1    **Genetic analysis reveals efficient sexual spore dispersal at a fine spatial scale in *Armillaria***

2    ***ostoyae*, the causal agent of root-rot disease in conifers**

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18    Keywords: Forest disease, isolation by distance, maritime pine, planted forest, population size,

19    single nucleotide polymorphism

## Abstract

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22  *Armillaria ostoyae* is a fungal species causing root diseases in numerous coniferous forests of the

23  northern hemisphere. The importance of sexual spores for the establishment of new disease centers

24  remains unclear, particularly in the large maritime pine plantations of south-western France. An

25  analysis of the genetic diversity of a local fungal population distributed over 500 ha in this French

26  forest showed genetic recombination between genotypes to be frequent, consistent with regular

27  sexual reproduction within the population. The estimated spatial genetic structure displayed a

28  significant pattern of isolation by distance, consistent with the dispersal of sexual spores mostly at

29  the spatial scale studied. Using these genetic data, we inferred an effective density of reproductive

30  individuals of 0.1 to 0.3 individuals/ha, and a second moment of parent-progeny dispersal distance

31  of 130 to 800 m, compatible with the main models of fungal spore dispersal. These results contrast

32  with those obtained for studies of *A. ostoyae* over larger spatial scales, suggesting that inferences

33  about mean spore dispersal may be best performed at fine spatial scales (i.e. a few kilometers) for

34  most fungal species.

35

36 **Introduction**

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38    During their life-cycle, fungal species may either alternate sexual and asexual reproduction, or

39    reproduce exclusively by one of these two modes of reproduction (e.g. Taylor et al. 1999). These

40    reproduction strategies have important consequences for fungal demographics and evolution. For

41    example, mostly asexual populations can produce large numbers of progenies rapidly, enabling

42    them to colonize new environments to which they are already adapted (e.g. Hovmøller et al. 2008,

43    Raboin et al. 2007). However, the absence of genetic recombination may decrease adaptation

44    potential in a changing environment, because new genotypes can emerge only by mutation (Crow &

45    Kimura 1965). Sexual and asexual propagules may also have different dispersal patterns, generating

46    different population genetic structures at different spatial scales (Barrès et al. 2012, Rieux et al.

47    2014). These different dispersal capacities may produce a spatial and temporal mosaic of genetic

48    diversity, allowing natural selection to act at different spatial and temporal levels (Thrall & Burdon

49    2002). Mode of reproduction is, therefore, an important biological trait that may vary with

50    ecological context, as predicted and observed in invasive species (Bazin et al. 2014, Gladieux et al.

51    2015).

52

53    It is not always easy to infer the mode of reproduction and dispersal processes within species,

54    or between populations of the same species. Sexual reproduction may be cryptic (Saleh et al. 2012),

55    or not very efficient at producing new recombining genotypes within populations (Dutech et al.

56    2010). Direct observations in the field may be rare or inconclusive. Population genetic analyses may

57    therefore be more relevant for estimating the contribution of sexual reproduction events within and

58    between populations (Halkett et al. 2005). First, analyses of genetic diversity performed at different

59    spatial scales may reveal the different reproductive processes within species (e.g. Barrès et al. 2012,

60    Dutech et al. 2008, Kohli et al. 1995). Second, methods using spatial genetic analysis to estimate

61    the genetic relatedness between individuals can be used to infer the spatial range of dispersal of

62    sexual and asexual spores (e.g. Dutech et al. 2008, Rieux et al. 2011, Travadon et al. 2012), under

63    the theoretical model of isolation by distance (IBD) (Rousset 1997, Vekemans & Hardy 2004,

64    Wright 1943). However, despite the common use of such methods on f fungal populations, the

65    conclusions drawn about dispersal processes may sometimes be irrelevant, due to the use of an

66    inappropriate spatial scale of investigation (see for example  Rousset 1997 for theoretical

67    considerations). Thus, conclusions that long-distance spore dispersal occurs based on an absence of

68    spatial genetic structure among populations may be incorrect for several fungal species, as already

69    shown for marine organisms with a large geographic distribution (Puebla et al. 2012). Puebla *et al*.

70    showed that genetic analyses over fine spatial scales (i.e. a few meters to a few kilometers), and

71    estimates of relatedness between genotypes, could, in some cases, better detect the effect of

72    restricted progeny dispersal than studies performed at population level.

73

74    *Armillaria ostoyae* is a fungal species for which the importance of sexual reproduction in the

75    life-cycle remains unclear (Baumgartner et al. 2011, Prospero et al. 2008). This pathogen species,

76    responsible for butt- and root-rot diseases of conifers throughout the northern hemisphere, has two

77    known modes of reproduction and dispersal. First, *A. ostoyae* produces a specific mycelium, known

78    as a rhizomorph, for dispersal in the soil from one infected root to another susceptible host root

79    **(**Lung-Escarmant & Guyon 2004, Redfern & Filip 1991). This vegetative propagation may lead to

80    the development of a single-genotype clonal patch in a forest stand, sometimes covering a very

81    large area (up to about 1000 ha; Ferguson et al. 2003). *A. ostoyae* also regularly produces fruiting

82    bodies during the fall, and these fruiting bodies can release sexual haploid spores (i.e. the

83    basidiospores) into the air. The germination of two compatible basidiospores and the fusion of their

84    hyphae gives rise to a new genotype able to infect a new host tree (Rishbeth 1988). It has been

85    assumed that sexual spores may occasionally be dispersed, by the wind, over tens of kilometers in

86   many fungal species (Aylor 1999, Halbwachs & Bässler 2015). This mode of reproduction should,

87   therefore, facilitate colonization by new genotypes and the development of new clonal patches some

88   distance away from the original disease focus. However, the fusion of two sexual basidiospores

89   leading to the colonization of a new host has rarely been observed or achieved in the field for *A.*

90   *ostoyae*, raising questions about the frequency with which this process occurs in natural conditions

91   for this species (e.g. Baumgartner et al. 2011, Rishbeth 1988).

92

93       Several studies on *A. ostoyae* across the northern hemisphere have identified different

94   population genetic structures potentially dependent on the ecological and climatic context of the

95   forests studied. For example, in extensively managed cold North American forests, clonal structures

96   of several tens to hundreds of hectares in size have frequently been reported (e.g. Dettman & Van

97   der Kamp 2001a, Fergusson et al. 2003). By contrast, in the conifer forests of European mountain

98   ranges, in which human activities have had a stronger impact on the ecosystem over a longer period

99   of time, *A. ostoyae* generally displays higher levels of genotypic diversity and forms smaller clonal

100  patches (e.g. Bendel et al. 2006, Legrand et al. 1996). In the maritime pine plantations of south-

101  western France, the Landes de Gascogne forest, small clonal patches associated with disease foci

102  have also been described (Prospero et al. 2008). This genetic structure would be expected for a

103  forest growing in a warm and wet climate, and with a rapid turnover of susceptible hosts due to

104  intensive management of the plantation (Fergusson et al. 2003, Risbeth 1988). As in previous

105  studies, no genetic relatedness was detected between clonal patches. Two mutually exclusive

106  hypotheses can be put forward to explain this absence of genetic structure. The first hypothesis is

107  that sexual spores are regularly dispersed over long distances (i.e. over several tens of kilometers),

108  with efficient germination and fusion of basidiospores, in this forest, resulting in a random genetic

109  structure (Prospero et al. 2008). However, this hypothesis is not consistent with the limited spread

110  of the disease observed over several decades in this forest. The disease is thought to have originated

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111  on the west coast of the region, where most of forest areas were located before the large maritime

112  pine plantations were established in the 19th century (Labbé et al. 2015, Prospero et al. 2008). The

113  second hypothesis is that the spatial scale of the previous study, covering the entire area of the

114  forest (i.e. around 1 x 10$^6$ ha) was inappropriate. Rare long-distance dispersal events can occur for

115  sexual spores, but the mean dispersal distance is probably of the order of a few kilometers, as

116  observed in other fungi (Rieux et al. 2014). In this context, as highlighted above, genetic studies at a

117  spatial scale similar to that over which dispersal occurs are required to detect any spatial genetic

118  structure associated with the dispersal process. We tested this hypothesis, by analyzing the spatial

119  genetic structure of *A. ostoyae* within a coastal forest in which several genotypes were identified in

120  a previous study (Prospero et al. 2008), expanding the sampled area from less than one hectare to

121  several hundred hectares.

122

123  Our main objectives were to use the recently developed molecular markers for *A. ostoyae*

124  (Dutech et al. 2016): 1) to estimate the importance of genetic recombination in the population

125  studied; 2) to characterize the spatial genetic structure of this population; 3) to test the hypothesis of

126  isolation by distance (Wright 1943), assuming that the mean dispersal distance of sexual spores is

127  between a few hundred meters and a few kilometers; 4) to estimate the spatial range over which

128  spores are dispersed from genetic data.

129

130  **Materials and Methods**

131

132  Study site

133

134  The study was performed in the western part of the Landes de Gascogne Forest, in La Forêt

135  Domaniale de Saint Julien en Born (1°,16',20"W – 44°,06',40"N) in southwestern France. This site

136    is located on sandy dunes 4 km from the Atlantic Ocean. The forest is composed mostly of maritime

137    pines (*Pinus pinaster*), with some oaks (*Quercus ilex, Q. robur*). Maritime pine is a local species

138    that has been cultivated intensively in plantations since the 19th century, to help drain the marshes

139    and swamps initially present in the area and for economic reasons (for details, see Labbé et al.

140    2015). The populations of maritime pine on the coast were present before these large plantations

141    were established. They formed small fragments of forest and were the first trees sown during this

142    period to stabilize the sandy coastal dunes (Labbé et al. 2015). The coastal part of the Landes de

143    Gascogne Forest is currently managed by the French National Forestry Office (*Office National des*

144    *Forêts*), which favors management by natural regeneration or the sowing of maritime pine seeds

145    after commercial logging.

146

147        We extended the initial sampling area used by Prospero et al. (2008), from the disease focus

148    named "Contis" to the south of the stand. We obtained 177 isolates from dead or dying maritime

149    pines, by collecting mycelium from under the cork as described by  Prospero et al. (2008). This

150    previous study clearly identified clonal patches that were generally less than 1 ha in diameter and

151    associated with a single disease focus. We maximized the chances of collecting different genotypes

152    for studies of their genetic relationships, by collecting isolates mostly from trees located at least 100

153    m apart (55% of the samples, and 98% of all pairs of distances). The minimum distance separating

154    two isolates was 16 m; the maximum distance was 3474 m, and the total area sampled was close to

155    500 ha. Samples were located with a GPS Trimble Geo7X, and GPS Pathfinder Office (Trimble

156    Navigation Ltd, USA) was used for the processing of spatial data.

157

158    Molecular analysis

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160    In the laboratory, we collected isolates from each infected piece of wood sampled in the field,

161    by detaching the mycelium from the cambium with a scalpel. These mycelial samples were freeze-

162    dried overnight (-45°C, 0.3 mbar), and then ground with metallic beads in 2 ml microtubes, with an

163    automatic grinder (GenoGrinder Sample Prep 2010, SPEX, USA), for 15 s at 1500 rpm. DNA was

164    extracted from the ground mycelium in a cetyltrimethyl ammonium bromide (CTAB) buffer,

165    according to the protocol described by Prospero et al. (2008).

166

167    Isolates were genotyped  for 27-single nucleotide polymorphisms (SNPs) identified in 24 genes

168    present as single copies in most fungal genomes, and isolated by the PHYLORPH method (see for

169    details, Feau et al. 2011). These 27 SNPs were selected from the list of 82 SNPs previously

170    validated in two coastal *A. ostoyae* populations (Dutech et al. 2016). These SNPs were multiplexed,

171    and genotyped in the medium-throughput MassARRAY iPLEX genotyping assay from Sequenom

172    (San Diego, CA, USA), as described by Chancerel *et al.* (2013) and Dutech et al. (2016). Details of

173    the 27 genotyped SNPs are provided in Table 1.

174

175    **TABLE 1: Details of the 27 single-nucleotide polymorphisms (SNPs) analyzed in the *A.***

176    ***ostoyae* population sampled from the St Julien en Born forest in southwestern France**

177

178    Data analyses

179

180    Repeated genotypes (i.e. identical for all 27 SNPs analyzed) were identified with GenoType

181    V.1.2, excluding missing loci for each comparison (Meirmans & Van Tienderen 2004). For each

182    SNP, allelic frequencies, genic diversity ($H_E$, Nei 1987) and intra-individual fixation index ($F_{IS}$)

183    according to the Weir and Cockerham (1984) method were estimated with Genepop V.4.3

184    (Raymond & Rousset 1995). Only one copy per genotype was retained, to exclude the effect of

185 clonal structure on the estimates of genetic diversity. Exact tests for Hardy-Weinberg equilibrium

186 within each population, and for linkage disequilibrium between SNPs were performed with

187 Genepop. Correction for multiple testing was performed according to the false discovery rate

188 method (FDR, Benjamini & Hochberg 1995), with the R package fdrtool V1.2.15 (Klaus &

189 Strimmer 2013).

190

191 Spatial genetic structure was analyzed by two methods. First, the kinship coefficient was

192 estimated for each pair of isolates, using the estimate proposed by Loiselle et al. (1995). The mean

193 per spatial distance classes was then plotted to generate a spatial autocorrelogram of the genetic

194 relationships between isolates. We defined the distance classes so as to obtain an even number of

195 pairs of isolates in each distance class. The first five distance intervals were each 150 m wide.

196 Beyond 750 m, each of the next five classes was 250 m wide and then, after 2000 m, there were 500

197 m-wide classes, making it possible to estimate kinship coefficients over distances of up to 3000 m.

198 Each distance class contained at least 210 pairs of isolates (obtained for the first distance class with

199 the genotypes in single copy). Random permutations of the spatial locations of isolates (1000

200 permutations) were performed to test the hypothesis that the mean kinship coefficient for each

201 distance class was significantly different from that expected for a random spatial genetic structure.

202 We investigated the effect of clonality on spatial genetic structure, by performing the analysis both

203 with all genotypes and with a single copy of each genotype randomly chosen from the copies.

204

205 The second method used to investigate spatial genetic structure was spatial principal component

206 analysis (sPCA, Jombart et al. 2008). This method can be used to identify various spatial genetic

207 structures (e.g. patches, clines, genetic barriers), without the need for assumptions concerning the

208 genetic model, whereas Bayesian genetic clustering methods are dependent on the assumption of

209 Hardy-Weinberg equilibrium. sPCA can be used to investigate the distribution of allelic diversity,

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210  by combining a principal component analysis (PCA) of allele frequencies and the spatial distance

211  between isolates. It can identify both global structures associated with the positive components of

212  the analysis, reflecting the decrease in similarity between individuals with increasing spatial

213  distance, and local structures associated with the negative components reflecting local

214  dissimilarities between individuals located close together spatially (Jombart et al. 2008). Each

215  significant structure detected was displayed by plotting the samples according to their geographic

216  coordinates, color-coding and sizing them according to their scores along the significant sPCA axes.

217  We determined whether the global and local structures were significantly different from a random

218  structure by performing random permutations of sPCA components (999 permutations), and

219  comparing the observed and simulated sPCA statistics as described by Jombart et al. (2008).

220  Estimation and testing were performed with the R package Adegenet V2.0.1 (Jombart 2008), using

221  a single copy per genotype to eliminate the effect of clonal structure on the analysis.

222

223      We tested the hypothesis of isolation by distance (IBD) associated with limited dispersal of the

224  progeny from the parents (Wright 1943), by assuming a spatial dispersal of sexual spores in two

225  dimensions. Under this hypothesis, the slope of the estimated kinship coefficient for each pair of

226  isolates decreases linearly with the logarithm of the spatial distance separating these pairs (Rousset

227  2000). The significance of the slope was tested by permutations of spatial distances between

228  isolates (1000 permutations) and Spearman's rank correlation analysis, as described by Rousset

229  (2000). All the estimations of kinship coefficient and tests were performed with SPAGeDi V.1.5a

230  (Hardy & Vekemans 2002).

231

232      We also estimated the effective size ($Ne$) of the *A. ostoyae* population by a method based on the

233  linkage disequilibrium between loci (Do et al. 2014, Waples & Do 2010). Estimates of $Ne$ may be

234  biased by a Wahlund effect in continuous populations, due to the limited dispersal of individuals or

235    their gametes (Neel et al. 2013). We therefore estimated this parameter for both the whole sample,

236    with a single copy per genotype, and for three subsamples of 30 genotypes each, with a maximum

237    distance of 1000 m between isolates from the same subsample. These subsamples, which were

238    smaller than the total sample, may be closer to the window in which most mating events occur, for

239    which the effective number of parents responsible for producing the sample is best estimated (Neel

240    et al. 2013). These three subsamples were evenly distributed over the sampling site, from north to

241    south. *Ne* was estimated with NeEstimator V.2.1 (Do et al. 2014).

242

243        Finally, we estimated the second moment of parent-progeny dispersal (Rousset 2000) with the

244    iterative procedure implemented in SPAGeDi V.1.5a (Hardy & Vekemans 2002). Assuming gene

245    dispersal in two dimensions and a genetic drift-dispersal equilibrium (Wright 1943, Rousset 2000),

246    the slope of the spatial autocorrelation of the kinship coefficient is $1/(4\pi D_e\sigma^2)$, where $D_e$ is the

247    effective density of reproductive individuals, and $\sigma$ is the second moment of parent-progeny

248    dispersal. However, this relationship should only be estimated in the range from $\sigma$ to around 10 or

249    $20\sigma$ (Rousset 2000). Outside this range, it may be biased by the shape of the dispersal function,

250    mutation and migration events. As this range is generally not known, the iterative procedure uses an

251    estimate of $D_e$, and yields a first estimate of $\sigma$ obtained from all the spatial distances analyzed. The

252    value of $\sigma$ is then re-estimated, considering a spatial range associated with this first estimate of $10\sigma$

253    or $20\sigma$, until $\sigma$ and the spatial range converge. For $D_e$, we used the density of genotypes obtained

254    from our sampling, or the estimate obtained from NeEstimator V.2.1.

255

256    **Results**

257

258        In total, 177 isolates were analyzed for the 27 SNPs, and 149 different genotypes were

259    detected. Seventeen genotypes occurred at least twice (eight genotypes occurred twice, seven

260    occurred three times and two occurred four times). The maximal distance between two identical

261    genotypes was between 27 m and 371 m, with a mean value of 120 m. Genetic diversity, estimated

262    with $H_E$, was between 0.046 (MS481_1) and 0.501 (FG691_1 and FG756_1), with a mean value of

263    0.36 (SE ± 0.02) (Table 1). The intra-individual fixation index ($F_{IS}$) was estimated at between -

264    0.151 (FG735_1) and 0.452 (MS467_8), with a mean value of 0.02 (SE ± 0.02). Overall, genotype

265    frequencies did not differ significantly from that expected under Hardy-Weinberg equilibrium. Two

266    loci (MS467_8 and FG529_1) displayed a significant departure of genotype frequencies from

267    Hardy-Weinberg expectations ($P$-values < 1 x 10$^{-4}$ and 0.008, respectively; Table 1). However, this

268    departure from expectations remained significant only for MS467_8 after correction for multiple

269    tests. In total, 351 comparisons were made for linkage disequilibrium, and 37 pairs of loci had

270    significant $P$-values (< 0.05). After correction for multiple testing, only four tests remained

271    significant. Two were associated with genes from the same locus (FG771_1 and FG771_3, and

272    FG848_1 and FG848_6). The other two significant tests concerned FG730_3 and MS452_3, and

273    FG893_1 and MS334_3. Finally, to avoid any effect of linkage disequilibrium or departure from

274    Hardy-Weinberg equilibrium on demographic inferences, we removed MS467_8, FG771_1,

275    FG848_1, FG730_3 and FG893_1, and used only 22 SNP loci for subsequent analyses.

276

277    **Figure 1: Relationships between kinship coefficients of pairs of *A*. *ostoyae* isolates and the**

278    **distance (in m) separating them**

279

280    The estimated kinship coefficient between pairs of *A*. *ostoyae* isolates decreased steadily with

281    increasing distance (Figure 1). Taking all the isolates into account, the estimated kinship coefficient

282    was 0.09 for the first distance class (0-150 m) and was close to zero beyond 450 m. With the

283    exception of two distance classes (1500-1750 m and 1750-2000 m) kinship coefficient estimates at

284    distances of more than 450 m were not significantly different from expectations for a random

285    genetic structure. For the two remaining distance classes, the kinship coefficient was estimated at -

286    0.01 and -0.025, respectively. A similar decrease in kinship coefficient with increasing distance was

287    observed when only one copy of each genotype was considered. The estimate was lower for the first

288    distance class (0.04), but identical for the second class and beyond, and not significantly different

289    from that expected under a random genetic structure, except, once again, for the 1500-1750 m and

290    1750-2000 m distance classes. The slope of the autocorrelation for one copy per genotype was –

291    0.012 (SE ± 0.003), which was significantly different from zero in Spearman's rank correlation

292    analysis on spatial locations (*p-value* < 0.01).

293        Tests on the spatial structure estimated from sPCA showed that only the global structure,

294    associated with positive eigenvalues, was significantly different from a random spatial structure

295    (999 permutations, *p*-value = 0.011). The local structure associated with negative eigenvalues (999

296    permutations, *p*-value =0.256) was not significant. Several spatial clusters of isolates with either the

297    most positive or the most negative values on the first principal axis were observed within the

298    population (Figure 2). Isolates with a value close to zero were generally located between these

299    clusters. These spatial clusters had a diameter of about 500 m-1000 m (Figure 2).

300

301    **Figure 2: Geographic distribution of sPCA values**

302

303        For the 149 genotypes sampled from an area of about 500 ha, the *Ne* estimate was 49.9 (95%

304    CI, determined by a jacknife procedure: 33.6 – 77.3), yielding a $D_e$ of 0.1 parents/ha. For each

305    subsample of 30 genotypes from about 100 ha, the estimated value of *Ne* ranged between 14.4 and

306    79.6 (mean = 40; $D_e$ = 0.4 parents/ha). We therefore used three effective densities of parents to

307    estimate σ, with the iterative procedure implemented in Spagedi. The first density used was that

308    estimated with NeEstimator V.2.1 and the 149 genotypes. The second was the density of sampled

309    genotypes over an area of 500 ha (0.3 parents/ha, similar to the density obtained with the three

13

310    subsamples and NeEstimator V.2.1), and the third was a density 10 times greater than the census

311    density, assuming an incomplete sampling of the area (3 parents/ha). The estimated value of σ was

312    between 130 and 162 m for the highest effective density of 3 parents/ha, depending on the

313    maximum distances considered for the regression (10 σ and 20σ, respectively). The corresponding

314    estimate was 830 m for the sampled density. No convergence was observed for density estimates

315    obtained for the 149 genotypes with NeEstimator.

316

317    **Discussion**

318

319        The observed clonal fraction in this study was lower than previously reported for other

320    European and North-American *A. ostoyae* populations (e.g. Bendel et al. 2006, Dettman & Van Der

321    Kamp 2001a, Prospero et al. 2003). In this forest in southwestern France, 90% of the genotypes

322    identified were sampled only once. By contrast, in most of the other forests studied to date, most, if

323    not all, genotypes were sampled several times. The sampling design of this study, with a minimal

324    distance of 100 m between most of the pairs of isolates, may account for the small number of

325    repeated genotypes. In a previous study on this maritime pine forest, most of the identical genotypes

326    associated with a single disease focus were sampled from trees less than this minimal distance apart

327    (Prospero et al. 2008). It was difficult to estimate the size of clonal patches from our sample, but

328    our results are consistent with an area of less than 1 ha, the estimate obtained in a previous study on

329    this forest (Prospero et al. 2008, Labbé 2015, Lung et al. unpublished). This mean area of disease

330    foci is among the smallest clonal patch sizes reported for *A. ostoyae* populations in Europe and

331    North America, where estimates of greater  than 1 ha, and up to 965 ha have been obtained (Bendel

332    et al. 2006, Dettman and Van Der Kamp 2001a, Ferguson et al. 2003). The large number of

333    different genotypes estimated to be present in an area of only 500 ha in this study may be due to the

334    more frequent production of fruiting bodies in coniferous forests in temperate climates than in those

335    growing in a boreal of alpine climate. This higher frequency of sexual reproduction would favor the

336    establishment of new genotypes over time, through the fusion of sexually compatible basidiospores,

337    resulting in the establishment of large numbers of small clonal patches at the local scale. This

338    hypothesis has been put forward and discussed before (Ferguson et al. 2003, Wargo & Shaw 1985),

339    but there is currently no evidence for such a variation of fruiting in *A. ostoyae* populations with

340    temperature. Additional studies are, therefore, required, for firm conclusions to be drawn on this

341    point. A second factor potentially explaining the higher level of local genotypic diversity is the

342    extinction-recolonization dynamics of genotypes associated with high levels of environmental

343    disturbance. In intensively managed forests, such as this single-species plantation, successive

344    planting events after the removal or destruction of stumps could eradicate clonal patches, or at least

345    initially decrease their size (Cleary et al. 2013). However, subsequently, as suggested in several

346    previous studies, clear-cutting and the planting of new stands of conifers would favor colonization

347    by new genotypes from basidiospores (see Risbeth 1988, Worrall 1994, Legrand et al. 1996). Fresh

348    dead wood remaining after logging, such as stumps, or the small pieces of root remaining in the soil

349    after stump removal, could serve as a substrate for the germination and fusion of basidiospores from

350    neighboring infected forest stands (Risbeth 1988), although this remains to be demonstrated.

351    Plantations of maritime pine seedlings, which are very sensitive to *A. ostoyae* in the first years of

352    their life (Lung-Escarmant & Guyon, 2004, Labbé et al. 2015), would also be favorable

353    environments for the rapid spread of these new genotypes. Furthermore, the coastal region in which

354    this study was performed, would have supported a large fungal population for several hundred

355    years, potentially accounting for the large number of genotypes observed. Maritime pine

356    populations have been present in this area for centuries and they may have hosted a large *A. ostoyae*

357    population, consistent with the substantial presence of disease due to this fungus observed in this

358    coastal area (Labbé et al. 2015). In addition, the first seeds of this tree species were sown at this

359    site, to stabilize the sandy dunes, in the middle of the 19th century, and this site has been managed

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360    ever since so as to keep the coastal forest intact, thereby decreasing the risk of local extinction for

361    fungal pathogen populations.

362

363    A role for basidiospores in the establishment of genotypes was strongly suggested, but not

364    clearly demonstrated, in a previous study on *A. ostoyae* in this planted forest of maritime pine

365    (Prospero et al. 2008). The results reported here provide even stronger support for the hypothesis

366    that the genotypes observed originated from the local dispersal of basidiospores rather than the

367    evolution of divergent clonal lineages present for long periods in the soil, as is sometimes assumed

368    (see, for example, Worall et al. 1994). We observed no strong genetic linkage disequilibrium among

369    the SNP loci, consistent with populations evolving sexually, at least occasionally, in the last few

370    years (Halkett et al. 2005, Tibayrenc et al. 1991). Furthermore, a long history of clonal evolution

371    should produce a Meselson effect, with allelic divergence leading to high levels of heterozygosity

372    within genotypes (e.g. Ali et al. 2014). Such an effect is inconsistent with the observed level of

373    homozygosity in this study, which was not significantly different from that expected. In addition,

374    the steady decrease in genetic relatedness with increasing spatial distance between the pairs of

375    isolates observed on the spatial correlogram is consistent with the isolation by distance model, and

376    the local dispersal of basidiospores, producing new genotypes. We cannot rule out the possibility

377    that basidiospores can also fuse with some genotypes already present in the stand, via Buller's

378    phenomenon, to produce new genotypes (Rizzo & May 1994). However, even if this process has

379    been described in the laboratory, there is no evidence to suggest that it occurs frequently in natural

380    conditions. The presence of these recombining genotypes in this French population suggests that

381    sexual reproduction is a key process in the population dynamics of *Armillaria* species. A similar

382    genetic IBD pattern was detected for an *A. mellea* population in California (Travadon et al. 2012),

383    and the authors also concluded that the local dispersal of basidiospores played an important role in

384    the observed spatial genetic structure. By contrast, although numerous genotypes were observed, no

16

385    IBD pattern was identified for *A. cepistipes* at nationwide scale in Switzerland (Heinzelmann et al.

386    2011), and a similar lack of IBD was also observed by Prospero et al. (2008) for *A. ostoyae* at the

387    scale of this French forest region. These conflicting results for IBD pattern highlight the importance

388    of choosing an appropriate spatial scale for inferences concerning the spatial dispersal of progenies

389    based on population genetic methods. If the spatial scale is too large relative to mean dispersal

390    distance, the spatial genetic structure may be missed due to the complex patterns associated with the

391    random effects of rare long-distance dispersal events, and genetic methods may not detect these

392    processes very efficiently (e.g. Schwartz &  McKelvey. 2009, Wingen et al. 2007). If the spatial

393    scale is too small relative to the mean dispersal distance, there may be too few comparisons

394    between genotypes, which may constitute a serious limitation to the detection of spatial genetic

395    structures, especially after removal of the clonal structure (e.g. Dutech et al. 2008). For example,

396    the use of too few comparisons may account for the random structure observed in a North-

397    American population of *A. ostoyae*, in which only a few transects of a few hundred meters in length

398    were studied (Dettman & Van der Kamp 2001b).

399

400    The IBD pattern observed in this study was evenly distributed over the site, as sPCA revealed

401    several spatial clusters of related genotypes with a diameter of about 1 km. This patchy genetic

402    structure is consistent with an IBD pattern (Jombart et al. 2008), and a dispersal of the sexual spores

403    over the spatial range of a few kilometers. This result suggests that there was no significant genetic

404    structure, such as a genetic cline or barrier, other than IBD at this spatial scale. Spatial Bayesian

405    clustering analyses detecting no significant genetic cluster also yielded similar conclusions (results

406    not shown). Assuming that the *A. ostoyae* population is close to the genetic drift-dispersal

407    equilibrium, then the genetic structure observed in this study can be attributed principally to IBD,

408    and gene flow can be estimated from the slope of the spatial autocorrelation (Rousset et al. 2000,

409    Vekemans & Hardy 2004). In the coastal area, the assumption of genetic equilibrium for *A. ostoyae*

17

410   populations is realistic, because maritime pines were present for many thousands of years before the

411   establishment of the large pine plantations in the 19th century (Paquereau 1964). Therefore, with an

412   estimated generation time of between 10 and 20 years (Labbé 2015), several hundreds of

413   generations of the fungus have probably developed in this area. Furthermore, simulations have

414   shown that the IBD pattern rapidly becomes established at fine spatial scales after tens of

415   generations for small effective population sizes, such as that estimated here (Bradbury & Bentzen

416   2007, Leblois et al. 2003). Assuming dispersal-drift equilibrium, we tried to estimate the second

417   moment of parent-offspring dispersal, using several estimates of the effective density of parents.

418   The method used to estimate this density yields robust estimates in isolated and panmictic

419   populations, but has some biases when applied to continuous populations with an IBD structure

420   (Neel et al. 2013). For example, when the sampling window is larger than the breeding window (i.e.

421   "the local area where most matings occur", Neel *et al*. 2013), the population size may be

422   underestimated for the sample. This estimated population size is often only one tenth the effective

423   population size of the sampled area (Neel et al. 2013). Finally, we obtained estimated densities

424   close to 0.3 parents/ha for the two types of sampling (i.e. total sample and subsamples), and close to

425   the number of sampled genotypes. These similar results suggest that this estimate for this *A. ostoyae*

426   population is relatively robust, and that the contribution of sexual reproduction is similar for each of

427   the 149 genotypes sampled in this area. Only a small number of studies have reported estimates of

428   effective population size for fungal populations. Ali et al. (2014) obtained an estimate of between

429   30 and 40 individuals for several Pakistani *Puccinia* populations, based on temporal genetic

430   sampling. These results, which are similar to our own, indicate that effective population size may

431   not always be as important as frequently assumed (e.g. McDonald & Linde 2002). Using this

432   estimate of effective population size and the iterative procedure, we obtained a second moment of

433   parent-descendant dispersal distance of between 100 and 800 m. This spatial range of dispersal is

434   consistent with direct estimates of the mean dispersal distances for spores obtained for other fungal

435   species and by direct methods (e.g. Rieux et al. 2014). All these results strongly suggest that long-

436   dispersal events do occur occasionally, as reported for many other fungal species (Barrès *et al*.

437   2008), but that spore dispersal is mostly local (over a few hundred meters). They also suggest that

438   adaptation for many biological traits may be local.

439

440   It is generally not possible to draw any firm conclusions about the effect of basidiospore

441   dispersal on the genetic structure of *A. ostoyae* populations from direct observations (Baumgartner

442   et al. 2011). By contrast, our study clearly shows that, for this species, as for many other fungi, an

443   analysis of spatial genetic structure can provide estimates of both sexual reproduction within

444   populations and the mean distance over which basidiospores disperse. However, we found no

445   evidence of the significant spatial genetic structure observed for this species at larger spatial scales

446   in this forest area (Prospero et al. 2008, Labbé 2015, Dutech et al. 2016). Our results are similar to

447   those obtained for maritime organisms, for which non-linear patterns of IBD have been observed,

448   depending on the spatial scale studied (Bradbury & Bentzen 2007). These studies have revealed

449   that, for organisms with large spatial distributions, such as many fungal species, limited dispersal

450   between the progenies and their parents is often estimated at fine spatial scales, but not at large

451   spatial scales. Rather than being associated with high levels of gene flow that are difficult to

452   estimate with genetic methods at large spatial scales (Wingen et al. 2007), these contrasting results

453   reveal that choosing the most appropriate spatial scale of investigation is crucial to the correct

454   estimation of dispersal processes by genetic methods, as pointed out by Rousset (1997).

455   Conclusions about long-distance spore dispersal may, therefore, often be incorrect for many fungal

456   species if only genetic differentiation between populations is considered. We advocated estimating

457   dispersal at different spatial scales, to avoid misleading conclusions likely to hinder our

458   understanding of fungal biology.

459

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621

622    **Figures**

623

624    **Figure 1: Relationships between the kinship coefficients of pairs of *A. ostoyae* isolates and the**

625    **distance (in m) separating them**

626    Square symbols represent the kinship coefficients estimated using all the isolates, and triangular

627    symbols represent those estimated from only one copy per genotype (i.e. clonal correction). The

628    light gray area corresponds to 95% of the expected kinship coefficient assuming a random spatial

629    genetic structure for all isolates considered, and the dark gray area corresponds to that based on

630    only one copy per genotype.

631

632 **Figure 2: Geographic distribution of individual scores on the first positive axis of the sPCA**

633 **for the *A. ostoyae* population of south-western France**

634 Positive and negative values are shown in black and white, respectively, and the size of the squares

635 is proportional to their absolute value. The eigenvalues of each sPCA axis are presented at the

636 bottom left of the figure.
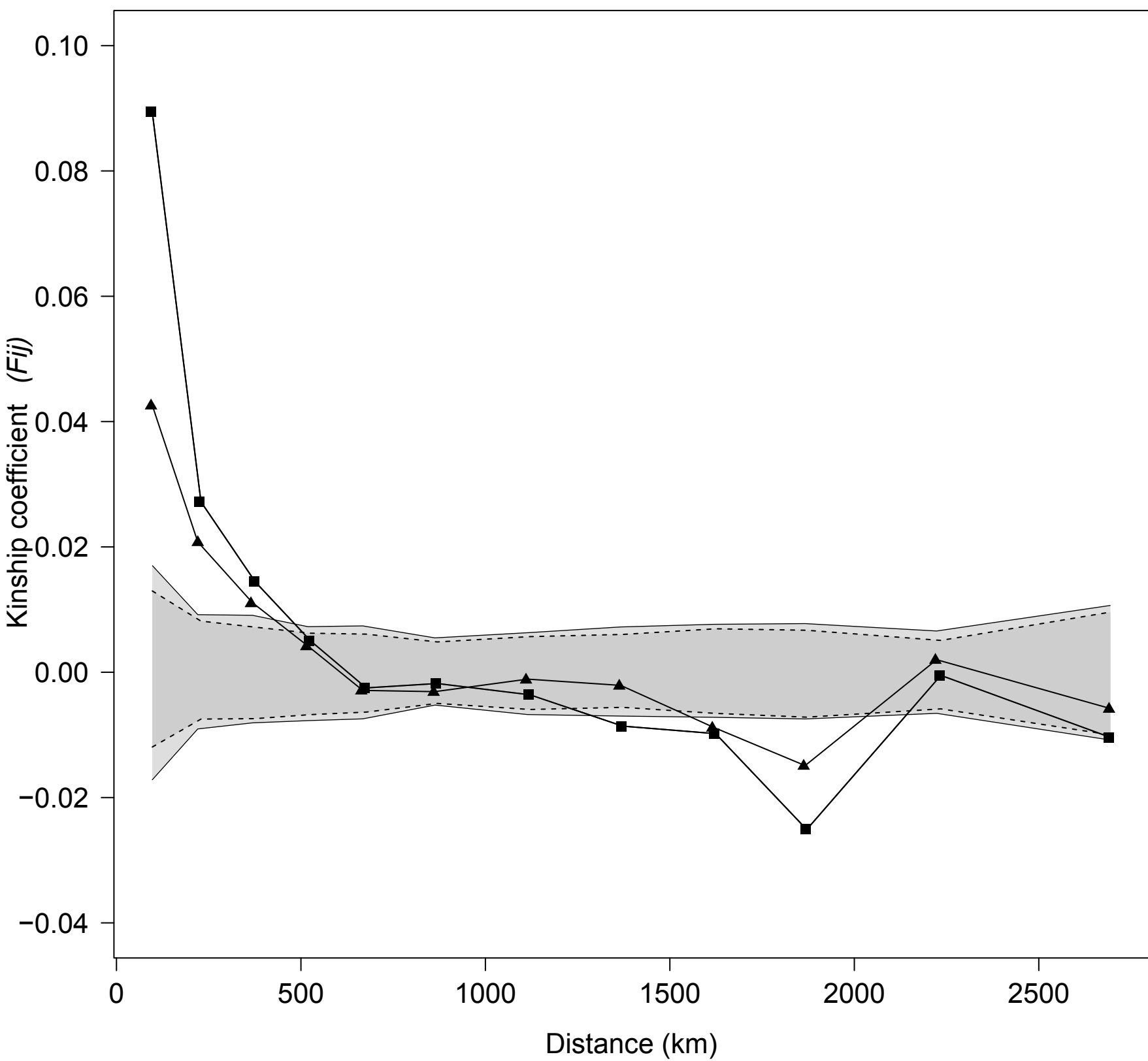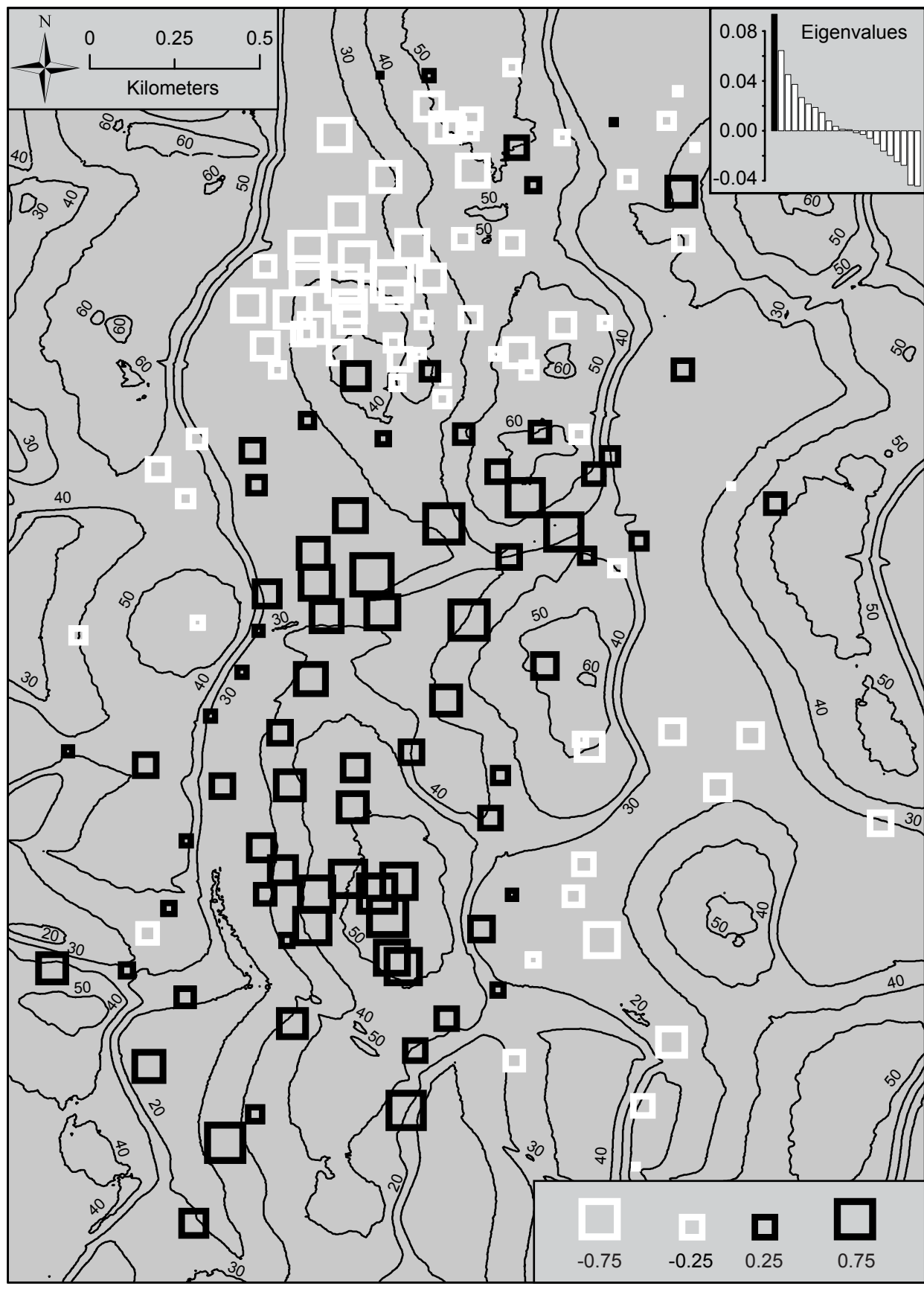
**TABLE 1: Details of the 27 single nucleotide polymorphisms (SNPs) analyzed in the *A. ostoyae* population sampled in the south-western French forest of St Julien en Born.**

| Gene | SNP_ID | 2nd-PCRP | 1st-PCRP | Lenght | SNP | He | Fis | P-val |
|------|--------|----------|----------|--------|-----|-----|-----|-------|
| FG487 | FG487_1 | ACGTTGGATGTCCCAAAGAACAGGGTTGTC | ACGTTGGATGAGCACTGCTAACACGGATAG | 103 | G/A | 0,364 | -0,125 | 0,174 |
| FG524 | FG524_3 | ACGTTGGATGTTCCTATGATGTCCAGGTA | ACGTTGGATGTTCGGTCAAACGAGCGAATC | 95 | A/G | 0,420 | -0,061 | 0,553 |
| FG529 | FG529_1 | ACGTTGGATGGAACGTCATCATCAAGTGGC | ACGTTGGATGAAAGCTCTTCCAGTCTTCCC | 117 | C/T | 0,420 | **0,253** | **0,008** |
| FG543 | FG543_5 | ACGTTGGATGACACCTCTGCAAAGAAAACC | ACGTTGGATGGCTGCCAAGGACTACGGTG | 113 | G/A | 0,298 | 0,053 | 0,579 |
| FG652 | FG652_1 | ACGTTGGATGTGTATCCCATCTTCATAAC | ACGTTGGATGAATTTGTTCACCCCCCATCG | 108 | C/T | 0,493 | -0,015 | 0,866 |
| FG686 | FG686_1 | ACGTTGGATGCAATCGTCTCGATCGCACTC | ACGTTGGATGGACAAGGTCAGTATTGGCAG | 120 | T/C | 0,243 | -0,050 | 0,739 |
| FG691 | FG691_1 | ACGTTGGATGTCCGGTAGCTTGTCAGTAAC | ACGTTGGATGAACCTCGAATCTGCTCGCAC | 117 | C/T | 0,501 | 0,096 | 0,411 |
| FG692 | FG692_2 | ACGTTGGATGCTAGGCTTCGAGTATTCGAG | ACGTTGGATGTGCGTCAGAATTTCTCGTCC | 100 | A/G | 0,497 | 0,029 | 0,738 |
| FG698 | FG698_3 | ACGTTGGATGCTTCGTATGCTACCGTTGTC | ACGTTGGATGCGTGATAAAGAATCGGCCTC | 100 | A/G | 0,466 | -0,050 | 0,596 |
| FG716 | FG716_1 | ACGTTGGATGTTGCTGGGGAAGACGGAAGT | ACGTTGGATGATATGTCTGCGGCTCAATGG | 112 | C/T | 0,303 | 0,140 | 0,101 |
|  | FG716_8 | ACGTTGGATGATTGAGCCGCAGACATATGG | ACGTTGGATGTACGGGTTTTTCCAATCCGC | 118 | A/G | 0,368 | -0,013 | 1,000 |
| FG730 | FG730_3 | ACGTTGGATGCAGAAGAATGTCTACCTCAG | ACGTTGGATGAAACATACTCCCACCTCTCC | 112 | C/T | 0,304 | 0,043 | 0,591 |
| FG735 | FG735_1 | ACGTTGGATGAGATGTCTCCGACGATGTTC | ACGTTGGATGAATAGCTGCACGATTCGAGG | 116 | C/T | 0,483 | -0,151 | 0,083 |
| FG747 | FG747_4 | ACGTTGGATGGCTACGGACATCCTCGATAT | ACGTTGGATGATTGTAGCCCAGGGATCCCA | 101 | T/C | 0,288 | -0,064 | 0,569 |
| FG756 | FG756_1 | ACGTTGGATGTAAACATCGAAGCCGCCTTG | ACGTTGGATGGCAGCGAGTGTGCAGAAAAC | 101 | C/T | 0,501 | 0,016 | 0,869 |
| FG762 | FG762_5 | ACGTTGGATGTGGTATCGCTATCGCTGGTC | ACGTTGGATGTACTGCTCCTAGCTTGAGAA | 118 | C/T | 0,386 | 0,043 | 0,667 |
| FG771 | FG771_1 | ACGTTGGATGGTCGAACCGTATTGTTACCG | ACGTTGGATGTAGGCTTCCAGCTACCATCG | 100 | C/G | 0,305 | -0,003 | 1,000 |
|  | FG771_3 | ACGTTGGATGACAAACTGCAATCGCGCTAC | ACGTTGGATGATTTGCTGTTGCGAGTGGTG | 108 | T/C | 0,478 | -0,011 | 1,000 |
| FG788 | FG788_1 | ACGTTGGATGACCCCGTAGCTGTTTCAGAC | ACGTTGGATGGGTCTGGACGATCGGACTTTG | 91 | C/T | 0,084 | -0,043 | 1,000 |
| FG848 | FG848_1 | ACGTTGGATGTTGACCATCGTATCGAGACC | ACGTTGGATGCGATTCTCTTGAGTCCCTAC | 99 | G/A | 0,388 | -0,108 | 0,209 |
|  | FG848_6 | ACGTTGGATGCAGCCTTGAATTTGCCAATG | ACGTTGGATGGCCATTCGCGTGACTGATCT | 114 | T/C | 0,254 | -0,064 | 0,743 |
| FG893 | FG893_1 | ACGTTGGATGTCTTGCGTTTTGCTAAGAGG | ACGTTGGATGAAACGAGCAGCCAACTCTTG | 116 | C/T | 0,362 | 0,030 | 0,819 |
| MS334 | MS334_3 | ACGTTGGATGAAACCAAGCATCGCGTCCTC | ACGTTGGATGAGTGAATGTTCAAGGCCAGC | 108 | C/T | 0,413 | -0,006 | 1,000 |
| MS441 | MS441_2 | ACGTTGGATGTCTAACTGGAGTGGCGGTGT | ACGTTGGATGATCAGTATCCAAAAGAAG | 118 | A/G | 0,303 | 0,049 | 0,583 |
| MS452 | MS452_3 | ACGTTGGATGTCCCAGAAATGAACCGAACC | ACGTTGGATGCGTTGTTGATGTCAACCCAG | 95 | T/C | 0,351 | 0,020 | 0,816 |
| MS467 | MS467_8 | ACGTTGGATGGCCAGTGGAACGAACCTAAA | ACGTTGGATGAGTTCTTCCGCAAGGTGCGA | 100 | C/A | 0,421 | **0,452** | **0,000** |
| MS481 | MS481_1 | ACGTTGGATGCTATCGATCAAGCACGACAC | ACGTTGGATGCGCGCGTTAATTGAGAAACC | 99 | C/T | 0,046 | -0,021 | 1,000 |

Gene and SNP_ID (the ID of the analyzed SNP for each gene) refer to the gene and SNP identification given in Dutech et al. (2016). 1rst- and 2ndPCRP refer to the primers used for the Sequenom PCR reaction for each SNP. Length and SNP are respectively the size of the PCR product and the type of nucleotide variation. He and Fis are the gene diversity (Nei, 1987) and the intra-individual genetic fixation index respectively. P-val are the probabilities of the exact test for Hardy-Weinberg expectations (Raymond & Rousset 1995). Values significantly different from a panmictic population are in bold.