Towards an Automated Population Genomics Pipeline for Microsatellite Screening and Primer Design

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Abstract

Analysis of intra- and inter-population diversity has become important for defining the genetic status and distribution patterns of a species and a powerful tool for conservation programs, since high levels of inbreeding could lead into a whole population extinction in few generations. Microsatellites (SSR) are commonly used in population studies, but discovering highly variable regions across species' genomes requires demanding computation and laboratorial optimization. In this work, we combine next generation sequencing (NGS) with automatic computing to develop a genomic-oriented tool for characterizing SSRs at the population level. Herein, we describe a new Python pipeline, named Micro-Primers, designed to identify and design PCR primers for amplification of SSR loci from a multi-individual enriched microsatellite library. The pipeline takes as input a fastq file containing sequences from NGS and returns a text file with information regarding the microsatellite markers, including number of alleles in the population, the melting temperature and the respective product of primer sets to easily guide the selection of optimal markers for the species. Experimental results show that Micro-Primers is able to reduce significantly a manual analysis that takes about 24 hours to 2 minutes, while keeping the same quality of the results.

INTRODUCTION

At the Omics' era the cost of sequencing and time required for getting useful information from different organisms, even uncultured, has been reduced drastically with the advances in technology (Ekblom & Galindo, 2011), allowing the broadening of the scientific scope worldwide. While traditional studies covered a gene region and/or a pathway with limited number of genes, next generation sequencing (NGS) has pushed the trend towards whole-genome analysis and population genetics, where the genome of several individuals of a species can be characterized at the same time (Bahassi & Stambrook, 2014). In this field, molecular techniques such as genotyping by sequencing (GBS) and marker-assisted selection (MAS) have gained prominence by not requiring a reference genome available (Collard & Mackill, 2008; He et al., 2014) and also by the possibility of characterizing a whole species at lower cost, providing comprehensive information for both evolution studies and conservation efforts (Khaing et al., 2013; Siadjeu, Mayland-Quellhorst, & Albach, 2018).

Genetic polymorphisms, such as single nucleotide polymorphisms (SNPs) or simple sequence repeats (SSRs), also known as microsatellites, have served the field of population genetics (Bruford & Wayne, 1993; Helyar et al., 2011). SSRs are repeated DNA motifs that occur in non-coding regions, evenly distributed throughout the genome. They are excellent markers for genotype identification, genetic diversity and genetic-phenotype mapping, at both species and population levels, due to their high levels of polymorphism (Morgante & Olivieri, 1993; Vieira, Santini, Diniz, & Munhoz, 2016). Traditional methods use a single individual per species for microsatellite library development, and the number of microsatellite loci genotyped afterwards needs to be limited as a balance between the cost associated with microsatellite design and optimisation.

The goal of this work is to design and implement an automated pipeline for screening SSRs from raw paired-end reads generated by the hybridization of a single-digest library enriched for di-, tri- and/or tetranucleotide motifs (adapted from Garrett, Dawson, Horsburgh and Reynolds (2017)) but using a multi-individual sample. The tool is able to detect the SSRs loci variation present in the population and design optimal primers per SSR marker. Highly polymorphic markers can be then used to genotype other individuals from the same species.

Currently, there are few programs that can detect microsatellites and that are able to design primers for later amplification such as SSR Pipeline (Miller, Knaus, Mullins, & Haig, 2013), GMATA (Wang & Wang, 2016), Full SSR (Metz, Cabrera, Rueda, Giri, & Amavet, 2016) and CandiSSR (Xia et al., 2016). However, to the best of our knowledge, none of them takes in consideration several important parameters for a multi-individual SSR identification. Although some of them can extract SSRs from sequence data and design primers (e.g., Full SSR and GMATA), they do not consider if, for example, an allele belongs to an existing SSR locus in the dataset. Micro-Primers (available at the GitHub repository https://github.com/FilAlves/micro-primers) integrates a set of external programs into an automated pipeline allowing the perfect communication between them by conditioning the input/output formats. Thus, Micro-Primers represents a unique and easy framework for microsatellite characterization.

MATERIALS AND METHODS

The main motivation of Micro-Primers is to eliminate issues regarding time and computational work, while doing a manual selection of microsatellites candidates. As such, several tools and scripts were integrated within Micro-Primers for discovering SSRs and designing the respective primers for further *in vitro* amplification.

Internal & External Components

The Micro-Primers pipeline was written in Python version 3.6. The two main internal components were implemented using the following scripts: (i) install.py that includes all necessary pre-requisites for a proper installation of Micro-Primers, and (ii) micro-primers.py, which is the main script that defined the pipeline. Analysis settings are described in the config.txt file, and parameters can be modified by the user accordingly to their own needs. The folder software, provided together with the Python scripts, holds all the scripts and external software employed by micro-primers.py.

The Micro-Primers pipeline integrates several external components, such as: (i) Trimmomatic (Bolger, Lohse, & Usadel, 2014) for the removal of the sequencing adapters; (ii) Cutadapt (Martin, 2011) for the removal of the technology-specific adapter; (iii) FLASH (Magoč & Salzberg, 2011) for the merging of paired-end reads (R1 and R2); (iv) MISA (Thiel, Michalek, Varshney, & Graner, 2003) for the SSR searching; (v) CD-HIT (Fu, Niu, Zhu, Wu, & Li, 2012; Li & Godzik, 2006) for the removal of redundancy; (vi) Primer3 (Rozen & Skaletsky, 2000) for the primer design.

Input Files & Pipeline

To run Micro-Primers, users only need to provide two FASTQ files corresponding to both ends of a paired-end sequencing run. Samples should come from a pool of (untagged) individuals of the same species so the microsatellite selection can be optimized. SSR selection will be performed based on the number of alleles of each SSR loci, so the more heterogeneous the sample is (i.e. containing individuals from distinct populations across the species distribution), the better the final result will be. Reads must come from a microsatellite library built using a restriction enzyme and following an enrichment protocol such as the one described in Garrett et al. (2017). The enrichment protocol is performed after digestion so the target SSR motifs are the most represented strands in the final library. A fragment size selection is then performed on the enriched

library to keep only fragments of an average length lower than the maximum sequencing length to allow both paired-ends reads overlap when merged later on. The final fragment size is important for microsatellite screening and must comprise the full SSR pattern (variable in length) and the two flanking regions with fair length for primer design.

Additionally, prior to the execution of Micro-Primers, the users must install all the external components and set the environment variables through the script install.py. Moreover, the users must also check the config.txt file (we will describe the configuration parameters in the next subsection) and then, they can execute the main script (micro-primers.py). Up on execution, Micro-Primers will follow the flowchart described in Figure 1. It begins using Trimmomatic and Cutadapt for the removal of sequencing and technology-specific adapters respectively, and both paired-end reads are merged via FLASH. Only sequence reads containing the restriction enzyme pattern are kept by the pipeline. Various parameters are then calculated and only the sequences that comply with the specifications of the users are selected. Next, the repeating region of SSRs is removed from sequences, and the flanking regions are aligned and assigned to a cluster using CD-HIT with the following parameters (-c=0.95 -n=10).

Sequences belonging to the same cluster are sorted and number of different alleles in the cluster are computed. Only clusters with a minimal number of alleles (set by the user at the config.txt file) are chosen and a random sequence among variants is selected as the representative of each SSR locus. Every representative is then parsed into primer3 and an output file with both, primer information and number of alleles for each sequence, will be created accordingly to the primer's specifications set by the user.

Execution Parameters

As described previously, all the parameters that Micro-Primers needs to perform the analysis properly, must be dully set at the config.txt file. In this file there are four sections with different parameters to be considered for the pipeline execution:

In the first section (Input Files), the user has to indicate the name of the paired-end files that will be used as input in the analysis.

In the second section (CUTADAPT), the sequence of adapters used after the restriction enzyme digestion is required. These adapters are necessary to transform the longer overhangs into blunt ends after the enzyme digestion. Only sequences with these adapters are considered 'true' digested sequences. In the third section (SSR), several parameters regarding the microsatellite selection are involved. The parameter MIN FLANK LEN indicates the minimum length accepted in both flanking regions where the primers will be designed on. The length of the flanking areas is critical to the final outcome since a very narrow window prevents the design of primers and subsequently causes the exclusion of the respective SSR. Thus, any sequence with shorter flanking region (in any of both ends) that the length specified will be discarded. The MIN MOTIF REP sets the minimum number of repeats that every SSR loci must have to be kept in the pipeline. Also, specific SSR motifs can be discarded from the output if indicated in the EXC MOTIF TYPE parameter. Options for this parameter are c (compound), c* (compound with imperfection) and p1 to p6 (repeated motif of 1 to 6 nucleotides) (Thiel et al., 2003). Motifs chosen to be discarded should be indicated separately with comma. The MIN ALLEL CNT option indicates the minimum number of alleles for a SSR locus to be selected and it is based on the observed alleles. In opposition, the parameter MIN ALLEL SPECIAL DIF indicates the minimum potential number of alleles desired for each loci, taking into account that not all alleles are represented in the multi-individual sample. Assuming that the difference between the alleles with higher and lower number of repeats, only loci that satisfy the minimum number of alleles indicated in the MIN ALLEL SPECIAL DIF are kept. The parameter MIN ALLEL SPECIAL is used to enable

(=1) or disable (=0) this option.

Finally, in the fourth section (PRIMER3), the config.txt file is used to implement PRIMER3, where the only requirement is to indicate the path to the Primer3 settings file containing the standard parameters of Primer3.

However, the parameters can be changed according with a user demands, e.g., PRIMER PRODUCT SIZE RANGE, PRIMER OPT SIZE, PRIMER OPT TM and/or PRIMER MAX POLY X, among others (find all parameters in https://primer3.org/manual.html). PCR amplification primers usually are designed with a length of 20-25 nucleotides and some particularities are required to avoid future problems during genotyping (Dieffenbach, Lowe, & Dveksler, 1993; Flores-Rentería & Krohn, 2013), like the presence of G or C at the 3' end, certain percentage of GC for a proper melting temperature and both primers having similar melting temperature for their hybridization to take place at the same time.

RESULTS

In this section, we show the experimental results obtained with our pipeline in a real case study and following the same procedure that would have been carried out in a manual analysis.

Micro-Primers' Output

The execution of Micro-Primers pipeline produces a single output file in plain text with useful information for the amplification of the SSR loci through its representative. Figure 2 shows a sample of file and how it is divided. It has eleven columns and each line represents the primers designed by Primer3 for each SSR recovered from the multi-individual sample. From left to right, the first column (in red) is the representative sequence of each cluster preceded by a unique index to easily identify them (sequence ID). Lines with same sequence ID represent different primer pairs for the same SSR loci. The second column has the size (or length) of the sequence resultant from PCR amplification using the respective primer pairs. The third and fourth columns are the forward primer sequence and its melting temperature. The fifth and sixth column are the equivalent but for the reverse primer. In the seventh column, the specific motif/allele found is shown with the number of repeats found in the SSR representative. Column marked as 'Range' shows the length rage for the PCR amplicon for all the alleles detected for the same SSR. Nineth column contains the total number of alleles for the specific SSR loci. Next column, the tenth, indicates the potential number of alleles to be found in the population estimated from the difference between the longest and shortest alleles found. Finally, the eleventh column indicates the best combination of primer pairs for each loci (coded as " | BEST | ") as provided by Primer3.

Performance Analysis

The Micro-Primers pipeline was tested with a dataset belonging to bats from two different populations, Namibia and Botswana, with a total of 15 and 21 individuals respectively (the dataset is also available in the GitHub repository together with the Micro-Primers' software). Samples were pooled, enriched for di- and tetra-repeat motifs separately following the protocol established by Garrett et al. (2017) and sequenced together on an Illumina MiSeq v2 kit (250 cycles, paired-end) targeting 300k reads.

Since the species is diploid, the maximum number of alleles to be found by locus is 72. The process was reproduced by both manually running each required program one after the other and executing the Micro-Primers pipeline with exactly the same parameters used in the manual run.

Results from both procedures were identical, as expected, being the only difference the time spent to complete them. The manual process took no less than 24 hours, mainly spent on the manual selection of the clusters. Some changes in input format were also required for the proper functioning of certain programs, such as Primer3 for which sequence identifiers were modified to include an index in the beginning to facilitate handling. The goal was to avoid problems with some software on dealing with long and redundant sequence names. On the other hand, the automatic pipeline took less than 2 minutes to execute the entire analysis, using a single core of an Intel i7 Octa-Core processor with 64 Gbytes of main memory. It should be noted

that the unique point where the memory is more demanding is at the trimming step carried out by the Trimmomatic component, so in general, minimal resources are needed.

In addition, four different parameters configurations were tested to check the performance of the pipeline and evaluate the differences in the number of microsatellites loci detected. The pipeline's execution was modified by changing the parameters at the configuration file or at the Primer3 settings file, and the number of sequences remaining after each step is presented in Table 1. The four configurations tested were: (i) the default; (ii) with activation of the special search with a minimal difference between extreme alleles of 8; (iii) with change of the flanking region length; and (iv) modifying the difference in melting temperature between forward and reverse primers at Primer3. As observed in Table 1, the numbers of sequences that comply the requirements in the first four pipeline steps are exactly the same since none of the tested configurations are applied in these levels. The pipeline output changes after Filter 2 depending on the configuration used.

The implementation of the special feature MIN ALLEL SPECIAL DIF, based on the potential number of alleles per loci, shows substantial impact on the final number of loci kept and subsequent number of primers selected in comparison with the default setting based on the observed number of alleles. When the special parameter is activated and the minimal difference between the extreme alleles is 8, the number of SSR loci increases from 26 to 104, producing a total of 83 primer pairs.

Variations on the minimal flanking region length at Filter 2 affect the number of sequences that will pass to the following steps, and thus the number of SSR markers at the end. Higher values in the flanking region parameter make the filter more restrictive, and less sequences are kept. There should be a compromise between the length of the flanking region and the capacity of Primer3 to design primers considering the parameter settings given. The shorter the flanking regions are the more sequences will pass through the Filter 2, although most will not be processed by Primer3 since they will not have enough length for the primers to be designed without overlapping with the microsatellite region.

At the end of the pipeline, changes in the maximum difference of melting temperature between primers in Primer3 (MAX DIFF TM) induces variation in the number of primer pairs designed as expected. Higher values in this parameter increase the capacity of Primer3 to find primer pairs in a sequence but, contrary to expectations, they may not necessarily be the most suitable and therefore less primers are selected at the end.

DISCUSSION

Nowadays, it can take up months for laboratories and researchers to analyze and select SSR loci and design primers for their projects. It requires several analyses of MISA without any kind of pre-processing and manual selection of sequence candidates for primer3. Hindered by the use of a multi-individual assay, most selected SSR loci and subsequent PCR amplifications will likely be mediocre if manual SSR selection is applied, but also if clustering of flanking regions and the number of putative alleles are not considered, generating markers with low variability across populations. These technical constraints imply more time and money, that could be easily overcome by the implementation of an automatic tool for selecting loci, based on their polymorphism, and designing suitable primers. This kind of tools allows users to test distinct parameters and select the most suitable SSRs for their experiment in a few seconds.

Specifically, there is a great advantage of implementing the automatic Micro-Primers pipeline since there are few time-consuming steps for the manual search that can be surpassed such as the input/output editing between software and the categorization and allele counting for every single SSR. First, all microsatellites individually identified need to be grouped by their 'true' locus, taking into account that they result from different individuals and can be represented in multiple sequences. After the identification of all copies of the same microsatellite loci, the user needs to count the number of different unique alleles for the subsequent selection. In some cases, this task can be very tedious as some of the locus can be represented by thousands of elements. Micro-Primers has a default configuration that has demonstrated to achieve a good balance

between the number of loci and primer designed, however users should modify these parameters to adequate the results to their requirements. For example, the inclusion of the *SPECIAL*parameter, which takes into account the maximum and minimum allele found in the SSR loci, incorporates in the decision-making the hypothesis of new alleles not yet discovered for the species maybe due to an insufficient sampling.

However, we advise the users not to consider the final output of Micro-Primers as ready-to-use and instead to scrutinise them prior to laboratorial processing. This is because, despite the imposed settings, the output from Primer3 include sometimes bad primer sequences (with repeated nucleotides) that should be immediately discarded by the user. Furthermore, other sources of variation have been detected in the tests performed, producing slight differences in the results when the process is replicated. The impact of these biases on the final output should not be considered as a performance deficiency of the Micro-Primers since they are intrinsic to the individual programs used, and they may also occur during manual processing. Specifically, in first place, since most SSR sequences occur on the overlapping region, the software FLASH can produce some length variation while merging the paired-end reads by not recognising the accurate position of the repeated pattern where sequences should be merged, thus creating 'fake' alleles. The second source of variation occurs in the software CD-HIT due to the fact that the creation of clusters based on the flanking regions is somewhat random. The group of sequences composing each cluster will depend on the seed sequence selected to originate each cluster. This variation can be justified by amplification and sequencing errors. The last source of variation is the random selection of a cluster representative for primer design. This fact can make a cluster to be discarded if the representative can not produce good enough primer pairs in Primer3, for example if the product size is out of the defined range, however its effect was found to be marginal.

Nevertheless, the use of Micro-Primers provides an unprecedented availability of candidate SSRs at a more reliable and faster pace than before. This strategy permits selection of markers that may be the most suitable for specific applications or particular organisms. The creation of an automatic pipeline is very interesting for the scientific community since it can speed up the process and overcome the biases associated with the manual processing, while allowing the user to test various parameter choices by automatically running the process several times on the same dataset.

CONCLUSIONS

We have presented Micro-Primers, a novel, portable and simple pipeline for microsatellite search and primer design. To the best of our knowledge, this is the first pipeline that is able to completely automatize the process, allowing any researcher to make a fast characterization of a species based on a multi-individual sample prior to any extensive genotyping. Since it is implemented in Python, it is portable enough to be used in any kind of operating system, such as Windows, Linux or MacOs. Micro-Primers also has a design simple enough that can be easily implemented in any kind of language, or included within any kind of library or complex software system.

Experimental results showed that Micro-Primers takes about 2 minutes to execute an entire analysis, using just a single core of an Intel i7 Octa-Core processor with 64 Gbytes of main memory while the manual execution took around 24 hours for the entire analysis. We have checked the accuracy of Micro-Primers, by confirming that the results from both procedures were similar.

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DATA ACCESSIBILITY

Data available at the GitHub repository https://github.com/FilAlves/micro-primers.

AUTHOR CONTRIBUTIONS

FMSM and AMM designed the research. FA and MA coded Micro-Primers pipeline. FA and AMM performed the analysis. FA, FMSM, MA and AMM wrote the paper.

TABLES

Table 1 Variation of the selected sequences during the steps of Micro-Primers

MIN_ALLEL_CNT	5	5	5	5	5	5	5	5
SPECIAL DIF	0	1(8)	0	0	0	0	0	0
MIN FLANK LEN	50	50	25	75	100	50	50	50
MAX DIFF TM	0.5	0.5	0.5	0.5	0.5	0.2	1	2
Original FASTQ File				259,506				
Trimming				188,843				
Pair-end Merge				130,603				
Filter 1				19,695				
Filter 2	8,083	8,083	9,568	6,616	3,752	8,083	8,083	8,083

MIN_ALLEL_CNT	5	5	5	5	5	5	5	5
Clusters	4,924	4,924	6,049	3,801	2,453	4,924	4,924	4,924
Filter 3	2,092	2,092	2,110	2,028	779	2,092	2,092	2,092
Unique loci	26	104	28	23	20	26	26	26
Primers Selected	23	83	23	20	15	25	21	21

FIGURES

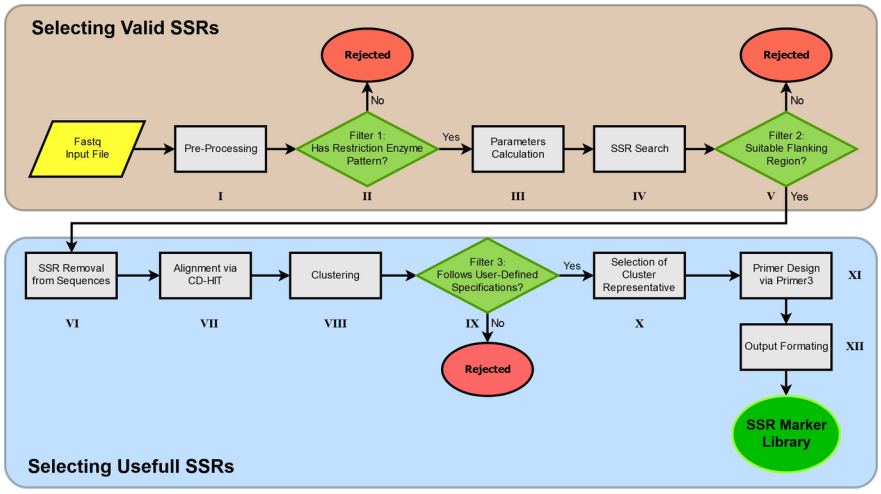
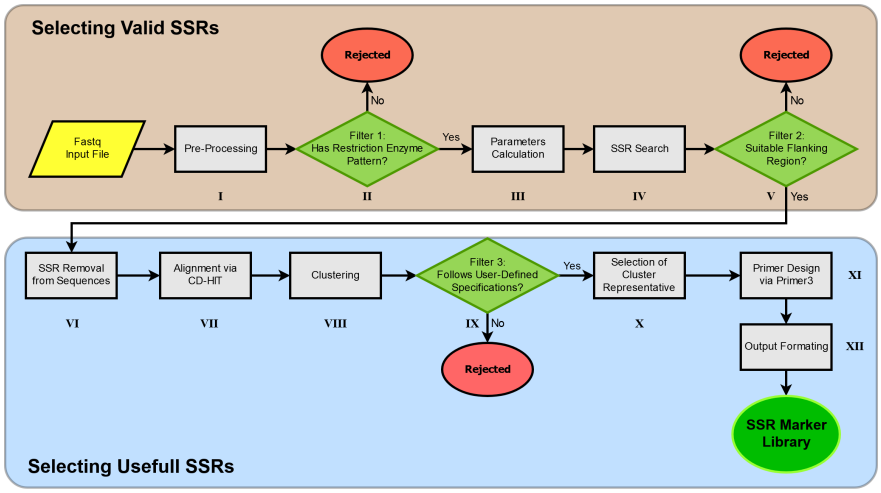


Figure 1 Flowchart of Micro-Primers. Green diamonds represent the different sequence filtering stages based on the parameters established by the user.

ID	Size	Fw Primer	Fw Tm	Rv Primer	Rv Tm	Motif	Range	Observed	Potential	Flag
BATS_Set1_Pair1	164	TCAATTGGCAAGATTTGCTTC	60.203	GTTCCCTGCAGGATTCTTTATG	59.966	(AC)13	[158,192]	16	18	| BEST |
BATS_Set1_Pair2	172	CATGTTGTTTCAATTGGCAAG	59.073	TTCCCTGCAGGATTCTTTATG	59.168	(AC)13	[166,200]	16	18	
BATS_Set1_Pair3	183	CTAGCACCATCCATGTTGTTTC	59.499	TTCCCTGCAGGATTCTTTATG	59.168	(AC)13	[177,211]	16	18	
BATS_Set1_Pair4	127	TCAATTGGCAAGATTTGCTTC	60.203	GTATGGAAGCATCATAGGTGTCC	59.744	(AC)13	[121,155]	16	18	
BATS_Set1_Pair5	128	TCAATTGGCAAGATTTGCTTC	60.203	GGTATGGAAGCATCATAGGTGTC	59.744	(AC)13	[122,156]	16	18	
BATS_Set2_Pair1	211	CACAACCAGGATATTGACGATG	60.249	AAGATTTCCACATGCCACTTG	59.985	(GT)12	[199,227]	14	15	| BEST |
BATS_Set3_Pair1	262	CTGGTCACAAAGGAGAAATGG	59.578	TGAACACTCTCCCAAATGGTC	59.958	(CT)28	[218,278]	14	31	| BEST |
BATS_Set3_Pair2	255	CTGGTCACAAAGGAGAAATGG	59.578	TCTCCCAAATGGTCACTGG	59.457	(CT)28	[211,271]	14	31	
BATS_Set3_Pair3	260	GGTCACAAAGGAGAAATGGAAG	59.976	TGAACACTCTCCCAAATGGTC	59.958	(CT)28	[216,276]	14	31	
BATS_Set4_Pair1	151	GTTTCTCCCAGCGGTAACAC	59.598	AGCAAACACACACACACACG	59.234	(GT)7	[151,183]	12	17	| BEST |
BATS_Set4_Pair2	148	CAGCGGTAACACCTTGTGG	60.159	CCCAGAGCAAACACACACAC	60.203	(GT)7	[148,180]	12	17	
BATS_Set4_Pair3	149	CCAGCGGTAACACCTTGTG	60.159	CCCAGAGCAAACACACACAC	60.203	(GT)7	[149,181]	12	17	
BATS_Set4_Pair4	255	GTTTCTCCCAGCGGTAACAC	59.598	GGAGGTCAAGGGACCTAAATG	59.813	(GT)7	[255,287]	12	17	
BATS_Set4_Pair5	157	CGTTTCTCCCAGCGGTAAC	60.642	CCCAGAGCAAACACACACAC	60.203	(GT)7	[157,189]	12	17	
BATS_Set5_Pair1	211	GTTCCCTGCAGGATTCTTTATG	59.966	ATGCCCTCTAGCACCATCC	60.050	(TG)11	[203,233]	12	16	| BEST |
BATS_Set5_Pair2	184	GTTCCCTGCAGGATTCTTTATG	59.966	TCAATTGGCAAGATTTGCTTC	60.203	(TG)11	[176,206]	12	16	
BATS_Set5_Pair3	210	GTTCCCTGCAGGATTCTTTATG	59.966	TGCCCTCTAGCACCATCC	59.743	(TG)11	[202,232]	12	16	
BATS_Set5_Pair4	174	GTATGGAAGCATCATAGGTGTCC	59.744	ATGCCCTCTAGCACCATCC	60.050	(TG)11	[166,196]	12	16	
BATS_Set5_Pair5	175	GGTATGGAAGCATCATAGGTGTC	59.744	ATGCCCTCTAGCACCATCC	60.050	(TG)11	[167,197]	12	16	

Figure 2 Micro-Primers' output file capture. Columns show sequence ID, PCR amplicon length for the corresponding primer pair (Size), sequence and melting temperature for forward and reverse primer, SSR pattern (Motif), range of sizes for the SSR loci including all alleles (Range), number of alleles found for the SSR (Observed), maximum number of alleles to expect from the difference between the longest and the shortest alleles (Potential) and best primer pair for the SSR loci respectively.



ID	Size	Fw Primer	Fw Tm	Rv Primer	Rv Tm	Motif	Range	Observed	Potential	Flag
BATS_Set1_Pair1	164	TCAATTGGCAAGATTTGCTTC	60.203	GTTCCCTGCAGGATTCTTTATG	59.966	(AC)13	[158,192]	16	18	| BEST |
BATS_Set1_Pair2	172	CATGTTGTTTCAATTGGCAAG	59.073	TTCCCTGCAGGATTCTTTATG	59.168	(AC)13	[166,200]	16	18	
BATS_Set1_Pair3	183	CTAGCACCATCCATGTTGTTTC	59.499	TTCCCTGCAGGATTCTTTATG	59.168	(AC)13	[177,211]	16	18	
BATS_Set1_Pair4	127	TCAATTGGCAAGATTTGCTTC	60.203	GTATGGAAGCATCATAGGTGTCC	59.744	(AC)13	[121,155]	16	18	
BATS_Set1_Pair5	128	TCAATTGGCAAGATTTGCTTC	60.203	GGTATGGAAGCATCATAGGTGTC	59.744	(AC)13	[122,156]	16	18	
BATS_Set2_Pair1	211	CACAACCAGGATATTGACGATG	60.249	AAGATTTCCACATGCCACTTG	59.985	(GT)12	[199,227]	14	15	| BEST |
BATS_Set3_Pair1	262	CTGGTCACAAAGGAGAAATGG	59.578	TGAACACTCTCCCAAATGGTC	59.958	(CT)28	[218,278]	14	31	| BEST |
BATS_Set3_Pair2	255	CTGGTCACAAAGGAGAAATGG	59.578	TCTCCCAAATGGTCACTGG	59.457	(CT)28	[211,271]	14	31	
BATS_Set3_Pair3	260	GGTCACAAAGGAGAAATGGAAG	59.976	TGAACACTCTCCCAAATGGTC	59.958	(CT)28	[216,276]	14	31	
BATS_Set4_Pair1	151	GTTTCTCCCAGCGGTAACAC	59.598	AGCAAACACACACACACACG	59.234	(GT)7	[151,183]	12	17	| BEST |
BATS_Set4_Pair2	148	CAGCGGTAACACCTTGTGG	60.159	CCCAGAGCAAACACACACAC	60.203	(GT)7	[148,180]	12	17	
BATS_Set4_Pair3	149	CCAGCGGTAACACCTTGTG	60.159	CCCAGAGCAAACACACACAC	60.203	(GT)7	[149,181]	12	17	
BATS_Set4_Pair4	255	GTTTCTCCCAGCGGTAACAC	59.598	GGAGGTCAAGGGACCTAAATG	59.813	(GT)7	[255,287]	12	17	
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BATS_Set5_Pair1	211	GTTCCCTGCAGGATTCTTTATG	59.966	ATGCCCTCTAGCACCATCC	60.050	(TG)11	[203,233]	12	16	| BEST |
BATS_Set5_Pair2	184	GTTCCCTGCAGGATTCTTTATG	59.966	TCAATTGGCAAGATTTGCTTC	60.203	(TG)11	[176,206]	12	16	
BATS_Set5_Pair3	210	GTTCCCTGCAGGATTCTTTATG	59.966	TGCCCTCTAGCACCATCC	59.743	(TG)11	[202,232]	12	16	
BATS_Set5_Pair4	174	GTATGGAAGCATCATAGGTGTCC	59.744	ATGCCCTCTAGCACCATCC	60.050	(TG)11	[166,196]	12	16	
BATS_Set5_Pair5	175	GGTATGGAAGCATCATAGGTGTC	59.744	ATGCCCTCTAGCACCATCC	60.050	(TG)11	[167,197]	12	16	