

Novel tools for analyzing genome-wide data of clonal populations

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2 ABSTRACT

To gain a detailed understanding of how plant microbe evolve and adapt to host and other factors such as pesticides, knowledge of the population dynamics and evolutionary history of populations is crucial. With the advent of high throughput sequencing technologies, obtaining genomic sequences for representative populations has become easier than ever before. A move towards open, reproducible science has provided impetus for developing population genetic analysis tools in R. We previously contributed the R package *poppr* specifically addressing issues with analysis of clonal populations. In this paper we provide several significant extensions to *poppr* with a focus on large, genome wide SNP data. Specifically, we provide analyses across any level of hierarchies, a new function to define clone boundaries we call `mlg.filter` allowing for inspection and definition of what is a clonal lineage, and the index of association for reduced representation genomic data, and modular bootstrapping of any genetic distance.

Keywords: clonality, population genetics, bootstrap, open source

INTRODUCTION

To paraphrase Dobzhansky, nothing in the field of plant-microbe interactions makes sense except in the light of population genetics (Dobzhansky, 1973). Genetic forces such as selection and drift act on alleles in a population. Thus, a true understanding of how plant pathogens evolve and adapt to crops, fungicides, or other factors, can only emerge in the context of population level phenomena given the demographic history of populations (McDonald and Linde, 2002; Grunwald and Goss, 2011; Milgroom et al., 1989). The field of population genetics, in the era of whole genome resequencing, provides unprecedented power to describe the evolutionary history and population processes that drive coevolution between pathogens and hosts. This powerful field thus critically enables effective deployment of R genes, design of pathogen informed plant resistance breeding programs, and implementation of fungicide rotations that minimize emergence of resistance.

Most computational tools for population genetics are based on concepts developed for sexual model organisms. Populations that reproduce clonally or are polyploid are thus difficult to characterize using classical population genetic tools because theoretical assumptions underlying the theory are violated. Yet,

28 many plant pathogen populations are at least partially clonal if not completely clonal (Milgroom, 1996;
29 Anderson and Kohn, 1995). Thus, development of tools for analysis of clonal or polyploid populations is
30 needed.

31 Genotyping by sequencing and whole genome resequencing provide the unprecedented ability to
32 identify >1,000 single nucleotide polymorphisms (SNPs) in populations (Elshire et al., 2011; Luikart
33 et al., 2003; Davey et al., 2011). Availability of these large SNP data sets provides new challenges for
34 data analysis. For example, it is not clear what a clone is in large SNP data where the chance of observing
35 variation at a given SNP locus within independent samples of the same clone are substantial enough that
36 novel tools for definition of clone boundaries are required. With traditional marker data (e.g., SSR, AFLP)
37 a clone was typically defined as a unique multilocus genotype (MLG). However, with large SNP data a
38 measure of genetic distance is required to define the boundary of an MLG (e.g., clone) or the boundaries
39 of a clonal lineage. Definition of a clone is further complicated by the presence of missing data that is
40 typical for reduced representation libraries used in GBS or genome re-sequencing. If two individuals are
41 identical for all observed SNPs except for one missing allele, should they be considered different?

42 The research community using the R statistical and computing language (R Core Team, 2015) has
43 developed a plethora of new resources for population genetic analysis (Paradis, 2010; Jombart, 2008).
44 Recently, we introduced the R package *poppr* specifically developed for analysis of clonal populations
45 (Kamvar et al., 2014b). *Poppr* previously introduced several novel features including the ability to conduct
46 a hierarchical analysis across unlimited hierarchies, test for linkage association, graph minimum spanning
47 networks or provide bootstrap support for Bruvo's distance in resulting trees. It was well received by
48 the community, garnering 14 citations in its first year of publication. Since it's first release, however,
49 limitations with speed, ease of use, and efficiency became more apparent as genomic data became more
50 readily available.

51 In version 1.1, to address difficulties with handling hierarchical and multilocus genotypic metadata,
52 a new S4 object called "genclone" was defined to expand the genind object of *aedgenet*. The genclone
53 object formalized the definitions of multilocus genotypes and population hierarchies by adding two slots
54 called mlg and hierarchy that carried a numeric vector and a data frame, respectively. These new slots
55 allow for increased efficiency and ease of use by allowing these metadata to travel with the genetic data.
56 The addition of the population hierarchies has proved to be advantageous enough that they have recently
57 been adopted into the more central *aedgenet* package (Jombart, 2008).

58 In version 1, *poppr* was appropriate for traditional markers systems, but not well suited to population
59 genomic data resulting from high throughput sequencing methods. The raw size of these data made
60 it difficult to conduct traditional analyses. Here, we introduce *poppr* 2.0, which provides a significant
61 update to *poppr* including novel tools for analysis of clonal populations specifically for large SNP data.
62 Significant novel tools include functions for calculating clone boundaries and collapsing individuals into
63 user-specified clones based on genetic distance, sliding window analyses, genotype accumulation curves,
64 reticulations in minimum spanning networks, and bootstrapping for any genetic distance.

CLONAL IDENTIFICATION

65 As highlighted in previous work, clone correction is an important component of population genetic
66 analysis of organisms that have cryptic growth or are known to reproduce asexually (Kamvar et al.,
67 2014b; Milgroom, 1996; Grnwald et al., 2003). This method removes bias that would otherwise affect
68 metrics that rely on allele frequencies. It was initially designed for data with only a handful of markers.
69 With the advent of large-scale sequencing and reduced-representation libraries, it has become easier to
70 sequence tens of thousands of markers from hundreds of individuals (Elshire et al., 2011; Davey et al.,
71 2011; Davey and Blaxter, 2010). With this larger number of markers, the genetic resolution is much
72 greater, but the chance of genotyping error is also greatly increased (Mastretta-Yanes et al., 2015). Taking
73 this fact and occasional somatic mutations into account, it would be impossible to separate true clones

74 from independent individuals by just comparing what multilocus genotypes are different. We introduce
75 a new method for collapsing unique multilocus genotypes determined by naive string comparison into
76 multilocus lineages utilizing any genetic distance given three different clustering algorithms: farthest
77 neighbor, nearest neighbor, and UPGMA (average neighbor) (Sokal, 1958).

78 The clustering algorithms act on a distance matrix that is either provided by the user or generated via a
79 function that will calculate a distance from genclone objects such as `bruvo.dist`, which in particular
80 applies to any level of ploidy (Bruvo et al., 2004). All algorithms have been implemented in C and
81 utilize the OpenMP framework for optional parallel processing (Dagum and Menon, 1998). Default is
82 the conservative farthest neighbor algorithm, which will only cluster samples together if all samples in
83 the cluster are at a distance less than the given threshold. By contrast, the nearest neighbor algorithm
84 will have a chaining effect that will cluster samples akin to adding links on a chain where a sample can
85 be included in a cluster if all of the samples have at least one connection below a given threshold. The
86 UPGMA, or average neighbor clustering algorithm is the one most familiar to biologists as it is often
87 used to generate preliminary ultra-metric trees based on genetic distance. This algorithm will cluster by
88 creating a representative sample per cluster and joining clusters if these representative samples are closer
89 than the given threshold.

DEMONSTRATION DATA: *P. INFESTANS*

90 We utilize data from the microbe *Phytophthora infestans* to show how the `mlg.filter` function
91 collapses multilocus genotypes with Bruvo's distance assuming a genome addition model (Bruvo et al.,
92 2004). *P. infestans* is the causal agent of potato late blight originating from Mexico and spread to Europe
93 in the mid 19th century (Goss et al., 2014; Li et al., 2013; Lees et al., 2006). *P. infestans* reproduces
94 both clonally and sexually. The clonal lineages of *P. infestans* have been formally defined into 18 separate
95 clonal lineages using a combination of various molecular methods including AFLP and microsatellite
96 markers (Lees et al., 2006). For these data, we used `mlg.filter` to detect all of the distance thresholds
97 at which 18 multilocus lineages would be resolved. We used these thresholds to define multilocus lineages
98 and create contingency tables and dendograms to determine how well the multilocus lineages were
99 detected.

100 For the *P. infestans* population, the three algorithms were able to detect 18 multilocus lineages at
101 different distance thresholds (Fig. 1). Contingency tables between the described multilocus genotypes
102 and the genotypes defined by distance show that most of the 18 lineages were resolved, except for US-8,
103 which is polytomic (Table 1).

DEMONSTRATION DATA: SIMULATED DATA

104 We utilized simulated data constructed using the `glSim` function in `adegenet` (Jombart and Ahmed, 2011)
105 to obtain a SNP data set for demonstration. Two diploid data sets were created, each with 10k SNPs (25%
106 structured into two groups) and 200 samples with 10 ancestral populations of even sizes. Clones were
107 created in one data set by marking each sample with a unique identifier and then randomly sampling
108 with replacement. It is well documented that reduced- representation sequencing can introduce several
109 erroneous calls and missing data (Mastretta-Yanes et al., 2015). To reflect this, we mutated SNPs at a rate
110 of 10% and inserted an average of 10% missing data for each sample after clones were created, ensuring
111 that no two sequences were alike. The number of mutations and missing data per sample were determined
112 by sampling from a poisson distribution with $\lambda = 1000$. After pooling, 20% of the data set was randomly
113 sampled for analysis. Genetic distance was obtained with the function `bitwise.dist`, which calculates
114 the fraction of different sites between samples, counting missing data as equivalent in comparison.

115 All three filtering algorithms were run with a threshold of 1, returning a numeric vector of length $n - 1$
116 where each element represented a threshold at which two samples/clusters would join. Since each data set
117 would have varying distances between samples, the clonal boundary threshold was defined as the midpoint
118 of the largest gap between two thresholds that collapsed less than 50% of the data.

119 Out of the 100 simulations run, we found that across all methods, detection of duplicated samples had
120 ~ 98% true positive fraction and ~ 0.8% false positive fraction indicating that this method is robust to
121 simulated populations.

INDEX OF ASSOCIATION

122 The index of association (I_A) is a measure of multilocus linkage disequilibrium that is most often used
123 to detect clonal reproduction within organisms that have the ability to reproduce via sexual or asexual
124 processes (Brown et al., 1980; Smith et al., 1993; Milgroom, 1996). It was standardized in 2001 as \bar{r}_d
125 by Agapow and Burt (2001) to address the issue of scaling with increasing number of loci. This metric is
126 typically applied to traditional dominant and co-dominant markers such as AFLPs, SNPs, or microsatellite
127 markers. With the advent of high throughput sequencing, SNP data is now available in a genome-wide
128 context and in very large matrices including thousands of SNPs. Thus, the likelihood of finding mutations
129 within two individuals of a given clone increases and tools are needed for defining clone boundaries. For
130 this reason, we devised two approaches using the index of association for large numbers of markers typical
131 for population genomic studies.

132 The first approach is a sliding window approach implemented in the function `win.ia`. It utilizes the
133 position of markers in the genome to calculate \bar{r}_d among any number of SNPs found within a user-
134 specified windowed region. It is important that this calculation utilize \bar{r}_d as the number of loci will be
135 different within each window (Agapow and Burt, 2001). This approach would be suited for a quick
136 calculation of linkage disequilibrium across the genome that can detect potential hotspots of LD that
137 could be investigated further with more computationally intensive methods assuming that the number of
138 samples << the number of loci.

139 As it would necessarily focus on loci within a short section of the genome that may or may not
140 be recombining, a sliding window approach would not be good for utilizing \bar{r}_d as a test for clonal
141 reproduction. A remedy for this is implemented in the function `samp.ia`, which will randomly sample
142 m loci, calculate \bar{r}_d , and repeat r times, thus creating a distribution of expected values of \bar{r}_d .

POPULATION STRATA AND HIERARCHIES

143 Assessments of population structure through methods such as hierarchical F_{st} and AMOVA benefit
144 greatly from multiple levels of population definition (Linde et al., 2002; Everhart and Scherm, 2015;
145 Grnwald and Hoheisel, 2006). With clonal organisms, basic practice has been to clone-censor data to
146 avoid downward bias in diversity due to duplicated genotypes that may or may not represent different
147 samples (Milgroom, 1996). Data structures for population genetic data mostly allow for only one level of
148 hierarchical definition. The impetus was placed on the researchers to provide the population hierarchies
149 for every step of the analysis. In `poppr` version 1.1, the `hierarchy` slot was introduced to allow unlimited
150 population hierarchies or stratifications to travel with the data. In practice, it is stored as a data frame
151 where each column represents a separate hierarchical level. This is then used to set the population factor
152 of the data by supplying a hierarchical formula containing one or more column names of the data frame
153 in the `hierarchy` slot. This functionality, developed in `poppr`, has been moved to the `aadegenet` package in
154 version 2.0 and the slot and methods have been renamed to `strata`.

GENOTYPE ACCUMULATION CURVE

155 Analysis of population genetics of clonal organisms often borrows from ecological methods such as
156 analysis of diversity within populations (Milgroom, 1996; Arnaud-Hanod et al., 2007; Grnwald et al.,
157 2003). When choosing markers for analysis, it is important to make sure that the observed diversity in your

158 sample will not appreciably increase if an additional marker is added (Arnaud-Hanod et al., 2007). This
 159 concept is analogous to a species accumulation curve, obtained by rarefaction. The genotype accumulation
 160 curve in *poppr* is implemented in the function `genotype_curve`. The curve is constructed by randomly
 161 sampling x loci and counting the number of observed MLGs. This repeated r times for 1 locus up to $n - 1$
 162 loci, creating $n - 1$ distributions of observed MLGs.

163 The following code example demonstrates the genotype accumulation curve for data from Everhart and
 164 Scherm (2015) showing that these data reach a small plateau and have a greatly decreased variance with
 165 12 markers, indicating that there are enough markers such that adding more markers to the analysis will
 166 not create very many new genotypes (Fig. 4).

```
library("poppr")
library("ggplot2")
data("monpop", package = "poppr")

set.seed(20150428)
genotype_curve(monpop, sample = 1000, quiet = TRUE)
p <- last_plot() + theme_bw() # get the last plot
p + geom_smooth(aes(group = 1)) # plot with a trendline
```

MINIMUM SPANNING NETWORKS WITH RETICULATION

167 In its original iteration, *poppr* introduced minimum spanning networks that were based on the *igraph*
 168 function `minimum.spanning.tree` (Csardi and Nepusz, 2006). This algorithm produces a minimum
 169 spanning tree with no reticulations where nodes represent individual MLGs. In other minimum spanning
 170 network programs, reticulation is obtained by calculating the minimum spanning tree several times and
 171 returning the set of all edges included in the trees. Due to the way *igraph* has implemented Prim's
 172 algorithm, it is not possible to utilize this strategy, thus we implemented an internal C function to walk
 173 the space of minimum spanning trees based on genetic distance to connect groups of nodes with edges of
 174 equal weight.

175 To demonstrate the utility of minimum spanning networks with reticulation, we used two clonal data
 176 sets: H3N2 flu virus data from the *aedegene* package using years of each epidemic as the population
 177 factor, and *Phytophthora ramorum* data from Nurseries and Oregon forests (Jombart et al., 2010; Kamvar
 178 et al., 2014a). Minimum spanning networks were created with and without reticulation using the *poppr*
 179 functions `diss.dist` and `bruvo.msn` for the H3N2 and *P. ramorum* data, respectively (Kamvar et
 180 al., 2014b; Bruvo et al., 2004). To detect mlg clusters, the infoMAP community detection algorithm was
 181 applied with 10,000 trials as implemented in the R package *igraph* version 0.7.1 utilizing genetic distance
 182 as edge weights and number of samples in each MLG as vertex weights (Csardi and Nepusz, 2006; Rosvall
 183 and Bergstrom, 2008).

184 To evaluate the results, we compared the number, size, and entropy (H) of resulting communities as we
 185 expect a highly clonal organism with low genetic diversity to result in a few, large communities. We also
 186 created contingency tables of the community assignments with the defined populations and used those
 187 to calculate entropy using Shannon's index with the function `diversity` from the R package *vegan*
 188 version 2.2-1 (Oksanen et al., 2015; Shannon, 2001). A low entropy indicates presence of a few large
 189 communities whereas high entropy indicates presence of many small communities.

190 The infoMAP algorithm revealed 63 communities with a maximum community size of 77 and $H = 3.56$
 191 for the reticulate network of the H3N2 data and 117 communities with a maximum community size of
 192 26 and $H = 4.65$ for the minimum spanning tree. The entropy across years was greatly decreased for all
 193 populations with the reticulate network compared to the minimum spanning tree (Fig. 2).

194 Graph walking of the reticulated minimum spanning network of *P. ramorum* by the infoMAP algorithm
 195 revealed 16 communities with a maximum community size of 13 and $H = 2.60$. The un-reticulated
 196 minimum spanning tree revealed 20 communities with a maximum community size of 7 and $H = 2.96$.
 197 In the ability to predict Hunter Creek as belonging to a single community, the reticulated network was
 198 successful whereas the minimum spanning tree separated one genotype from that community. The entropy
 199 for the reticulated network was lower for all populations except for the Coast population (supplementary
 200 information).

BOOTSTRAPPING

201 Calculating genetic distance for among samples and populations is very important method for assessing
 202 population differentiation through methods such as G_{st} , AMOVA, and Mantel tests (Nei, 1973; Excoffier
 203 et al., 1992; Mantel, 1967). Confidence in distance metrics is related to the confidence in the markers to
 204 accurately represent the diversity of the data. Especially true with microsatellite markers, a single hyper-
 205 diverse locus can make a population appear to have more diversity based on genetic distance. Using a
 206 bootstrapping procedure of randomly sampling loci with replacement when calculating a distance matrix
 207 gives confidence in hierarchical clustering. Because genetic data in a genind object is represented as a
 208 matrix with samples in rows and alleles in columns, bootstrapping is a non-trivial task as all alleles in
 209 a single locus need to be sampled together. To remedy this, we have created an internal S4 class called
 210 “bootgen”, which extends the internal “gen” class from *aedegenet*. This class can be created from any
 211 genind, genclone, or genpop object, and allows loci to be sampled with replacement. To further facilitate
 212 bootstrapping, a function called *aboot*, which stands for “any boot”, is introduced that will bootstrap
 213 any genclone, genind, or genpop object with any genetic distance that can be calculated from it.

214 To demonstrate calculating a dendrogram with bootstrap support, we used the *poppr* function *aboot*
 215 on population allelic frequencies derived from the data set *microbov* in the *aedegenet* package with 1000
 216 bootstrap replicates (Jombart, 2008; Lalo et al., 2007). The resulting dendrogram shows bootstrap support
 217 values > 50% (Fig. 3).

```
library("poppr")
data("microbov", package = "aedegenet")
strata(microbov) <- data.frame(other(microbov))
setPop(microbov) <- ~coun/spe/breed
bov_pop <- genind2genpop(microbov, quiet = TRUE)

set.seed(20150428)
pop_tree <- aboot(bov_pop, sample = 1000, cutoff = 50, quiet = TRUE)
```

AVAILABILITY

218 As of this writing, the *poppr* R package version 2.0 containing all of the features described here is located
 219 at <https://github.com/grunwaldlab/poppr>. It is necessary to install *aedegenet* 2.0 before
 220 installing *poppr*. It can be found at <https://github.com/thibautjombart/aedegenet>. Both
 221 of these can be installed via the R package *devtools* (Wickham and Chang, 2015):

```
library("devtools")
install_github("thibautjombart/aedegenet")
install_github("grunwaldlab/poppr")
```

DISCUSSION

222 We have presented here new model-free tools for the analysis of clonal populations with emphasis on
223 genomic-scale data. Especially important is `mlg.filter`, which

224 Creating structures like minimum spanning networks and dendrograms allow researchers to distill the
225 most important information from large distance matrices, revealing patterns that could support hypotheses
226 of differentiation or the lack thereof. Bifurcating dendrograms are most familiar to biologists as the
227 interpretation of them is straightforward and bootstrap confidence values can easily be obtained due to the
228 basic structure of the tree. Minimum spanning networks allow for a different view into populations, where
229 samples themselves can be treated as internal nodes connecting other samples, which could effectively
230 describe populations sampled through time. The drawback to these is that there is no clear method for a
231 bootstrap procedure to obtain confidence intervals.

232 Reticulate minimum spanning networks are very important for clonal organisms where a minimum
233 spanning tree would become a chain, implying that the clones were derived in a progressive and linear
234 fashion. This presents but one potential scenario for clonal organisms, but does not account for any other
235 biologically relevant process. Reticulations in the minimum spanning networks allow for a representation
236 of uncertainty that goes along with clonal organisms. The current implementation in `poppr` has been
237 successfully used in analyses such as reconstruction of the *P. ramorum* epidemic in Curry County, OR
238 (Kamvar et al., 2014a, 2015). Reticulated networks also allow for the application of graph community
239 detection algorithms such as the infoMAP algorithm (Rosvall and Bergstrom, 2008). As shown in the *P.*
240 *ramorum* and H3N2 data, while it is possible to utilize these graph walking algorithms on non-reticulate
241 minimum spanning trees, the results derived from these are limited to explain populations derived from
242 serial cloning events.

- 243
- bootstrapping methods encourage future developers to write distance implementations in common
244 format
 - moving towards open source, modular tools is the direction that population genetics and plant
245 pathology needs to go.
- 246

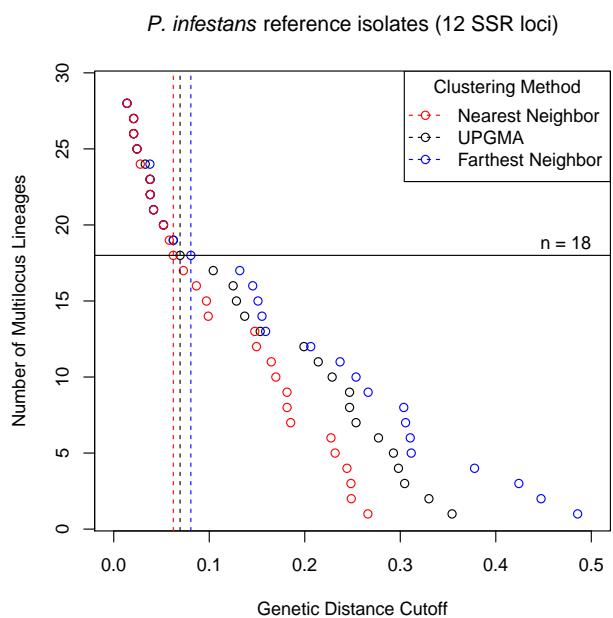
FIGURES AND TABLES**FIGURE 1**

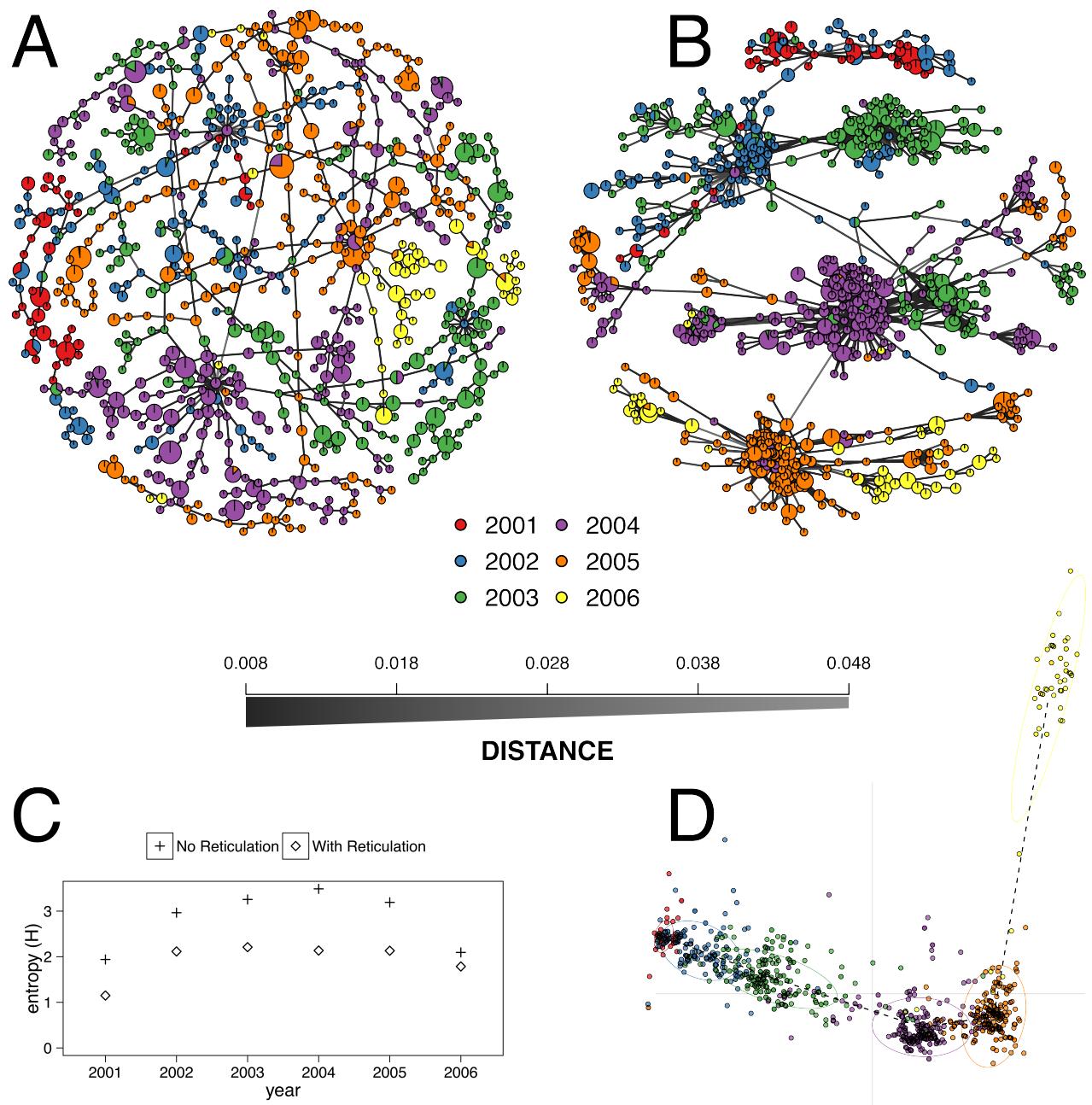
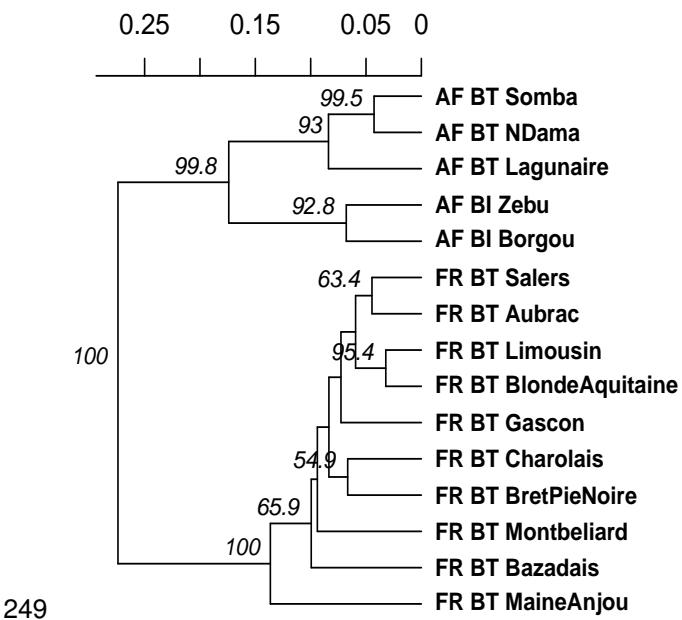
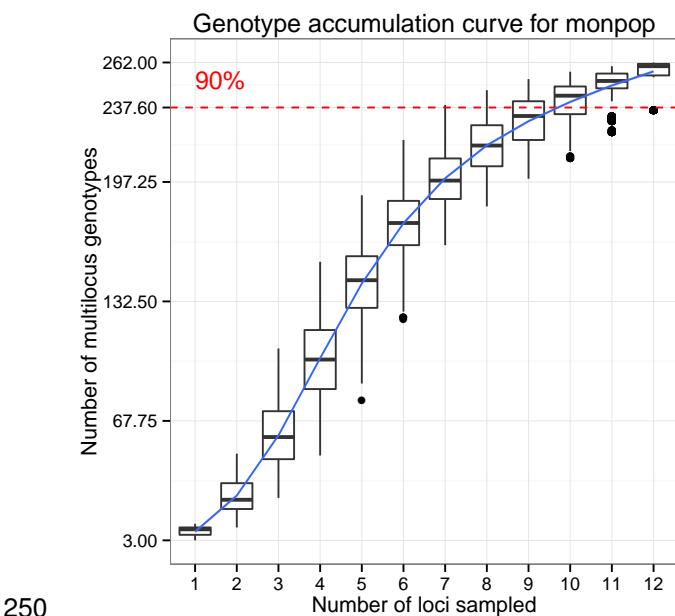
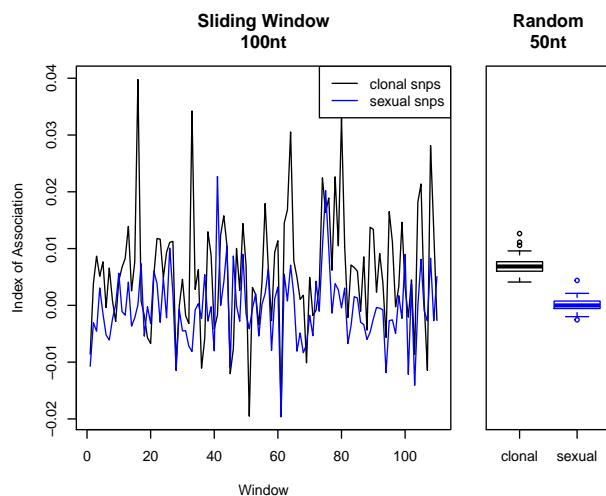
FIGURE 2

FIGURE 3**FIGURE 4**

250

FIGURE 5

251

TABLE 1

	3	4	5	6	8	10	12	15	16	17	18	20	21	22	24	25	27	28
B	1	.	.
C	1	.	.	.
D.1	1	.	.	.
D.2	1	.	.	.
EU-13	1
EU-4	1
EU-5	2
EU-8	1
US-11	2	.	.
US-12	.	1
US-14	1
US-17	1
US-20	2
US-21	2	.	.
US-22	2
US-23	3
US-24	.	.	.	1	3
US-8	.	.	1	1	.	2

FIGURE AND TABLE LEGENDS**FIGURE 1**

252 Graphical representation of three different clustering algorithms collapsing multilocus genotypes for 12
 253 SSR loci from *Phytophthora infestans* representing 18 clonal lineages. The horizontal axis is Bruvo's
 254 genetic distance assuming the genome addition model. The vertical axis represents the number of
 255 multilocus lineages observed. Each point shows the threshold at which one would observe a given number
 256 of multilocus genotypes. The horizontal black line represents 18 multilocus genotypes and vertical dashed
 257 lines mark the thresholds used to collapse the multilocus genotypes into 18 multilocus lineages.

FIGURE 2

258 (A-B) Minimum spanning networks of the hemagglutinin (HA) segment of H3N2 viral DNA from the
259 *adegenet* package representing flu epidemics from 2001 to 2006 with (B) and without (A) reticulations
260 (Jombart, 2008; Jombart et al., 2010). Each node represents a unique multilocus genotype, colors represent
261 epidemic year, and edge color represents absolute genetic distance. (C) Shannon entropy values for
262 population assignments compared with communities determined by the infoMAP algorithm on (A) and
263 (B). (D) Graphic reproduced from Jombart et al. (2010) showing that the 2006 epidemic does not cluster
264 neatly with the other years.

FIGURE 3

265 UPGMA dendrogram generated from Nei's genetic distance on 15 breeds of *Bos taurus* (BT) or *Bos indicus*
266 (BI) from Africa (AF) or France (FR). These data are from Lalo et al. (2007). Node labels represent
267 bootstrap support > 50% out of 1,000 bootstrap replicates.

FIGURE 4

268 Genotype accumulation curve for 694 isolates of the peach brown rot pathogen, *Monilinia fructicola*
269 genotyped over 13 loci from Everhart and Scherm (2015). The horizontal axis represents the number
270 of loci randomly sampled without replacement up to $n - 1$ loci, the vertical axis shows the number of
271 multilocus genotypes observed, up to 262, the number of unique multilocus genotypes in the data set. The
272 red dashed line represents 90% of the total observed multilocus genotypes. A trendline (blue) has been
273 added using the *ggplot2* function `stat_smooth`.

TABLE 1

274 Contingency table comparing multilocus lineages assigned based on average neighbor clustering
275 (columns) vs. multilocus lineages defined in Li et al. (2013) and Lees et al. (2006).

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