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

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

3 Mauro SIMONATO<sup>1</sup>, Marco PILATI<sup>1,2</sup>, Emmanuelle MAGNOUX<sup>1</sup>, Claudine COURTIN<sup>1</sup>, Laure SAUNÉ<sup>3</sup>,  
4 Jérôme ROUSSELET<sup>1</sup>, Andrea BATTISTI<sup>2</sup>, Marie-Anne AUGER-ROZENBERG<sup>1\*</sup> & Carole KERDELHUE<sup>3\*</sup>

5

6 <sup>1</sup> URZF, INRA Val de Loire, Orléans, France

7 <sup>2</sup> DAFNAE, Università degli Studi di Padova, Italy

8 <sup>3</sup> CBGP, INRA CIRAD IRD Montpellier SupAgro Université de Montpellier, France

9 \* These authors contributed equally to the work

10

11 **Email addresses:**

12 msimonato73@gmail.com

13 marcopilaz@hotmail.it

14 Emmanuelle.Magnoux@inra.fr

15 Claudine.Courtin@inra.fr

16 Laure.Saune@inra.fr

17 Jerome.Rousselet@inra.fr

18 Andrea.Battisti@unipd.it

19 Marie-Anne.Auger-Rozenberg@inra.fr

20 Carole.Kerdelhue@inra.fr

21

22

23 **Corresponding author**

24 Carole Kerdelhue, CBGP, 755 avenue du Campus Agropolis, CS30016, F-34988 Montferrier-sur-  
25 Lez cedex

26 email Carole.Kerdelhue@inra.fr

27

28 **Declarations of interest:** none.

29

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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39

40 **Abstract**

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

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60  
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62 **Keywords (max 6)**

63 Hymenoptera; Eulophidae; microsatellites; pine processionary moth; *Thaumetopoea*; *Rickettsia*

64

## 65 **1. Introduction**

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

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

200 Biosystems). The PCR products were visualized using an ABI 3500 Genetic Analyzer (Applied  
201 Biosystems) with the 600 LIZ™ GeneScan™ size standard and scored with the GENEMAPPER™  
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  $\text{Log } P(X|K)$  as well  
219 as using the  $\Delta K$  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 ( $H_o$ ) 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

#### 303 **4. Discussion**

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

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

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

331 In the case of *B. servadeii*, rare heterozygous loci in some of the MLG confirmed that females are  
332 actually diploid, even if homozygosity is very high. More, we showed that all populations were  
333 infected by a *Rickettsia* symbiont, even if the PCR diagnostic test was negative for some  
334 individuals, which could be either due to technical issues (low bacterial DNA obtained after  
335 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

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401

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554

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560

561

562 **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

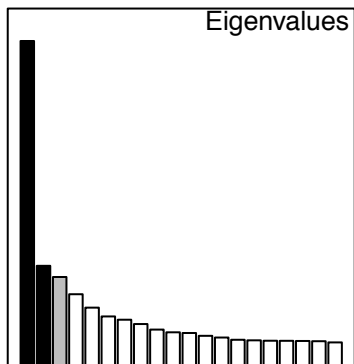
PCA  
axes 1-2

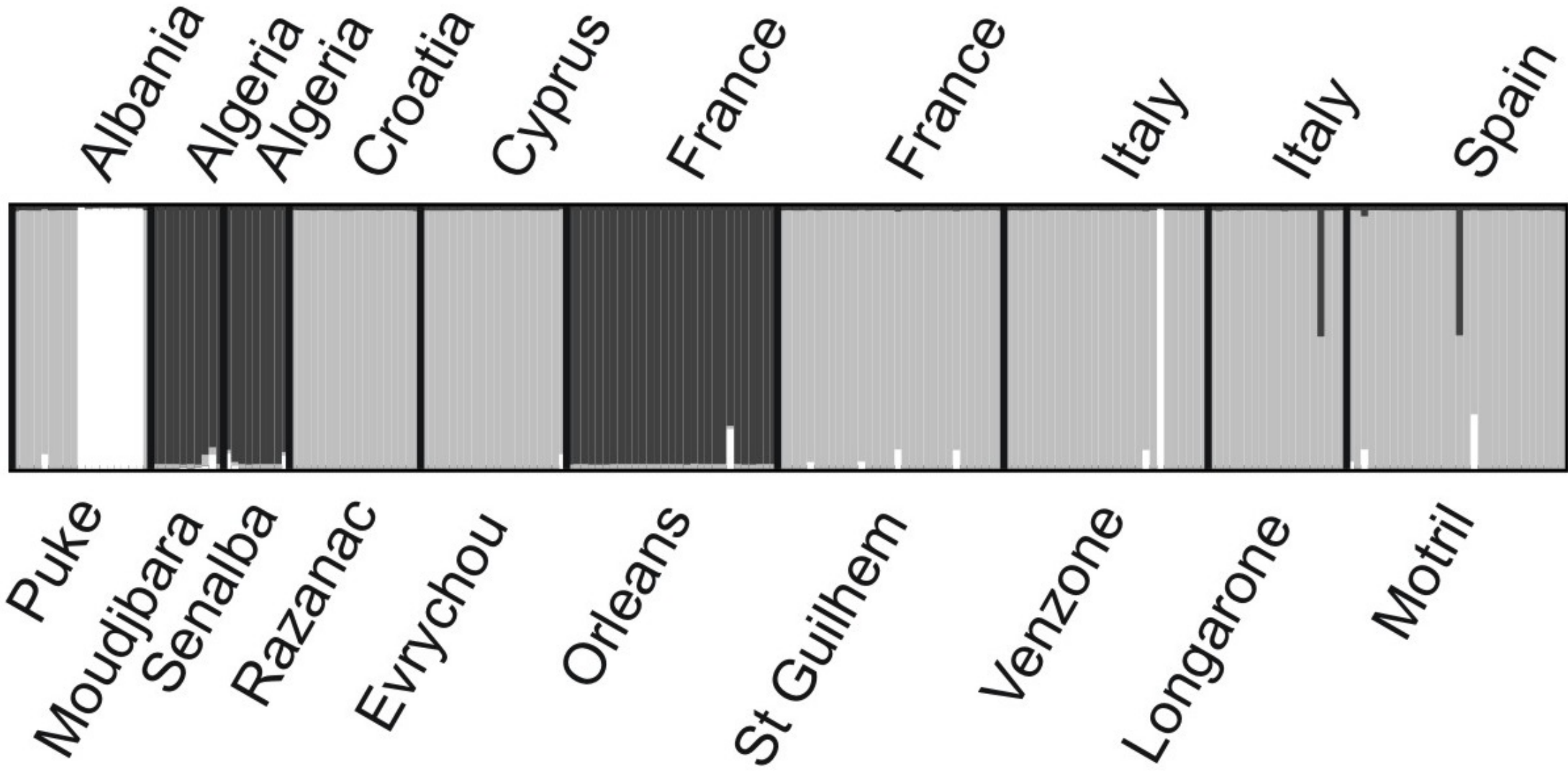
Group G1  
*B. transversalis* (11 indiv)

Group G2  
*B. servadeii* (230 indiv)

PCA 1 (27.5%)

PCA 2 (8.4%)





1 **Table 1.** Characterization of sampling sites and mitochondrial clades (following Auger-Rozenberg  
2 et al. 2015b) of the three sets used for microsatellite development and genotyping. PP: *Pinus*  
3 *pinaster*; PN: *P. nigra*; PS: *P. sylvestris*; PA: *P. halepensis*; PC: *P. canariensis*; PB: *P. brutia*; CA:  
4 *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)
c	Algeria	Moudjebara	MOU	34.641	3.317	1200	PA	10	Clade I (2)
c	Algeria	Senalba	SEN	34.616	3.098	1320	PA	9	Clade II (4)
c	Croatia	Razanac	RAZ	44.308	15.289	10	PA	18	Clade II (2)
c	Cyprus	Evrychou	EVR	35.072	32.876	310	PB	20	Clade I (9)
c	France	Orléans	ORL	47.845	1.942	100	PN	29	Clade I (5)
c	France	St Guilhem le Désert	StGUI	43.769	3.557	640	PN	31	Clade I (4)
c	Italy	Longarone	LANG	46.267	12.306	435	PN/PS	19	Clade I (1)
c	Italy	Venzone	VEN	46.316	13.133	350	PN	28	Clade I (5)
c	Spain	Motril	MOT	36.784	-3.541	60	PC	30	Clade I (5)

5  
6

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

<b>Locus name</b>	<b>Size range</b>	<b>Primers (5' – 3') F: Forward; R: Reverse</b>	<b>Repeat</b>	<b>Multiplex</b>
Bary-02	80 - 100	F: GGAGGGAAAAAGGACGTTTC R: GGAAGGACAAATGAGCCAAA	(ACG) <sub>8</sub>	1
Bary-06	140 - 190	F: CCGTTCTCCATGATCTCGTT R: GCTGCAATCGGAGGAAAGTA	(GTC) <sub>8</sub>	1
Bary-07	140 - 190	F: CGGAAGTCGACAGTAAAGGG R: ACTCGACGAACGACGAGAGT	(TCC) <sub>6</sub>	1
Bary-12	80 - 100	F: TCATTTGCTTAATCGAGCGTT R: ATACATCGACACCCTCGACC	(GA) <sub>8</sub>	2
Bary-13	80 - 100	F: AAGCCTTGGCTATTCAACGA R: AGGACAACGAATTACGCGAT	(AG) <sub>9</sub>	2
Bary-14	140 - 180	F: TCTCAGCCGCAGTAATCCTT R: GGATGAAGTGGGCTACCAGA	(CA) <sub>10</sub>	1
Bary-19	260 - 280	F: TCCTGCCATACTTTACCTACCC R: ATCCAGTAAGAATCGCGACG	(CTATC) <sub>4</sub>	2
Bary-21	200 - 250	F: AGAATCACTGCAACCCGATAA R: TATATCGTCATTCCCTCGGC	(CT) <sub>4</sub>	2

3  
4



1 **Table 3.** Pairwise *Fst* values (below diagonal) and Cavallis & Sforza chord distance (above  
2 diagonal) between populations of Set-c. Individuals from Pukë (Albania) were separated in two  
3 populations according to their species, and the *B. transversalis* individual found in Venzone was  
4 omitted.  
5

	1	2	3	4	5	6	7	8	9	10	11
<b>1- <i>B. transversalis</i></b>	-	0.74	0.74	0.76	0.75	0.75	0.75	0.76	0.76	0.75	0.74
<b>2- Moudjebara</b>	0.62	-	0.06	0.14	0.16	0.07	0.17	0.15	0.13	0.16	0.16
<b>3- Senalba</b>	0.61	-0.03	-	0.16	0.18	0.08	0.19	0.17	0.15	0.18	0.18
<b>4- Razanac</b>	0.74	0.84	0.87	-	0.02	0.13	0.03	0.01	0.01	0.04	0.02
<b>5- Evrychou</b>	0.74	0.80	0.83	0.02	-	0.14	0.03	0.03	0.03	0.05	0.01
<b>6- Orleans</b>	0.79	0.12	0.07	0.96	0.93	-	0.14	0.14	0.12	0.14	0.14
<b>7- St Guilhem</b>	0.80	0.81	0.83	0.00	-0.01	0.91	-	0.04	0.04	0.04	0.03
<b>8- Venzone</b>	0.79	0.85	0.88	-0.01	0.02	0.95	0.01	-	0.02	0.05	0.04
<b>9- Longarone</b>	0.74	0.81	0.84	0.00	0.02	0.94	0.00	0.00	-	0.04	0.04
<b>10- Motril</b>	0.78	0.76	0.78	0.00	0.00	0.88	0.00	0.01	0.00	-	0.06
<b>11- Pukë</b>	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
<i>B. servadeii</i>										
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	94	186	183	172	90	88	258	242
		PUKĚ								
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
<b>MLG23</b>	<b>40</b>	<b>(1)</b>	<b>94</b>	<b>186</b>	<b>183</b>	<b>172</b>	<b>94</b>	<b>90</b>	<b>258</b>	<b>242</b>
MLG24	2	LONG	94	186	183	172	94	88/90	258	242
		MOT								
MLG25	1	MOT	94	186	183	172	94	84/88	258	242
<b>MLG26</b>	<b>143</b>	<b>(2)</b>	<b>94</b>	<b>186</b>	<b>183</b>	<b>172</b>	<b>94</b>	<b>88</b>	<b>258</b>	<b>242</b>
MLG27	2	MOT	94	186	183	172	92/94	88	258	242
		StGUI								
<i>B. transversalis</i>										
MLG1	1	PUKĚ	85/88	189	180	164/170	90	102/138	263	248
MLG2	1	PUKĚ	85/88	186/189	180/183	164/170	94	98/126	263	250
MLG3	1	PUKĚ	79	189	181/183	170	94	102/140	263	248
MLG4	1	PUKĚ	88	186/189	181	168/172	90	140/144	263	248
MLG5	1	PUKĚ	88	189	180/181	168/172	94/96	138/140	263	250
MLG6	1	PUKĚ	85	189	183	164/170	94/96	112/130	263	250
MLG7	1	PUKĚ	79	186	180	164	98	112/112	263	246
MLG8	1	PUKĚ	79	189	180/183	164/174	94/96	138/140	263	248
MLG9	1	PUKĚ	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), PUKĚ (9)

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