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

Mauro SIMONATO[1], Marco PILATI[1,2], Emmanuelle MAGNOUX[1], Claudine COURTIN[1], Laure SAUNÉ[3], Jérôme ROUSSELET[1], Andrea BATTISTI[2], Marie-Anne AUGER-ROZENBERG[1*] & Carole KERDELHUÉ[3*]

[1] URZF, INRA Val de Loire, Orléans, France

[2] DAFNAE, Università degli Studi di Padova, Italy

[3] CBGP, INRA CIRAD IRD Montpellier SupAgro Université de Montpellier, France

* These authors contributed equally to the work

Email addresses:
msimonato73@gmail.com
marcopilaz@hotmail.it
Emmanuelle.Magnoux@inra.fr
Claudine.Courtin@inra.fr
Laure.Saune@inra.fr
Jerome.Rousselet@inra.fr
Andrea.Battisti@unipd.it
Marie-Anne.Auger-Rozenberg@inra.fr
Carole.Kerdelhue@inra.fr

Corresponding author
Carole Kerdelhué, CBGP, 755 avenue du Campus Agropolis, CS30016, F-34988 Montferrier-sur-Lez cedex
email Carole.Kerdelhue@inra.fr

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Highlights

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

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.

Keywords (max 6)

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

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 could efficiently reduce the damage caused by the PPM (Auger-Rozenberg et al. 2015a). Both species occur across the Mediterranean Basin and are present all over the distribution of their main host; they both mostly reproduce through thelytokous parthenogenesis (Battisti et al. 1990). In this mode of reproduction, males are extremely rare and diploid females are produced from the development of unfertilized eggs. Diploidy can be restored by diverse mechanisms leading to different genetic outcomes (Rabeling and Kronauer 2013). Depending on the mechanism at play, the offspring can be clonally identical to the mother and heterozygozity is maintained; or they can be fully homozygous. Thelytoky can either have an intrinsic genetic basis, or it can be induced by a microbial infection (Ma and Schwander 2017). Three genera of parthenogenetic-inducing bacteria are known to occur in Hymenoptera, namely *Wolbachia*, *Rickettsia,* and *Cardinium*. The first one is by far the most frequently insect-associated symbiont inducing parthenogenesis, in particular in chalcid wasps (Rabeling and Kronauer 2013; Ma and Schwander 2017).

In a recent study, Auger-Rozenberg et al. (2015b) investigated the phylogeographical structures of *B. servadeii* and *O. pityocampae* over the Mediterranean Basin using mitochondrial and nuclear sequences. They showed contrasting levels of diversity in both species, the specialist *B. servadeii* having 87 haplotypes whereas only 16 were found in *O. pityocampae* over the same spatial scale. The two species were found to have drastically different Quaternary evolutionary histories. Interestingly, *B. servadeii* showed a strong geographical structure, with 3 main clades partially mirroring the genetic structure found in its host insect (Kerdelhué et al. 2009); a fourth, strongly divergent mitochondrial clade, linked to a similarly strong differentiation of the nuclear marker, was suggested to correspond to a congeneric hyperparasitoid, *Baryscapus transversalis* Graham. This species is difficult to differentiate based on morphological characters. It occurs in the Balkans, Spain and Turkey (Auger-Rozenberg et al. 2015b; Boyadzhiev et al. 2015). Contrary to *B. servadeii*, males are frequent and probably involved in sexual reproduction. Presence of males is actually one of the cues used to suggest occurrence of *B. transversalis*. Yet, complementary data using polymorphic nuclear loci are still needed to confirm this result.

At a finer geographical scale, studies carried on latitudinal and altitudinal gradients along the expanding front of the PPM suggested that the egg parasitoids tend to track the expansion of their PPM host, yet with a time lag (Auger-Rozenberg et al. 2015a; Robinet et al. 2012; Zovi et al. 2006). Using mitochondrial markers, a strong genetic structure was found along these gradients for *B. servadeii* (Auger-Rozenberg et al. 2015a), with a higher variability in the core areas compared to the expansion areas, as it is expected in case of diffusive expansion. On the other hand, a study carried on a few populations in northeastern Italy, using variable genetic markers (AFLP), underlined the lack of geographical patterns at a finer geographical scale, which could be due to higher dispersion of *B. servadeii* compared to *O. pityocampae* (Simonato et al. 2012). Characterizing population structure and dispersal modes using polymorphic and codominant nuclear markers is now necessary to compare host and parasitoid spatial expansions, in particular in the case of the specialist species, which strictly depends on its insect host.

Obtaining a set of variable microsatellite loci is thus a crucial step for future studies on the expanding patterns of *B. servadeii* at a finer geographical scale in the PPM expanding areas, as well as to characterize the divergence between *B. servadeii* and *B. transversalis*. Here we report the development of a set of 8 polymorphic microsatellite markers, retrieved by high throughput genomic sequencing. Our primary goal was to use these markers to characterize genetic diversity and structure in populations chosen to represent south – north gradients in Western Europe, and to include sites in the Balkans where *B. transversalis* is expected to occur. Yet, given the very low genetic diversity found in *B. servadeii*, we completed the sampling design by adding sites from North Africa, Cyprus and Crete, where the *B. servadeii* mitochondrial clades II and III occur (Auger-Rozenberg et al. 2015b). A further goal was to determine if both the parasitoid and the hyperparasitoid were associated with parthenogenesis-inducing bacterial symbionts that could explain the different reproduction type.

2. Materials and Methods

2.1. Sampling

Samples were collected from egg masses of the pine processionary moths *Thaumetopoea pityocampa* and *T. wilkinsoni* following the procedure described in Auger-Rozenberg et al. (2015b), and adults were morphologically identified as *Baryscapus* spp. as discrimination between *B. servadeii* and the hyperparasitoid *B. transversalis* is difficult. Three successive sampling were used during the course of the project (hereafter named Sets-a, -b and -c, see Table 1):

Set-a: a pool of individuals chosen from Auger-Rozenberg et al. (2015b) to represent the diversity of *B. servadeii* in Europe was used for microsatellite library construction. This pool was constituted from five localities from France, Spain and Portugal;

Set-b: 28 individuals sampled in 14 localities chosen all over the distribution range of *B. servadeii* and belonging to the 4 main mitochondrial clades identified in this species were used to test the microsatellite primer pairs designed during marker development;

Set-c: Ten populations (9 to 31 individuals each) were finally genotyped using the validated microsatellite loci. The initial objective was to use Western European samples along north-south gradients to test for signs of geographic expansion and host tracking. The sampling was enlarged to

Eastern Europe and North Africa in a second step, when the first results suggested that this species has an extremely low genetic diversity.

2.2. DNA extraction and mitochondrial characterization

For each population except Longarone (Italy), we used the DNA of individuals from Auger-Rozenberg et al. (2015b) and completed the sampling using other individuals from the same localities, emerged from different egg batches to avoid siblings. Individuals from Longarone originated from the study of Simonato et al. (2012). Genomic DNA was extracted using NucleoSpin® Tissue XS kit (Macherey-Nagel, Germany), according to the manufacturer's protocol. Quantification and quality check of the extracted DNA were assessed by a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Within each sampling site, a sub-sample of 1 to 12 individuals was further sequenced using the same mitochondrial marker as in Auger-Rozenberg et al. (2015b) to ascertain identification and define their mitochondrial clades (CI to CIV, see Auger-Rozenberg et al. 2015b). In particular, all individuals belonging to the G1 group defined from microsatellite analyses (see below) were sequenced.

2.3. Microsatellite isolation and primer development

The microsatellite library was constructed following Malausa et al. (2011) as detailed in Retamal et al. (2016). Library enrichment and sequencing were carried out by GenoScreen (Lille, France) on a Roche 454 GsFLX Titanium system (Brandford, USA) using 1 µg of the pooled DNAs from Set-a individuals. Possible primer pairs were identified using the QDD software (Meglécz et al. 2010) and chosen using the following parameters (modified from Retamal et al. 2016): target PCR product size between 80 and 300 bp, perfect and various microsatellite motifs with at least 4 repetitions, primer annealing temperature close to 60°C. In total, 42 primer pairs were selected and first tested using Set-b individuals with non-fluorescent primers to determine if amplification was successful for all individuals before proceeding with microsatellite development. The PCR program consisted in an initial step of denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 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 72°C for 10 min. PCR products were then run on a 3% agarose gel stained with ethidium bromide, and DNA bands visualized on a UV transluminator. The loci for which the amplification produced bands in the expected size range for all individuals of Set-b and showed some variability (at least between *B. servadeii* and *B. transversalis*) were then selected for a further amplification with forward primers labeled with the fluorescent dyes 6-FAM, PET, NED or VIC (Applied

Biosystems). The PCR products were visualized using an ABI 3500 Genetic Analyzer (Applied Biosystems) with the 600 LIZ^{TM} GeneScan^{TM} size standard and scored with the GENEMAPPER^{TM} 4.1 software. The loci showing unambiguous genotype patterns for the individuals of Set-b were then organized in 2 multiplex and used to genotype individuals from Set-c. Primer sequences and multiplex compositions are given in Table 2.

2.4. Genetic data analyses

Number of alleles and observed/expected heterozygosities were estimated using Arlequin 3.5 (Excoffier et al. 2005; Excoffier and Lischer 2010). The number and frequency of the different multilocus genotypes (MLG) found in each population were estimated using the package 'poppr' (Kamvar et al. 2014) of the R software (R Core Team 2018).

Using genotyping data obtained for Sets-b and -c, we performed a Principal Component Analysis (PCA) to explore population genetic structure using the R package adegenet 1.4-2 (Jombart 2008). Individuals from Set-c were further assigned to genetic clusters using the Bayesian clustering method implemented in Structure 2.3.4 (Pritchard et al. 2000) under the model with admixture. We set K, the number of clusters, varying from 1 to 6, and a burn-in of 100,000 steps followed by 100,000 iterations of the Markov Chain. To assess the consistency of results, we performed 10 independent runs for each value of K and compared the obtained individual Q-values. The number of clusters K that best explained the data was chosen by examining the curve of Log P(X|K) as well as using the ΔK method described in Evanno et al. (2005) and implemented in Structure Harvester (Earl and vonHoldt 2012). Pairwise F_{ST} values were estimated using Arlequin. Cavalli-Sforza and Edwards chord distances were calculated using Populations 1.2.30 (Olivier Langella, http://bioinformatics.org/Btryphon/populations/).

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

Given the extremely low genetic diversity found in *B. servadeii*, all individuals of Set-c were tested for the presence of *Wolbachia* using the same primers as in Boivin et al. (2014), amplifying the surface protein, namely wsp81F TGGTCCAATAAGTGATGAAGAAAC and wsp691R AAAAATTAAACGCTACTCCA. They were also tested for *Rickettsia* using the primers Rct1-F CCGCGTCAGATTAGGTAGTT and Rct1-R TCAGTTGTAGCCCAGATGAC that specifically amplify the 16S region of this endosymbiont (Simonato 2010). PCR cycles were set up as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min) and elongation at 72°C (1 min). PCR products were then run on a 1.5% agarose gel stained with ethidium bromide and visualized by a UV transluminator. A subset of 19

Rickettsia PCR products (1-3 from each population) were then purified and Sanger sequenced following the same protocol as in Auger-Rozenberg et al. (2015b). Three *Rickettsia* sequences obtained from the insect hosts *Neochrysocharis formosa* (Westwood), *Pnigalio soemius* (Walker) and *Bembidion articulatum* (Panzer) were retrieved from GenBank (accession numbers AB231472.1, JN182552.1 and MK078278.1), aligned to the sequences obtained from *Baryscapus servadeii*, and p-distances were calculated using MEGA3 (Kumar et al. 2004).

3. Results

3.1. Microsatellite library and marker development

Using QDD, we identified 1101 microsatellite loci for which satisfactory primer pairs could be designed, and we further selected 42 candidates using the criteria described above. After testing these primer pairs on Set-b, 17 loci were monomorphic and 15 could not be successfully genotyped for some individuals. Hence, 10 loci were selected and further amplified using fluorescent primers. After genotyping of Set-c individuals, 8 loci were finally selected as resulting in 100% genotyping success and were organized in 2 multiplex (Table 2). Number of alleles ranged from 2 to 15 across loci.

3.2. Genotyping and population genetics results

All 241 individuals (Set-b and Set-c) were successfully genotyped, without any missing data. The PCA clearly showed that individuals were grouped in 2 clusters separated along axis 1 (Fig. 1). One group (hereafter G1) had negative coordinates along that axis and gathered 11 individuals (9 from Pukë, Albania; 1 from Venzone, Italy; 1 from Lilyanovo, Bulgaria) distributed along axis 2, while the other group (G2) contained all 230 remaining individuals, all located very close to the origin of the PCA. Structure results suggested that K = 3 was the best solution (Fig. 2). One cluster corresponded to the 11 individuals from G1, while the two other clusters corresponded respectively to (i) all individuals from Algeria, Morocco, Orléans (France) as well as 1 individual from Chania (Crete) and (ii) all remaining individuals from G2. Sequencing of COI haplotypes of all individuals from G1 and a subset of individuals from G2 showed that all G1 individuals belonged to Clade IV sensu Auger-Rozenberg et al. (2015b), while the individuals from G2 belonged to the other 3 clades. These results consistently suggested that all individuals belonging to G1 corresponded to the species *B. transversalis*. We thus proceeded with population analyses of Set-c after splitting the individuals from Pukë in two groups corresponding to *B. servadeii* (10 individuals) and *B. transversalis* (9 individuals), and excluding the *B. transversalis* individual found in Venzone, Italy.

Observed heterozygosity (Ho) was null in almost all populations and loci in *B. servadeii*, and it reached 0.22 at most in Senalba (Algeria). In the only population of *B. transversalis*, it ranged between 0 (Bary19) and 0.89 (Bary13). The genetic differentiation between *B. servadeii* and *B. transversalis* populations was high and significant (pairwise F_{ST} spanning between 0.61 and 0.80, Cavalli-Sforza and Edwards chord distance between 0.74 and 0.76). Among *B. servadeii* populations, high and significant differentiation was found between populations from Algeria and Orléans vs. all other populations (pairwise F_{ST} spanning between 0.73 and 0.96 and Cavalli-Sforza and Edwards chord distance between 0.13 and 0.19, see Table 3). Interestingly, all 9 *B. transversalis* individuals from Pukë had different genotypes (9 MLG for 9 individuals), while 90% of *B. servadeii* individuals (183/203) belonged to 2 MLG, one mostly found in Algeria (Moudjbara and Senalba) and in Orléans (France), and the second in all the other populations. The remaining 16 MLG were rare and each was found in 1 or 2 individuals only. Note that most MLG found in *B. servadeii* only slightly differed from each other, and one single allele was fixed for most loci; for instance the major MLG23 and MLG26 only differed by a different allele fixed for locus Bary-13 (allele 88 vs. allele 90), the very same alleles being present in both MLG for all other loci. Detailed distributions and genotypes of the 27 MLG are given in Table 4. The populations characterized by the occurrence of MLG23 actually corresponded to cluster 2 in Structure, while the populations characterized by MLG26 corresponded to cluster 3.

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

The PCR screening performed to detect the presence of *Wolbachia* returned only negative results in *Baryscapus* spp., while a positive control taken from Boivin et al. (2014) was successfully amplified. The screening performed to detect *Rickettsia* showed that this bacterium was absent from all *B. transversalis* individuals whereas it was present in all *B. servadeii* populations and in 89% of the studied individuals for that species. The rates of association were generally very high (100% in Motril (30/30 individuals), Evrychou (20/20) and Longarone (19/19); 97% in St Guilhem (30/31); 90% in Moudjbara (9/10) and Pukë (9/10); 89% in Senalba (8/9); 86% in Orléans (25/29)) but were somewhat lower in Razanac and in Venzone (67% in both sites). We obtained 426 bp long sequences from the obtained amplicons, corresponding to 2 alleles differing by only 1 mutation (G/A at position 94) for *B. servadeii*. One allele was found in Algeria whereas the other was found in all other sequenced individuals. The *Rickettsia* sequences obtained from *B. servadeii* were slightly different from the ones retrieved from GenBank for different hosts (p-distance between 0.9 and 1.4%). Sequences of the two alleles found in this study were deposited in GenBank (accession numbers MN400060 and MN400061).

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 involved in the studied system. We thus suggest that thelytokous parthenogenesis in *B. servadeii* is induced by *Rickettsia* and probably corresponds to a mode of automictic parthenogenesis involving gamete duplication, which leads to full homozygosity in the progeny. This hypothesis should now be confirmed by detailed cytological studies.

Wolbachia frequently occurs in a wide range of hymenoptera families, particularly within the Chalcidoidea superfamily, and was expected to be the main parthenogenesis-inducing bacterium in the case studied here (Rabeling and Kronauer 2013). Moreover, even if *Rickettsia* bacteria are described from chalcid wasps, they are much rarer and the only case in which the cytological mechanisms were described concluded that *Rickettsia* induces heterozygous offspring identical to the mother through a single equational division during meiosis (Adachi-Hagimori et al. 2008). The case of *B. servadeii* thus raised original results, both concerning the type of endosymbiont identified and the cytological mechanisms suggested, which is very similar to *Wolbachia*-induced thelytoky. Mutation is then the single strength potentially inducing some diversity, and would explain the occurrence of the rare MLG documented in this study.

The very strong reduction of diversity and large-scale homozygosity also suggest that thelytoky is by far the most frequent reproduction mode in this species. In a recent review, Ma & Schwander (2017) showed that endosymbiont-induced parthenogenesis tends to facilitate the maintenance of both sexual and parthenogenetic strains. Yet, our work suggests that *B. servadeii* has a parthenogenetic reproduction at a large geographical scale, encompassing all the mitochondrial lineages identified in this species (clades I, II and III from Auger-Rozenberg et al. 2015b). This hypothesis is supported by the contrasting results we obtained for the sexually-reproducing *B. transversalis* (Boyadzhiev et al. 2015), for which no association with *Rickettsia* was found and genetic diversity and heterozygosity were documented using the same microsatellite markers. Microsatellite genotypes and mitochondrial sequences both confirmed the occurrence of *B. transversalis* in the Balkans (Albania and Bulgaria), but also in Italy where this species was not mentioned so far but was suspected to occur based on the high number of males (unpublished results). Indeed, both *B. servadeii* and *B. transversalis* can be found in PPM eggs, but males are known mostly from the second species, which is likely to reproduce sexually rather than through thelytoky.

The genetic pattern found may have some profound implications in the regulation of the PPM populations across the range. *B. servadeii* is tracking the host in the expansion range in a homogenous way, ensuring a relatively quick density-dependent control and bringing the initially explosive population growth observed at range edge to the levels observed in the core range (Auger-Rozenberg et al. 2015a). Yet, lack of genetic diversity in the nuclear genome suggests limited adaptation capacities. This could negatively affect the ability of *B. servadeii* to adapt to the new environments colonized by the PPM, such as urban areas, northern regions or new host trees, and the efficiency of biological control. The lack of genetic diversity may also hamper the response to the current global changes (climate warming, increased risks of extreme climatic events, land-use changes...). On the other hand, the fact that *B. servadeii* is associated to a *Rickettsia* symbiont and has a pure thelytokous reproduction can ease colonization or recolonization of certain environments, as a single female can produce many offspring. Successful expansion can thus originate from a limited number of dispersing individuals, as was already evidenced in an invasive chalcid wasp (Auger-Rozenberg et al. 2012).

The hyperparasitoid *B. transversalis* does not seem to track the egg parasitoid as it could be expected, occurring sporadically and in limited number. This lack of specialization can be explained by the generalist trait of the hyperparasitoid, known to be associated also with other egg parasitoids of the PPM, such as *O. pityocampae* (Tsankov et al. 1996). In addition, *B. transversalis* is characterized by multivoltinism whereas *B. servadeii* is generally univoltine, matching perfectly the annual appearance of the PPM eggs.

Altogether the results found here are striking, and suggest that microsatellite nuclear markers will not be useful in future studies to decipher the demography and expansion routes of *B. servadeii*, while they could be used for the hyperparasitoid *B. transversalis*. Interestingly, mutations seem to accumulate in the mitochondrial DNA that is directly inherited from mother to daughters, and this organelle DNA could be sufficiently informative to study spatial patterns of expansion in Europe, even though it will not allow to test contrasting scenarios as it can be done with Approximate Bayesian Computation approaches using nuclear markers (Estoup et al. 2018). High-throughput sequencing and SNP genotyping technologies will be necessary to confirm that the high homozygosity documented here is a genome-wide characteristic, and to use a larger portion of the mitochondrial genome in future population studies. Finally, *B. servadeii* should now be seen as an interesting example to study the mechanisms inducing thelytoky in an original *Rickettsia* – chalcid parasitoid association.

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FIGURE CAPTIONS

Figure 1: Graph of the principal component analysis (PCA, axes 1 and 2) ran on microsatellite genotypes of 241 individuals from Set-b and Set-c.

Figure 2: Graphical representation of the individual genetic assignments given by the individual Q-values inferred by STRUCTURE at K=3 for the 213 individuals from Set-c. White cluster: *B. transversalis* (same individuals as in group G1 in Fig. 1). Dark-grey cluster: *B. servadeii* cluster 2, mostly characterized by MLG23. Light-grey cluster: *B. servadeii* cluster 3, mostly characterized by MLG26.

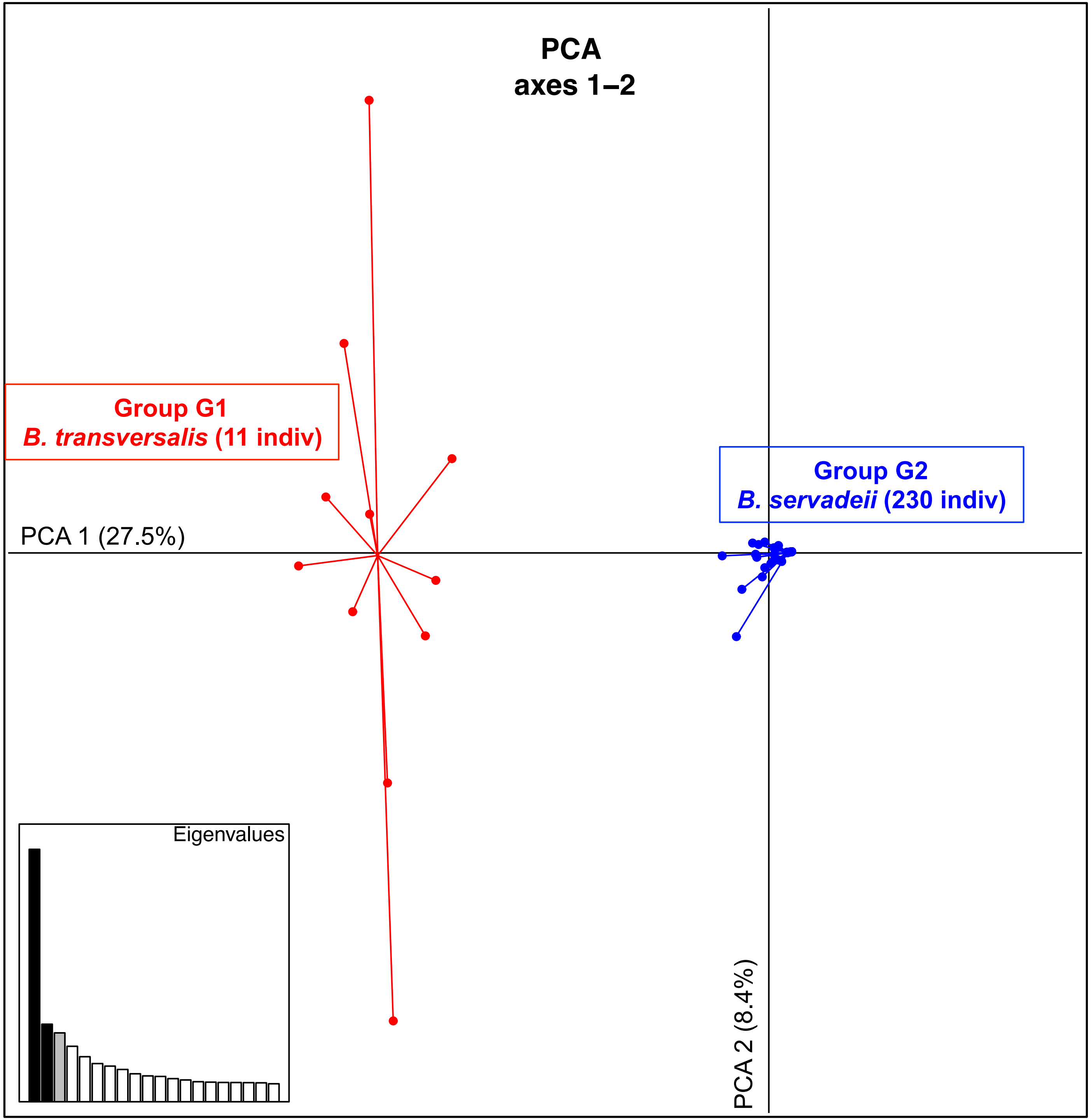
TABLE CAPTIONS

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*

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

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

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


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

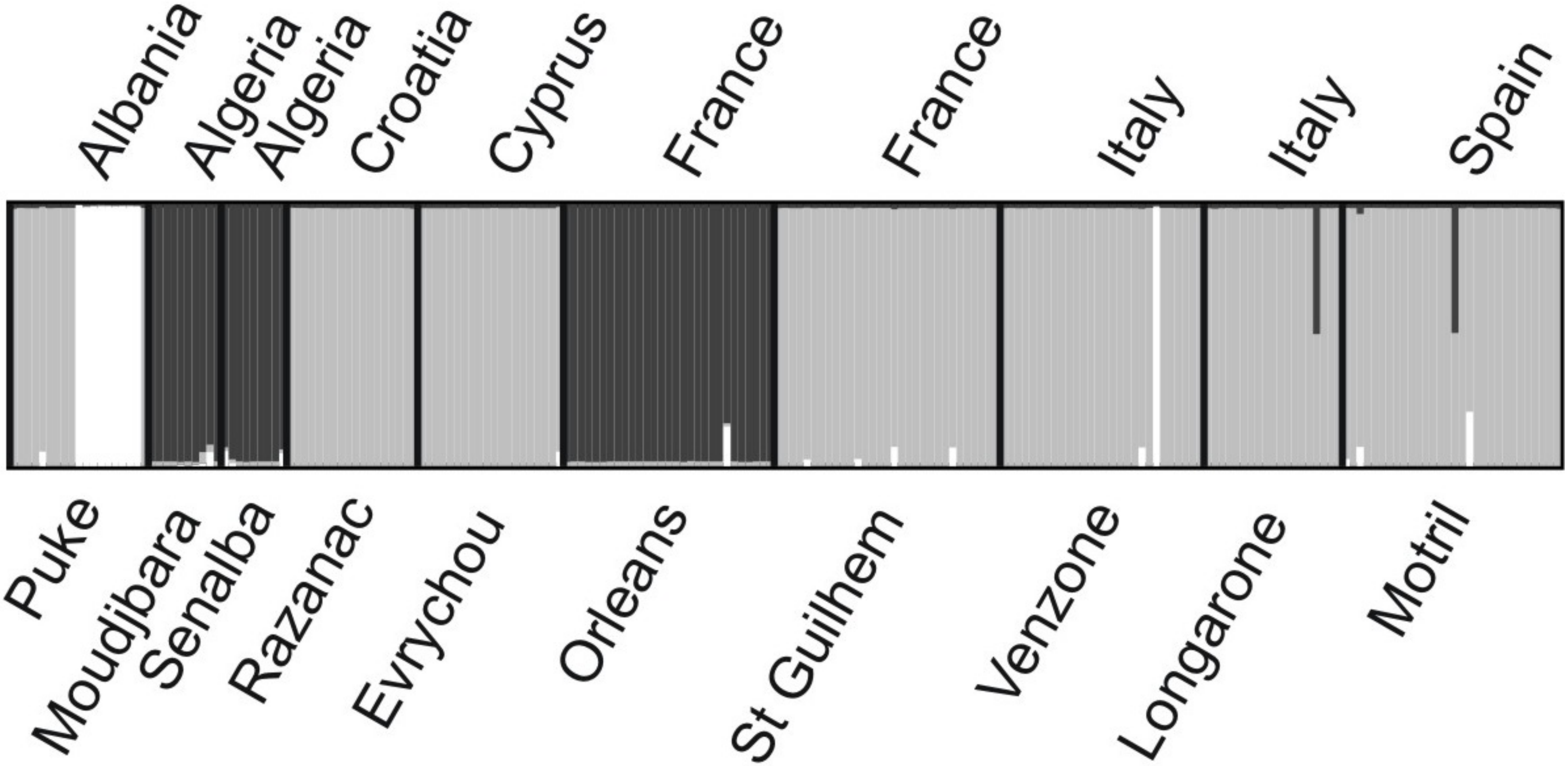

Albania
Algeria
Algeria
Croatia
Cyprus
France
France
Italy
Italy
Spain
Puke
Moudjbara
Senalba
Razanac
Evrychou
Orleans
St Guilhem
Venzone
Longarone
Motril

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)

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

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

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.

	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	-

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

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

(1): MOU(6), SEN(6), ORL(28)

(2): RAZ (18), EVR(19), StGUI (27), VEN(26), LONG(18), MOT(26), PUKË (9)