

Multiplexing with three-primer PCR for rapid and economical microsatellite validation

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The next generation sequencing revolution has enabled rapid discovery of genetic markers, however, development of fully functioning new markers still requires a long and costly process of marker validation. This study reports a rapid and economical approach for the validation and deployment of polymorphic microsatellite markers obtained from a 454 pyrosequencing library of Atlantic cod, *Gadus morhua*, Linnaeus 1758. Primers were designed from raw reads to amplify specific amplicon size ranges, allowing effective PCR multiplexing. Multiplexing was combined with a three-primer PCR approach using four universal tails to label amplicons with separate fluorochromes. A total of 192 primer pairs were tested, resulting in 73 polymorphic markers. Of these, 55 loci were combined in six multiplex panels each containing between six and eleven markers. Variability of the loci was assessed on *G. morhua* from the Celtic Sea ($n = 46$) and the Scotian Shelf ($n = 46$), two locations that have shown genetic differentiation in previous studies. Multilocus F_{ST} between the two samples was estimated at 0.067 ($P = 0.001$). After three loci potentially under selection were excluded, the global F_{ST} was estimated at 0.043 ($P = 0.001$). Our technique combines three-primer and multiplex PCR techniques, allowing simultaneous screening and validation of relatively large numbers of microsatellite loci.

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Thirty years after their discovery in the 1980s, microsatellite-based genetic markers are still extensively used in studies of population structure, parentage analysis, genetic mapping, evolutionary processes and forensics (BRUFORD and WAYNE 1993; BROCKMANN et al. 1994; KNAPIK et al. 1998; GOLDSTEIN et al. 1999; PRIMMER et al. 2000). These markers have a wide application due to high allelic diversity and co-dominance of alleles (CHAMBERS and MACAVOY 2000). Many methodologies have been established in order to discover such markers, but it is only following the recent advent of next generation sequencing (NGS) technology that large amounts of markers can be increasingly rapidly and economically developed from non-model organisms. NGS approaches allow the fast discovery of large amounts of microsatellite-containing sequences, however mining such data for suitable DNA fragments and validation of candidate markers are still posing challenges prior to the utilisation of fully operating new markers.

The most common approach to date for de novo microsatellite marker development includes creation of repeat-enriched DNA libraries, fragment replication by cloning, and Sanger sequencing of clones containing potential microsatellites (ZANE et al. 2002). These processes are laborious and time consuming, and typically have low marker yield, with the percentage of positive clones averaging 2–3% (ASHWORTH et al. 2004). The final marker yield is even lower with a large portion of markers discarded during the isolation–characterisation process (SQUIRRELL et al. 2003). Alternatively, microsatellite-containing sequences can be mined from existing molecular data such as genomic DNA or expressed sequence tag (EST) sequences (LI et al. 2004). These approaches are limited by the paucity of data on non-model organisms. EST-linked microsatellites can be relatively easy to identify but have a higher probability of being affected by selective processes, and hence may not be suitable for

population analyses that assume that loci are selectively neutral (ELLIS and BURKE 2007). Microsatellite markers developed for one species may also be applied to closely related species (SCHLÖTTERER et al. 1991). However, this approach is limited by varying levels of successful cross-species amplification between species (MOORE et al. 1991). Even when cross-species amplification is successful, levels of variability tend to be lower compared with the species for which the markers were developed (PRIMMER et al. 1996). Because of these limitations, it may be preferable to develop markers de novo for a species or population of interest to ensure optimal power of newly discovered markers (CARLSSON et al. 2013).

Recently, several approaches have been presented for discovery of microsatellites using next generation sequencing (NGS)-generated data (ABDELKRIM et al. 2009; ALLENTOFT et al. 2009). Large amounts of sequence data can be generated from either genomic DNA or microsatellite enriched libraries (GUICHOUX et al. 2011; MALAUSA et al. 2011) and then mined for microsatellite loci. With this approach, thousands of potential loci can be rapidly identified (GUICHOUX et al. 2011). Large-scale microsatellite identification has several advantages, including the ability to rigorously screen individual loci for presence of optimal primer-binding sites in flanking regions (GUICHOUX et al. 2011; ZALAPA et al. 2012; FERNANDEZ-SILVA et al. 2013). Additionally, deployment criteria (e.g. higher levels of variability, neutrality and low linkage) are study-specific and cannot be assessed until the markers have been validated (SELKOE and TOONEN 2006). Validation of a large number of markers enables selection of most suitable loci (SELKOE and TOONEN 2006). Even with the NGS approach, validation is labour intensive and a limiting bottleneck in microsatellite marker development (SQUIRRELL et al. 2003; MALAUSA et al. 2011; FERNANDEZ-SILVA et al. 2013).

Typically, methods for validation and genotyping of microsatellites involve capillary gel electrophoresis with fluorescence-based amplicon detection (EDWARDS et al. 1991; GUICHOUX et al. 2011). The three-primer PCR method can be used to reduce the expense associated with fluorescently labelled primers (sensu SCHUELKE 2000; DINIZ et al. 2007; RUBIN et al. 2009). In three-primer PCR, the primers comprise an unlabelled forward primer with a universal tail attached to its 5' end, a labelled universal primer matching the tail sequence and an unlabelled reverse primer (STEFFENS et al. 1993; OETTING et al. 1995; NEILAN et al. 1997; SCHUELKE 2000). The labelled universal primer can be used in combination with any appropriately tailed forward primer, thereby eliminating the need to synthesize a fluorescently labelled forward primer for every unique locus during the validation phase, in which a large proportion of loci may be excluded because of problems with amplification.

Following initial identification and PCR optimisation of successful markers, sets of primers are usually labelled with a fluorescent label either on the forward or the reverse primer (GUICHOUX et al. 2011). Markers are then amplified in single PCR reactions or combined into a multiplex PCR containing multiple markers (GUICHOUX et al. 2011). Improvement of the traditional multiplex PCR technique (MISSIAGGIA and GRATTAPAGLIA 2006) employed human microsatellite primer sequences as universal tails and combined three universal tails with three dyes in a true multiplex PCR (sensu GUICHOUX et al. 2011). However, despite the obvious cost benefits of the three primer PCR approach in combination with multiplexing, few studies have employed the method (LANGEN et al. 2011; BLACKET et al. 2012). This may be attributable to poor amplification or poor quality chromatograms, resulting in difficulty in accurate genotyping of individuals (HAGELL et al. 2013).

Here we present the development and application of a method for rapid validation and genotyping of novel microsatellites in Atlantic cod, *Gadus morhua*, Linnaeus 1758, using the three primer approach with multiplex PCR. The main aim of this study is the fast and economic development and deployment of microsatellite multiplexes from raw NGS data applicable for studies on a wide range of organisms.

MATERIAL AND METHODS

Sampling

Gadus morhua were obtained by trawling in 2009 and 2011 from the Celtic Sea, south of Ireland (n = 7, n = 46, respectively) and in 1996 from the Scotian Shelf, off Nova Scotia in eastern Canada (n = 46). Previous research has shown that these two populations are genetically differentiated (HUTCHINSON et al. 2001; O'LEARY et al. 2007). Fin clip samples were preserved in 100% ethanol.

DNA extraction

DNA was extracted from fin clips using a Chelex protocol as described in MIRIMIN et al. (2011). DNA from the Scotian Shelf samples was extracted using a standard phenol–chloroform method (O'LEARY et al. 2007). DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and normalised to a concentration of 50 ng μl^{-1} .

Microsatellite selection

The unpublished sequence data used here were generated for a previous study (CARLSSON et al. 2013), in which microsatellite containing sequences were obtained from five of the 2009 Celtic Sea individuals also used in the

present study, using 454 pyrosequencing of a reduced representation library. CARLSSON et al. (2013) identified a total of 11 341 microsatellite containing sequences as suitable for primer design using the Primer3 plug-in (ROZEN and SKALETSKY 2000) for MISA ver. 1.0 (<<http://pgrc.ipk-gatersleben.de/misa>>). Of these, 6424 were estimated to be unique. These microsatellite-containing sequences were used in the present study.

To avoid excessive homoplasy (alleles identical in state but not in descent, cf. ESTOUP et al. 2002) and to ensure ease of genotyping, complex repeat motifs (i.e. compound and imperfect motifs) were excluded. To ensure sufficient space for primer design, reads that had less than 50 bp of sequence before and after the repeat-containing region were removed. In addition, to avoid excessively large allele size ranges, repeat sequences of more than 100 bp, and penta- and hexanucleotide repeats were excluded. A subsample of the remaining microsatellite sequences ($n = 1309$) were visually inspected for primer design.

Primer design

Primers were designed using Primer3Plus (ROZEN and SKALETSKY 2000; UNTERGASSER et al. 2007) with optimal primer length as 20bp and optimal T_m at 60°C. Two sets of three size classes were used: the first set of size classes was separated by 30 bp (100–150, 180–250, 280–450 bp), and the second set separated by 50 bp (100–150, 200–250, 300–450 bp). Equal numbers of markers were designed for each size class. Only primer pairs with a T_m difference of less than 1°C were accepted in order to facilitate PCR multiplexing.

Designed primers were cross-referenced with the original sequence data set to identify primers that annealed to multiple regions (not unique) or originated from redundant sequences (different reads of the same sequence). Redundant sequences not detected in the previous steps (due to sequencing error in the primer regions) were identified by performing a de novo assembly with the remaining candidate loci sequences using Geneious ver. 6.1.5 (created by Biomatters; available from <www.geneious.com>), CAP3 plug-in (default settings; i.e. min overlap length = 40 bp, min overlap identity = 90%; HUANG and MADAN 1999). If two or more reads assembled together they were considered redundant and only one of them was kept for future analysis.

To minimise the risk that primer sequences were derived from contaminants, expressed *G. morhua* gene regions or previously published *G. morhua* microsatellites, microsatellite-containing sequences and primers were subjected to a BLAST search in the GenBank nucleotide database (ALTSCHUL et al. 1990). For possible contaminants, we considered a match with $\geq 95\%$ coverage

and 100% identity as a threshold for excluding reads. No threshold was employed for matches on *G. morhua* sequences. When such matches were encountered, primers were excluded from further analyses. In addition, validated primer sequences were subjected to BLAST searches against the *G. morhua* genome (STAR et al. 2011) in the whole-genome shotgun contigs database in GenBank.

Universal primers

The universal dye-labelled primers used were T3: PET-5' AATTAACCCTCACTAAAGGG 3', M13 Reverse: NED-5' GGATAACAATTCACACAGG 3' (DINIZ et al. 2007), Hill: 6FAM-5' TGACCGCAGCAAAATTG 3' (TOZAKI et al. 2001) and Neomycin rev: VIC-5' AGGTGAGATGACAGGAGATC 3'. Each forward primer had one of the above universal primer sequences added to its 5' end. PIG-tails were added to the 5' end of all the reverse primers. PIG-tailing leads to an addition of a non-templated adenosine nucleotide to the 3' end on nearly 100% of PCR products which reduces stutter caused by random addition of dATP (BROWNSTEIN et al. 1996). The tails were matched with the primers using OligoAnalyzer ver. 3.1 (<www.idtdna.com>) to ensure the least amount of different secondary structures. Equal numbers of primers were paired with each of the four different universal primers.

Microsatellite validation

Primers were combined into twelve multiplex PCR reactions containing 12 markers each (12-plex) and validated using all seven 2009 Celtic Sea individuals. Loci that amplified successfully and showed polymorphism were combined into further multiplexes. The construction of multiplexes was done by means of successive attempts of adding and removing loci from sets of markers that had amplified together in the initial test panels until at least six loci were successfully combined in a panel. When amplified loci were monomorphic, the procedure was repeated on seven Scotian Shelf samples to assess whether they were monomorphic in these individuals.

Multiplex PCRs were performed in 5 μ l reactions with 50 ng template DNA, 1 \times Multiplex PCR Master Mix (Qiagen), 0.2 μ M of each reverse primer, 0.05 μ M of each unlabelled forward primer (modified with the appropriate universal tail) and 0.2 μ M of labelled universal primer for each forward primer labelled with matching universal tail. Further adjustments made to optimize concentrations of primers in the PCR reactions are given in Table 1. PCR thermal cycling conditions were as follows: 1 \times 95°C (15 min); 30 \times 94°C (30 s), 60°C (90 s), 72°C (60 s); 8 \times 94°C (30 s), 53°C (90 s), 72°C (60 s); 1 \times 60°C (30 min). No-template controls were included to monitor for potential contamination.

A total of 1 μ l of the multiplex PCR product was added to 9 μ l of Super-DI Formamide (MCLAB) with 0.01 μ l of Orange DNA Size Standard (MCLAB) and run on an ABI 3130xl Genetic Analyzer according to manufacturer's recommendations. GeneMarker ver. 1.97 (<www.softgenetics.com>) was used for fragment length analysis.

Microsatellite genotyping

All 46 Celtic Sea and 46 Scotian Shelf samples were genotyped with multiplex panels. Genotype data were inspected with Micro-Checker ver. 2.2.3 for genotyping errors and presence of null-alleles (VAN OOSTERHOUT et al. 2004) using default settings. The 99% confidence interval was used when checking for null alleles to avoid false positives resulting from multiple tests. MSAnalysér ver. 4.05 (DIERINGER and SCHLÖTTERER 2003), using default settings, was used to assess the number of alleles, allelic richness, allele size ranges, F_{ST} estimates and expected and observed heterozygosity. Data were analysed for possible departure from Hardy–Weinberg equilibrium, linkage disequilibrium, and excess and deficit of heterozygotes using Genepop ver. 4.2 with default settings (RAYMOND and ROUSSET 1995; ROUSSET 2008). False discovery rate (FDR) was used to correct for multiple comparisons (BENJAMINI and YEKUTIELI 2001) with initial $\alpha = 0.05$. Lositan (ANTAQ et al. 2008) was used to detect loci that could be under positive or balancing selection (settings ‘Neutral’ mean F_{ST} and ‘Force mean F_{ST} ’ with 10 000 simulations were used under both the infinite allele model and stepwise mutation model).

F_{ST} replicate sampling

The current study purposefully aimed to validate more markers than required for accurate evaluation of population differentiation (i.e. multilocus F_{ST}). To estimate the number of microsatellite loci future studies on cod population structure may require we investigated how many markers were needed to accurately estimate multilocus F_{ST} . Data sets were generated by randomly drawing 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 loci from the 55-locus dataset with each condition (number of loci) replicated ten times. Average F_{ST} and 95% confidence interval of the ten replicates were calculated and plotted to visualise the variability of average F_{ST} estimates as a function of numbers of markers (Fig. 1).

RESULTS

Primer design

Of 1309 candidate microsatellite loci, 559 were determined to be suitable for primer design upon visual inspection. A total of 349 primer pairs were rejected based on

T_m difference, sequence redundancy or secondary structure with the 5' tail. Another 18 were excluded due to a BLAST match (one match to a *Gadus morhua* microsatellite, nine to a *G. morhua* gene and eight to a possible contaminant). The remaining 192 primer pairs were chosen for validation. Of those, 51 failed to amplify and 45 were excluded due to low scorability. Unambiguous amplification of PCR products in the expected size range was successful in 96 of the 192 markers tested (50%), of which 73 showed polymorphism (38%). The 73 polymorphic loci were used to build multiplex panels. Of these 13 were not included in the final multiplexes because of incompatible size, associated fluorochrome or failure to amplify with the other markers in a panel. As a result 60 markers were combined into six multiplex panels ranging between eight and twelve loci. Five markers were not used in the final analysis due to ambiguous genotyping leading to high chance of scoring errors, resulting in a final panel of 55 polymorphic markers combined into six multiplexes (Table 1). The results of the BLAST search on the validated primers against the *G. morhua* genome are presented in the Supplementary material Appendix 1 Table A1.

Application of markers to test populations

The mean allelic richness (R_s) was 7.1 (SD = 4.11) in the Celtic Sea sample and 7.2 (SD = 4.24) in the Scotian Shelf sample. The minimum number of alleles was two for both the Celtic Sea and Scotian Shelf samples; the maximum number of alleles was 21 and 25, respectively (Supplementary material Appendix 1 Table A2). Micro-Checker analyses indicated no genotyping errors. However, ten loci had a different repeat pattern than the motif originally identified from the raw sequence (Supplementary material Appendix 1 Table A3). Null alleles were observed in 13 loci in the Celtic Sea sample and 11 in the Scotian Shelf sample (Supplementary material Appendix 1 Table A4). Twelve and eleven loci deviated significantly from Hardy–Weinberg equilibrium (after FDR correction) in Celtic Sea and Scotian Shelf samples, respectively. Linkage disequilibrium was observed (after FDR correction) in locus pair A43_T3 x C01_M13 in the Celtic Sea sample; and locus pairs A43_T3 x B19_T3 and C15_Hill x C17_M13 in the Scotian Shelf sample. Lositan identified loci A11_Hill, C40_M13, C42_M13 and D14_Hill as being potentially affected by positive selection, (both under IA and SMM). Only C40_M13 (global $F_{ST} = 0.581$), C42_M13 (global $F_{ST} = 0.301$) and D14_Hill (global $F_{ST} = 0.246$) remained significant after correction for multiple comparisons.

Global multilocus F_{ST} was estimated at 0.067 ($P = 0.001$). After the three outlier loci identified by Lositan were excluded, the global F_{ST} was estimated at

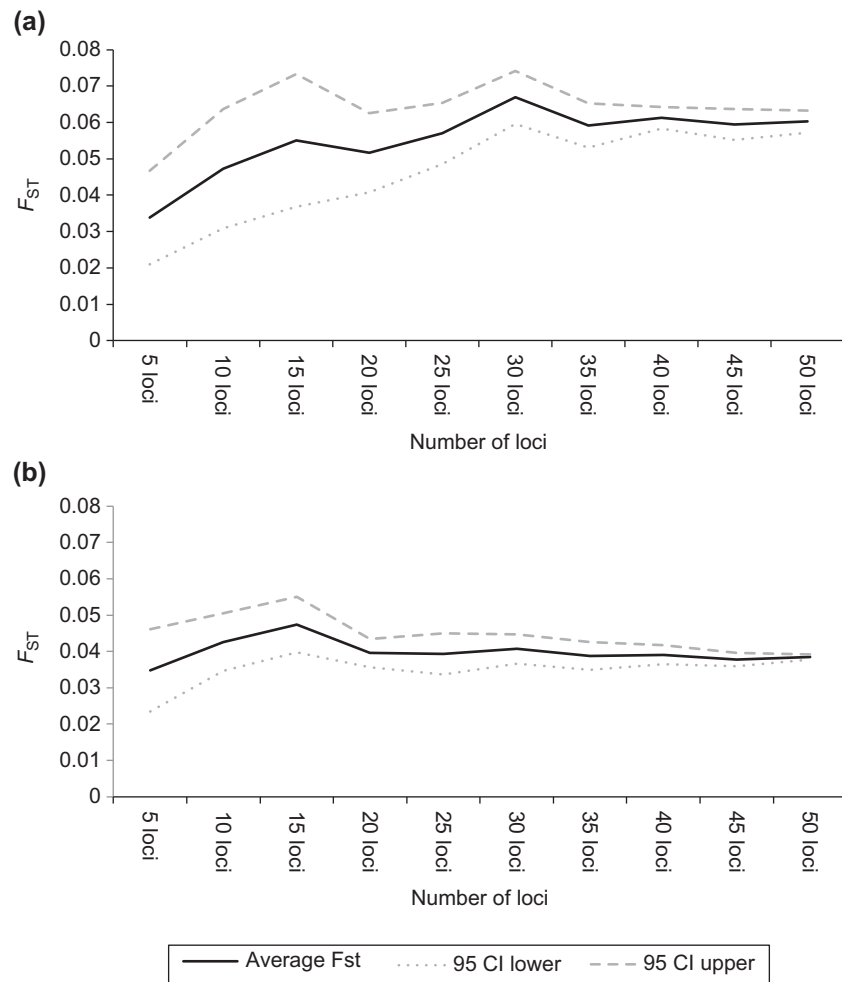


Fig. 1a–b. F_{ST} replicate sampling with all 55 loci (a) and with outliers excluded (b). 95% confidence interval is displayed.

0.043 ($P=0.001$). Global F_{ST} values of individual loci are presented in Supplementary material Appendix 1 Fig. A1.

Replicate sampling of loci to visualise the effect of increasing numbers of loci on F_{ST} estimates and their variances is presented in Fig. 1. This was done in order to see how many loci were needed to reach F_{ST} point estimates with low variances to accurately describe the level of genetic variability between the Celtic Sea and Scotian Shelf samples. The procedure was performed both with and without loci under potential selection. In both cases, increased number of markers reduced the variation in multilocus F_{ST} estimates.

DISCUSSION

Since the initial reports of three-primer PCR (STEFFENS et al. 1993; OETTING et al. 1995; NEILAN et al. 1997; SCHUELKE 2000) the approach has gained wide acceptance, particularly for initial validation while using conventional

two-primer PCR for genotyping (GUICHOUX et al. 2011; HUNTER and HART 2013; OLAFSDOTTIR et al. 2013; SKIRNISDOTTIR et al. 2013). Similarly, multiplex amplification of microsatellites is now commonly employed. In a few instances, these two techniques have been combined for microsatellite deployment (MISSIAGGIA and GRATTAPAGLIA 2006; LANGEN et al. 2011; BLACKET et al. 2012). However, the combined three primer/multiplex PCR approach, as used here for both microsatellite development and deployment, has not to our knowledge been previously reported. The lack of such studies may reflect conservative views on multiplexing and/or the limited availability of suitable universal primers (GUICHOUX et al. 2011; BLACKET et al. 2012). The three-primer/multiplex PCR approach for validation and genotyping has several characteristics that facilitate cost savings (consumables and labour) relative to other approaches. Fluorescently labelled primers are typically an order of magnitude more expensive than unlabelled primers. Therefore, direct modification of locus specific

Table 1. Six multiplex panels for amplifying 55 loci. Underlined sequence in the forward primer signifies the universal tail sequence, whereas underlined sequence in the reverse primer signifies the PIG-tail sequence. Adjustment to primer concentration applies for all three primers used to amplify a locus (forward, reverse and universal primer). SRA accession number for the sequences is SRP041380.

Multiplex	Name	Motif	Size range	Adjustment to primer concentration	Forward primer	Reverse primer	
1	A08_T3	tetra	163–187		<u>AATTAACCCCTCACTAAAGGGATCTCGAGTGGCGCAGTAG</u>	<u>GTTTCTTGACAGCGTGACAAGTGAAGAG</u>	
	A16_M13	tetra	156–194		<u>GGATAACAATTCACACAGGGCTCCCTCAGCTTGCTCT</u>	<u>GTTTCTTCGTCCACACAGATCTATGCCAATC</u>	
	A43_T3	tetra	309–369		<u>AATTAACCCCTCACTAAAGGGCCACTTAACTGCGGTTTC</u>	<u>GTTTCTTCGTCCGTGTATACGCTGA</u>	
	B19_T3	tri	242–320		<u>AATTAACCCCTCACTAAAGGGAAAAGGATCTGCTTG</u> CCTCA	<u>GTTTCTTCGTGAGCTCAGTTTTGGCTA</u>	
	B38_Neo	tri	388–415		<u>AGGTGAGATGACAGGAGATCGAATTGAGGAGGC</u> ATGGGTA	<u>GTTTCTTGGTTAATTCAGGCCGTAGAGG</u>	
	C15_Hill	tetra	169–205		<u>TGACCGGCAGCAAAAATTGCCCTTTCGTTCTCTCCGTGAG</u>	<u>GTTTCTTGAGGATTTGGTGGGATGAT</u>	
	C28_Neo	tri	255–282		<u>AGGTGAGATGACAGGAGATCCAGCACAAAGTGGT</u> AGGGTCA	<u>GTTTCTTGGGATCAGAAATGTGCTT</u>	
	C36_Hill	tri	319–352		<u>TGACCGGCAGCAAAAATTGGTGGTCCACACAATCATCG</u>	<u>GTTTCTTATCCTTCAAACAGCCCTCAA</u>	
	C40_M13	tri	276–277		<u>GGATAACAATTCACACAGGGGTCTTTGGGAGGTCTTCCCT</u>	<u>GTTTCTTCAATCTGCTTGGGACTTAT</u>	
	D14_Hill	tetra	228–276		<u>TGACCGGCAGCAAAAATTGGGGATAATCTGCCAATTTG</u>	<u>GTTTCTTCGTGACAAAGTGCATTTGC</u>	
	D30_M13	tetra	317–333		<u>GGATAACAATTCACACAGGTTTCAAAAACGGGAACACTAGA</u> TCA	<u>GTTTCTTGACAAGTCCAAAGATGTGTC</u>	
	2	A18_M13	tetra	203–243		<u>GGATAACAATTCACACAGGGACTGTCCGTTGAG</u> GGTGT	<u>GTTTCTTGGGTGCAACTGGTCTGGTTA</u>
		A19_T3	tetra	242–274		<u>AATTAACCCCTCACTAAAGGGTCTCTGGTCCAACACATGAC</u>	<u>GTTTCTTAGCCAATGGTGC AAGT</u>
		A34_M13	tetra	297–321		<u>GGATAACAATTCACACAGGTCCTTAAACGACAGGCACCTT</u>	<u>GTTTCTTCCTGACTGTGTCGTTCCAG</u>
A37_Neo		tetra	265–489		<u>AGGTGAGATGACAGGAGATCCCGGCAGTACAGC</u> TAATGAA	<u>GTTTCTTAAATGCTCAACCCATGGAC</u>	
B03_T3		di	147–161		<u>AATTAACCCCTCACTAAAGGGGATAATAGCGTTCCTCCATCC</u>	<u>GTTTCTTGGGGTACCTTGTGACCTGT</u>	
B12_T3		di	346–398	2x	<u>AATTAACCCCTCACTAAAGGGGCTTTGGCAACACTGTTTGA</u>	<u>GTTTCTTGTGAGCAGACCAAGAACCC</u>	
B30_Neo		tri	151–169		<u>AGGTGAGATGACAGGAGATCTTGTACGGACAGG</u> AAGTCCA	<u>GTTTCTTGCGAACAGTGTGTAATGAA</u>	
3	C01_M13	tetra	115–195		<u>GGATAACAATTCACACAGGACCAGGAGTTGGATCAGTGTG</u>	<u>GTTTCTTCCAATTAATTCATTCGTCAITCCA</u>	
	C14_Hill	tetra	146–166		<u>TGACCGGCAGCAAAAATTGAGGTTTCCAGCCAGAAGCTGAT</u>	<u>GTTTCTTCCAATGGTTGTCCGGTGAITA</u>	
	C20_Neo	tri	227–239		<u>AGGTGAGATGACAGGAGATCCCGCCTATCACCCCTA</u> AATCTG	<u>GTTTCTTCGTCTACATGTCTGTGGTAGGG</u>	
	D12_Hill	tetra	251–291		<u>TGACCGGCAGCAAAAATTGAACGGCTCTCTCAAGACAAAC</u>	<u>GTTTCTTAGGCAATCTGCGTCCATACTC</u>	
	A33_M13	tetra	233–297		<u>GGATAACAATTCACACAGGAGACACTGAGCTC</u> GACAGCA	<u>GTTTCTTATCAATGATCCCAGGCCAAAC</u>	
	A39_Hill	tetra	356–416		<u>TGACCGGCAGCAAAAATTGCCGTCTCCAAATGCACACAAG</u>	<u>GTTTCTTAGTGTGGATGGTGTGATG</u>	
	B01_Hill	di	129–213		<u>TGACCGGCAGCAAAAATTGTAGACTCTGGGGCTGGGTA</u>	<u>GTTTCTTGCATCCGAGACTCTTGTTC</u>	
B15_T3	di	170–178		<u>AATTAACCCCTCACTAAAGGGCGATGGGATTTCTT</u> GGTAAATG	<u>GTTTCTTGGTGTCTCATCCCTCTTCA</u>		

Continued

Table 1. Continued.

Multiplex	Name	Motif	Size range	Adjustment to primer concentration	Forward primer	Reverse primer
4	B29_Neo	tri	262–281		<u>AGGTGAGATGACAGGAGATCGGGAAAGAGCCGGA</u> AAAGTA	<u>GTTTCTTGCTAATGTTGGCAGAACCA</u>
	C13_Neo	tri	158–176		<u>AGGTGAGATGACAGGAGATCGGGTGAITGAGGTTGGGATA</u>	<u>GTTTCTTGCTCACACATCCTACGAGCA</u>
	C17_M13	tetra	154–190		<u>GGATAACAATTCACACAGGCTTCGATGGCATGTTCC</u>	<u>GTTTCTTCCTGGACAATGATCTGCAT</u>
	C22_Hill	tetra	225–273		<u>TGACCGGCAGCAAAATGGGCTTGCTGGTTCCTT</u>	<u>GTTTCTTGTTGAATGCAACCCCTCAGT</u>
	C30_Neo	tri	337–343		<u>AGGTGAGATGACAGGAGATCAGGTGGTCCGAGTGA</u> AGAAG	<u>GTTTCTTGGGTGAATGCTCTTAATCG</u>
	C42_M13	tri	345–358		<u>GGATAACAATTCACACAGGGCTGAGGGGATGC</u> GATAATA	<u>GTTTCTTAGCCAAGGGTGAAGTGTGT</u>
	D37_T3	tetra	313–333		<u>AATTAACCCCTACTAAAGGGATGTGACACCCGAATCACAGC</u>	<u>GTTTCTTAGCCGCTCCTGTACGTGAACT</u>
	A11_Hill	tetra	163–175		<u>TGACCGGCAGCAAAATTCGGACAGGGAGGCA</u> TAAAGAC	<u>GTTTCTTGTTACCTCCCTGGCTCTT</u>
	A22_Neo	tetra	214–243		<u>AGGTGAGATGACAGGAGATCGGTGAGGTTCTT</u> GAGGGTCA	<u>GTTTCTTGATTAATTCGCCCTGCTG</u>
	A31_T3	tetra	314–386		<u>AATTAACCCCTACTAAAGGGGATATGTGGGG</u> ATGAGCAC	<u>GTTTCTTATGGGTCTTCTCCTTTGGT</u>
	B33_M13	tri	120–135	0.5x	<u>GGATAACAATTCACACAGGTACAGCAGGGG</u> TTCTCAG	<u>GTTTCTTGTTGTTGCTCCGATGGACT</u>
	C08_T3	tetra	145–185		<u>AATTAACCCCTACTAAAGGGCTCGGACCCAGA</u> GATCAAAA	<u>GTTTCTTGCAGCACTGAACTGAAACG</u>
	D15_Hill	tetra	244–256		<u>TGACCGGCAGCAAAATGTGACTCAACGGAGGTACGTG</u>	<u>GTTTCTTCCATCAGGATCAGGACCACT</u>
	D21_Neo	tetra	337–389		<u>AGGTGAGATGACAGGAGATCACACGTTGCT</u> GGACTAC	<u>GTTTCTTCACTGGAGTACGGTCTCTGA</u>
	D46_Neo	tetra	139–155		<u>AGGTGAGATGACAGGAGATCCCTCCCTAATACCA</u> TGTACCA	<u>GTTTCTTCGTCTGTTACGGATGCAC</u>
5	A04_Neo	tetra	143–211		<u>AGGTGAGATGACAGGAGATCAATCAACCCCTCAA</u> ACTCG	<u>GTTTCTTCAGGTCCCGAATATCAAGG</u>
	B07_M13	di	163–187		<u>GGATAACAATTCACACAGGTGGACAATACATGAAA</u> ATCACAG	<u>GTTTCTTCCTGAACTGCCTGTCAATG</u>
	B28_Neo	tri	226–238		<u>AGGTGAGATGACAGGAGATCCCAACCCCTTAATGTTCAA</u>	<u>GTTTCTTGGGCTAATCTCTTTGATG</u>
	D10_Neo	tetra	308–312	2x	<u>AGGTGAGATGACAGGAGATCCGCAATGCAAAATCTTTT</u>	<u>GTTTCTTATCTGAGTGGCTCGAGTGC</u>
6	D35_M13	tetra	316–360		<u>GGATAACAATTCACACAGGTCCACACTTGGTCCGATGAAA</u>	<u>GTTTCTTGACGAGTGCAGCAGGTGTG</u>
	D43_T3	tetra	260–292	2x	<u>AATTAACCCCTACTAAAGGGTGGCCGCTCACGCTACTAAT</u>	<u>GTTTCTTCGTGATCGCTCTCGATTCT</u>
	A03_Hill	tetra	158–170		<u>TGACCGGCAGCAAAATGGAGCTGTTGAACGACTTGA</u>	<u>GTTTCTTCCTGAGCAGTGGAGTGACAA</u>
	A20_Neo	tetra	164–182		<u>AGGTGAGATGACAGGAGATCCGAGGCTACACAGCCTGTAA</u>	<u>GTTTCTTACTGTGGGCATGTAACAGCA</u>
6	A30_T3	tetra	245–269		<u>AATTAACCCCTACTAAAGGGATGAACTGCGGGTCTGT</u>	<u>GTTTCTTGCACGATGTACAGCTGAT</u>
	B36_Hill	tri	384–396		<u>TGACCGGCAGCAAAATTCGCCAGACATAATAAGA</u>	<u>GTTTCTTAGCTCAGCCTCCACATCA</u>
	C31_Neo	tri	332–341		<u>AGGTGAGATGACAGGAGATCCCAAGCAAGCAATTCAT</u>	<u>GTTTCTTCGAGCCAGGCTTACTTCTC</u>
	C35_M13	tri	233–348		<u>GGATAACAATTCACACAGGGGCAATGTGTACACCTCAA</u>	<u>GTTTCTTCTGAAACGGCAACACTTCGTA</u>
	D05_Hill	tetra	230–270		<u>TGACCGGCAGCAAAATGACTGCCCTGTATAACAATGC</u>	<u>GTTTCTTAGGCATCGACCAATTTGTAGC</u>
	D16_T3	tetra	424–436		<u>AATTAACCCCTACTAAAGGGCCAGCAGCTTCTGGGTAGT</u>	<u>GTTTCTGAAAGCTTACTGCAGACAG</u>

primers substantially increases project costs, especially when markers must be excluded due to low scorability and/or bias (SELKOE and TOONEN 2006). The use of fluorescently labelled universal primers avoids these potential complications and further decreases project costs, as a limited number of these primers can be purchased at large synthesis scales. Further multiplexing six to eleven amplicons per ABI capillary lane reduces PCR and genotyping costs as well as labour effort.

Primers were designed to amplify loci in three non-overlapping allele size ranges per dye as reported by NEFF et al. (2000), however, larger size separations between ranges were employed. Overlapping size ranges have a disadvantage in that only one marker can be used per dye (MILLER et al. 2013a, 2013b). Because actual allele sizes were not known in advance of capillary separation, two gap sizes (30 bp and 50 bp) between marker class size ranges were used to minimize overlap chances within a dye set. In practice, only a single overlap between markers was observed in the combined set of gap sizes, while the remaining markers were separated by at least 8 bp. However, microsatellites generated using the 50 bp gap size were more easily combined in multiplex PCR. We therefore recommend that marker size classes are set apart by at least 50 bp during primer

design (e.g. 100–150, 200–250, 300–450 bp) to facilitate combining loci in multiplexes.

Both raw reads and contigs have been used with similar success for microsatellite discovery (Table 2). Contigs can yield more robust primers because increased sequencing depth can be used to detect sequencing errors or genetic variation in the primer binding region (FERNANDEZ-SILVA et al. 2013; ZALAPA et al. 2012). However, repeat-containing reads may fail to assemble during contig construction, preventing discovery of some valid microsatellite loci (sensu CAVAGNARO et al. 2010). Also, if the assembler is not able to distinguish the repeat and uses it as the basis for alignment, the unique flanking regions can easily be erroneously collapsed (TREANGEN and SALZBERG 2012). We used raw reads to maximise microsatellite yield in this study and were able to achieve a 50% amplification success rate for trialled primers.

The design of a multiplex panel usually starts with evaluation of loci in single locus PCR reactions (NEFF et al. 2000; GUICHOUX et al. 2011). For this study, evaluating 192 loci in single PCR reactions would have required 192 additional PCRs on the validation panel of seven individuals and a negative control, and the analyses of the resulting 1536 amplicons via capillary electrophoresis. The elimination of this step reduced primer validation

Table 2. Comparison of previous studies using either contigs or raw reads in microsatellite discovery via 454 pyrosequencing. The studies employed 454 GS-FLX Titanium chemistry, apart from the publications marked with * which used the 454 GS-FLX chemistry.

Data	Taxon name	Primers screened	Polymorphic	Primer-to-polymorphic marker proportion	Reference		
Contig	<i>Neophoca cinerea</i>	28	12	0.43	Ahonen et al. 2013	Average	0.45
	<i>Cyanoramphus malherbi</i>	35	18	0.51	Andrews et al. 2013*	Median	0.43
	<i>Catha edulis</i>	63	27	0.43	Curto et al. 2013*		
	<i>Stylissa carteri</i>	96	12	0.13	Giles et al. 2013		
	<i>Python molurus bivittatus</i>	26	18	0.69	Hunter and Hart 2013		
	<i>Popenaias popeii</i>	28	20	0.71	Inoue et al. 2013		
	<i>Isoodon obesulus</i>	46	9	0.20	Li et al. 2013		
	<i>Antilocapra americana sonoriensis</i>	100	14	0.14	Munguia-Vega et al. 2013		
	<i>Scomber scombrus</i>	80	30	0.38	Olafsdottir et al. 2013		
	multiple species	16–81	8–25	0.15–0.88	Schoebel et al. 2013		
	<i>Unio crassus</i>	77	11	0.14	Sell et al. 2013*		
	<i>Cyclopterus lumpus</i>	48	22	0.46	Skirnisdottir et al. 2013		
	<i>Kunzea pulchella</i>	27	10	0.37	Tapper et al. 2013		
Raw	<i>Gadus morhua</i>	15	6	0.40	Carlsson et al. 2013	Average	0.44
	<i>Mulloidichthys flavolineatus</i>	24	23	0.96	Fernandez-Silva et al. 2013	Median	0.38
	<i>Pleuromamma xiphias</i>	15	8	0.53	Fernandez-Silva et al. 2013		
	<i>Brachyptera braueri</i>	30	5	0.17	Geismar and Nowak 2013		
	<i>Euastacus bispinosus</i>	40	15	0.38	Miller et al. 2013a*		
	<i>Neophema chrysogaster</i>	55	14	0.25	Miller et al. 2013b*		
	<i>Prionace glauca</i>	100	12	0.12	Taguchi et al. 2013		
	<i>Silurus asotus</i>	70	47	0.67	Xu et al. 2013		

time and lowered consumable and labour costs. The present approach will yield markers for use in multiplex panels. However we recognise that some markers that would amplify in single locus PCR may fail in multiplex, therefore potentially lowering the conversion proportion from tested loci to polymorphic loci. Nevertheless, we contend that the increase in speed outweighs the possible loss of potential markers.

The conversion proportion from tested loci to polymorphic loci in this study was 38% (73/192). This value was similar to the conversion proportion of 40% (6/15) observed in the initial small scale validation by CARLSSON et al. (2013) using the same data, and is consistent with recent studies using raw reads from 454 GS-FLX sequencing of genomic DNA (Table 2). Considerable variability of primer-to-polymorphic marker proportion has been observed among studies (Table 2). A portion of this variation can be attributed to differences in the genome composition of the study organisms (SCHOEBEL et al. 2013), for example, PCR amplification success is lower in organisms with comparatively large genomes (GARNER 2002; SCHOEBEL et al. 2013). This can be due to larger genomes typically harbouring more repetitive elements (HANCOCK 2002). Microsatellite discovery strategies, such as differences in search parameters and algorithms, or using contigs versus raw sequence reads, can possibly affect the conversion from tested loci to polymorphic loci as well. Variation is also likely caused by different strategies in selection of loci to be validated (FERNANDEZ-SILVA et al. 2013).

Implementation in *G. morhua*

As a proof of concept we applied the described approach to samples of *G. morhua* from the Celtic Sea and the Scotian Shelf. *G. morhua* from the Celtic Sea were used for initial microsatellite development (CARLSSON et al. 2013) and the Scotian Shelf *G. morhua* form a genetically distinct population from eastern Atlantic *G. morhua* (HUTCHINSON et al. 2001; O'LEARY et al. 2007). The present study estimated F_{ST} between Celtic Sea and Scotian Shelf *G. morhua* at 0.067 when 55 loci were employed. After exclusion of three loci that were potentially under positive selection, F_{ST} was estimated at 0.043. The reduction in F_{ST} is consistent with previous studies that have demonstrated that inclusion of outlier loci that are potentially under selection can markedly affect F_{ST} estimates (NIELSEN et al. 2006; ALLENDORF et al. 2010). The presence and scale of population structure between Celtic Sea and Scotian Shelf *G. morhua* in the present study concurs with previous studies that examined these populations (HUTCHINSON et al. 2001; O'LEARY et al. 2006, 2007; PAMPOULIE et al. 2008), and with additional studies that demonstrated population differentiation

between the eastern and western Atlantic *G. morhua* (i.e. allozymes, MORK et al. 1985; minisatellite, GALVIN et al. 1995; nuclear RFLPs, POGSON et al. 1995, 2001; microsatellites, BENTZEN et al. 1996; HUTCHINSON et al. 2001; O'LEARY et al. 2007; PAMPOULIE et al. 2008; single nucleotide polymorphisms, O'LEARY et al. 2006; NIELSEN et al. 2009; BRADBURY et al. 2010).

The combined microsatellite validation and genotyping approach presented here was designed to be a fast and cost-effective means for developing and deploying large numbers of microsatellite markers. Using larger numbers of genetic markers confers considerable advantages of increased precision and statistical power when assessing intra- and inter-population genetic parameters such as population structure and gene flow, as well as when inferring demographic parameters, such as effective population size, population expansions and bottlenecks (NEI and TAJIMA 1981; RYMAN et al. 2006). This improved precision allows for more robust and trustworthy management advice based on genetic data. In the present case, the rate of reduction of multilocus F_{ST} variability decreased after 20–30 loci suggesting that this is the point where using more loci only slightly improves the precision of the multilocus F_{ST} estimate. The point of diminishing returns may not be the same for other populations, other geographic scales or other organisms. The advantage of the method presented here is that more loci can be effectively genotyped, ensuring that the point of diminishing returns has in fact been reached and the most precise estimate of population genetic parameter acquired.

Conclusions

The current study combines three-primer PCR with multiplexing to allow for more economical, rapid development and deployment of microsatellite markers discovered from high throughput sequencing data. Fifty-five polymorphic *G. morhua* microsatellites were combined into six PCR multiplexes, which allowed for determination of F_{ST} between two populations with high precision. This approach is transferable to any species, including those for which extensive sequence resources are not available, and will allow for large and robust population genetic studies while minimising expensive and labour intensive capillary sequencing runs.

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Supplementary material (available online as Appendix hrd.00044 at <www.hereditasjournal.org/readers/appendix>). Appendix 1.

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