Supplementary Material on the Methodology Part VI

Protocol for analysis of microsatellite loci in sardine

Technical document with detailed information of the protocol developed for the analysis of 12-16 microsatellite loci in Sardina pilchardus.

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Material and Methods

Twenty microsatellites loci were selected, which were used in studies of sardine populations (Table 1), for the evaluation of amplification conditions in DNA of sardine. The samples were provided by the sampling campaign in charge of the CopeMed II project (TRANSBORAN) and extracted using the FENOLSAT method (Pérez and Presa, 2011).

Single microsatellite amplification by PCR.

Each locus was amplified individually using the polymerase chain reaction (PCR). PCR reactions were performed in a 20 µL volume and contained about 25 ng of genomic DNA previously extracted from the sardine samples (see report *Samples_DNA_Report_MNande*). The reaction contained 1 X PCR buffer (10x NH4 5x MyTaq™ Reaction Buffer Bioline), 1.5 mM $MgCl_2$ (50mM MgCl2 solution, Bioline), 0.5 mM di-nucleotide triphosphate (dNTP NZYSet, NZYTech), 0.5 µM of each primer (forward and reverse) and 0.2 U Taq DNA polymerase (BIOTAQ ™ DNA polymerase, Bioline). The following PCR profile was used: 5 min at 95 ° C, 40 cycles consisting of 20 sec at 95 ° C, 25 sec at the annealing temperature (see below), 45 sec at 72 ° C, and at the end extension at 72 ° C for 7 min. Different temperatures of annealing (Table 1) were used in order to optimize the amplification efficiency for each one of the loci separately and to establish the common amplification conditions in a multiplex PCR.

The results of the PCR amplification were observed by electrophoresis on to 2% (w/v) agarose gel and stained with ethidium bromide to check for size and amplification specificity.

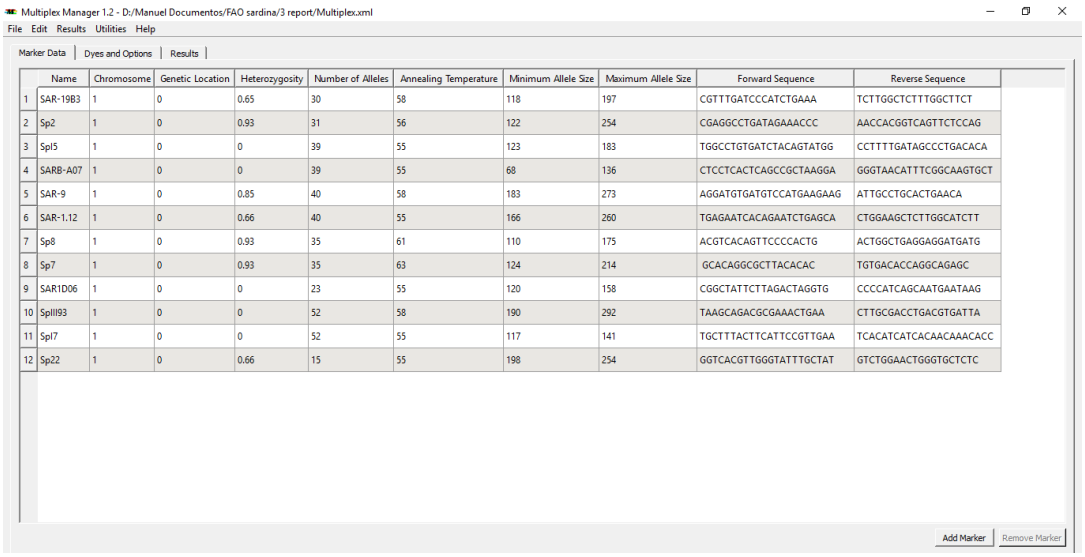
Table 1. Summary of the 20 microsatellite loci described in studies of sardine populations. Ta, annealing temperature in ° C, TaS, different annealing temperature in ° C used in this study.

Microsat Loci	Product size (bp)	Primer Sequences 5'-3'	Ta (°C)	Reference	TaS (ºC)
SAR-9	183-273	F :AGGATGTGATGTCCATGAAGAAG R : TTATTGCCTGCACTGAACA	55	González et al 2007a González et al 2007b Ruggeri et al 2012 Ruggeri et al 2013	52, 55 and 58
SAR-1.5	128–208	F : AGCTAAAAAGAAAACACACAG R: CTTCCTTCATGACCCAAGGTGA	54	González et al 2007a González et al 2007b Ruggeri et al 2012 Ruggeri et al 2013	52, 55 and 58
SAR-1.12	166–292 183-273	F : TGAGAATCACAGAATCTGAGCA R : CTTCTGGAAGCTCTTGGCATCTT	54 55	González et al 2007a González et al 2007b Ruggeri et al 2013	52, 55 and 58

SAR-2.18	176–242	F : CTGCGTGTGAATGTAGTCTG R : CTTCCATTTCTCACCATTTCTT	55	González et al 2007a González et al 2007b	52, 55 and 58
SAR-A2F	185–269	F : GCAGTAGTGAACCATCCCCTA R : TTGACACACGCGTACCACCA	63	González et al 2007a González et al 2007b	58, 61 and 63
SAR-A3C	101–311	F : GGGGCTTTTCATTATTCATCAG R : CTTCCTGTGCCCAAATGACACT	63	González et al 2007a González et al 2007b	58, 61 and 63
SAR-19B3	118–197	F : CGTTTGATCCCATCTGAAA R : TCTTGGCTCTTTGGCTTCT	58	González et al 2007a González et al 2007b	55, 58 and 61
SAR-19B5	147–337	F : AATGGCTTTCATCTGACCTTG R : TGGGACGACACCTTTAGTGAT	58	González et al 2007a González et al 2007b	55, 58 and 61
Sp22	194-232 198-230	F : GGTCACGTTGGGTATTTGCTAT R : GTCTGGAACTGGGTGCTCTC	49 50	Kasapidis et al 2012. Thesis E. Cilli.	52 and 55
SpI5	123-183	F: TGGCCTGTGATCTACAGTATGG R: CCTTTTGATAGCCCTGACACA	55	Ruggeri et al 2012 Ruggeri et al 2013	52, 55 and 58
Sp2	138-254 146-238 122-274	F : CGAGGCCTGATAGAAACCC R : AACCACGGTCAGTTCTCCAG	56 59 54	Kasapidis et al 2012 Thesis E. Cilli 2009 Baibai et al 2012	52, 55 and 58
Sp7	124-212 125-169 112-262	F : GCACAGGCGCTTACACAC R : TGTGACACCAGGCAGAGC	62 62 61	Kasapidis et al 2012 Thesis E. Cilli 2009 Baibai et al 2012	58, 61 and 63
Sp8	109-211 110-160 111-327	F : ACGTCACAGTTCCCCACTG R : ACTGGCTGAGGAGGATGATG	62 62 63	Kasapidis et al 2012 Thesis E. Cilli 2009 Baibai et al 2012	58, 61 and 63
Sp10	148-248	F: GCAAAAGTGCTCGAAGACG R:CGCTTTTGTTGGCTAAAACAT	58	Ruggeri et al 2012 Ruggeri et al 2013	55, 58 and 61
Sp45	160-216	F : TAGGAATTGAGCCATGACCTTT R :TGAAAACACCACTCATCAGGAC	ND	Thesis E. Cilli 2009	52 and 55
Sp5	84-90	F : ATAAGAGTTACGGGCCACCC R :TATGCCTACTGGCCTCAATG	57	Thesis E. Cilli 2009	55, 58 and 61
SAR1D06	120-158	F: CGGCTATTCTTAGACTAGGTG R: CCCCATCAGCAATGAATAAG	50	Ruggeri et al 2012 Ruggeri et al 2013	52 and 55
SpI7	117-141	F: TGCTTTACTTCATTCCGTTGAA R: TCACATCATCACAACAAACACC	52	Ruggeri et al 2012 Ruggeri et al 2013	52 and 55
SpIII93	170-292	F:TAAGCAGACGCGAAACTGAA R:CTTGCGACCTGACGTGATTA	58	Ruggeri et al 2013	55, 58 and 61
SARB-A07	68-136	F:CTCCTCACTCAGCCGCTAAGGA R:GGGTAACATTTCGGCAAGTGCT		Ruggeri et al 2013	52 and 55

Multiplex PCR design "in silico".

The Multiplex Manager 1.0® software (Holleley and Geerts, 2009) was used to group in multiplex the selected microsatellites markers previous the amplification separately. The selection criteria were the amplification in a single band, being discarded those microsatellites markers with non-specific amplification (several bands) or without amplification band. Four fluorophore dyes (6-FAM, VIC, NED, PET) will be used for primer marker (Fig. 1).



	Name	Chromosome	Genetic Location	Heterozygosity	Number of Alleles	Annealing Temperature	Minimum Allele Size	Maximum Allele Size	Forward Sequence	Reverse Sequence
1	SAR-19B3	1	0	0.65	30	58	118	197	CGTTTGATCCCATCTGAAA	TCTTGGCTCTTTGGCTTCT
2	Sp2	1	0	0.93	31	56	122	254	CGAGGCCTGATAGAAACCC	AACCACGGTCAGTTCTCCAG
3	Spl5	1	0	0	39	55	123	183	TGGCCTGTGATCTACAGTATGG	CCTTTTGATAGCCCTGACACA
4	SARB-A07	1	0	0	39	55	68	136	CTCCTCACTCAGCCGCTAAGGA	GGGTAACATTTCGGCAAGTGCT
5	SAR-9	1	0	0.85	40	58	183	273	AGGATGTGATGTCCATGAAGAAG	ATTGCCTGCACTGAACA
6	SAR-1.12	1	0	0.66	40	55	166	260	TGAGAATCACAGAATCTGAGCA	CTGGAAGCTCTTGGCATCTT
7	Sp8	1	0	0.93	35	61	110	175	ACGTCACAGTTCCCCACTG	ACTGGCTGAGGAGGATGATG
8	Sp7	1	0	0.93	35	63	124	214	GCACAGGCGCTTACACAC	TGTGACACCAGGCAGAGC
9	SAR1D06	1	0	0	23	55	120	158	CGGCTATTCTTAGACTAGGTG	CCCCATCAGCAATGAATAAG
10	SpIII93	1	0	0	52	58	190	292	TAAGCAGACGCGAAACTGAA	CTTGCGACCTGACGTGATTA
11	Spl7	1	0	0	52	55	117	141	TGCTTTACTTCATTCCGTTGAA	TCACATCATCACAACAAACACC
12	Sp22	1	0	0.66	15	55	198	254	GGTCACGTTGGGTATTTGCTAT	GTCTGGAACTGGGTGCTCTC

Figure 1. Data on microsatellites loci used for the design of two multiplex PCR "*in silico*" using six loci by PCR.

Amplification conditions for the selected microsatellites markers by PCR-multiplex.

PCR multiplex consisted of the combination of 6 loci per reaction. PCR optimization was carried out by testing buffers with different concentrations of $MgCl_2$ among 1.5 to 2 mM (50mM MgCl2 solution, Bioline), and concentration of primers from 0.1 to 0.30. Others conditions were constants, 1 X PCR buffer (10x NH4 5x MyTaq™ Reaction Buffer Bioline), 10 mM di-nucleotide triphosphate (dNTP NZYSet, NZYTech), 0.1 to 0.3 µM of each primer and 0.2 U of polymerase (BIOTAQ ™ DNA polymerase, Bioline). The following PCR profile was used: 5 min at 95 ° C, 40 cycles consisting of 20 sec at 95 ° C, 25 sec at consensus annealing temperature provided by Multiplex Manager 1.0® software of 55 ºC for Multiplex 1 and 59 ºC for the Multiplex 2, 45 sec at 72 ° C, and at the end extension at 72 ° C for 7 min.

The different microsatellite markers were amplified at the same annealing temperature than multiplex and all were observed in the same agarose gel at 3% (w/v) to evaluate the amplification of all loci in the multiplex and separately PCR.

Results

Single microsatellite amplification by PCR.

All loci showed bands of amplified except three (Sp 45, Sp5 and SAR19B5). Several bands were observed at the microsatellite markers Sp22, SAR-1-5, SAR 2.18, Sp10, SAR-A2F, and SAR-A3C (Fig 2). For the Sp7 microsatellite marker, two bands are observed with an annealing temperature of 58 °C and a single one at 63 °C (Fig 2, D). The microsatellite markers selected to amplify in PCR multiplex were SAR19B3, Sp2, Spl5, SARB-A07, SAR-9, SAR-1.12, Sp8, Sp7, SAR1D06, SPIII93, Spl7 and Sp22.

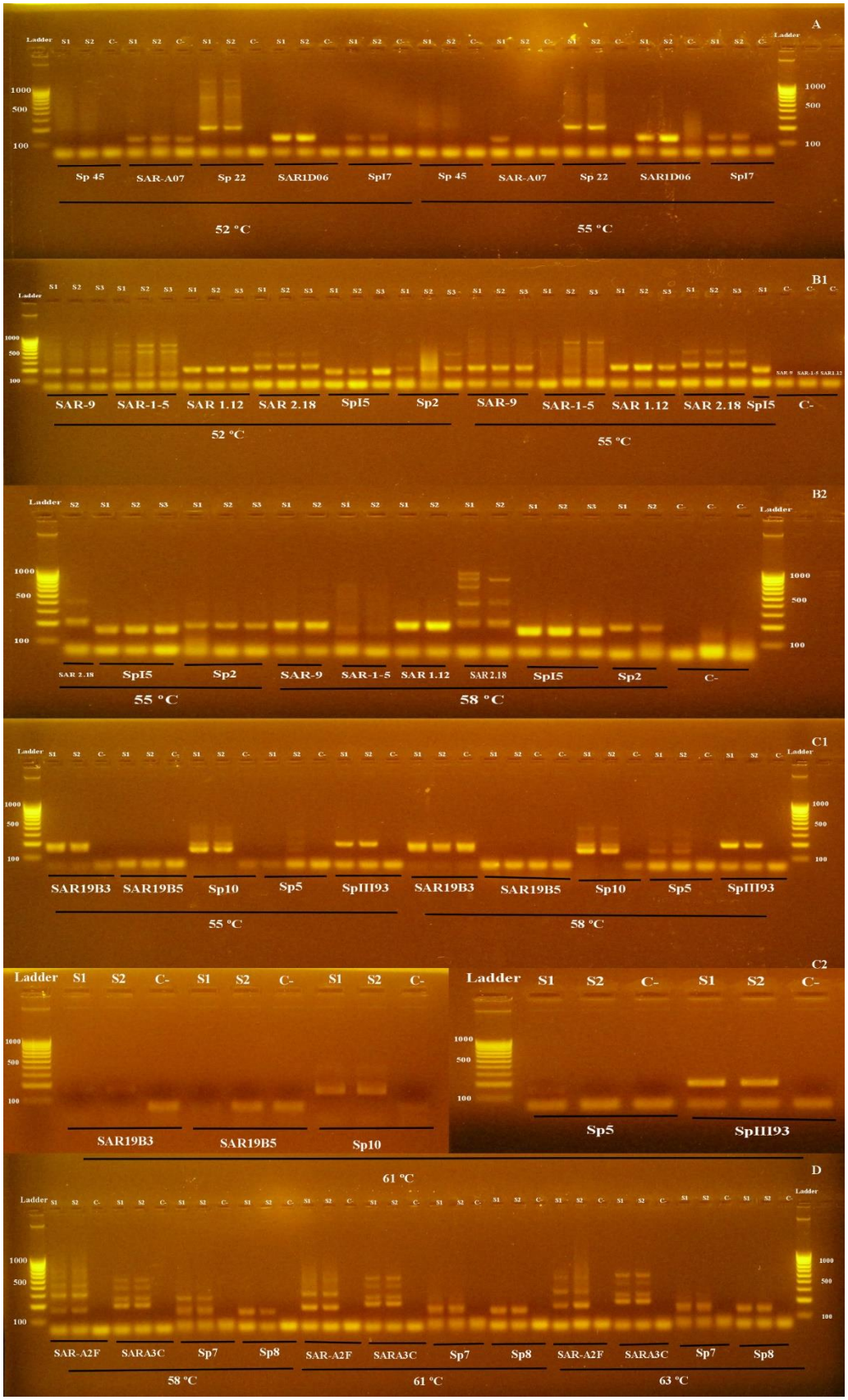


Figure 2. PCR products of the 20 microsatellite markers amplified at different annealing temperatures (Table 1) in 2% (w/v) agarose gel.

Multiplex PCR design "in silico".

Results of grouping microsatellite markers in two PCR multiplex were Spl5, Spl7, SARB-A07, SAR-1.12, Sp22 and SAR19B3 with an annealing temperature of 55 ° C and SAR1D06, SpIII93, Sp8, Sp7, Sp2 and SAR-9 on a temperature of 59 ° C (Fig. 3).

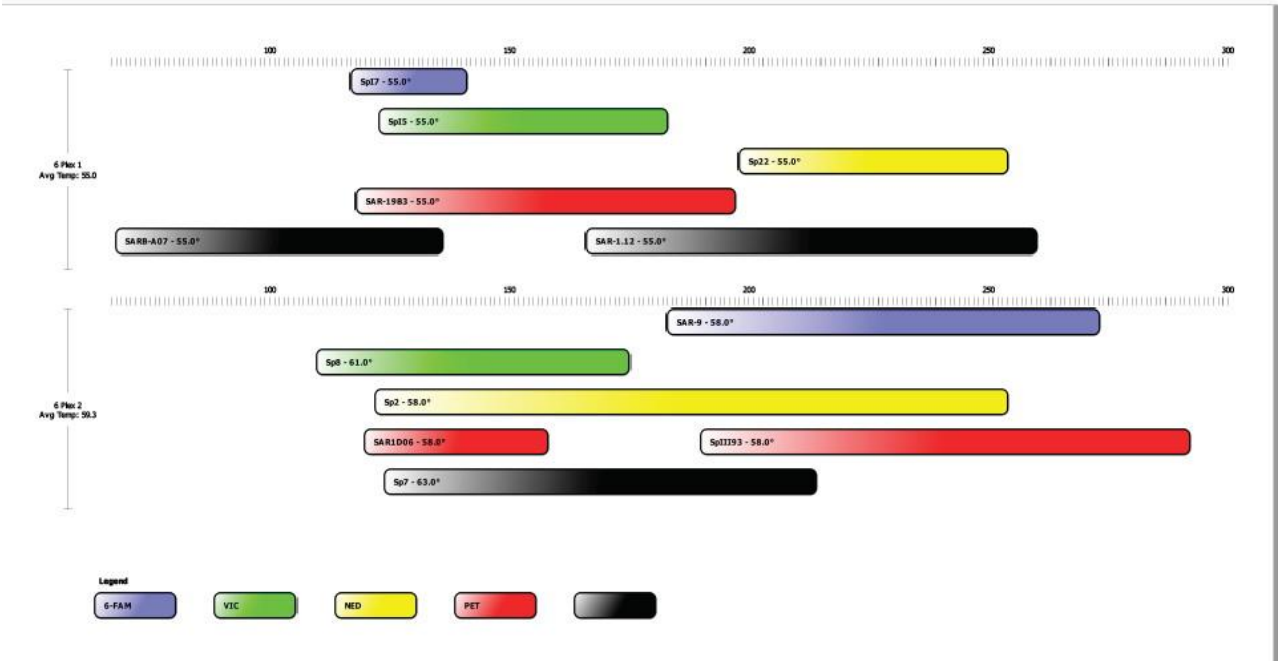


Figure 3. Detail of microsatellite that constitute the multiplex 1 at an annealing temperature of 55 ºC and the multiplex 2 of 59 ºC.

Amplification conditions for the selected microsatellites markers by PCR-multiplex.

In the agarose gel, three amplification bands were observed for multiplex 1 with 0.1 µM of each primer, coinciding with the sizes (bp) of the expected amplicons, while only two were observed with a concentration of 0.3 µM of each primer (Fig. 4). For multiplex 2, only two bands are observed regardless of the concentration of the primers (Fig 4).

In the individual amplification the loci Sp7, SAR19B3, and SAR-9 showed two bands so they were discarded for the final multiplex design (Fig. 5). No differences were observed in the intensity in the bands for multiplex 1 to 1.5 and 2 mM $MgCl_2$ (Fig 5, A). For multiplex 2 highest intensity is observed with 2 mM $MgCl_2$ than 1.5 and 1.75 mM $MgCl_2$ (Fig 5, B).

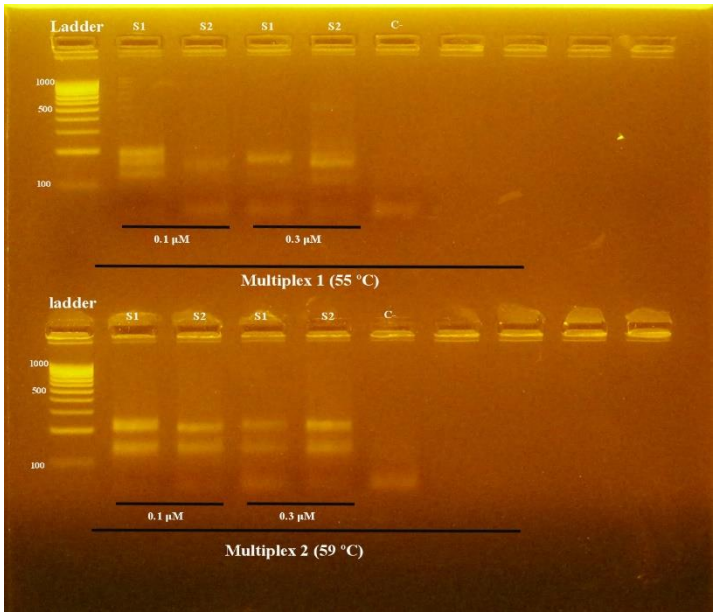


Figure 4. Amplification fragments of multiplex 1 and 2 with different concentrations of primers (0.1 and 0.3 µM of each primer) in agarose gel 3% (w/v).

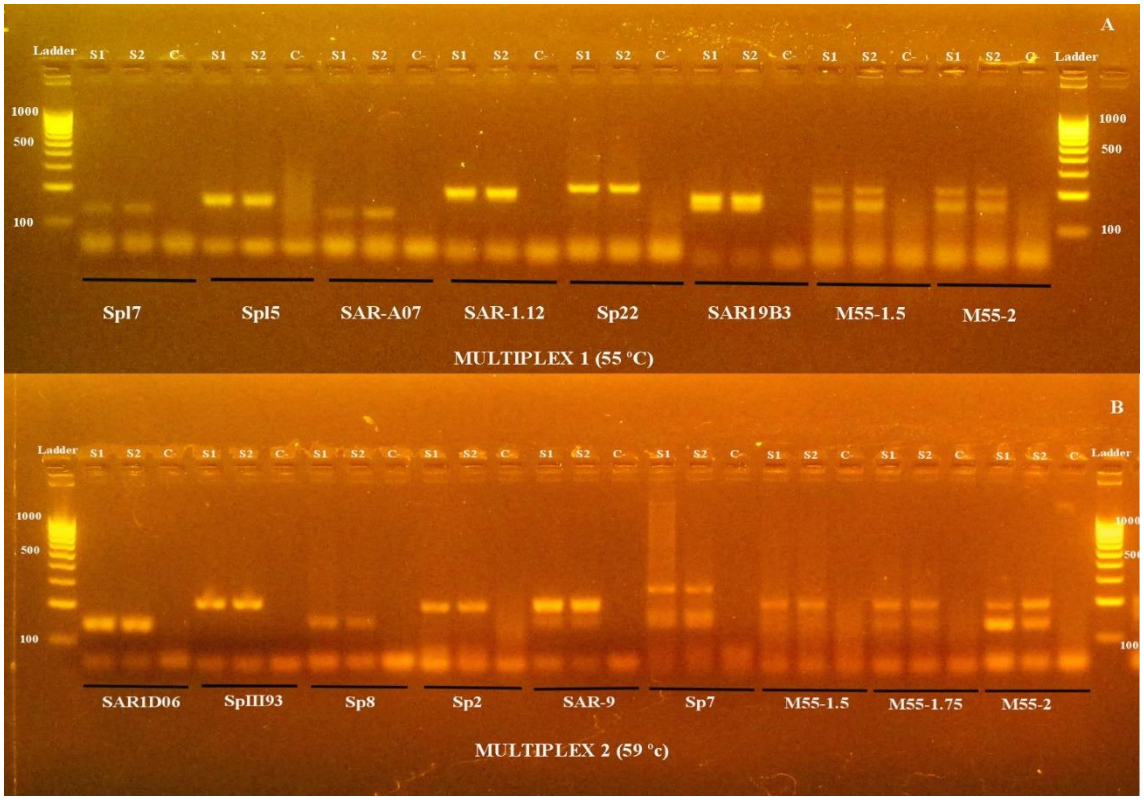


Figure 5. Products of each single microsatellite PCR and PCR-multiplex are observed in the agarose gel 3% (w/v). M55-1.5, 1.5 mM $MgCl_2$; M55-1.75, 1.75 mM $MgCl_2$ and M55-2, 2 mM $MgCl_2$.

Conclusion

Having analyzed 20 initial loci, 12 were selected for first PCR-multiplex design. Finally, a total of 9 microsatellites markers was grouped in two multiplex PCR consist of 4 (Multiplex 1) and 5 (Multiplex 2) loci (Fig. 6). For the labeling, fragments of amplification sizes were taken into account for each PCR-multiplex.

Because the agarose gel does not allow in this case to differentiate by size different bands that would correspond to each microsatellite, it is recommended to mark the primers in order to be able to genotype the amplified fragments in a sequencer and then analyzed the efficiency of PCR-multiplex for all microsatellites. The primers will be marked with different fluorophores dye (6-FAM, VIC, MED, and PET) following figure 6 and will be ordered Company NZYTech, Lda.

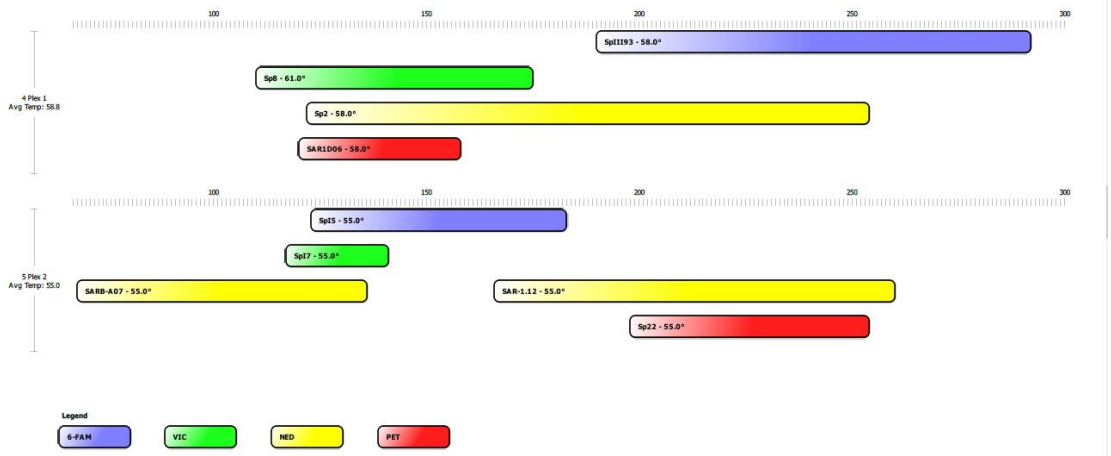


Figure 6. Final design of the two PCR-multiplex consisting of four (multiplex 1) and five (multiplex 2) microsatellites markers with a different fluorescents dye (6-FAM, VIC, MED and PET).

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