Methods for 12-plex Microsatellite (SSR) Genotyping of *Phytophthora infestans*

Microsatellites can be used for genotyping lineages of *P. infestans*. Li and Cooke (2013) have developed a protocol that multiplexes 12 diagnostic SSR primer sets in a single tube for more rapid analysis and genotyping. The protocol uses fluorescently labeled primers, which can then be read by a capillary analyzer for analysis. This protocol is optimized for use with an ABI 3730xl DNA analyzer with a 5 dye set (6-FAM, VIC, NED, PET, and LIZ size standard). The following protocol is from Li and Cooke, with modifications implemented by the lab of Bill Fry at Cornell University and Anne Njoroge.

Primers (5' – 3')

Locus	Dye	Product size range (bp)	Primer sequence
PiG11	NED	130-206	FwdNED-TGCTATTTATCAAGCGTGGG Rev-GTTTCAATCTGCAGCCGTAAGA
Pi02	NED	255-275	FwdNED-ACTTGCAGAACTACCGCCC Rev-GTTTGACCACTTTCCTCGGTTC
PinfSSR11	NED	325-360	FwdNED-TTAAGCCACGACATGAGCTG Rev-GTTTAGACAATTGTTTTGTGGTCGC
D13	FAM	100-210	FwdFAM-TGCCCCCTGCTCACTC Rev-GCTCGAATTCATTTTACAGACTTG
PinfSSR8	FAM	250-275	FwdFAM-AATCTGATCGCAACTGAGGG Rev-GTTTACAAGATACACACGTCGCTCC
PinfSSR4	FAM	280-305	FwdFAM-TCTTGTTCGAGTATGCGACG Rev-GTTTCACTTCGGGAGAAAGGCTTC
Pi04	VIC	160-175	FwdVIC –AGCGGCTTACCGATGG Rev-GTTTCAGCGGCTGTTTCGAC
Pi70	VIC	185-205	FwdVIC – ATGAAAATACGTCAATGCTCG Rev-CGTTGGATATTTCTATTTCTTCG
PinfSSR6	VIC	230-250	FwdVIC-GTTTTGGTGGGGCTGAAGTTTT Rev - TCGCCACAAGATTTATTCCG
Pi63	VIC	265-280	FwdVIC – ATGACGAAGATGAAAGTGAGG Rev-CGTATTTTCCTGTTTATCTAACACC
PinfSSR2	PET	165-180	FwdPET-CGACTTCTACATCAACCGGC Rev-GTTTGCTTGGACTGCGTCTTTAGC
Pi4B	PET	200-295	FwdPET – AAAATAAAGCCTTTGGTTCA Rev-GCAAGCGAGGTTTGTAGATT

Instead of individually pipetting each primer into the master mix, a 10X multiplex primer mix is made that includes all primers. The primer mix is made as follows (makes 400µl):

Primer	Volume of 100µM primer stock (µl)
PiG11F	6
PiG11R	6
Pi02F	6
Pi02R	6
PinfSSR11F	6
PinfSSR11R	6
PinfSSR4F	6
PinfSSR4R	6
Pi04F	6
Pi04R	6
Pi70F	6
Pi70R	6
PinfSSR6F	6
PinfSSR6R	6
Pi63F	6
Pi63R	6
PinfSSR2F	6
PinfSSR2R	6
D13F	6.4
D13R	6.4
PinfSSR8F	12
PinfSSR8R	12
Pi4BF	12
Pi4BR	12

Combine with 231.2µL of 10 mM Tris buffer (pH=8.0) to make 400µl of primer mix.

The master mix can be made using either the Qiagen multiplex PCR kit (Qiagen, cat. No 206145) or the Qiagen Type-it Microsatellite PCR kit (Qiagen, cat. No. 206243). For the purposes of this protocol we use the Type-it microsatellite PCR kit.

SSR master mix:

Reagent	Volume per reaction (µl)
2X Type-it master mix	6.25
10X multiplex primer mix	1.3
PCR grade water	1.95
Total reaction mix volume per sample (µl)	11.5

1µl of template DNA is added to bring the total volume per sample to 12.5 µl. If more DNA is desired, adjust the volume of reaction mix per sample.

Thermal cycler program:

1 cycle	95C	5 min.
	95C	30 sec.
33 cycles	58C	90 sec.
	72C	20 sec.
1 cycle	60C	30 min.

Before loading on a DNA analyzer, samples must be prepared with the LIZ size standard (Applied Biosystems LIZ500, cat. No. 4322682) and suspended in an appropriate loading solution. For use on an ABI 3730xl DNA analyzer we use highly deionized formamide (hi di formamide, Applied Biosystems, cat. No. 4311320). Check with your local source for fragment analysis for preparation and submission protocols specific to their facilities.

Master mix for analysis preparation:

Reagent	Volume per reaction (µl)
Hi-di formamide	10
LIZ500 size standard	0.3
Total volume per sample (µl)	10.3

Add 0.5 µl of template reaction product to bring the total volume per sample to 10.8 µl. More product may be used if desired, up to 3 µl.

An optional denaturation step can be employed after plate prep to increase peak resolution by heating the prepared plate at 95C for 3 min, then chilling on ice.

Interpreting results

This data will be available as a trace file (.fsa), which will contain colored peaks corresponding to lengths of different genetic fragments. The 12-plex protocol described at the link above uses five dyes and generates thirteen series of fragments in total. The 12 microsatellite loci are represented by four different dye colors, with fragment length between those with like colors dissimilar enough to allow for differentiation. There is also a ladder included, which will have its own color of dye. The raw reads visible in this trace file can be scored using several different bioinformatics programs, including Geneious, a popular paid program, and STRand, a free resource provided by UC Davis. Using one of these programs or something similar, the researcher identifies the peak (or peaks if heterozygous) of fluorescence for a particular color in a particular region.

Identifying true peaks is a skill that takes practice. David Cooke has developed an allele calling guide to assist new researchers with getting familiar with the behavior of loci associated with the 12-plex *P. infestans* system: Help notes for scoring SSR alleles in *Phytophthora infestans*

Scoring a trace file typically has three stages: Cleaning the ladder (if needed), identifying true peaks, and designating alleles.

Cleaning the ladder

Before identifying peaks, the ladder should be inspected to ensure the program you are using has called the ladder peaks directly. See the table below for the LIZ500 expected peak sizes within the ladder. If your program is not correctly identifying the ladder as LIZ500, examine the called peaks and look for peaks identified that do not fit the expected fragment pattern. Ladder peaks are typically very regular both in size and distance (i.e. the 150bp peak will appear equidistant between the 100bp peak and the 200bp peak), and this can be used to identify and deselect errant peaks.

NOTE: LIZ500 has been identified by Applied Biosystems to have some degree of variability at the 250 peak and recommends removing it from analysis. We follow this recommendation and ensure the 250 peak has been deleted before designating alleles.

Identifying true peaks

Dye traces typically consist of a combination of true peaks and false peaks that can arise via various processes (e.g. spectral overlap, peak stutter). The degree and intensity of false peaks varies between loci. See David Cooke's scoring SSR alleles notes linked above for more information on learning the difference between structures observed in a trace.

Designating alleles

The fragment length for a particular locus is expected to have some degree of variability between runs, with fragment lengths identified with decimal points and without consideration

for the repeat motif. Since it is impossible to have 0.5 of a base pair, and in order to make sure alleles follow the known motif (e.g. repeats of 2), allele names are used.

The exact method for designating alleles can vary depending on the program used. In programs such as GeneMapper, alleles are named at a single point. In other programs such as Geneious, bin ranges are used, which are user-designated ranges that designate an allele name for peaks that fall within its size range.

In order to compare different data sets, the same allele names and naming strategy should be used to avoid artificially inflating the distance between samples. For information on the SSR binning scheme used by this tool, see "Population structure of *Phytophthora infestans* collected on potato and tomato in Italy" by Saville et al. 2021, Supplemental Table 3. This scheme was adapted from the Euroblight naming strategy for use in Geneious.

LIZ500 size standard peaks

Peak sizes/names	
35	250 (remove)
50	300
75	340
100	350
139	400
150	450
160	490
200	500

12-plex SSR *Phytophthora infestans* locus information

Locus	Dye	Expected number of peaks	Repeat unit	Range begin	Range end
D13	FAM	3	2	96	221
PinfSSR8	FAM	3	2	255	270
PinfSSR4	FAM	4	2	276	320
Pi04	VIC	2	2	154	181
Pi70	VIC	2	3	183	210
PinfSSR6	VIC	3	2	220	263
Pi63	VIC	3	3	264	290
PiG11	NED	3	2	120	239
Pi02	NED	3	2	252	280
PinfSSR11	NED	3	2	320	360
PinfSSR2	PET	2	2	160	180
Pi4B	PET	4	2	200	295

References

Li Y, Cooke DE, Jacobsen E, van der Lee T. Efficient multiplex simple sequence repeat genotyping of the oomycete plant pathogen Phytophthora infestans. Journal of microbiological methods. 2013;92(3):316-22.

Njoroge AW et al. 2019. Genotyping of *Phytophthora infestans* in eastern Africa reveals a dominating invasive European lineage. Phytopathology. 2019;109: 670-680.

Saville A, Ristaino, JB. Genetic structure and subclonal variation of extant and recent US lineages of *Phytophthora infestans*. Phytopathology. 2019;109(9):1614-27.

Saville, A.C., La Spada, F., Faedda, R, Migheli, Q., Scanu, B, Ermacora, et al. Population structure of *Phytophthora infestans* in Italy, Algeria and Tunisia. Plant Pathol. 2021; 7: 2165-2178.

Other Resources

USABlight: Genotype tracking website for North America
EuroBlight: Genotype tracking website for Europe
Tizón Latino: Genotype tracking website for Latin America