frontiers

Novel tools for analyzing genome-wide data of clonal populations

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ABSTRACT

To gain a detailed understanding of plant microbe interactions, adaptation of pathogens to hosts, develop reactive plant breeding programs, and to deploy R genes effectively, 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, many plant pathogen populations are at least partially clonal if not completely clonal (Milgroom, 1996; Anderson and Kohn, 1995). Thus, development of tools for analysis of clonal or polyploid populations is needed.

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

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

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

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

CLONAL IDENTIFICATION

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

this fact and occasional somatic mutations into account, it would be impossible to separate true clones from independent individuals by just comparing what multilocus genotypes are different. We introduce a new method for collapsing unique multilocus genotypes determined by naive string comparison into multilocus lineages utilizing any genetic distance given three different clustering algorithms: farthest neighbor, nearest neighbor, and UPGMA (average neighbor) (Sokal, 1958).

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

DEMONSTRATION DATA: *P. INFESTANS*

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

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

DEMONSTRATION DATA: SIMULATED DATA

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

All three filtering algorithms were run with a threshold of 1, returning a numeric vector of length $n - 1$ where each element represented a threshold at which two samples/clusters would join. Since each data set

would have varying distances between samples, the clonal boundary threshold was defined as the midpoint of the largest gap between two thresholds that collapsed less than 50% of the data.

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

INDEX OF ASSOCIATION

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

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

As it would necessarily focus on loci within a short section of the genome that may or may not be recombining, a sliding window approach would not be good for utilizing $\bar{r}_d$ as a test for clonal reproduction. A remedy for this is implemented in the function `samp.ia`, which will randomly sample 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

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

GENOTYPE ACCUMULATION CURVE

Analysis of population genetics of clonal organisms often borrows from ecological methods such as analysis of diversity within populations (Milgroom, 1996; Arnaud-Hanod et al., 2007; Grnwald et al., 2003). When choosing markers for analysis, it is important to make sure that the observed diversity in your sample will not appreciably increase if an additional marker is added (Arnaud-Hanod et al., 2007). This concept is analogous to a species accumulation curve, obtained by rarefaction. The genotype accumulation curve in *poppr* is implemented in the function `genotype_curve`. The curve is constructed by randomly sampling x loci and counting the number of observed MLGs. This repeated r times for 1 locus up to $n-1$ loci, creating $n-1$ distributions of observed MLGs.

The following code example demonstrates the genotype accumulation curve for data from Everhart and Scherm (2015) showing that these data reach a small plateau and have a greatly decreased variance with 12 markers, indicating that there are enough markers such that adding more markers to the analysis will 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

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

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

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

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

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

BOOTSTRAPPING

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

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

```
library("poppr")
data("microbov", package = "adegenet")
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

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

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

DISCUSSION

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

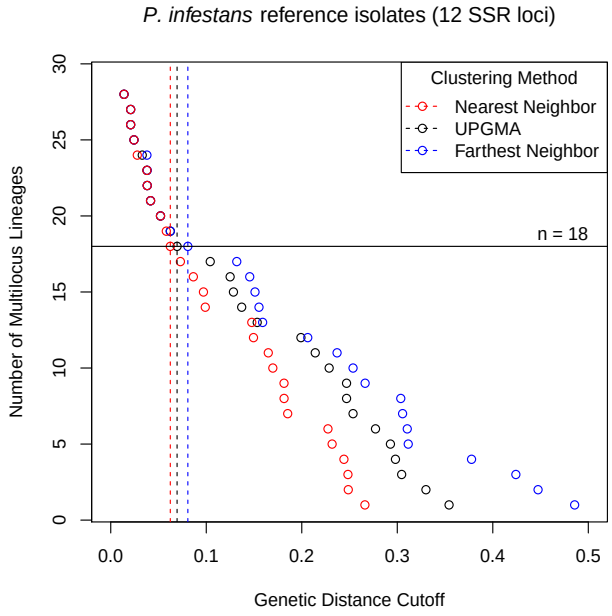
Creating structures like minimum spanning networks and dendrograms allow researchers to distill the most important information from large distance matrices, revealing patterns that could support hypotheses of differentiation or the lack thereof. Bifurcating dendrograms are most familiar to biologists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

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

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

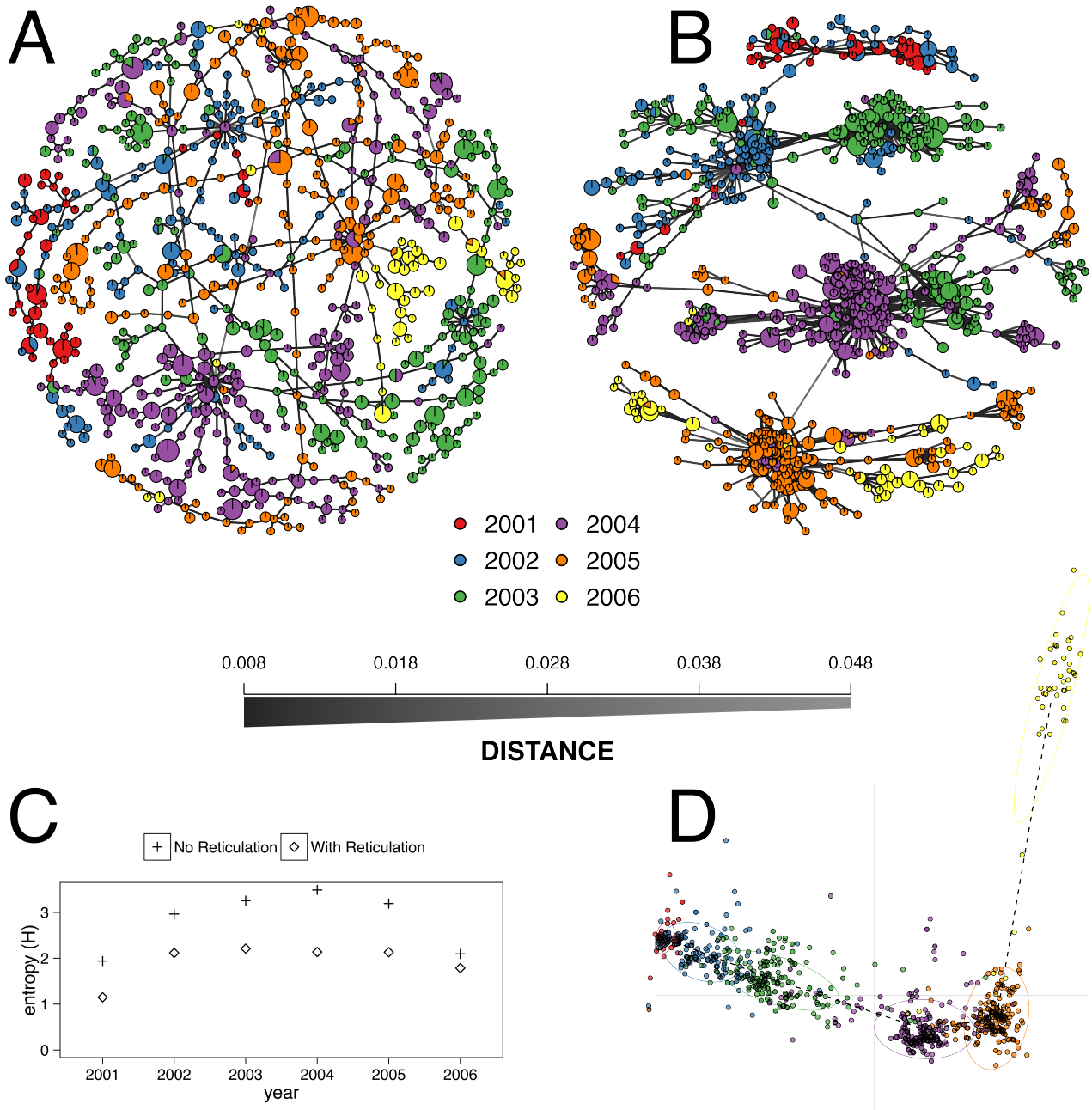
FIGURES AND TABLES

FIGURE 1



248

FIGURE 2


A
B
2001
2002
2003
2004
2005
2006
0.008
0.018
0.028
0.038
0.048
DISTANCE
C
No Reticulation
With Reticulation
entropy (H)
0
1
2
3
2001
2002
2003
2004
2005
2006
year
D

249

FIGURE 3

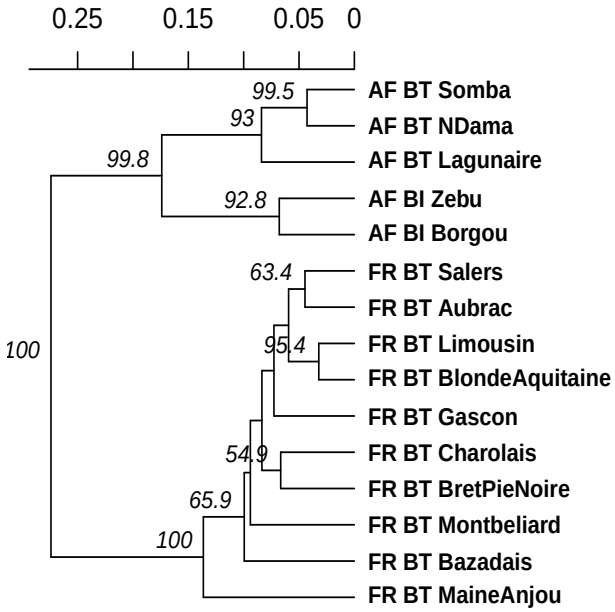


FIGURE 4

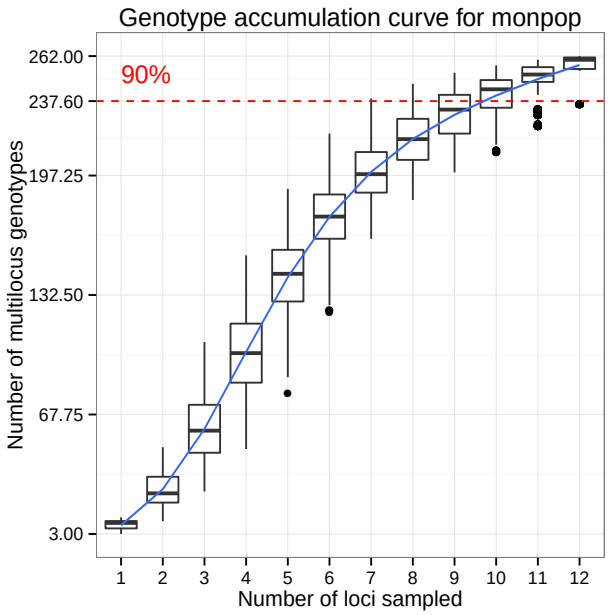


TABLE 1

FIGURE AND TABLE LEGENDS

FIGURE 1

Graphical representation of three different clustering algorithms collapsing multilocus genotypes for 12 SSR loci from *Phytophthora infestans* representing 18 clonal lineages. The horizontal axis is Bruvo's genetic distance assuming the genome addition model. The vertical axis represents the number of

	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	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.
US-8	.	.	1	1	.	2	.	.	.	.	.	.	.	.	.	.	.	.

multilocus lineages observed. Each point shows the threshold at which one would observe a given number of multilocus genotypes. The horizontal black line represents 18 multilocus genotypes and vertical dashed lines mark the thresholds used to collapse the multilocus genotypes int 18 multilocus lineages.

FIGURE 2

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

FIGURE 3

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

FIGURE 4

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

TABLE 1

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

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