costedoat C., Dubut V., Pech N.,
503   Castagnone-Sereno P., Délye C., Feau N., Frey P., Gauthier P., Guillemaud T., Hazard L.,
504   Le Corre V., Lung-Escarmant B., Malé P.-J.G., Ferreira S. & Martin J.-F. 2011. High-
505   throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of
506   enriched DNA libraries. *Molecular Ecology Resources* 11(4): 638-644
507   http://dx.doi.org/10.1111/j.1755-0998.2011.02992.x

508   Meglécz E., Costedoat C., Dubut V., Gilles A., Malausa T., Pech N. & Martin J.-F. 2010. QDD: a
509   user-friendly program to select microsatellite markers and design primers from large
510   sequencing projects. *Bioinformatics* 26(3): 403-404
511   http://dx.doi.org/10.1093/bioinformatics/btp670

512   Pritchard J.K., Stephens M. & Donnely P. 2000. Inference of population structure using
513   multilocus genotype data. *Genetics* 155: 945-959

514   R Core Team 2018. R: a language and environment for statistical computing. R Foundation for
515   Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0,

516   Rabeling C. & Kronauer D.J.C. 2013. Thelytokous parthenogenesis in eusocial hymenoptera.
517   *Annual Review of Entomology* 58(1): 273-292 http://dx.doi.org/10.1146/annurev-
518   ento-120811-153710

519   Rafferty N.E., CaraDonna P.J., Burkle L.A., Iler A.M. & Bronstein J.L. 2013. Phenological overlap
520   of interacting species in a changing climate: an assessment of available approaches.
521   *Ecology and Evolution* 3(9): 3183-3193 http://dx.doi.org/10.1002/ece3.668

522   Retamal R., Zaviezo T., Malausa T., Fauvergue X., Le Goff I. & Toleubayev K. 2016. Genetic
523   analyses and occurrence of diploid males in field and laboratory populations of
524   *Mastrus ridens* (Hymenoptera: Ichneumonidae), a parasitoid of the codling moth.
525   *Biological Control* 101: 69-77 http://dx.doi.org/10.1016/j.biocontrol.2016.06.009

526   Robinet C., Imbert C.E., Rousselet J., Sauvard D., Garcia J., Goussard F. & Roques A. 2012.
527   Human-mediated long-distance jumps of the pine processionary moth in Europe.
528   *Biological Invasions* 14(8): 1557-1569 http://dx.doi.org/doi:10.1007/s10530-011-
529   9979-9

530 Robinet C., Rousselet J. & Roques A. 2014. Potential spread of the pine processionary moth in
531   France: preliminary results from a simulation model and future challenges. *Annals of*
532   *Forest Science* 71: 149-160 http://dx.doi.org/doi:10.1007/s13595-013-0287-7

533 Simonato M. (2010) *Genetics and genomics of pine processionary moths and their parasitoids.*
534   PhD Thesis, University of Padova, Italy,

535 Simonato M., Battisti A., Zovi D. & Medina R.F. 2012. Testing for host-associated differentiation
536   in two egg parasitoids of a forest herbivore. *Entomologia Experimentalis et Applicata*
537   145(2): 124-133 http://dx.doi.org/doi:10.1111/j.1570-7458.2012.01321.x

538 Thompson R.M., Beardall J., Beringer J., Grace M. & Sardina P. 2013. Means and extremes:
539   building variability into community-level climate change experiments. *Ecology Letters*
540   16(6): 799-806 http://dx.doi.org/10.1111/ele.12095

541 Thomson L.J., Macfadyen S. & Hoffmann A.A. 2010. Predicting the effects of climate change on
542   natural enemies of agricultural pests. *Biological Control* 52: 296–306
543   https://doi.org/10.1016/j.biocontrol.2009.01.022

544 Tsankov G., Schmidt G.H. & Mirchev P. 1996. Parasitism of eggbatches of the pine
545   processionary moth *Thaumetopoea pityocampa* (Den. & Schiff.) (Lep.,
546   Thaumetopoeidae) in various regions of Bulgaria. *Journal of Applied Entomology* 120:
547   93–105.

548 Tsutsui Y., Maeto K., Hamaguchi K., Isaki Y., Takami Y., Naito T. & Miura K. 2014. Apomictic
549   parthenogenesis in a parasitoid wasp *Meteorus pulchricornis*, uncommon in the
550   haplodiploid order Hymenoptera. *Bulletin of Entomological Research* 104(3): 307-313
551   http://dx.doi.org/10.1017/S0007485314000017

552 Zovi D., Battisti A., Hellrigl K. & Minerbi S. 2006. Egg parasitoids of the pine processionary
553   moth and their occurrence in Venosta/Vinschgau. *Forest Observer* 2: 81-88

554

555 **7- Funding**

560

561

FIGURE CAPTIONS

563 **Figure 1**: Graph of the principal component analysis (PCA, axes 1 and 2) ran on microsatellite

564   genotypes of 241 individuals from Set-b and Set-c.

565 **Figure 2**: Graphical representation of the individual genetic assignments given by the individual Q-

566   values inferred by STRUCTURE at K=3 for the 213 individuals from Set-c. White cluster: *B.*

567   *transversalis* (same individuals as in group G1 in Fig. 1). Dark-grey cluster: *B. servadeii* cluster

568   2, mostly characterized by MLG23. Light-grey cluster: *B. servadeii* cluster 3, mostly

569   characterized by MLG26.

570

571

572 **TABLE CAPTIONS**

573 **Table 1**. Characterization of sampling sites and mitochondrial clades (following Auger-Rozenberg

574 et al. 2015b) of the three sets used for microsatellite development and genotyping. PP: *Pinus*

575 *pinaster*; PN: *P. nigra*; PS: *P. sylvestris*; PA: *P. halepensis*; PC: *P. canariensis*; PB: *P. brutia;* CA:

576 *Cedrus atlantica*

577 **Table 2.** Primer sequences for the 8 microsatellites developed for *B. servadeii* and *B. transversalis*

578

579 **Table 3**. Pairwise *Fst* values (below diagonal) and Cavalli-Sforza & Edwards chord distance (above

580 diagonal) between populations of Set-c. Individuals from Pukë (Albania) were separated in two

581 populations according to their species, and the *B. transversalis* individual found in Venzone was

582 omitted.

583

584 **Table 4.** Distribution and characteristics of the 27 Multi-locus genotypes (MLG) identified in this

585 study. Only one allele is mentioned at homozygous loci. N: number of individuals; Sites: code of

586 sampling site as in Table 1.

587

588

PCA axes 1–2

Group G1
B. transversalis (11 indiv)

Group G2
B. servadeii (230 indiv)

PCA 1 (27.5%)

PCA 2 (8.4%)
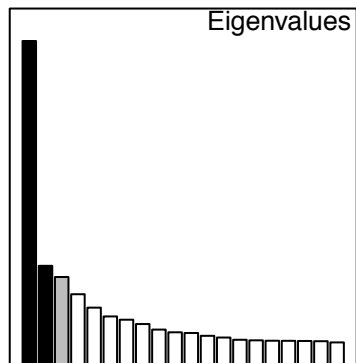
Eigenvalues

Albania   Algeria   Algeria   Croatia   Cyprus   France   France   Italy   Italy   Spain

Puke   Moudjbara   Senalba   Razanac   Evrychou   Orleans   St Guilhem   Venzone   Longarone   Motril
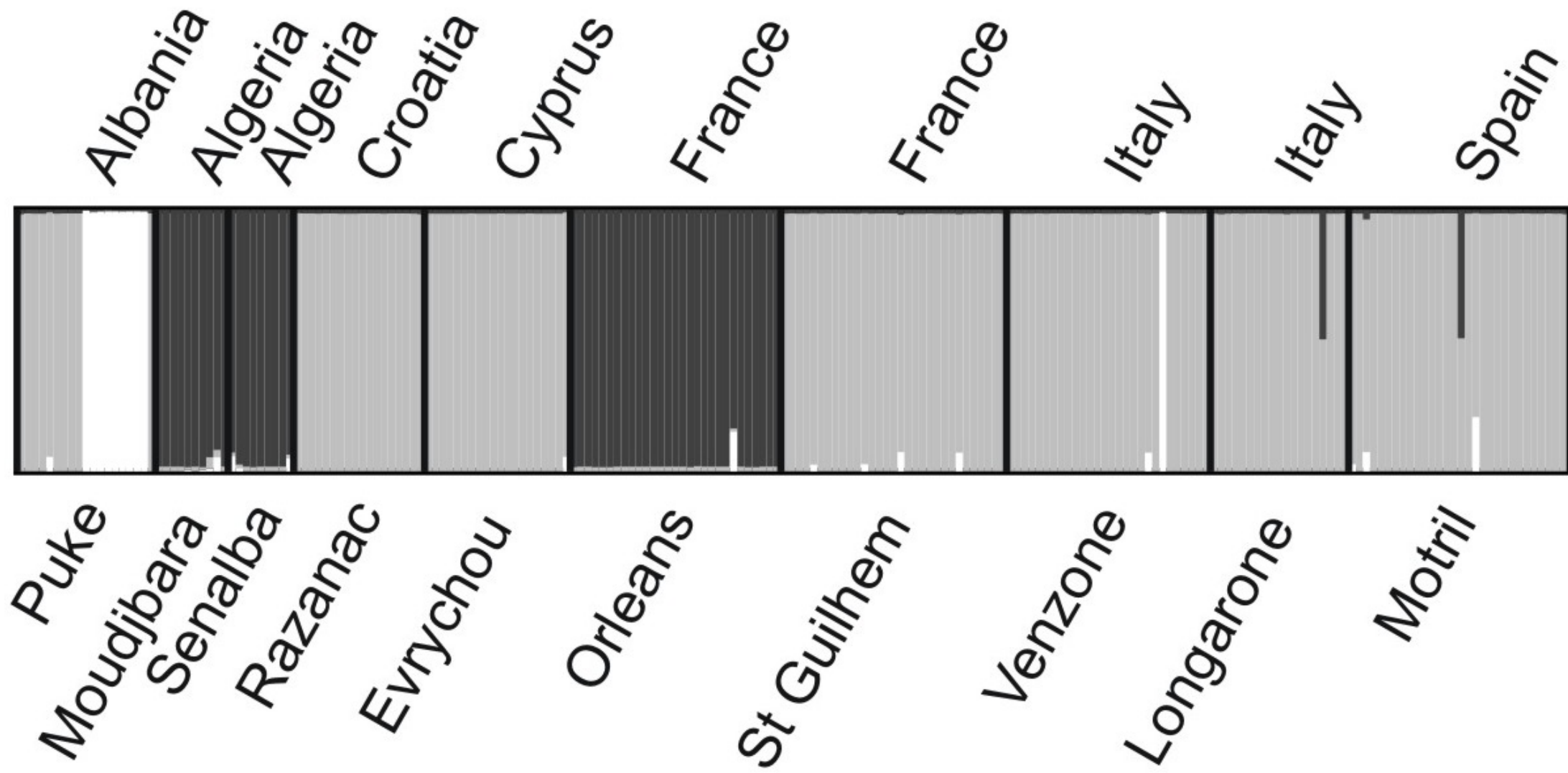
Table 1. Characterization of sampling sites and mitochondrial clades (following Auger-Rozenberg et al. 2015b) of the three sets used for microsatellite development and genotyping. PP: *Pinus pinaster*; PN: *P. nigra*; PS: *P. sylvestris*; PA: *P. halepensis*; PC: *P. canariensis*; PB: *P. brutia;* CA: *Cedrus atlantica*

Set	Country	Locality	Site code	Lat.	Long.	Elev. (m)	Host plant	N	Mt clade (N)
a	France	Mont-Dauphin		44.676	6.625	1025	PS	10	Clade I
a	France	Pennautier		43.258	2.312	192	PN	10	Clade I
a	Portugal	Pinhas del Freiras		38.583	-9.117	0	PP	2	Clade I
a	Spain	Burgos		42.380	-3.584	900	PN	10	Clade I
a	Spain	Soria		41.811	-2.451	1000	PN	10	Clade I
b	Algeria	Theniet el Had		35.8551	2.002	1465	CA	1	Clade II
b	Bosnia	Borci		43.580	18.017	1010	PN	1	Clade I
b	Bulgaria	Banya		42.557	24.843	342	PN	1	Clade I
b	Bulgaria	Lilyanovo		41.624	23.312	585	-	1	Clade IV
b	France	Pennautier		43.258	2.312	192	PN	1	Clade I
b	France	Serre-Ponçon		44.5278	6.336	1250	PN/PS	4	Clade I (4)
b	Greece	Chania		35.517	24.050	150	PA	4	Clade III (4)
b	Italy	Pradis		46.273	12.887	1050	PN	2	Clade I
b	Italy	Tugliezzo		46.367	13.183	1510	PN	1	Clade I
b	Italy	Venosta		46.640	10.749	1360	PS	1	Clade I
b	Macedonia	Kadrifakovo		41.188	21.922	456	PN	2	Clade I (2)
b	Morocco	Oukaimeden		31.229	-7.824	2400	PM	1	Clade II (1)
b	Spain	Burgos		42.380	-3.584	900	PN	5	Clade I (5)
b	Spain	Soria		41.811	-2.451	1000	PN	3	Clade I (3)
c	Albania	Pukë	PUKË	42.041	19.876	800	PN	19	Clade IV (9) Clade I (2)
c	Algeria	Moudjebara	MOU	34.641	3.317	1200	PA	10	Clade II (4)
c	Algeria	Senalba	SEN	34.616	3.098	1320	PA	9	Clade II (2)
c	Croatia	Razanac	RAZ	44.308	15.289	10	PA	18	Clade I (9)
c	Cyprus	Evrychou	EVR	35.072	32.876	310	PB	20	Clade I (5)
c	France	Orléans	ORL	47.845	1.942	100	PN	29	Clade I (4)
c	France	St Guilhem le Désert	StGUI	43.769	3.557	640	PN	31	Clade I (1)
c	Italy	Longarone	LANG	46.267	12.306	435	PN/PS	19	Clade I (5)
c	Italy	Venzone	VEN	46.316	13.133	350	PN	28	Clade I (5) Clade IV (1)
c	Spain	Motril	MOT	36.784	-3.541	60	PC	30	Clade I (5)

1 **Table 2.** Primer sequences for the 8 microsatellites developed for *B. servadeii* and *B. transversalis*

2

Locus name	Size range	Primers (5' – 3') F: Forward; R: Reverse	Repeat	Multiplex
Bary-02	80 - 100	F: GGAGGGAAAAAGGACGTTTC R: GGAAGGACAAATGAGCCAAA	$(ACG)_8$	1
Bary-06	140 - 190	F: CCGTTCTCCATGATCTCGTT R: GCTGCAATCGGAGGAAAGTA	$(GTC)_8$	1
Bary-07	140 - 190	F: CGGAAGTCGACAGTAAAGGG R: ACTCGACGAACGACGAGAGT	$(TCC)_6$	1
Bary-12	80 - 100	F: TCATTTGCTTAATCGAGCGTT R: ATACATCGACACCCTCGACC	$(GA)_8$	2
Bary-13	80 - 100	F: AAGCCTTGGCTATTCAACGA R: AGGACAACGAATTACGCGAT	$(AG)_9$	2
Bary-14	140 - 180	F: TCTCAGCCGCAGTAATCCTT R: GGATGAAGTGGGCTACCAGA	$(CA)_{10}$	1
Bary-19	260 - 280	F: TCCTGCCATACTTTACCTACCC R: ATCCAGTAAGAATCGCGACG	$(CTATC)_4$	2
Bary-21	200 - 250	F: AGAATCACTGCAACCCGATAA R: TATATCGTCATTCCCTCGGC	$(CT)_4$	2

3

4

Table 3. Pairwise *Fst* values (below diagonal) and Cavallis & Sforza chord distance (above
diagonal) between populations of Set-c. Individuals from Pukë (Albania) were separated in two
populations according to their species, and the *B. transversalis* individual found in Venzone was
omitted.

5

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1- *B. transversalis*	-	0.74	0.74	0.76	0.75	0.75	0.75	0.76	0.76	0.75	0.74
2- Moudjebara	0.62	-	0.06	0.14	0.16	0.07	0.17	0.15	0.13	0.16	0.16
3- Senalba	0.61	-0.03	-	0.16	0.18	0.08	0.19	0.17	0.15	0.18	0.18
4- Razanac	0.74	0.84	0.87	-	0.02	0.13	0.03	0.01	0.01	0.04	0.02
5- Evrychou	0.74	0.80	0.83	0.02	-	0.14	0.03	0.03	0.03	0.05	0.01
6- Orleans	0.79	0.12	0.07	0.96	0.93	-	0.14	0.14	0.12	0.14	0.14
7- St Guilhem	0.80	0.81	0.83	0.00	-0.01	0.91	-	0.04	0.04	0.04	0.03
8- Venzone	0.79	0.85	0.88	-0.01	0.02	0.95	0.01	-	0.02	0.05	0.04
9- Longarone	0.74	0.81	0.84	0.00	0.02	0.94	0.00	0.00	-	0.04	0.04
10- Motril	0.78	0.76	0.78	0.00	0.00	0.88	0.00	0.01	0.00	-	0.06
11- Pukë	0.65	0.73	0.76	0.10	-0.02	0.91	0.00	0.09	0.07	0.02	-

6

1 **Table 4.** Distribution and characteristics of the 27 Multi-locus genotypes (MLG) identified in this
2 study. Only one allele is mentioned at homozygous loci. N: number of individuals; Sites: code of
3 sampling site as in Table 1.
4

MLG	N	Sites	Bary-2	Bary-6	Bary-7	Bary-14	Bary-12	Bary-13	Bary-19	Bary-21
B. servadeii										
MLG10	1	SEN	88/94	186	183	172	94	90	258	242
MLG11	1	VEN	94	169/186	183	172	94	88	258	242
MLG12	1	MOT	94	186	180	172	94	88	258	242
MLG13	1	StGUI	94	186	183	172	90/94	88	258	242
MLG14	1	SEN	94	186	183	170/172	94	90/92	258	242
MLG15	1	SEN	94	186	183	172/176	94	90	258	242
MLG16	2	MOU	94	186	183	170/172	94	90	258	242
MLG17	2	EVR PUKË	94	186	183	172	90	88	258	242
MLG18	1	ORL	94	186	183	172	96/98	90	258	242
MLG19	1	StGUI	94	186	183	172	94/96	88	258	242
MLG20	1	StGUI	94	186	183/186	172	94	88	258	242
MLG21	1	MOU	94	186	183	172	94	92	258	242
MLG22	1	MOU	94	186	183	172	94	90/96	258	242
MLG23	**40**	**(1)**	**94**	**186**	**183**	**172**	**94**	**90**	**258**	**242**
MLG24	2	LONG MOT	94	186	183	172	94	88/90	258	242
MLG25	1	MOT	94	186	183	172	94	84/88	258	242
MLG26	**143**	**(2)**	**94**	**186**	**183**	**172**	**94**	**88**	**258**	**242**
MLG27	2	MOT StGUI	94	186	183	172	92/94	88	258	242
B. transversalis										
MLG1	1	PUKË	85/88	189	180	164/170	90	102/138	263	248
MLG2	1	PUKË	85/88	186/189	180/183	164/170	94	98/126	263	250
MLG3	1	PUKË	79	189	181/183	170	94	102/140	263	248
MLG4	1	PUKË	88	186/189	181	168/172	90	140/144	263	248
MLG5	1	PUKË	88	189	180/181	168/172	94/96	138/140	263	250
MLG6	1	PUKË	85	189	183	164/170	94/96	112/130	263	250
MLG7	1	PUKË	79	186	180	164	98	112/112	263	246
MLG8	1	PUKË	79	189	180/183	164/174	94/96	138/140	263	248
MLG9	1	PUKË	80	189	180	170/172	94	119/144	263	248/254

5 (1): MOU(6), SEN(6), ORL(28)
6 (2): RAZ (18), EVR(19), StGUI (27), VEN(26), LONG(18), MOT(26), PUKË (9)
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