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**Towards unravelling the Rosette agent enigma: Spread and emergence of the co-invasive host-pathogen complex, *Pseudorasbora parva*-*Sphaerothecum destruens***

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50 **Abstract**

51 The emergence of non-native fungal pathogens is a growing threat to global health,  
52 biodiversity, conservation biology, food security and the global economy. Moreover, a  
53 thorough understanding of the spread and emergence of pathogens among invasive and  
54 native host populations, as well as genetic analysis of the structure of co-invasive host  
55 populations, is crucial in terms of conservation biology and management strategies. Here we  
56 combined extensive catchment sampling, molecular detection tools and genomic signatures  
57 to i) assess the prevalence of the rosette agent *Sphaerothecum destruens* in invasive and  
58 native fish populations in contrasting french regions, and ii) characterize the genetic diversity  
59 and population structure of its co-invasive and asymptomatic carrier *Pseudorasbora parva*.  
60 Although *S. destruens* was not detected in all the fish collected its presence in contrasting  
61 freshwater ecosystems suggests that the disease may already be widespread in France.  
62 Furthermore, our results show that the detection of *S. destruens* DNA in its asymptomatic  
63 carrier *P. parva* is still limited. Finally, we found that *P. parva* populations show a  
64 homogeneous genetic and geographical structuring, which raises the possibility of the  
65 occurrence of successive introduction events in France from their native and invasive range.

66

67 **Keywords:** Biological invasions, fungal pathogens, aquatic disease emergence, population  
68 genomics

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78 **Introduction**

79 Species dispersal and habitat colonization are key ecological processes for maintaining  
80 species diversity and population persistence (Hubbell, 2001; Lande et al., 2003). However,  
81 the dramatic increase in species translocations and biological invasions over the last century  
82 due to human activities (Lawler et al., 2006), such as the increase in global trade and  
83 transportation networks, has led to a global redistribution of biodiversity (including  
84 pathogens) (Ricciardi, 2007) as well as an irrevocable loss of ecosystem functions (Chapin et  
85 al., 2000; Al-Shorbaji et al., 2016), the decline in wildlife biodiversity (Gozlan et al., 2006)  
86 and the emergence of diseases in livestock and wildlife populations worldwide (Daszak et  
87 al., 2000; Woolhouse et al., 2005; Gozlan et al., 2005; Jones et al., 2008; Lymbery et al.,  
88 2014).

89 Emerging fungal pathogens pose a growing threat to global health, ecosystems, food  
90 security and the world economy (Fisher et al., 2012). The introduction and spread of non-  
91 native fungal pathogens is a serious threat to biodiversity and a major concern for  
92 conservation biology. Indeed, the co-introduction of a host and a pathogen can dramatically  
93 affect the invaded ecosystem through biodiversity losses, destabilization of foodwebs and  
94 the spread of emerging infectious diseases to native host species (Stiers et al., 2011;  
95 Gallardo et al., 2016; Crowl et al., 2008; Hatcher et al., 2012). These co-introductions of non-  
96 native host-pathogen systems can also create new host-pathogen interactions. For example,  
97 if native species are susceptible to the pathogen, the pathogen may become a powerful  
98 asset allowing the invading host to win competition for niche space (Price et al., 1986). This  
99 is illustrated by the co-introduction in the UK of squirrel pox virus and the invasive grey  
100 squirrel (*Sciurus carolinensis*), its asymptomatic carrier, which resulted in high mortality of  
101 the native red squirrel (*S. vulgaris*) and accelerated its replacement by the invader  
102 (Tompkins et al., 2003). Similarly, the extinction of the native white-clawed crayfish  
103 (*Austropotamobius pallipes*) caused by crayfish plague is the result of the co-introduction of  
104 the fungus *Aphanomyces astaci* and its healthy carrier crayfish (*Pacifastacus leniusculus*)

105 into Europe (Holdich & Pöckl, 2007). In addition, to understand the processes leading to  
106 successful invasion, a thorough understanding of the spread of pathogens and their  
107 virulence among invasive and native host populations, as well as genetic analysis of the  
108 structure of co-invasive host population, is crucial in terms of conservation biology and  
109 management strategies.

110 Among the well-known invasions of host and pathogen, the rise of the rosette agent in  
111 Europe remains an epidemiological enigma (Combe & Gozlan, 2018). Indeed, there is still a  
112 discrepancy between the magnitude of observed and predicted fish mortalities at specific  
113 sites where *S. destruens* was introduced and the low or absent level of monitoring and  
114 reporting of *S. destruens* across Europe (Combe & Gozlan, 2018). The topmouth gudgeon  
115 *Pseudorasbora parva*, a small freshwater cyprinid fish native to East Asia (East China,  
116 Taiwan, Korea and Japan), has been the fastest fish invasion worldwide (Gozlan, 2012;  
117 Zhang & Zhao, 2016). In particular, *P. parva* is also the asymptomatic carrier of the rosette  
118 agent (*Sphaerothecum destruens*), a generalist parasite, so far phylogenetically located at  
119 the fungal-animal boundary, which has proven to be highly virulent to many native European  
120 fish species and responsible for major ecological and economic impacts worldwide (Gozlan  
121 et al., 2005; Sana et al., 2017; Combe & Gozlan, 2018). Their co-introduction into European  
122 countries is the result of accidental translocations from their native range via aquaculture  
123 trade of Chinese carps between China and former USSR countries (Gozlan et al., 2010).  
124 Then, during the 1960s multiple introductions of *P. parva* took place around the Black Sea,  
125 followed by further introductions in the 1980's in Eurasia and North Africa (reviewed in  
126 Combe & Gozlan, 2018). Following these initial human-mediated introductions, natural local  
127 spread took place across the major European rivers to the Middle-East. While previous  
128 studies suggested that European populations of *P. parva* were introduced from two of four  
129 distinct native lineages (Sana et al., 2017; Hardouin et al., 2018; Combe & Gozlan, 2018),  
130 we recently found that the current genetic clustering of native *P. parva* range was shaped by  
131 waves of gene flow from population in southern and northern China and that the invasive  
132 genetic diversity was the result of multiple invasion pathways of already admixed native

133 populations (Brazier et al., 2021). In France, *P. parva* has been observed for the first time in  
134 northwestern rivers in 1978-1979 (Allardi & Chancerel, 1988; Poulet et al., 2011), and *S.*  
135 *destruens* has been detected in asymptomatic populations of topmouth gudgeon in  
136 Southwestern France (Charrier et al., 2016) and has been suspected of being responsible  
137 for two episodes of mortality of brown and rainbow trout in farms and experimental facilities  
138 in western France (Boitard et al., 2017). As *P. parva* is now reported in almost all french  
139 regions, including the island of Corsica, the potential spread and virulence of the rosette  
140 agent in native and invasive fish populations represent a major ecological, health and  
141 economic issue for aquatic biodiversity and fish conservation. For example, its co-  
142 introduction into a catchment area in southeast Turkey almost led to the total extinction (up  
143 to 80%-90% of mortalities) of native fish populations of conservation or economic importance  
144 such as sea bass (Ercan et al., 2015; Combe & Gozlan, 2018). However, to date, the extent  
145 of distribution of *S. destruens* in any country and the genetic structuring of co-invasive host-  
146 pathogen populations have not yet been identified in relation to their native range and to  
147 other invasive European countries. This information would be of primary importance to  
148 assess whether there is a risk of disease emergence in wild and farmed fish populations at a  
149 country level and to better understand the historical connections between the different host-  
150 pathogen.

151 Here we combined catchment-scale sampling, molecular detection tools and genomic  
152 signatures to i) assess the presence and prevalence of *S. destruens* in invasive and native  
153 fish populations at a country level, and ii) characterize the genetic diversity and population  
154 structure of co-invasive *P. parva* populations to ascertain whether they constitute a  
155 heterogeneous genetic pool derived from admixed native populations that could have major  
156 implications for the genetic diversity and virulence of the pathogen.

157

## 158 **Materials & Methods**

### 159 **Sampling**

160 Fish sampling was conducted in France between March and November in 2017, 2018 and  
161 2019 in collaboration with seven French Department Angling Associations, such as  
162 Fédération Départementale de Pêche et de Protection du Milieu Aquatique FDPPMA de l'Ain  
163 (Ain), FDPPMA de l'Indre (Indr), FDPPMA de Haute-Savoie (HSAv), FDPPMA de Seine-  
164 Maritime (Smar), FDPPMA de Gironde (Gir), FDPPMA des Pyrénées-Atlantiques (PyrA),  
165 FDPPMA de Loire-Atlantique (LoirA); the Provence Alpes Côte d'Azur regional direction of  
166 the French Biodiversity Agency (OFB) (Cors); and two research institutes: the Tour du Valat  
167 (Brhon) and INRAE (Vauc). For each angling association, specific considerations were  
168 followed such as conservation, target species and sensible sites. Fish were collected using  
169 nets, traps or even electrofishing. Sampled fish were immediately euthanized with an  
170 anesthetic (120 mg/L benzocaïne) and dissected either at the sampling site or at the  
171 Laboratory des Pyrénées et des Landes (LPL). Ten freshwater sites, five in northern France  
172 and five in southern France, were selected. At each sampling site, 50 individuals of *P. parva*  
173 and other native fish species corresponding to a total of 100 individuals were collected and  
174 analyzed at the LPL, although for some sites we could not reach this exact number of  
175 individuals. For all fish, liver, kidney and spleen were collected and preserved in 70%  
176 ethanol to test for the presence of the parasite *S. destruens* and the prevalence of the  
177 disease. For each *P. parva*, fin clips were collected, preserved in 70% ethanol and used to  
178 assess the genetics of the host population.

179

## 180 **DNA extractions**

181 Total DNA was extracted from internal organs (kidney, liver and spleen) previously pooled  
182 from all individuals belonging to the same species and sampling site. Two commercial kits,  
183 selected on the basis of their column capacity, were used according to the weight of the  
184 organs collected and therefore the size of the fish. The NucleoSpin® Tissue kit (Macherey-  
185 Nagel) was used for weights up to 25 mg while the NucleoSpin® Soil kit (Macherey-Nagel)  
186 was used for higher weights up to 250 mg. Prior to extraction, samples were ground in lysis  
187 buffer using a tissue homogenizer (Precellys® 24, Bertin). Extractions were performed



188 according to the manufacturer's instructions for both kits. A negative control was added (kit  
189 reagents without sample) for each set of extractions to ensure that the kit reagents were not  
190 contaminated.

191

## 192 **Real-time quantitative PCR**

193 *Primers and probe design.* The primers and probes for the real-time qPCR for the detection  
194 of *S. destruens* were designed from the 18S rRNA gene sequences obtained from GenBank.  
195 The accession numbers for the *S. destruens* sequences were: AY267344, AY267345,  
196 AY267346 and FN996945. Sequences of closely related organisms such as *Dermocystidium*  
197 *salmonis* (U21337), *Dermocystidium spp.* (U21336), *Amphibiocystidium ranae* (AY550245),  
198 *Rhinosporidium seeberi* (AF118851) and *Ichthyosporea* (KM213082) were aligned with the  
199 18S sequences of *S. destruens* in order to find regions specific to *S. destruens*, using Clustal  
200 Omega (Sievers et al., 2011). Primers and probe were then designed using Primer3  
201 software (Koressaar & Remm, 2007; Untergasser et al., 2012) and synthesized by Eurofins  
202 Genomics, with the following sequences: forward primer (SdACH F) 5'-  
203 ACCGCCCGTCGCTACTAC-3', reverse primer (SdACH R) 5'-AACTTTTCGGCAGCCTCAC-  
204 3' and probe (SdACH P) 5'-(FAM)-TGGCCCTGTACCG-(MGBEQ)-3'.

205 *TaqMan qPCR reaction.* Amplification reactions were performed in a QuantStudio™ 5 Real-  
206 Time PCR System (ThermoFisher Scientific). The qPCR reaction mixes (25 µL) consisted of  
207 12.5 µL of GoTaq® Probe qPCR Master Mix (Promega), 5 µL of DNA template, and primers  
208 and probe at the same final concentration of 200 nM each. Water was added up to 25 µL.  
209 Cycling conditions were as follows: 95°C for 2 min, then 40 cycles of 95°C for 15 sec, 60°C  
210 for 1 min. A negative control (5 µL of Nuclease-free water instead of DNA template) was  
211 added at each qPCR run.

212 *Specificity of the qPCR assay.* The specificity of this new qPCR design was determined  
213 using DNA extracts of various fish pathogens, obtained from collections or isolated in the  
214 LPL laboratory from naturally infected fish and identified by MALDI-TOF mass spectrometry  
215 (Bruker Daltonics) (see Supplementary Table S1). DNA was extracted from pure cultures

216 using the NucleoSpin<sup>®</sup> Tissue kit (Macherey-Nagel) following the manufacturer's  
217 recommendations. For Ichthyosporea species related to *S. destruens* (*Dermocystidium*  
218 *salmonis*, *Dermocystidium spp.*, *Rhinosporidium seeberi* and *Amphibiocystidium ranae*),  
219 synthetic genes (Eurofins Genomics) containing the targeted sequences of 18S rRNA were  
220 synthesized and freeze-dried plasmids were resuspended in 1 mL of 10 mM Tris-HCL pH 8  
221 and used in qPCR assays. The DNA concentrations used for this specificity test were 100  
222 ng/ $\mu$ L for all extracts and  $10^6$  copies for the plasmids.

223 *qPCR assay performance.* Our protocols are based on the french PCR standard for animal  
224 health NFU47-600. A synthetic gene (Eurofins Genomics) containing the *S. destruens* 18S  
225 rRNA sequence targeted by our TaqMan assay was synthesized to determine qPCR assay  
226 performance criteria such as efficiency, slope, intercept and linear regression coefficient ( $r^2$ ).  
227 Standard curves were generated from 10-fold serial dilutions in 10 mM Tris-HCl, pH 8 of the  
228 plasmid DNA from  $10^6$  to 1 copy used as PCR template, allowing the determination of the  
229 limit of quantification (LOQ) and the identification of the gene copy number. The LOQ is the  
230 cut-off point below which three replicates of CT-values tend to disperse one from each other  
231 or are indeterminate. The standard curves were performed on three separate days (see  
232 Supplementary Fig. S1). The threshold values (CT) were plotted against the corresponding  
233 gene copy numbers.

234 *Limit of Detection (LOD).* The french PCR standard for animal health NFU47-600 states that  
235 the LOD should be determined by 6 serial dilutions from the identified LOQ (here LOQ = 100  
236 copies) with 8 replicates of each dilution, repeated three times independently. Considering  
237 the 95% confidence interval, the LOD should correspond to the number of plasmid copies  
238 giving 23/24 positive results. For this purpose, the synthetic *S. destruens* 18S rRNA gene  
239 was diluted by half from the solution corresponding to 100 copies (LOQ) to 3.125 copies to  
240 identify the limit of detection (LOD). In total of six dilutions (100, 50, 25, 12.5, 6.25 and  
241 3.125) were tested in a first experiment, which consisted of serial dilutions in 10 mM Tris-  
242 HCl, pH 8. In a second experiment three serial dilutions (50, 25 and 12.5) were performed in  
243 an uninfected fish DNA solution at 100 ng/ $\mu$ L to further determine the LOD (see

244 Supplementary Table S2). The fish DNA solution was obtained from the EPC (epithelioma  
245 papulosum cyprini ATCC® CRL-2872™) cell line after DNA extraction using the NucleoSpin®  
246 Soil kit (Macherey-Nagel).

247

#### 248 **Disease Prevalence**

249 Each individual sampled was tested by qPCR assays (kidney, liver, spleen being pooled  
250 together), thus indicating how many individuals were found infected out of the total number  
251 of individuals sampled. The prevalence of the disease was calculated as follows: (number of  
252 positive individuals / total number of individuals tested) × 100.

253

#### 254 **GBS sequencing**

255 A total of 420 DNA samples (410 individuals in total, with 10 individuals replicated on the  
256 sequencing lanes) were genotyped for single nucleotide polymorphism (SNP) markers by  
257 first digesting the genomic DNA with PstI and then genotyping-by-sequencing (GBS),  
258 resulting in an average of 2,225,123 (±1,108,497) raw sequencing reads per sample (Table  
259 1; see Supplementary Information). Native samples from Brazier et al. (2021) supplemented  
260 with individuals from Taiwan were also included in the subsequent analysis to place the  
261 french population back into the native range (see Supplementary Table S3). SNP calling was  
262 performed using the denovo\_map.pl pipeline implemented in Stacks (v2.55) (Catchen et al.,  
263 2013). The same combination of parameters used by Brazier et al. (2021) was applied  
264 (ustacks; m=10, M=3, maximum of 2 loci per stack and cstacks; n=3). A minimum read  
265 depth of 20 was required for each marker. To avoid bias due to large proportions of missing  
266 data and maintaining a large sample size, loci with more than 45% missing data were  
267 removed from the dataset, resulting in 5028 validated SNP markers with an overall missing  
268 data rate of 36%. After trimming, the final dataset contained 409 french individuals from the  
269 sampled sites, with an average of 45 individuals per site (Table 1) and 300 individuals from  
270 the native range (Table S3).

271

## 272 **Genotype phasing and imputation**

273 Haplotype phasing and missing data were inferred for the 708 individuals analyzed using  
274 Beagle 5.1 (Browning & Browning, 2007; Browning et al., 2018). Beagle uses the localized  
275 haplotype-cluster model and applies an iterative approach to infer the most likely haplotype  
276 pair for each individual. At each iteration, phased input data is used to build a localized  
277 haplotype-cluster model. Once the model is constructed, the phased haplotypes of each  
278 individual are sampled from the induced diploid HMM, conditional on the individual's  
279 genotypes. The sampled haplotypes form the input for the next iteration, and so on. In the  
280 final iteration, the Viterbi algorithm selects the most likely haplotypes for each individual,  
281 conditional on the diploid HMM and the individual's genotype data. For each copy of each  
282 individual, missing alleles are randomly imputed according to allele frequencies, and the  
283 data for each individual are phased by randomly ordering the genotypes (Browning &  
284 Browning, 2007).

285

## 286 **Genetic diversity and population structure**

287 A hierarchical approach was adopted to study and place back the french *P. parva* diversity  
288 within the native range. First, the genotypes from France were analyzed to test whether the  
289 french group is a continuous homogeneous population or a structured population. Secondly,  
290 the analyses were performed at the level of the area of origin (France vs. native range).

291 Expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ), diversity ( $\pi$ ), private alleles  
292 ( $P_a$ ), variant sites ( $V_s$ ), inbreeding coefficient ( $F_{is}$ ) and population differentiation indices  $F_{st}$   
293 and  $\Phi_{st}$  on haplotype data were estimated by kernel-smoothed calculations with 1000  
294 bootstrap resamples using the populations program implemented in Stacks (v2.55) (Catchen  
295 et al., 2013). In addition to allele occurrence frequencies, pairwise  $\Phi_{st}$  analysis takes  
296 advantage of the evolutionary distance between alleles and provides insight into the patterns  
297 of relationships between populations (Holsinger & Weir, 2009).

298 The genetic distance between individuals based on dissimilarity was estimated by  
299 calculating the number of allelic differences between two samples using the bitwise.dist

300 function in the popprv2.9.1 package (Kamvar et al., 2014). To visualize the population  
301 structure, a minimum spanning network (MSN) clustering multilocus genotypes (MLG) by the  
302 previously estimated genetic distances was constructed using the popr.msn function  
303 (popprv2.9.1 package).

304 The relationship between individuals was inferred by an Identity-By-State (IBS) analysis  
305 through a genome-wide average IBS pairwise distances using the function snpgdsIBS from  
306 the SNPRelate package and followed by a hierarchical cluster analysis using the  
307 snpgdsHCluster function (SNPRelate Package). A multidimensional scaling analysis (MSA)  
308 based on IBS pairwise distances was also performed using the cmdscale function of the R  
309 stats package (v3.6.2) (R Core Team, 2020).

310 Principal component analysis (PCA) and discriminant analysis of principal components  
311 (DAPC) were used to estimate clustering first between the french groups, then between the  
312 french population and the native groups. The find.clusters algorithm was run with 10 K and  
313 ten repetitions for each value of K. The optim.a.score function was used to identify the  
314 optimal number of principal components to support the DAPC. The Bayesian information  
315 criterion was used to select the most likely number of genetic clusters. All these analyses are  
316 implemented in the adegenet package (v 2.1.3) (Jombart & Ahmed, 2011). The R-dependent  
317 analyses were run in R version 4.0.5 (R Core Team, 2020).

318 The fastSTRUCTURE program (Raj et al., 2014) was used to estimate the most likely  
319 number of genetic clusters K among the french and native groups. Structure\_threader (Pina-  
320 Martins et al., 2017) program was used to parallelize and automate fastSTRUCTURE runs.  
321 Both simple prior and logistic prior models were used for ancestry estimates, the latter being  
322 recommended for identifying subtle structure (Raj et al., 2014). K values ranging from 1 to 10  
323 were analyzed. The best value was evaluated in two ways: using the chooseK algorithm  
324 implemented in fastSTRUCTURE and the Puechmaille statistics adapted to uneven  
325 sampling (Puechmaille, 2016), implemented in the STRUCTURE SELECTOR web interface  
326 (Li & Liu, 2018). Then, CLUMPAK (Kopelman et al., 2015) implemented in STRUCTURE  
327 SELECTOR was used with the default options to summarize the results and represent

328 graphically the sample assignment probability for each K.

329

## 330 **Results**

### 331 **Validation of the qPCR assay for *S. destruens* in fish tissues**

332 The first step in validating the specificity of this new qPCR assay was to verify that  
333 amplification reactions for *S. destruens* DNA could not induce cross-amplification reactions  
334 of other fish pathogens. As this generalist parasite is now well known to infect a wide variety  
335 of fish species (Combe & Gozlan, 2018), we targeted bacterial strains infecting salmonids  
336 (Harrell et al., 1986; Hedrick et al., 1989; Arkush et al., 1998; Boitard et al., 2017) such as  
337 *Aeromonas salmonicida* subsp. *salmonicida*, *Renibacterium salmoninarum*,  
338 *Tetracapsuloides bryosalmonae*, *Flavobacterium psychrophilum* and *Yersinia ruckeri*, but  
339 also bacteria infecting a wider spectrum of fish species (*Photobacterium damsela* subsp.  
340 *damsela* and *Photobacterium damsela* subsp. *piscicida*) as well as closely related species  
341 (18S rRNA synthetic gene from *Amphibiocystidium ranae*, *Rhinosporidium seeberi*,  
342 *Dermocystidium* spp., *Dermocystidium salmonis* and other *Ichthyosporidia* spp.) and showing  
343 95% sequence identity with the 18S rRNA targeted gene from *S. destruens* when subjected  
344 to NCBI BLAST analysis. All DNA extracts tested and the negative control resulted in no  
345 amplification, while DNA from the North American isolate RA-1 (ATCC® 50643™) of *S.*  
346 *destruens* produced efficient qPCR amplification ( $C_T = 20.63$ ) (Table S1).

347 Next, qPCR performance (efficiency, slope, intercept and linear regression correlation) was  
348 assessed using 10-fold serial dilutions of a plasmid solution harboring the *S. destruens* 18S  
349 rRNA gene sequence ranging from  $10^6$  copies to 1 copy. Three separate qPCR runs were  
350 performed to validate the reproducibility of the results. Each standard curve was obtained by  
351 plotting the threshold values ( $C_T$ ) against the corresponding copy numbers. Here the linear  
352 range of this plot was validated over the range of  $10^2$  to  $10^6$  copies. The limit of quantification  
353 (LOQ), defined by the smallest amount of analyte that can be quantified, corresponds here  
354 to  $10^2$  copies (mean  $C_T$  value = 33.6) (Fig. S1). The PCR amplification efficiencies obtained

355 were 102.96, 99.13 and 98.31% and were calculated using the following formula:  $E = (10^{(1/\text{slope})} - 1)$ , using the slope values for each standard curve. For each assay, the intercepts are  
356 also shown (b) with the linear regression correlation ( $R^2$ ), which are very close to 1 in all  
357 cases. All these criteria demonstrate a good performance of qPCR and validate the use of  
358 this new tool for the detection of *S. destruens* DNA in fish tissues.

360 By definition, the limit of detection (LOD) is the smallest amount of analyte in a sample that  
361 can be detected with a 95% confidence interval. To determine this LOD, it is necessary to  
362 test different concentrations of plasmids with at least 8 technical replicates per dilution and to  
363 repeat the experiment on three separate days. Thus, each dilution tested gave 24 results.  
364 Given the 95% confidence interval, LOD should correspond to the plasmid copy numbers  
365 giving 23/24 positive results. LOD was estimated to be between 25 (24/24 positive results)  
366 and 12.5 (20/24 positive results) copies of the target 18S rRNA (Table S2). In order to  
367 resolve the exact gene copy number that corresponds to the LOD and to more accurately  
368 assess the exact qPCR conditions, another set of assays was carried out by replacing the  
369 Tris buffer with uninfected fish DNA extract (Table S2). In this second assay, the 25 copies  
370 dilution gave 23/24 positive results and is therefore considered to represent the LOD of *S.*  
371 *destruens* qPCR system. These parameters were taken into account when analyzing the  
372 samples by differentiating between *S. destruens* positive samples that are detected and  
373 quantifiable ( $C_T < LOQ$  ( $C_T$  33.6) or detected but not quantifiable ( $C_T > LOD$  (25 DNA copies)  
374 but  $C_T > LOQ$  ( $C_T$  33.6)).

375

### 376 **Detection of *S. destruens* in native and invasive fish samples in France**

377 While fish sampling was spread out between 2017-2019, all angling associations sampled  
378 during the same period of the year, between March and November. Whilst May-September  
379 represents the optimal period for proliferation, transmission and thus detection of *S.*  
380 *destruens* (Ercan et al., 2015), we acknowledge that sampling in March, April, October and  
381 November may have impacted the efficiency of *S. destruens* detection and thus lead to an  
382 underestimation of the prevalence of this parasite. Only the angling associations of Ain,

383 Cors, Gir, Indr, HSAv, Smar sent samples strictly following the protocol detailed above. In  
384 some cases, either fewer *P. parva* individuals or fewer native fish species were sampled and  
385 therefore analyzed (Table 2). *S. destruens* DNA of *S. destruens* was detected in fish  
386 samples collected in Ain, Indr, Cors, Brhon and Gir, indicating thus a wide spread of this  
387 parasite across freshwater rivers in France (Fig. 1). We found at least 6 different fish species  
388 infected with *S. destruens*; its co-invasive asymptomatic carrier *P. parva* (prevalence  
389 between 2-4%) as previously reported (Charrier et al., 2016); 3 native fish species found  
390 infected with this parasite for the first time such as *Alburnus alburnus* (bleak, 9%  
391 prevalence), *Rhodeus amarus* (European bitterling, 20% prevalence) and *Rutilus rutilus*  
392 (roach, 4% prevalence); 2 Genus for which specific species were not determined such as  
393 *Gobio sp.* (3 species of gudgeons are reported in France, 10% prevalence) and *Phoxinus*  
394 *sp.* (5 species of minnows are reported in France, 2% prevalence).

395

#### 396 **Genetic diversity and structure of *P. parva* populations at country level**

397 Analysis of the genetic diversity of the nine groups sampled from the northern (Smar),  
398 central (Indr), central-eastern (Ain, HSAv), south-eastern (Brhon, Vauc, Cors) and south-  
399 western (PyrA, Gir) regions of France showed low overall diversity within the groups (Table  
400 1). The estimated percentages of polymorphic loci ranged from 8.59% to 13.4%, with the  
401 Smar group and Indr group having the lowest and the highest rate, respectively (Table 1).  
402 Low values of observed heterozygosity ( $H_o$ ) and nucleotide diversity ( $\Pi$ ) were also found in  
403 the analyzed groups.

404 The lowest values of  $H_o$  and  $\Pi$  were observed in the Cors group (0.009 and 0.0116,  
405 respectively), while the Vauc group had the highest values ( $H_o$ : 0.0166 and  $\Pi$ : 0.0151)  
406 (Table 1). To test whether the french population is structured, the genetic differentiation and  
407 relationship between the nine french groups were assessed using four complementary  
408 analyses. First, the pairwise  $F_{st}$  between groups showed little genetic differentiation between  
409 the french groups with values ranging from 0.0064 to 0.0199 and the highest value being  
410 observed between the PyrA and Gir groups (Fig. 2A). Secondly, the genetic distance



411 between individuals and the MSN showed that the samples do not cluster by groups, without  
412 geographical differentiation (Fig. 2B). Finally, this lack of genetic and geographical structure  
413 was also observed by hierarchical clustering based on the pairwise IBS and by PCA analysis  
414 (Fig. 2C and 2D). Our results showed unambiguously and surprisingly that individuals  
415 sampled across the country cluster in a unique homogeneous deme rather than in regionally  
416 associated demes.

417

#### 418 **Relationship of french deme *P. parva* and native range**

419  $\Phi_{st}$  values ranged from 0.009 to 0.146 (Fig. 3A; See Supplementary Table S4). The pairwise  
420  $\Phi_{st}$  values between the french deme and each native deme were relatively high and ranged  
421 from 0.040 to 0.146. Japan and Taiwan showed the greatest differentiation with the french  
422 deme with  $\Phi_{st}$  equal to 0.146 and 0.097, respectively. On the other hand, the lowest  
423 differentiation values (French vs. native) were observed with the North China (0.040) and  
424 Northeast China (0.047) demes. The differentiation between the french deme and South and  
425 Southeast China demes were equivalent (0.058) (Fig. 3A; See Supplementary Table S4).  
426 The IBS pairwise distances used to perform a multidimensional scaling analysis showed a  
427 clear compact cluster with predominantly french individuals also containing several  
428 individuals from each native deme except Japanese and South China demes (Fig. 3B).

429 We sought to determine the genetic structure and relationship between the french deme and  
430 the native range. To select the most plausible number of genetic clusters  $K$ , several criteria  
431 were evaluated. The values of the Bayesian Information Criterion for the K-means clustering  
432 suggested a  $K=8-9$  (see Supplementary Information). The chooseK algorithm based on  
433 marginal likelihood and Puechmaille's statistics suggested a  $K=9$  (see Supplementary Fig.  
434 S2, S3) for fastSTRUCTURE analysis. For all values of  $K$ , the results converged to  
435 aggregate french samples to one independent cluster (Fig. 3C). The results of all the  
436 analyses show a french cluster different from the native clusters (Fig. 3). Admixtures and  
437 lower genetic differentiation between the french deme and several native demes exist  
438 however (Fig. 3C; Fig. S3).

440 **Discussion**

441 This is the first study assessing the distribution and the prevalence of the rosette agent *S.*  
442 *destruens* among invasive and native fish species in different regions in France. We found  
443 that *S. destruens* is widely distributed throughout France, in regions such as Ain (central-  
444 eastern France), Indre (central France), Gironde (south-western France), Bouches-du-  
445 Rhône and Corsica island (south-eastern France) and exhibiting a range of climates going  
446 from temperate, oceanic and Mediterranean, respectively, but also in contrasting freshwater  
447 habitats such as lotic and lentic ones. Moreover, our broad environmental screening of *S.*  
448 *destruens* distribution in France shows new native fish species infected with this parasite.  
449 The disease prevalence ranging between 2% and 20% is in the range of those previously  
450 found in wild fish in other countries (Combe & Gozlan, 2018), such as in the Netherlands  
451 where a prevalence of *S. destruens* of about 25% has been found in the native *G. aculeatus*  
452 (Spikmans et al., 2020), and other countries (Combe & Gozlan, 2018). However, the rosette  
453 agent was not detected in all host populations of *P. parva* despite a homogeneous genetic  
454 background between french populations and a similar invasion history. These findings lead  
455 us to raise two hypothesis: 1) a loss of pathogen has occurred due to its absence in the  
456 founding *P. parva* population, either because a small number of pathogen-free founders (i.e.  
457 *P. parva* hosts for which the pathogen was not able to survive/replicate in their native range,  
458 highly unlikely) has invaded the new location or 2) because although present in *P. parva*  
459 founder hosts *S. destruens* was unable to survive translocation (i.e. actual absence, unlikely)  
460 or 3) the presence of the rosette agent in each *P. parva* individual (i.e. asymptomatic  
461 carrier) is of such low prevalence and potentially associated with a non-homogeneous  
462 distribution in fish organs that our detection protocol could not detect its presence at a site  
463 (likely). Based on its co-introduction with *P. parva* into Europe, the wide distribution of *P.*  
464 *parva* in France and previous reports of *S. destruens*'s presence and impact on freshwater  
465 fish biodiversity (reviewed in Combe & Gozlan, 2018) the first scenario seems highly

466 unlikely. Interestingly, we found *P. parva* hosts infected with the rosette agent in Ain (central-  
467 eastern, France), Bouches-du-Rhône and Corsica island (south-eastern France) and Indre  
468 (central France), regions that are far away from each other, notably for Corsica island that is  
469 separated from the continent by the Mediterranean Sea and where high virulence levels of  
470 *S. destruens* were reported (personal communication). Surprisingly, while in some regions  
471 neither the host *P. parva* nor native fish species were infected with the rosette agent, in  
472 Gironde *P. parva* individuals seem parasite-free although native species such as *R. rutilus*  
473 and *Gobio sp.* were *S. destruens* positive. Taken together, our findings along with the results  
474 from Spikmans et al. (2020) showing the strong correlation between *S. destruens*  
475 occurrence and *P. parva* presence via water body analyses, tend to indicate that *S.*  
476 *destruens* is widely distributed across freshwater ecosystems in France. Furthermore,  
477 despite its presence and likely introduction by *P. parva* hosts, detecting *S. destruens* in  
478 asymptomatic carriers who typically have a low copy number of the infective agent remains  
479 challenging. Moreover, it is worth noting that prior to the study of Ercan et al. (2015) showing  
480 large fish population collapses in Turkey, no one had noticed the significant increase in fish  
481 mortality rates across Europe following *P. parva* and *S. destruens* co-invasion, despite  
482 laboratory evidence. This lack of disease detection can also be attributed to 1) the chronic  
483 nature of the disease leading to slow and periodic mortality rates in the wild, 2) the difficulties  
484 in monitoring fish mortality in freshwater systems and 3) the non-inclusion of this parasite in  
485 the existing list of noticeable diseases in Europe, including France (Combe & Gozlan, 2018).  
486 Here our results clearly highlight the risk of disease emergence in wild fish in France and  
487 beyond, and potentially in fish of economic importance. Thus, it is necessary for  
488 policymakers and stakeholders to react quickly to control its spread from already  
489 contaminated reservoirs to still healthy ecosystems.

490 It has been hypothesized that different *S. destruens* strains (i.e. isolates) could have been  
491 co-introduced in Europe with distinct *P. parva* lineages and sequencing of the ITS-1 genetic  
492 marker of *S. destruens* strains has shown that European strains (UK and Turkey) cluster

493 with respect to North-American strains but do not intermingle with each other and are closely  
494 related to Chinese strains (Ercan et al., 2015; Sana et al., 2017; Hardouin et al., 2018). This  
495 was consistent with the invasion history of *P. parva* populations in Europe (Combe & Gozlan,  
496 2018; Brazier et al., 2021), thus raising the possibility that host-parasite populations  
497 eventually invaded northern and southern France from at least two sources of native  
498 population, originating from north and south of the Yangtze River, respectively. Such  
499 scenarios of an ancient host-parasite coevolution and co-introduction could have direct  
500 implications on disease risk, e.g. increased transmission and virulence of the pathogen to  
501 naive fish populations. For example, all identified strains of *S. destruens* have been shown to  
502 be highly virulent to local fish populations in England, Turkey and the USA, although they are  
503 asymptomatic in *P. parva* populations (Combe & Gozlan, 2018). Due to the absence of *S.*  
504 *destruens* strains in France, we performed here an analysis of the genetic diversity of french  
505 *P. parva* populations to check their potential heterogeneity and admixed origin of  
506 introduction, as recently found for some other European populations (Brazier et al., 2021).  
507 Surprisingly, our molecular screening and genetic assignment of the nine sampled groups  
508 revealed a lack of genetic and geographic structure within and between *P. parva*  
509 populations, with the French populations representing a single homogeneous gene pool.  
510 Furthermore, no strong links are shared between french and native populations, with the  
511 entire French range constituting an independent genetic pool. Therefore, our results suggest  
512 that the French (continental and insular) population of *P. parva* could not have originated  
513 from a direct introduction from the native range, but is likely to have originated from a  
514 successful invasive population through an invasive bridgehead effect. Indeed, bridgehead  
515 effects could stimulate the invasion process and could be due to either the evolution of  
516 higher invasiveness in the primary introduced population (e.g. acquisition of new traits  
517 increasing establishment and spread success), or to an increased abundance in the  
518 bridgehead region, leading to a higher probability of movement to new non-native habitats  
519 compared to native populations (Lombaert et al., 2010; Bertelsmeier & Keller, 2018).  
520 However, empirical evidence to confirm these assumptions is still lacking. Nevertheless,

521 evidence of successful invasive populations becoming the source of new introductions in  
522 distant territories is increasingly available for several invasive species such as the red  
523 swamp crayfish (*Procambarus clarkii*), grapevine downy mildew (*Plasmopara viticola*) and  
524 common ragweed (*Ambrosia artemisiifolia*) (van Boheemen et al., 2017; Oficialdegui et al.,  
525 2019; Fontaine et al., 2021). Recently, Brazier et al. (2021) found a similar homogeneous  
526 and specific pattern for *P. parva* populations in Italy, raising the question of the genetic  
527 structure of other invaded Mediterranean regions such as Spain or Morocco.

528 Although our results require further analysis by adding the range of invasions, they provide  
529 insight into the complex origin of the French *P. parva* populations, and allow us to infer the  
530 origin of *S. destruens* strains within a country, as observed for France. Overall low but  
531 equivalent genetic diversity was observed within each French group (Table 1). Nevertheless,  
532 there was a clear reduction in genetic diversity within the French deme compared to each  
533 native deme, highlighting that the invasion of *P. parva* in France was accompanied by a  
534 substantial loss of genetic diversity (Fig. 3D). Therefore, if the co-introduction of *P. parva*/*S.*  
535 *destruens* is considered as a host-pathogen complex, we can assume that French  
536 populations of the rosette agent should also show low genetic variation. However, further  
537 genetic analysis will be required to clearly assess the potential pathway of introduction of *P.*  
538 *parva* populations into France from primary European invasive demes, the resulting genetic  
539 diversity of the co-invasive pathogen and the potential high virulence of *S. destruens* strains  
540 in France.

541

## 542 **Conclusion**

543 Although showing relatively low levels of prevalence, *S. destruens* is widespread among  
544 invasive and native fish populations from contrasting regions of France, suggesting that  
545 there is a high risk of disease emergence. We also found that co-introduced *P. parva*  
546 populations represent a homogeneous gene pool between these regions, indicating a

547 potential comparable homogeneity of the rosette agent population. Overall, our results  
548 provide a better understanding of pathogen emergence resulting from secondary dispersal of  
549 a successful invasive population in a host-pathogen co-introduction context, paving the path  
550 for the transition from invasion biology to invasion epidemiology to assess risks of disease  
551 emergence associated with biological invasions.

552

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559

### 560 **Conflict of interest**

561 The authors declare no conflict of interest.

562

### 563 **Credit author statement**

564 **Combe Marine:** Conceptualization, Data curation, Original draft preparation

565 **Cherif Emira:** Data curation, Software, Formal analysis, Original draft preparation

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576

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794 **Figures caption**

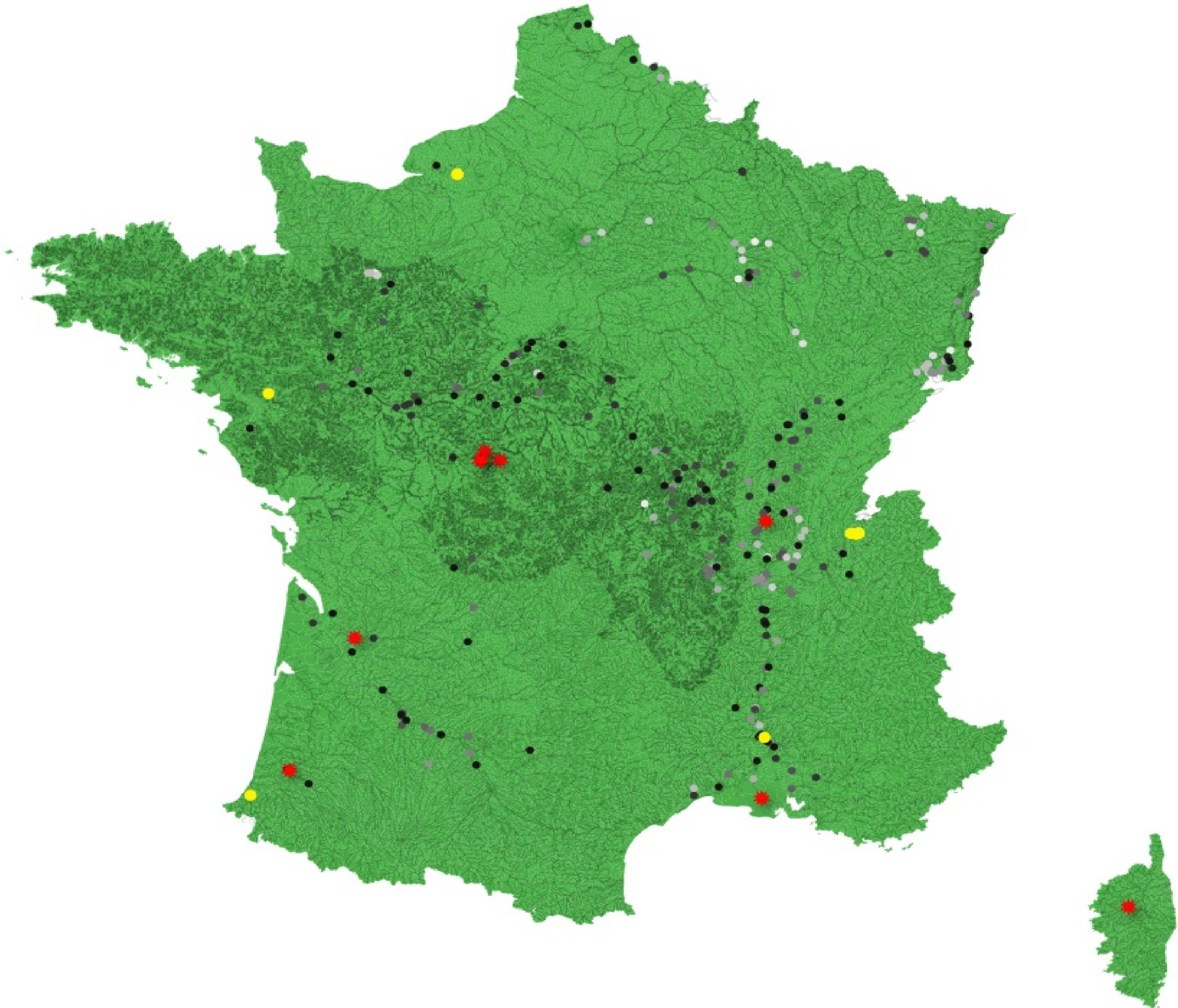
795 **Fig. 1.** Distribution of *Pseudorasbora parva* and the fish pathogen *Sphaerothecum destruens*  
796 in France. The gradient from light grey dots to black dots indicates the distribution of *P.*  
797 *parva* populations in 1995, 2000, 2005 and 2007 respectively; Yellow dots are sites where *S.*  
798 *destruens* was tested and not detected. Red dots are sites where *S. destruens* was tested  
799 and found in fish species. Rivers systems are shown in light grey.

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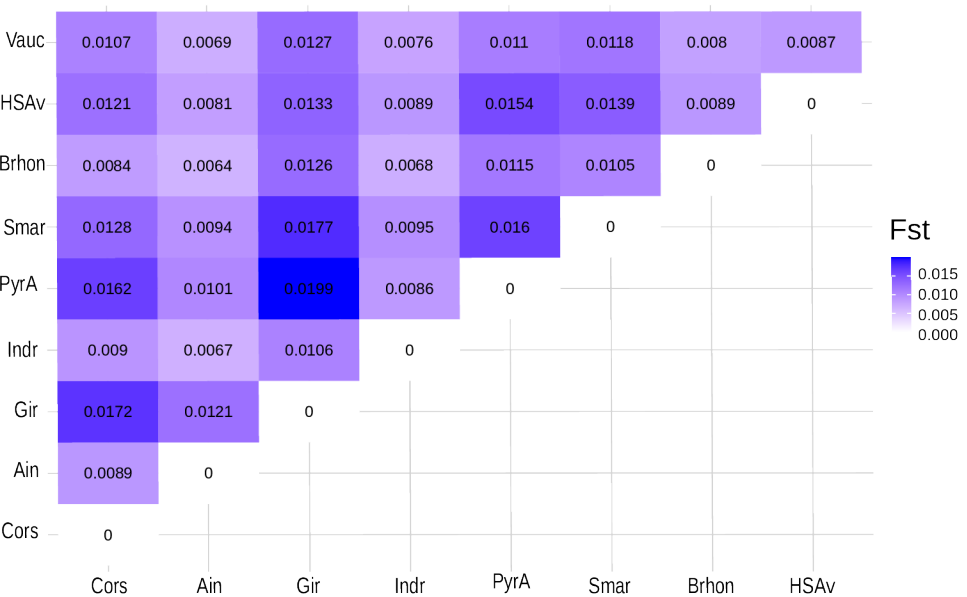
801 **Fig. 2.** Genetic diversity and structure of the French *P. parva* groups. (A) Pairwise  $F_{st}$ . (B)  
802 Minimum spanning network (MSN), each node is a genotype and the edges represent the  
803 genetic distance, nodes with identical genetic distance are connected, lighter the edges, the  
804 longer the distance. (C) Hierarchical clustering. (D) Principal component analysis.

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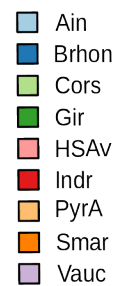
806 **Fig. 3.** Genetic clustering and relationship of the french deme of *P. parva* with the native  
807 range. (A) Pairwise  $\Phi_{st}$ . (B) Multidimensional scaling plot of the french deme and native  
808 range. based on the identity by state (IBS) distance (C) Discriminant analysis of principal  
809 components of the french and native range populations, axes represent the first two linear  
810 discriminants (LD). (D) Nucleotide diversity in the french deme and in each native range.



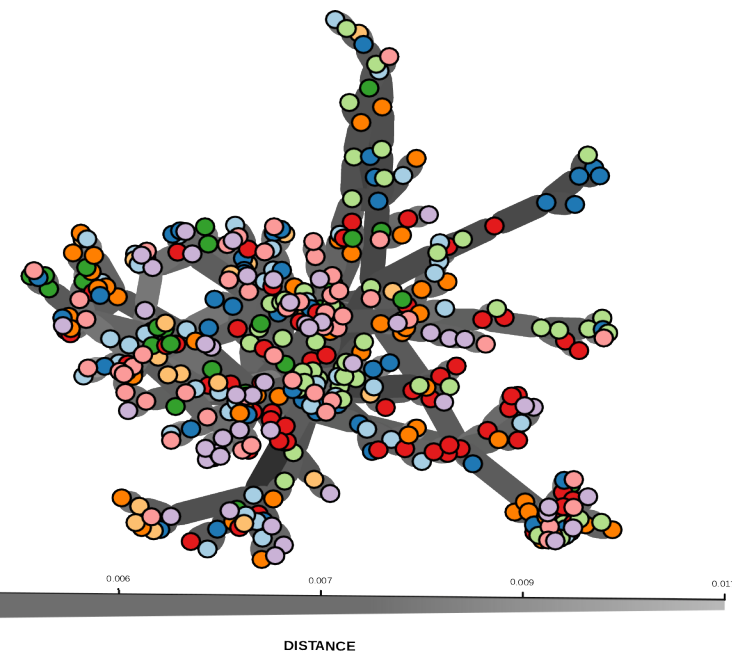
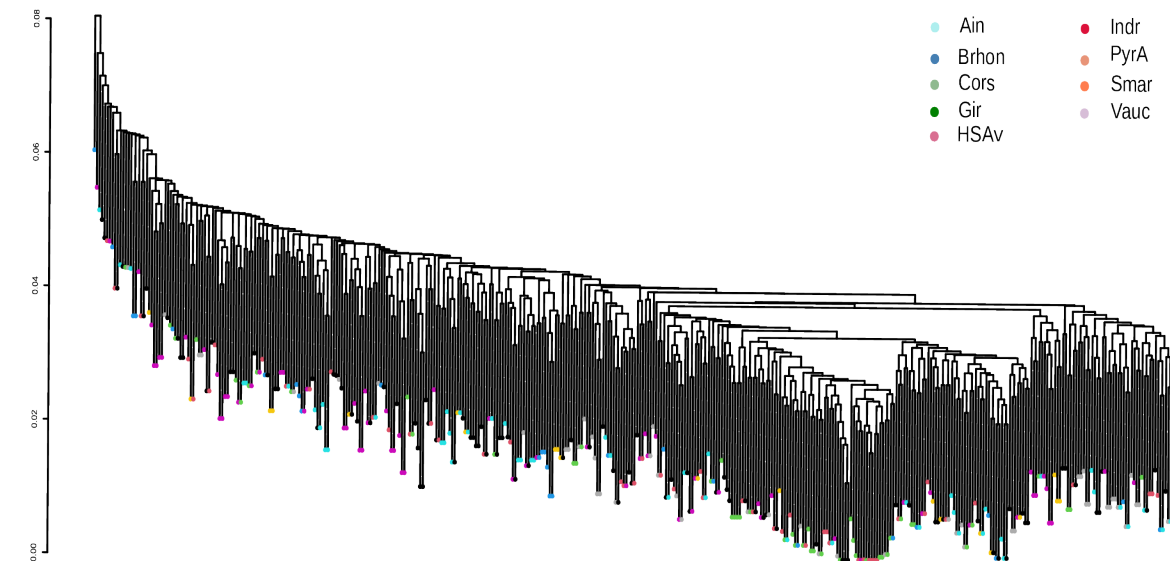


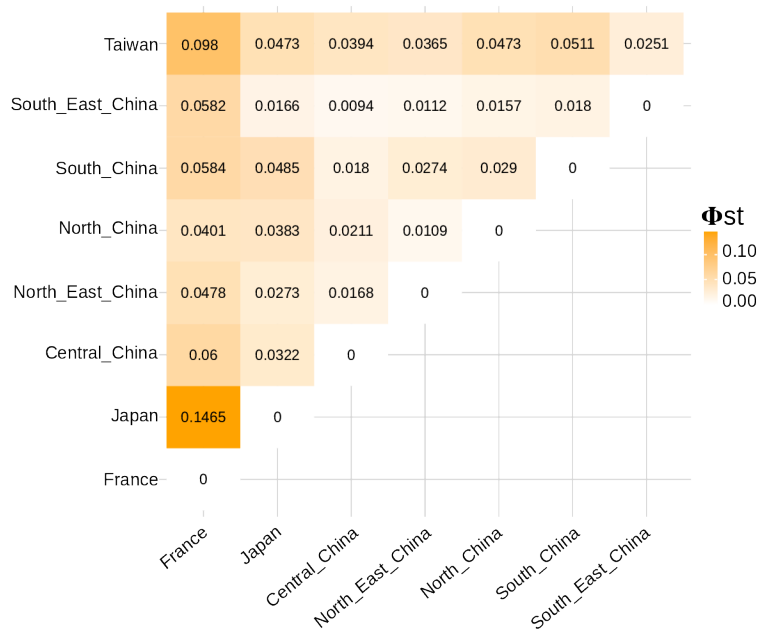
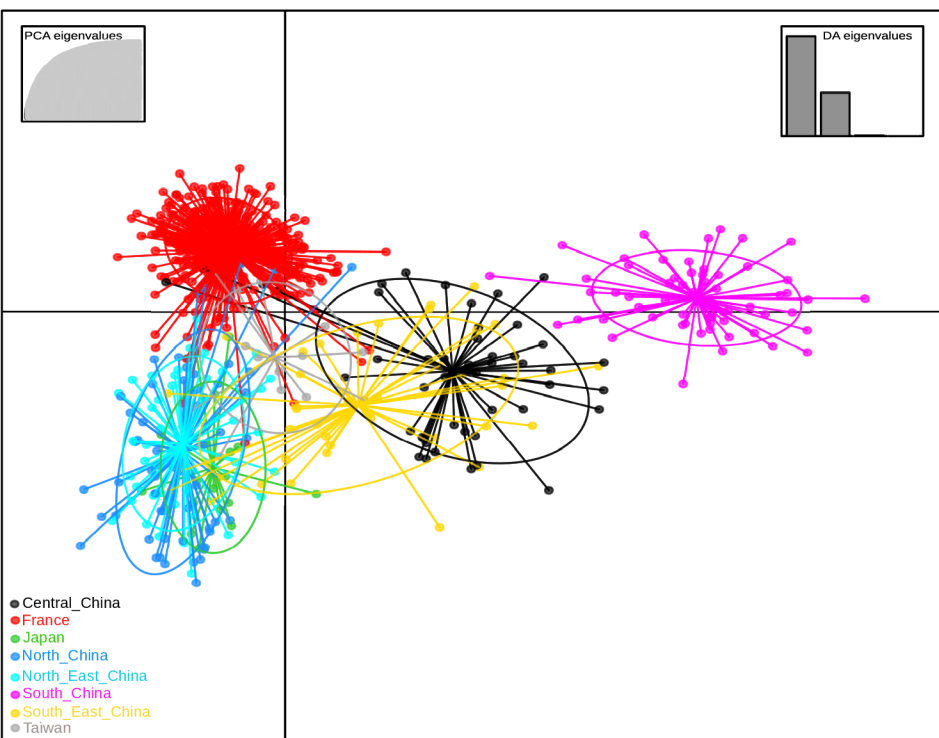
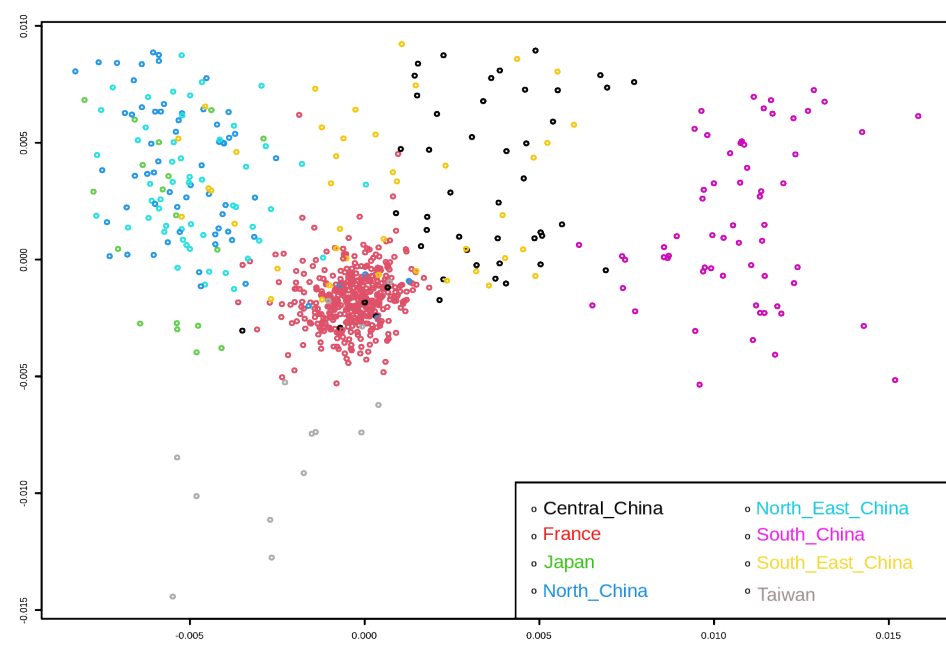
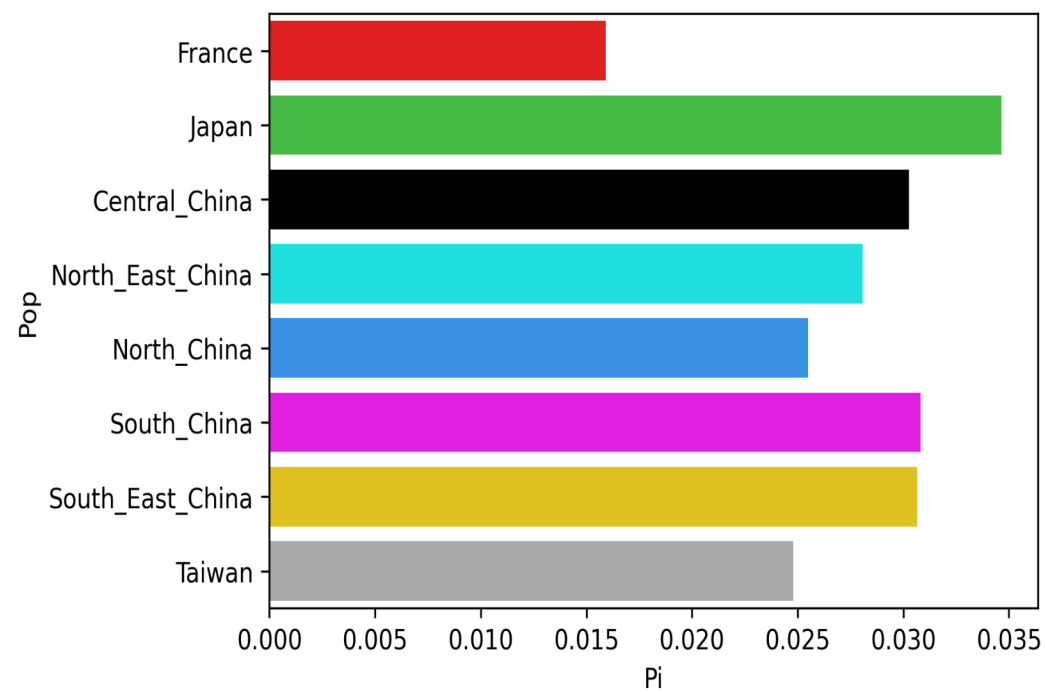
**A****B**

POPULATION



Samples/Node

**C****D**

**A****B****C****D**

**Table 1.** French population properties with geographic origin and diversity indices.

Grp	Region	N	Sites	%Polymorphic_loci	Pa	Ho (SD)	Pi	Fis
Cors	Corse	48	5028	8.8106	31	0.0096(0.0547)	0.0116	0.01696
Ain	Ain	50	5028	12.0326	55	0.0134(0.0734)	0.0131	0.01047
Gir	Gironde	26	5028	8.7509	34	0.0156(0.0821)	0.0150	0.00765
Indr	Indre	69	5028	13.4049	86	0.0162(0.0831)	0.0151	0.01079
PyrA	Pyrénées-Atlantiques	20	5028	6.4240	14	0.0134(0.0801)	0.0132	0.00652
Smar	Seine-Maritime	49	5028	8.5918	54	0.0129(0.0763)	0.0127	0.01095
Brhon	Bouches-du-Rhone	50	5028	11.1177	37	0.0116(0.0613)	0.0127	0.01527
HSAv	Haute-Savoie	50	5028	9.8050	33	0.0145(0.0791)	0.0138	0.00868
Vauc	Vaucluse	47	5028	12.6690	41	0.0166(0.0848)	0.0151	0.00469

Note: Grp IDs will be used as tags in the manuscript.

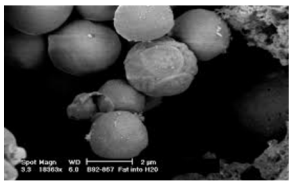
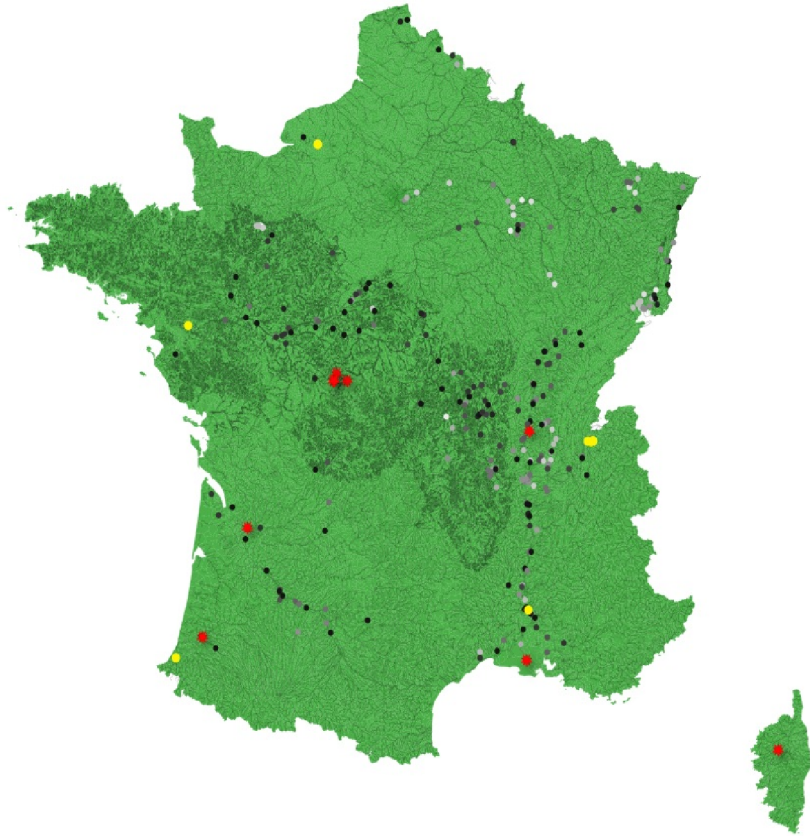
Abbreviations: Grp, population; N, number of genotyped individuals; Sites, number of fixed and variant sites; %Polymorphic, percentage of polymorphic loci; PA, number of private alleles within each population; Ho, observed heterozygosity; SD, Standard Deviation; Pi, nucleotide diversity.

**Table 2.** Detection of *S. destruens* DNA in invasive and native fish populations in France.

Region: angling association (abbreviation); Date: sampling date; N: number of individuals; qPCR: number of positive individuals; Ct: Ct-value; DV: disease prevalence (%); NA: missing data. Whilst three species of gudgeon and 5 species of minnows are reported in France, *Phoxinus sp.* and *Gobio sp.* were not identified at the species level here.

Region	Date	Genus/Species	N	qPCR	Ct	DV (%)	
Ain (Ain)	06/09/17	<i>Pseudorasbora parva</i>	50	2	33.7	4	
					31.1		
		<i>Phoxinus sp.</i>	50	0	0	0	
		<i>Perca fluviatilis</i>	18	0	0	0	
		<i>Rutilus rutilus</i>	13	0	0	0	
		<i>Chondrostoma nasus</i>	19	0	0	0	
Corse (Cors)	19/09/18	<i>Pseudorasbora parva</i>	50	1	33.6	2	
		<i>Phoxinus sp.</i>	50	1	29.2	2	
		<i>Salmo trutta</i>	38	0	0	0	
Bouches-du-Rhône (Brhon)	01/10/18	<i>Pseudorasbora parva</i>	50	1	36.7	2	
		<i>Lepomis gibbosus</i>	36	0	0	0	
		<i>Abramis brama</i>	22	0	0	0	
		<i>Rutilus rutilus</i>	2	0	0	0	
		<i>Scardinius erythrophthalmus</i>	1	0	0	0	
	26/06/19	<i>Pseudorasbora parva</i>	50	0	0	0	
		<i>Lepomis gibbosus</i>	29	0	0	0	
		<i>Alburnus alburnus</i>	11	1	36.1	9	
		<i>Abramis brama</i>	1	0	0	0	
		<i>Cyprinus carpio</i>	1	0	0	0	
Gironde (Gir)	15/11/18	<i>Pseudorasbora parva</i>	26	0	0	0	
		<i>Rutilus rutilus</i>	50	2	33.9	4	
					36.5		
	<i>Gobio sp.</i>	10	1	37.8	10		
	<i>Squalius cephalus</i>	11	NA	NA	NA		
	<i>Lepomis gibbosus</i>	11	NA	NA	NA		
	<i>Phoxinus sp.</i>	11	NA	NA	NA		
	<i>Anguilla anguilla</i>	6	NA	NA	NA		
Indre (Indr)	26/09/17	<i>Pseudorasbora parva</i>	50	1	36.64	2	
		<i>Rutilus rutilus</i>	50	0	0	0	
		<i>Abramis brama</i>	10	0	0	0	
		<i>Carassius carassius</i>	10	0	0	0	
		<i>Rhodeus amarus</i>	10	2	36.1	20	
						36.2	
		<i>Ameiurus melas</i>	10	0	0	0	
<i>Perca fluviatilis</i>	10	0	0	0			

Loire Atlantique (LoirA)	15/03/18	<i>Pseudorasbora parva</i>	9	0	0	0
Pyrénées Atlantiques (PyrA)	20/10/17	<i>Pseudorasbora parva</i>	20	0	0	0
Haute Savoie (HSAv)	24/10/17	<i>Pseudorasbora parva</i>	50	0	0	0
	09/10/17	<i>Salmo trutta</i>	20	0	0	0
	09/10/17	<i>Squalius cephalus</i>	20	0	0	0
	09/10/17	<i>Chondrostoma nasus</i>	5	0	0	0
	09/11/17	<i>Cyprinus carpio</i>	4	0	0	0
	09/11/17	<i>Barbus barbus</i>	20	0	0	0
Seine Maritime (Smar)	05/09/17	<i>Pseudorasbora parva</i>	49	0	0	0
		<i>Lepomis gibbosus</i>	80	0	0	0
		<i>Scardinius erythrophthalmus</i>	7	0	0	0
		<i>Abramis brama</i>	2	0	0	0
		<i>Carassius carassius</i>	7	0	0	0
		<i>Rutilus rutilus</i>	2	0	0	0
Vaucluse (Vauc)	17/07/18	<i>Rutilus rutilus</i>	27	0	0	0
		<i>Blicca bjoerkna</i>	18	0	0	0
		<i>Squalius cephalus</i>	50	0	0	0
	15/05/19	<i>Pseudorasbora parva</i>	47	0	0	0
		<i>Chondrostoma nasus</i>	8	0	0	0
		<i>Rutilus rutilus</i>	13	0	0	0
		<i>Gobio sp.</i>	22	0	0	0
		<i>Rhodeus amarus</i>	20	0	0	0
		<i>Blicca bjoerkna</i>	8	0	0	0
		<i>Scardinius erythrophthalmus</i>	3	0	0	0
		<i>Alburnus alburnus</i>	30	0	0	0
		<i>Chondrostoma toxostoma</i>	2	0	0	0

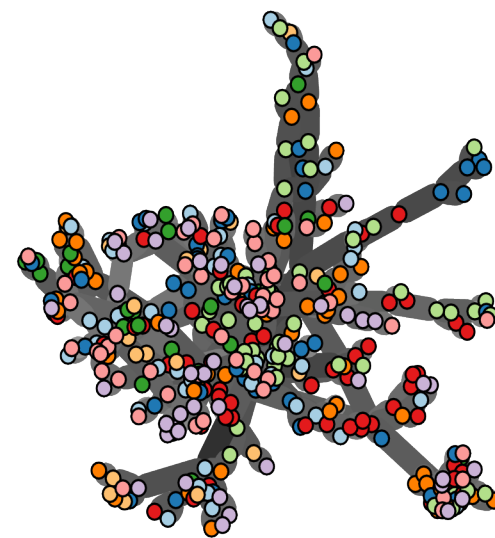


POPULATION

- Ain
- Brhon
- Cors
- Gir
- HSAv
- Indr
- PyrA
- Smar
- Vauc

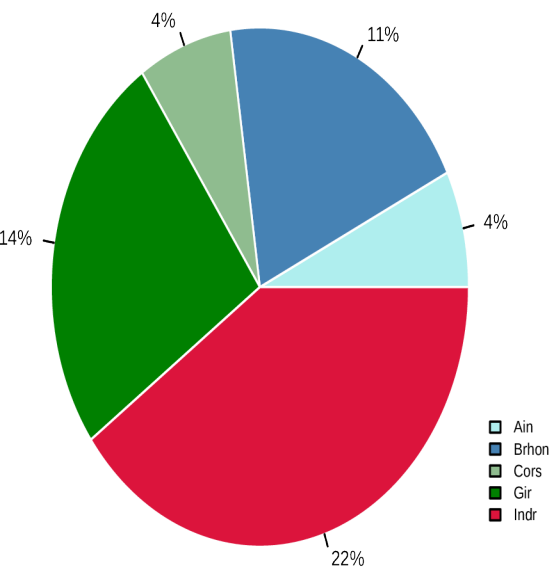
Samples/Node

○ 4



0.003 0.006 0.007 0.008 0.01

DISTANCE



- Ain
- Brhon
- Cors
- Gir
- Indr