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High-throughput microsatellite genotyping in ecology: improved accuracy, efficiency, standardization and success with low-quantity and degraded DNA

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Abstract

Microsatellite markers have played a major role in ecological, evolutionary and conservation research during the past 20 years. However, technical constrains related to the use of capillary electrophoresis and a recent technological revolution that has impacted other marker types have brought to question the continued use of microsatellites for certain applications. We present a study for improving microsatellite genotyping in ecology using high-throughput sequencing (HTS). This approach entails selection of short markers suitable for HTS, sequencing PCR-amplified microsatellites on an Illumina platform and bioinformatic treatment of the sequence data to obtain multilocus genotypes. It takes advantage of the fact that HTS gives direct access to microsatellite sequences, allowing unambiguous allele identification and enabling automation of the genotyping process through bioinformatics. In addition, the massive parallel sequencing abilities expand the information content of single experimental runs far beyond capillary electrophoresis. We illustrated the method by genotyping brown bear samples amplified with a multiplex PCR of 13 new microsatellite markers and a sex marker. HTS of microsatellites provided accurate individual identification and parentage assignment and resulted in a significant improvement of genotyping success (84%) of faecal degraded DNA and costs reduction compared to capillary electrophoresis. The HTS approach holds vast potential for improving success, accuracy, efficiency and standardization of microsatellite genotyping in ecological and conservation applications, especially those that rely on profiling of low-quantity/quality DNA and on the construction of genetic databases. We discuss and give perspectives for the implementation of the method in the light of the challenges encountered in wildlife studies.

Keywords: faecal DNA, high-throughput sequencing, individual identification, parentage analysis, short tandem repeat (STR), *Ursus arctos*

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Introduction

Short tandem repeats (STR), commonly known as microsatellites (Goldstein & Schlötterer 1999; Ellegren 2004), have played a major role in ecological, evolutionary and conservation research during the past 20 years (Scribner & Pearce 2000; Sunnucks 2000; Avise 2004; DeSalle & Amato 2004; Selkoe & Toonen 2006; Kim & Sappington 2013). The codominant nature, biparental mode of inheritance and elevated levels of polymorphism (Goldstein & Schlötterer 1999; Schlotterer 2000; Ellegren 2004) have made them particularly informative and powerful for individual identification, parentage and kinship determination, and for investigating genetic and demographic processes, especially at fine scales, and over recent time periods (Luikart & England 1999; Blouin 2003; Manel *et al.* 2003; Avise 2004; Randi 2008; Jones *et al.* 2010; Kim & Sappington 2013). In addition, successful cross-amplification of assays in populations of the same species and closely related species, and high reproducibility with low-

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quantity/quality DNA have further contributed to their popularity in the molecular ecology arena (Selkoe & Toonen 2006; Barbara *et al.* 2007). In view of these features, STRs are still the most widely used molecular markers in noninvasive genetic studies of elusive and endangered species and in forensic identifications from degraded samples (Alacs *et al.* 2010; Lampa *et al.* 2013).

Because of this extensive utilization, researchers working with microsatellites have also long known their limitations (Bonin *et al.* 2004; Pompanon *et al.* 2005; Selkoe & Toonen 2006; Putman & Carbone 2014). The main criticisms are often of technical nature (Guichoux *et al.* 2011) and relate to low throughput and automation, difficulty of scoring and lack of transferability of genotype data between platforms. Whereas some of these are partly due to the hypervariable and repetitive nature of microsatellites, all are almost entirely dependent on the method currently used for genotyping, which is based on size polymorphism of amplified microsatellites determined through capillary electrophoresis (CE). This system is easy to implement, and all steps can be carried out in standard genetic laboratories, but it poses limitations on the number of samples and fluorescently labelled markers that could be run in parallel and for complete automation of sample processing and analysis (Guichoux *et al.* 2011). Microsatellite patterns on an electropherogram can be difficult to interpret and to score consistently, for example PCR artefacts such as stutter bands, varying degrees of PCR product adenylation, or differential DNA migration between runs complicate standardized and automated allele calling, and may also inflate genotyping errors especially with low-quantity and degraded DNA sources (Pompanon *et al.* 2005; Guichoux *et al.* 2011). Practitioners have been using highly trained technicians and strict protocols for ensuring data quality, but this involves significant amounts of time and effort, which in turn affects the cost/sample. In addition, because DNA electrophoretic migration is affected by experimental and platform-specific conditions, results produced by different laboratories, or at long-time intervals cannot be directly compared, which impedes wider data utilization (Guichoux *et al.* 2011).

High-throughput sequencing (HTS) technologies (Glenn 2011) offer a mean to overcome most of these technical limitations. In the last years, HTS has been applied to STR profiling in human forensics (Fordyce *et al.* 2011, 2015; Bornman *et al.* 2012; Van Neste *et al.* 2012, 2014), opening a completely new paradigm for STR genotyping. Enhanced throughput is a first improvement associated with this approach, as the massive parallel sequencing ability of HTS sequencers greatly expands the potentials for marker and sample multiplexing, much beyond the capacities of CE. The main progress, however, consists of enabling direct access to the sequences of amplified microsatellites, and therefore, profiling to be performed on the basis of the sequences of the alleles without indirect sizing through electrophoresis. Unambiguous allele identification by the sequence, both the nucleotide sequence and the length of the microsatellite, allows greater accuracy of allele determination, because allele calling is not affected by variation in experimental conditions, and allelic variants including SNPs, indels and complex repeat structure can be distinguished and characterized. In addition, because alleles are handled as discrete sequence data, the genotyping process becomes amenable to full automation through bioinformatics, and genotype data are platform independent.

The first studies using HTS to type human forensic DNA samples at core STR loci have shown results comparable to the electrophoresis method, with high levels of correct allele detection and resolution of DNA mixtures of multiple contributors (Fordyce *et al.* 2011, 2015; Bornman *et al.* 2012; Van Neste *et al.* 2012, 2014). HTS-based genotyping also showed higher sensitivity for low DNA amounts and greater ability to generate full STR profiles with degraded DNA compared to CE (Fordyce *et al.* 2015). As a consequence, the human forensic community is now evaluating HTS for routine forensic STR applications (Børsting & Morling 2015). It is clear that transitioning to the new approach would have revolutionizing implications also for ecological research relying on DNA profiling of biological samples, as anticipated by very recent applications in nonmodel species (Darby *et al.* 2016; Suez *et al.* 2016; Vartia *et al.* 2016; Zhan *et al.* 2016). Such technical shift would not only result in higher throughput and accuracy of microsatellite data available for ecological investigations, but has also the potential to further enable studies involving the genotyping of low-quantity and degraded DNA samples. Moreover, it will address the critical need in conservation research for increased cost- and time-effectiveness of genotype information production and sharing.

In this article, we present a strategy for implementing HTS-based microsatellite genotyping in ecological studies. We validate the approach for individual identification and parentage analysis using brown bear (*Ursus arctos*) tissue and hair samples of good DNA quality and illustrate results of its application for genotyping DNA of lower quantity/quality from brown bear hair and faecal samples. We discuss aspects of the implementation and performance of the method in comparison with traditional CE and in the light of the challenges typically encountered in wildlife genotyping in ecological and conservation studies, particularly those based on the collection of low amount and degraded DNA samples.

Material and methods

Brown bear samples and DNA extraction

Samples used for this study included tissue samples from hunted brown bears in Sweden, and brown bear hair and faecal samples collected in the field in the French Pyrenees. Tissue biopsies and ~1 cm^3 of scat material were collected and stored in 95% ethanol. Hair samples were collected and stored in paper envelopes in silica desiccant.

Genomic DNA was extracted from tissue and hair samples with the DNeasy Blood and Tissue Kit (QIAgen GmbH) using 25 mg of tissue material and up to 10 hair follicles, and from faecal samples with the Qiamp DNA stool kit (Qiagen GmbH) using 0.1–0.2 mL of faeces. DNA was eluted in 300 μL volume. A negative control was included in each extraction to monitor contamination. Hair and faecal DNA extractions were performed in a dedicated room.

Brown bear STR markers development

We aimed to design microsatellite markers to be specifically used for genotyping low-quantity and degraded DNA samples with HTS. For this reason, we targeted (i) mainly tetranucleotide loci because they present reduced strand slippage during PCR compared to dinucleotide repeats (Ghebranious *et al.* 2003); (ii) short markers (<150 bp) for robust amplification of degraded DNA; and (iii) markers enabling the highest level of PCR multiplexing to increase efficiency of laboratory processing.

The DNA extracted from one Scandinavian brown bear tissue sample was used for preparing a shotgun library for STR identification (Fig. 1). Following published protocols (Margulies *et al*. 2005), total DNA was fragmented into 300–800 bp by nebulization, adaptors were ligated to the fragments' 3′ and 5′ ends to perform emulsion PCR and amplified fragments were sequenced on the 454 sequencing system (Roche). Software Msatcommander v.1.08 (Faircloth 2008) was used to screen reads containing perfect tetranucleotides with 12–14 repeats and with at least 40 nucleotides on each flanking region. These reads were used in program Primer3Plus v.2.4.0 (Untergasser *et al.* 2012) to design primer pairs for PCR amplification based on default parameters except (i) product size range of 80–150 bp, (ii) primer size of 17–24 bp, (iii) melting temperature of 58–62 °C with maximum 2 °C degree difference between paired primers and (iv) 40%–60% GC content. For each read, we selected the primer pair with the lowest penalty score assigned by Primer3Plus. From the list of automatically generated primers, we further selected primers that had or could be manually adjusted to obtain most of the following, while ensuring initial melting temperature and primer size requirements: C or G within the last five 3′ nucleotide positions, no homopolymers of four or more nucleotides, minimum distance from the microsatellite repeats to reduce the length of amplified fragments and no secondary structure formation.

Selection of suitable STRs for multiplexing

Short tandem repeats markers designed in the previous step were tested for coamplification in PCR multiplexes (Fig. 1) of 20 using the Platinum Multiplex PCR Master Mix (Life Technologies) and hair DNA extracts from four Pyrenean brown bears. Markers were pooled in different PCR multiplexes in an attempt to minimize cross-dimer formation based on Primer Tools (http://yellow.nist.gov:8444/dnaAnalysis/primerToolsPage.do). Reactions were carried out in a 50 μL volume and contained 1× concentrated Platinum Multiplex PCR Master Mix, 1% GC enhancer, 0.5 μL each primer and 5 μL DNA extract, as by the manufacturer's instructions. Thermocycling profile had an initial denaturation step of 2 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, 90 s at 57 °C, 60 s at 72 °C and a final elongation step of 10 min at 72 °C. PCR products were purified using the MinElute PCR purification kit (QIAGEN GmbH), visualized using QIAxel (QIAgen GmbH) and quantified with Qubit 2.0 Fluorometer (Life Technologies). A different library was constructed for each multiplex and sequenced on an Illumina MiSeq platform (2 × 150 bp). Library construction and sequencing were carried out using a commercial service (http://www.fasteris.com). The resulting sequence reads were sorted by marker and inspected using Unix commands. Polymorphic markers with the highest specificity (i.e. producing only the target sequence) and read count were retained to be integrated into a single multiplex PCR. This step was crucial for ensuring a robust marker set for optimal multiplex amplification for HTS genotyping. Loci selected for the final multiplex PCR were mapped against the polar bear (*Ursus maritimus*) genome to exclude physical proximity on the chromosomes.

Validation of HST STR genotyping for individual identification and parentage assignment

Brown bear samples of good DNA quality from the Scandinavian (80 tissues) and Pyrenean (17 hair) populations were genotyped at the selected marker panel to validate their application with HTS for individual identification and parentage analysis (Fig. 1). The Pyrenean samples were reference hair samples previously genotyped in our laboratory using dinucleotide microsatellites and CE and

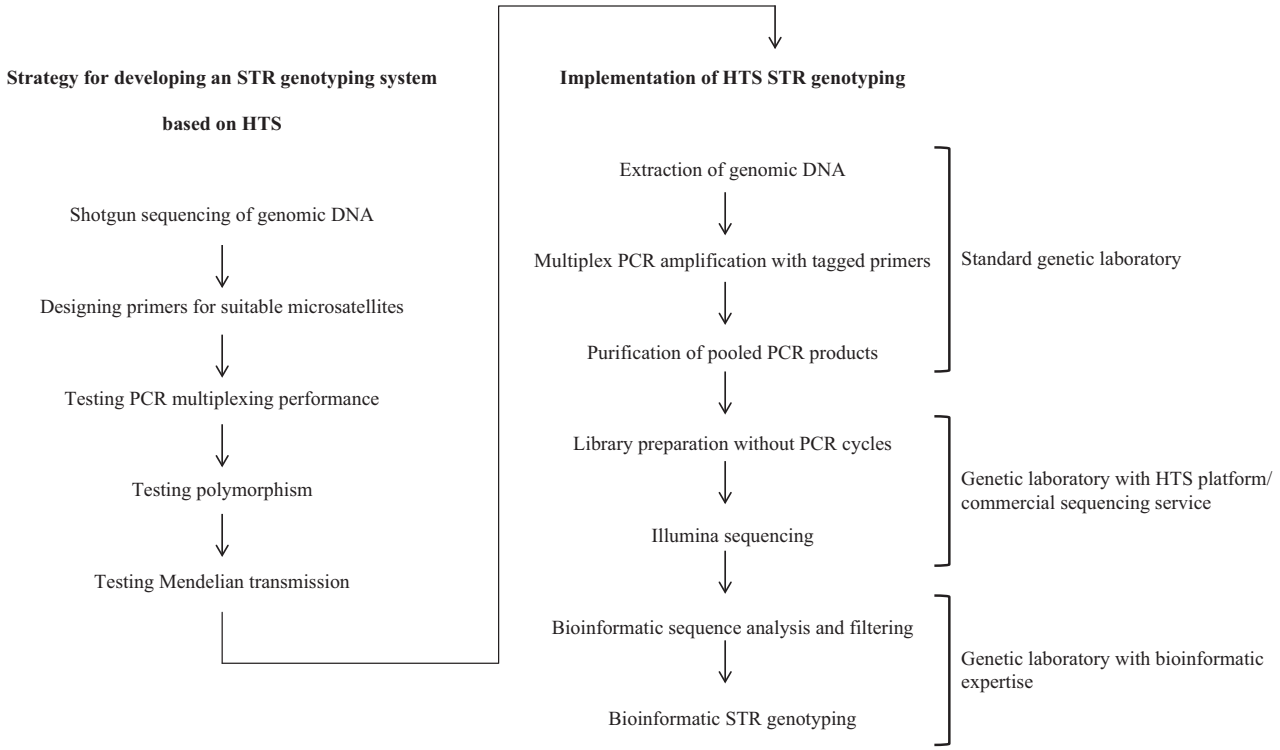


Fig. 1 Development strategy and workflow of high-throughput microsatellite genotyping.

were from different bears, including 7 males, 10 females and 6 known parent-offspring pairs. After extraction, DNA concentrations of tissue samples were quantified using Qubit 2.0 Fluorometer and equalized to 10 ng/μL. Amplifications were performed as reported in the previous section, except adjusting primer concentrations (Table 1) and using 2 μL DNA extract in 20 μL reaction volume. A sex marker targeting a short fragment of the ZF gene on the X and Y sexual chromosomes in Ursids (Pagès *et al.* 2009) was added to the multiplex PCR (Table 1). All forward and reverse primers in each individual multiplex reaction were modified by the addition of molecular identifier tags (Coissac 2012) on the 5′, to obtain unique tag combinations for any given PCR product and retrieving the respective sequence data in postsequencing bioinformatic analysis. Amplifications were performed in four replicates for tissue samples and eight replicates for reference hair samples. For each data set (Scandinavian and Pyrenean samples), PCR products were mixed equivolume and then purified and quantified as above. Separate libraries were prepared for each data set, according to the MetaFast protocol, a PCR-free procedure enabling a significant reduction in bias associated with library preparation and sequencing (http://www.fasteris.com/metafast). Each library was sequenced on a MiSeq (2 × 150-bp, targeting approximately 2000 reads/marker/PCR).

DNA sequence data analysis was performed in two steps (Fig. 1), outlined in Fig. 2. First, DNA reads were treated to generate tabular files containing filtered microsatellite sequence counts (Fig. 2a); then, genotypes were extracted (Fig. 2b). In the initial treatment (Fig. 2a), the sequence read output was processed using the OBITools (Boyer *et al.* 2016), a set of bioinformatic programs designed to handle HTS sequences (http://metabarcoding.org/obitools). Filtered sequences include microsatellite allele sequences (the amplification targets) and various less abundant artefact sequences. These latters comprise stutter sequences (sequences identical to a parental microsatellite allele sequence but one repeat motif shorter in length and less abundant), and other sequences originating from PCR and sequencing errors. Microsatellite genotypes were generated from the observed sequence reads and relative counts, using R and python scripts, implementing a set of rules and user-defined thresholds (Fig. 2b). Complete description of the bioinformatic pipeline is available in Supporting Information; scripts are provided in Dryad repository.

Amplification and genotyping performance were assessed based on the reads contributing to allele sequences, stutter sequences and other sequences for a given marker in each PCR. For each locus, we derived measures of locus balance, as the deviation of the allele read count for a locus in a PCR from the average allele read count across loci in that PCR, standardized by the

Table 1 Markers used in this study and polymorphism observed in the Scandinavian brown bear tissue and Pyrenean brown bear reference hair samples

Locus	Repeat motif	Primer sequence 5′–3′	Concentration (μM)	Overall size range	Number of alleles Scandinavia Pyrenees	H_O Scandinavia Pyrenees	H_E Scandinavia Pyrenees
UA03	CTAT	F: GCTCCCATAACTGCATAAGGTC	0.1	55–75	5	0.45	0.69
		R: CTGGCTGGCTGGCTAGG			4	0.71	0.67
UA06	AAGG	F: CCTCACTTAGCAGCCTACTTG	0.1	63–79	5	0.73	0.73
		R: TGCTCTTCTCTTCAAACTGAGC			5	0.77	0.73
UA14	TTTTA	F: CCACATTACTGCCAGATAGAGC	0.1	83–128	12	0.89	0.87
		R: ACATCAAACACTAATGATGCACTG			5	0.69	0.74
UA16	CTTT	F: CCCCCAAGTCAATTTCTAATATG	0.1	59–87	7	0.65	0.63
		R: CCTTTAGTTTAGTGGCCATCAATC			5	0.59	0.69
UA17	CTTT	F: AAGGGTCAGAATTAGGTATCTGTC	0.07	85–101	5	0.46	0.52
		R: TGCTATTTCCATCTTCAACCTGAC			3	0.71	0.64
UA25	CTTT	F: CTCCATTTGGGGTCTGTTGT	0.1	61–78	10	0.61	0.72
		R: GATTGCTTCATGCACGCTTA			4	0.29	0.47
UA51	CTTT	F: ACCACTTTACTTCCTCATGTCTG	0.1	68–80	4	0.60	0.64
		R: GTGAGTTCAAGCACCACGTAG			5	0.71	0.71
UA57	CATT	F: ACATCTAGGACCAAGCATTGC	0.07	70–78	3	0.40	0.46
		R: GTCTGCCTCTTAACCATGGC			3	0.65	0.51
UA63	TCCA	F: TATCCACTCACCATCCACCA	0.1	69–88	5	0.49	0.66
		R: CCAGGAAGCGTAACTCCAGA			6	0.47	0.77
UA64	TTTA	F: CATGCACTCTCTGTATCCTGCT	0.1	63–71	6	0.69	0.76
		R: CCTCTACCCTCTGCCTCGAC			4	0.53	0.62
UA65	GATA	F: TCAGGGTTCTCCAAAGAAACA	0.07	85–112	8	0.70	0.73
		R: CTGGGCCTCCACTATCATGT			3	0.59	0.63
UA67	ATTT	F: TCCTGCTTACCGCACTTCTT	0.1	85–101	4	0.48	0.58
		R: GAGGACACCAGCTGTGAGAA			3	0.35	0.58
UA68	ATCT	F: TTCCCAACTTCCAAACACCC	0.1	66–113	9	0.66	0.82
		R: GGTAGGTAAGAAGGCATGCATG			5	0.65	0.71
UAZF		F: GACAGCTGAACAAGGGTTG	0.08	51	2		
		R: GCTTCTCGCCGGTATGGATG			2		

F, forward primer; R, reverse primer; H_O, observed heterozygosity; H_E, expected heterozygosity.

PCR allele reads average across loci; heterozygote balance, as the read count ratio of the high and low molecular weight allele in a PCR product of heterozygote genotypes; and stutter ratios, as the stutter read percentages of the corresponding parent allele in a PCR product (Fordyce *et al.* 2015). We estimated per locus amplification success, as the proportion of PCR amplifications for each marker yielding reads assigned to at least one allele sequence, and per locus genotyping success, as the proportion of samples yielding a consensus genotype at a locus (refer to step 4 of the pipeline in Supporting Information). Per locus per reaction error rates due to allelic dropout and false alleles were derived following Broquet & Petit (2004) by comparing alleles scored in each PCR replicate to the consensus genotype at each locus.

Program GenAlEx v6.5 (Peakall & Smouse 2006) was used to estimate the number of alleles per locus and observed and expected heterozygosity, and to evaluate the power of the marker set for individual identification, by examining match probabilities (P_{ID} Woods *et al.* 1999; $P_{ID(sib)}$ Waits *et al.* 2001) and genotype mismatches (Paetkau 2003). Marker Mendelian inheritance and parentage assignment were verified on known parent-offspring pairs using program Gimlet (Valière 2002). The presence of null alleles was assessed using program MicroChecker (Van Oosterhout *et al.* 2004) and linkage disequilibrium tested using Genepop v. 4.2 (Raymond & Rousset 1995), with $\alpha = 0.05$ and Bonferroni corrections for multiple comparisons.

Application of HTS STR genotyping to low-quantity and degraded DNA samples

We evaluated the applicability of the HTS approach for genotyping biological samples containing poor-quality and low-quantity DNA. For this purpose, we used hair ($n = 51$) and faecal ($n = 31$) samples noninvasively collected in the Pyrenees during 2012–2014 for genetic monitoring of the Pyrenean brown bear. Samples were processed and data analysed as described for hair samples

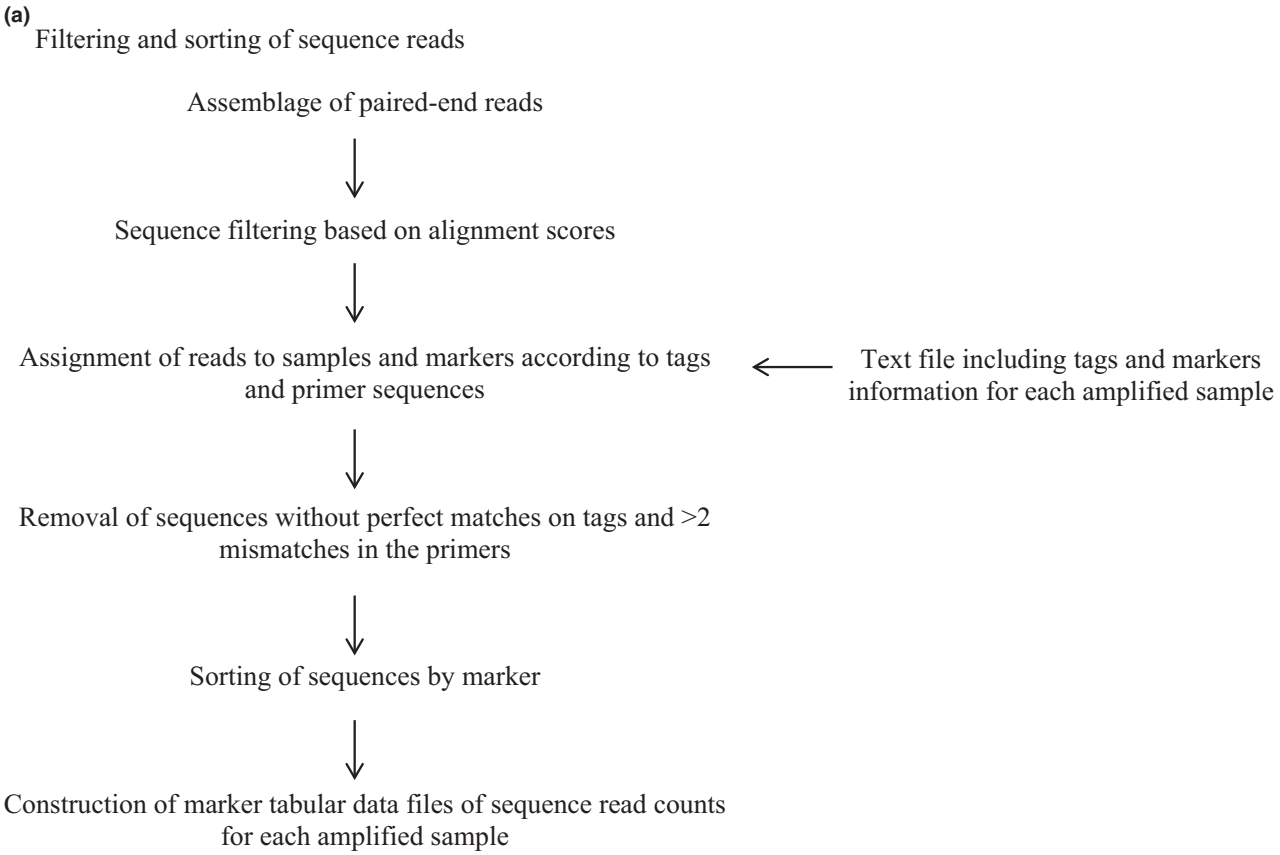


Fig. 2 Outline of the bioinformatic pipeline used to generate genotypes from STR HTS outputs. (a) Filtering and sorting of sequence reads, (b) locus genotype assignment. [Colour figure can be viewed at wileyonlinelibrary.com]

in the previous section. Program GenAlEx was used to group samples with identical genotypes, match sample genotypes to genotypes determined from the Pyrenean reference hair and flag similar sample genotypes possibly containing undetected genotyping errors to be accounted for. Per locus amplification and genotyping success were estimated as described above. In addition, we calculated multilocus genotyping success, as the proportion of samples for which a consensus genotype was obtained at a minimum number of loci, based on $P_{ID(sib)}$ and genotype mismatch considerations (see Results). Per locus genotyping error rates were estimated as previously described, but in addition, we used the reference genotypes determined from the Pyrenean hair samples for detecting errors by comparison with the sample genotypes.

Results

Brown bear STR marker development and selection for HTS genotyping

Shotgun sequencing of the brown bear tissue sample generated a total of 277 901 sequences, with 427 bp mean read length. Totally, 112 sequences contained perfect tetranucleotides with 12–14 repeats, with at least 40 nucleotides on the flanking regions. From these sequences, 70 primer pairs were identified that fulfilled our criteria for enabling suitable markers. Based on marker performance and polymorphism (results not shown), a final set of 12 tetranucleotides and one pentanucleotide (Table 1) was selected for HTS genotyping. Markers were mapped on different scaffolds of the polar bear genome (Table S1, Supporting information).

High-throughput STR genotyping for individual identification and parentage assignment

Sequencing of the Scandinavian and Pyrenean samples amplified at the selected panel of 13 microsatellites, and the sex marker generated an average of 36 056 and 11 153 reads/PCR, respectively, that were used for genotyping (Tables 2 and 3). We report on amplification performance based on 10 PCR products randomly selected from each data set (Table S2, Supporting information, results for a sample of each type are summarized in Table 4). The majority of reads of each amplification

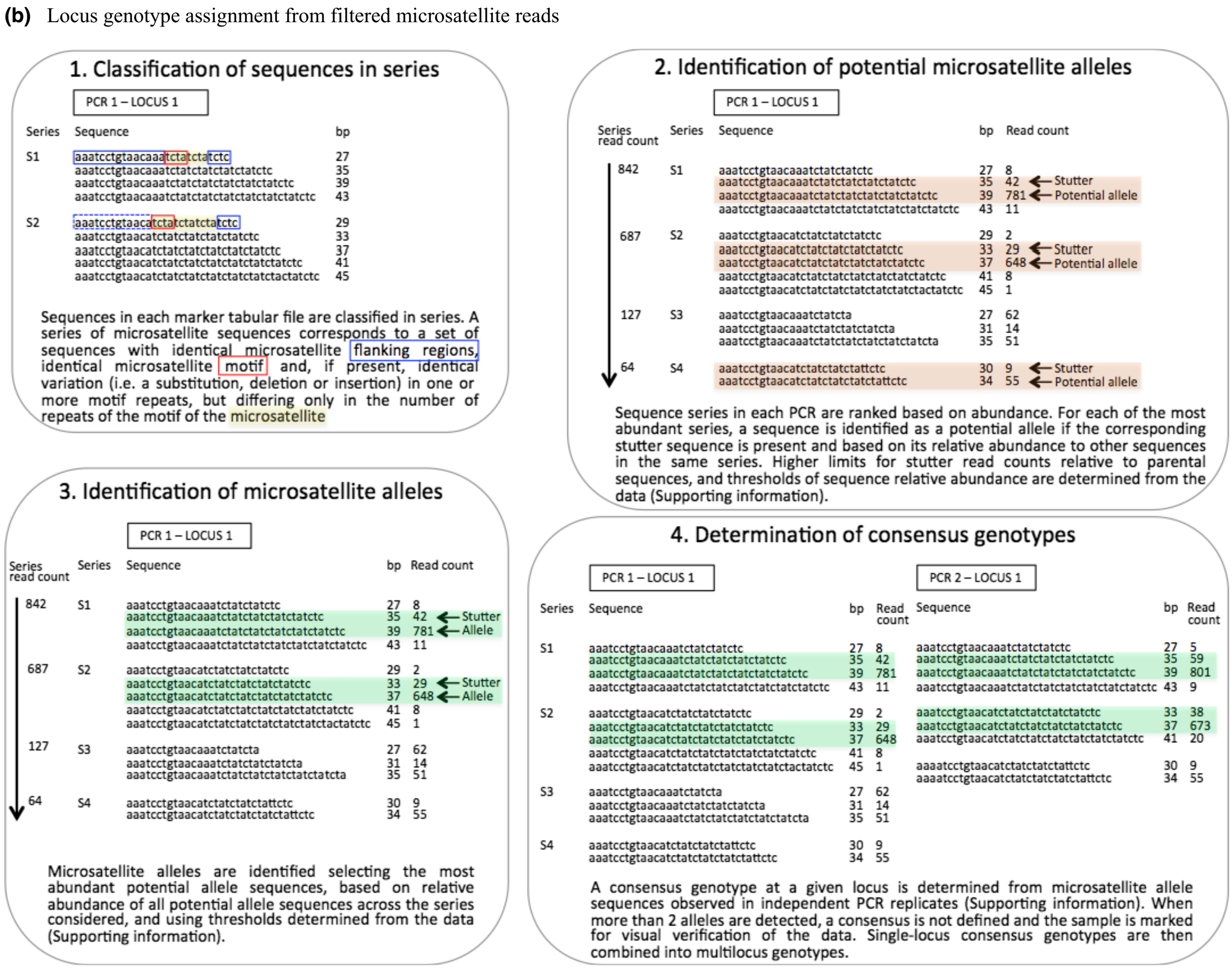


Fig. 2 Continued. [Colour figure can be viewed at wileyonlinelibrary.com]

product were sorted into alleles (48%–84%), while remaining reads corresponded to stutters (0.2%–9%) and other sequences (15%–50%). Proportions of reads assigned to different categories of sequences (alleles, stutters, other sequences) in each PCR were similar among sample types, markers and replicates of the same sample (Table S2, Supporting information, Table 4). Patterns of allele amplification differed to some degree among markers and between the two data sets. However, most loci had comparable measures of locus balance, except for a few loci that had allele read counts above average (Fig. 3a), most microsatellite markers seemed to amplify the lowest molecular weight allele preferentially (Fig. 3b) and stutter ratios were generally around or below 5% of the parent allele for most markers (Fig. 3c). The pentanucleotide (UA14) had the lowest stutter ratio. Overall, wider variation was apparent for hair samples (Table S2, Supporting information, Table 4, Fig. 3).

Table 2 Summary of number of reads of the Miseq runs for samples analysed in this study

	Scandinavian tissues	Pyrenean reference hair	Pyrenean hair and scats
Total Miseq reads	2 134 9346	20 814 043*	23 208 795
Reads assigned to markers and samples	12 402 179	2 235 960	5 748 489
Average number of reads/PCR	36 056	11 153	8910

*Reference hair samples were included in a library with other samples; therefore, this count refers to the whole library, as it is not possible to distinguish among samples at this stage.

Average per locus amplification and genotyping success of microsatellites markers were 93.3%–100% (Table 3). Average rates of allelic dropout were 0.004 for tissues and 0.039 for reference hair; average false allele

Table 3 Average number of reads per PCR (Count) and rates of amplification success (AS), genotyping success (GS), allelic dropout (ADO) and false allele (FA) by locus for each data set

	Good DNA quality samples										Low DNA quantity/quality samples								
	Scandinavian brown bear tissues					Pyrenean brown bear reference hair					Pyrenean brown bear hair					Pyrenean brown bear scat			
Locus	Count	AS	ADO	FA	GS	Count	AS	ADO	FA	GS	Count*	AS	ADO	FA	GS	AS	ADO	FA	GS
UA03	2238	1	0	0	1	443	0.934	0.023	0	1	592	0.836	0.131	0.013	0.863	0.855	0.126	0.005	0.871
UA06	1719	1	0.004	0	1	528	0.926	0.031	0	1	337	0.819	0.145	0.015	0.843	0.810	0.127	0.025	0.839
UA14	3214	1	0.004	0	1	1215	0.831	0.055	0	0.941	1036	0.828	0.103	0.034	0.843	0.742	0.202	0.006	0.774
UA16	2062	1	0	0	1	781	0.978	0.026	0	1	697	0.821	0.009	0	0.863	0.823	0.056	0.010	0.839
UA17	1755	1	0	0	1	830	0.956	0	0	1	595	0.809	0.087	0.006	0.824	0.798	0.262	0.021	0.871
UA25	2169	1	0	0	1	515	0.912	0.185	0	1	477	0.841	0.104	0.003	0.863	0.867	0.025	0.010	0.903
UA51	2196	1	0	0	1	673	0.904	0.013	0	1	259	0.716	0.199	0.004	0.784	0.718	0.116	0.011	0.839
UA57	3217	1	0	0	1	1142	0.941	0	0	1	762	0.846	0.048	0.003	0.863	0.819	0.122	0.005	0.871
UA63	2506	1	0	0	1	566	1	0.032	0.016	1	354	0.789	0.112	0.007	0.804	0.806	0.122	0	0.903
UA64	2737	1	0	0	1	668	0.846	0.017	0	1	735	0.868	0.082	0.003	0.843	0.831	0.092	0.005	0.871
UA65	2199	1	0.013	0	1	903	0.993	0.013	0	1	738	0.797	0.078	0.003	0.824	0.778	0.163	0	0.839
UA67	3853	1	0	0	1	1338	0.978	0	0	1	1159	0.836	0.095	0.003	0.843	0.786	0.186	0	0.839
UA68	3898	1	0.031	0.006	1	834	0.934	0.114	0.024	1	606	0.811	0.187	0.010	0.843	0.811	0.187	0.010	0.843
STR average	2597	1	0.004	0.000	1	803	0.933	0.039	0.003	0.995	642	0.817	0.106	0.008	0.839	0.803	0.137	0.008	0.854
ZF	2293	1	0	0	1	717	0.971	0	0.008	1	563	0.782	0.019	0	0.804	0.758	0.120	0	0.839

*The count refers to both hair and scat samples as they were analysed in the same run

Table 4 Percentage of reads assigned to allele, stutter and other sequences for a sample of different types

Locus	Sample type	% Allele sequences	% Stutter sequences	% Other sequences
UA03	Tissue	80.01 (0.42)	3.15 (0.33)	16.84 (0.31)
	Reference hair	78.41 (4.39)	3.83 (0.94)	17.76 (3.65)
	Scat	76.24 (1.06)	5.39 (0.69)	18.37 (0.50)
UA06	Tissue	68.21 (0.81)	4.62 (0.28)	27.17 (0.61)
	Reference hair	70.55 (1.62)	7.89 (1.10)	21.57 (2.28)
	Scat	66.31 (2.55)	9.19 (0.78)	24.49 (2.59)
UA14	Tissue	73.73 (0.90)	1.00 (0.23)	25.27 (0.99)
	Reference hair	76.55 (1.37)	1.80 (0.52)	21.65 (1.50)
	Scat	75.52 (1.66)	0.96 (0.32)	23.52 (1.69)
UA16	Tissue	69.09 (1.57)	4.50 (0.27)	26.41 (1.51)
	Reference hair	72.02 (1.18)	4.42 (0.99)	23.56 (1.30)
	Scat	69.89 (2.45)	3.98 (0.41)	26.13 (2.54)
UA17	Tissue	71.54 (2.42)	2.38 (0.52)	26.08 (1.92)
	Reference hair	71.83 (2.55)	3.53 (0.76)	24.64 (2.53)
	Scat	67.60 (3.54)	4.46 (0.95)	27.93 (3.55)
UA25	Tissue	69.98 (1.36)	5.68 (0.31)	24.33 (1.60)
	Reference hair	70.93 (2.24)	3.74 (1.33)	25.32 (2.74)
	Scat	72.05 (1.29)	3.06 (0.88)	24.89 (1.40)
UA51	Tissue	74.07 (0.77)	2.72 (0.31)	23.20 (0.94)
	Reference hair	72.51 (1.49)	5.12 (0.92)	22.37 (1.62)
	Scat	71.32 (1.96)	3.84 (0.40)	24.85 (2.03)
UA57	Tissue	77.58 (2.23)	1.44 (0.20)	20.98 (2.07)
	Reference hair	77.43 (2.81)	2.61 (0.41)	19.96 (2.97)
	Scat	76.91 (3.15)	1.45 (0.32)	21.64 (3.33)
UA63	Tissue	71.18 (0.55)	2.59 (0.02)	26.23 (0.56)
	Reference hair	75.48 (1.67)	3.26 (0.79)	21.26 (1.87)
	Scat	70.03 (2.52)	4.76 (0.67)	25.20 (2.64)
UA64	Tissue	78.85 (1.06)	3.17 (0.34)	17.98 (0.76)
	Reference hair	80.67 (1.90)	2.77 (0.80)	16.56 (2.25)
	Scat	79.87 (1.25)	1.44 (0.26)	18.68 (1.18)
UA65	Tissue	78.14 (1.69)	2.47 (0.26)	19.39 (1.65)
	Reference hair	75.25 (1.55)	3.99 (0.66)	20.75 (1.22)
	Scat	75.04 (2.52)	1.76 (0.22)	23.20 (2.53)
UA67	Tissue	76.55 (0.86)	2.05 (0.24)	21.40 (0.98)
	Reference hair	79.10 (1.21)	1.26 (0.39)	19.63 (1.42)
	Scat	74.73 (2.20)	2.57 (0.72)	22.70 (1.93)
UA68	Tissue	72.10 (1.52)	3.20 (0.15)	24.69 (1.41)
	Reference hair	78.61 (1.25)	4.01 (0.91)	17.39 (2.02)
	Scat	71.09 (2.50)	3.60 (0.52)	25.31 (2.40)
ZF	Tissue	77.50 (0.90)	na	22.50 (0.90)
	Reference hair	76.74 (1.15)	na	23.25 (1.15)
	Scat	75.97 (2.81)	na	24.02 (2.81)

Standard deviation across PCR replicates is indicated in parentheses.

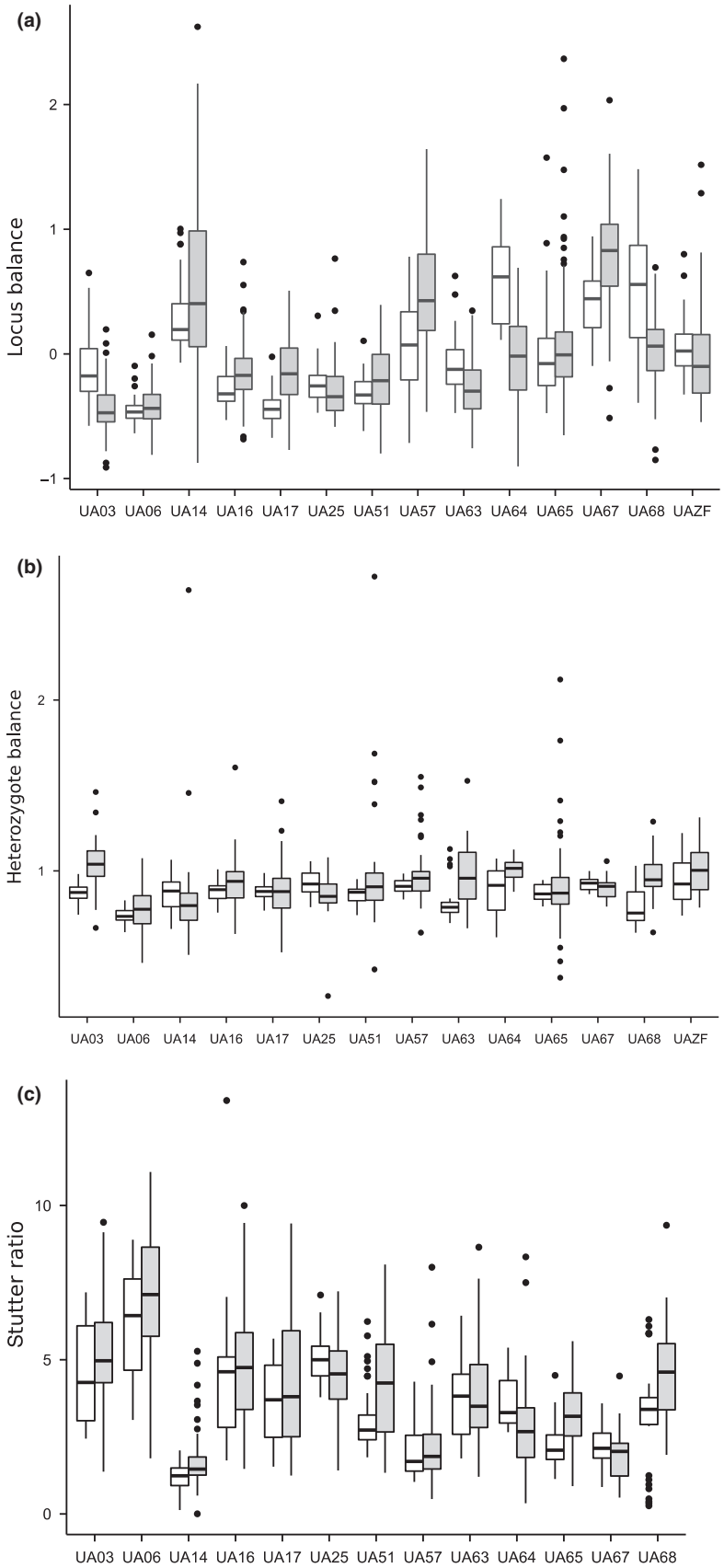
rates were 0.0005 for tissues and 0.003 for reference hair. Amplification and genotyping success of the sex marker were 97.1%–100%. Dropout rate for the X- and Y-amplified fragment was 0, whereas rates of false alleles were 0 for tissues and 0.008 for reference hair (Table 3).

Genotypes of the Scandinavian samples had 6.4 mean alleles per locus (range 3–12), mean observed heterozygosity H_O = 0.60 and expected heterozygosity H_E = 0.68 (Table 1). Genotypes of the Pyrenean reference hair had 4.2 mean alleles per locus (range 3–6), mean observed heterozygosity H_O = 0.59 and expected heterozygosity H_E = 0.65 (Table 1). Allele variants containing SNPs and indels, both in the repeat unit and the flanking regions, were found at several loci (Table S3, Supporting information). Cumulative P_{ID} and $P_{ID(sib)}$ were 4.8E−12 and 2.7E−05 in the Scandinavian genotypes and 1.2E−10 and 5.1E−05 in the Pyrenean genotypes, respectively, and multilocus profiles differed at ≥3 microsatellite loci. Mendelian inheritance of alleles was confirmed in all known parent-offspring pairs of the Pyrenean samples. MicroChecker indicated null alleles at five loci in the Scandinavian samples (UA03, UA25, UA63, UA67 and UA68); however, most null allele signals disappeared when samples were analysed according to sampling locations. Linkage disequilibrium was detected ($P < 0.05$) in 31 (13 with Bonferroni correction) and 19 (two with Bonferroni correction) of 78 locus combination tests for the Scandinavian and Pyrenean genotypes, respectively.

High-throughput STR genotyping for low-quantity and degraded DNA samples

Sequencing of Pyrenean scats and hair samples generated an average of 8910 reads/PCR used for genotyping (Table 2). Amplification performance and repeatability were comparable to tissue and reference hair samples (Tables 3 and 4). Average per locus amplification and genotyping success of microsatellites markers were 80.3%–85.6%; same rates for the sex marker were 75.8%–83.9% (Table 3). We retained samples for which a consensus genotype was attained for at least 10 microsatellite loci ($P_{ID(sib)}$ 4.9E−04), resulting in a multilocus genotyping success of 82% for hair and 84% for scats. Among these sample genotypes, four were identified in only one sample and differed at a minimum of three markers from other genotypes. All others matched genotypes of reference hair (n = 6) and/or were found in multiple samples (n = 12). Average rates of allelic dropout were 0.11 for hair and 0.14 for scats; average rates of false alleles were 0.008 for hair and 0.01 for scats (Table 3). For the sex marker, dropout rate was 0.018 for

Fig. 3 Marker amplification performance for the Scandinavian brown bear tissues and Pyrenean brown bear reference hair: (a) locus balance (deviation of the allele read count for a locus in a PCR from the average allele read count across loci in that PCR, standardized by the PCR allele reads average across loci), (b) heterozygote balance (read count ratio of the high and low molecular weight allele in a PCR product of heterozygote genotypes), (c) stutter ratios (stutter read percentages of the corresponding parent allele in a PCR product).



hair and 0.12 for scats, and rate of false alleles was 0 (Table 3).

Discussion

We have presented a strategy for microsatellite genotyping based on HTS for ecological studies. It entails the extraction of genomic DNA from biological samples, sequencing of PCR coamplified microsatellite markers of similar short size on an Illumina sequencing platform and bioinformatic treatment of the sequence data output to obtain multilocus genotypes. Our results are in accordance with recent studies that explored similar approaches in human forensics and nonmodel species. In addition to these previous works, we addressed challenges specific to the application of HTS-based STR genotyping for ecological and conservation research, particularly in the context of wildlife studies based on the analysis of low amount and degraded DNA samples, highlighting the vast potential for improving genotyping yield, accuracy, efficiency and standardization.

Genotyping success and accuracy

Using the HTS genotyping approach, we accurately sequenced microsatellite amplicons with levels of correct allele detection comparable to human forensic studies (Fordyce *et al.* 2015), in tissue and noninvasively collected hair and faecal samples, encompassing the gradient of DNA quality and quantity typically encountered in wildlife studies. Accuracy of allele detection and reliable genotyping was confirmed by reproducible results in independent replicate PCRs and correct Mendelian inheritance of alleles for all known parent-offspring pairs considered. Marker amplification and per locus genotyping success were high for all sample categories, including hair and scat samples with poor DNA quality/quantity from the Pyrenean bears, which resulted in individual identification ≥82%. Direct comparisons with the CE method were not possible because we did not analyse the same samples and markers with CE. Nonetheless, this represents a significant improvement, and one of the most relevant results of this study, as rates of individual identification of hair and faeces reported in multiyear genetic monitoring of the Pyrenean brown bear using CE were consistently <50% (C. Miquel, unpublished). Similar progresses can be reasonably deduced for genotyping error rates, especially allelic dropout for scat samples, because rates were lower than derived using the same error estimation method, for other European brown bears in comparable environments, both using similar collection protocols (De Barba *et al.* 2010), and upon collection of solely fresh samples (Skrbinšek *et al.* 2010).

Increasing genotyping yield and accuracy is a priority in wildlife research, which often must rely on DNA profiling of degraded and low-quantity DNA sources (i.e. faeces, shed hair, feathers, saliva, ancient and historical samples). The development of new methods for genotyping large number of single-nucleotide polymorphisms (SNPs) has generated expectations for improved analysis of these types of samples (Kraus *et al.* 2015). However, SNP genotyping assays may still present limits for use with low amount of degraded template in some species, and RADseq genotyping-by-sequencing protocols require large amount of good starting DNA (Graham *et al.* 2015). Improved STR genotyping will therefore particularly benefit and further enable wildlife noninvasive genetic studies and forensic identifications from degraded samples, for which microsatellites are still the main markers of choice (Alacs *et al.* 2010; Lampa *et al.* 2013).

The improvements observed in this study can be explained by the combination of the new marker set and the HTS strategy adopted. We designed new microsatellite markers to be used specifically for the analysis of degraded samples with HTS. Improved genotyping success and error rates, particularly for scat samples, were likely a consequence of robust amplification of degraded DNA by short markers and the enhanced sensitivity of HTS that has the ability to generate reads from single DNA molecules, therefore increasing detection with limiting amount of template. In addition, the use of markers with overlapping amplification products (total observed allele size range for all markers 51–128 bp) and selection of a panel with compatible primer properties contributed to maximize amplification performance in a single multiplex PCR by reducing bias between markers. The preferential use of tetranucleotide repeats allowed a reduction of the amount of PCR-induced stutter sequences in HTS outputs of amplified microsatellites, facilitating the extraction of allele reads. Pentanucleotide loci may have lower stutter proportions compared to tetranucleotides. This may affect allele detection with our pipeline because the categorization of a sequence as an allele is dependent upon the presence of the relative stutter sequence, possibly resulting in allele dropout if stutters are absent due to low DNA quantity or low sequencing coverage. Although this did not seem to be a problem for the pentanucleotide used in our study, a possible solution could be adjusting the bioinformatic pipeline to use marker specific thresholds or, alternatively, perform allele detection based on allele reads distribution (i.e. Suez *et al.* 2016; but see Zhan *et al.* 2016 for a comparison between this distribution based approach and a sequence string based approach). We found that identification of stutter sequences was useful to distinguish true alleles from artefact sequences and provided a conservative

validation of new and rare alleles especially in the absence of allele databases, as could be the case in wildlife genetics where new loci and alleles are often characterized during the course of studies. Read counts were lower in hair and scat samples compared to tissues, probably reflecting lower DNA quantity. However, sequencing coverage per se did not explain the ability to obtain accurate genotypes, and loci with lowest counts did not systematically correspond to those with the poorest genotyping success and error rates. Finally, the MetaFast PCR-free procedure employed for library preparation was a key technical adjustment for significantly decreasing the amount of chimeric sequences originating from tag jumping (Schnell *et al.* 2015) and improving sequencing quality.

One of the greatest advantages of HTS for STR genotyping is allowing direct access to microsatellite allele sequences. This provides greater accuracy of allele determination, because alleles are identified unambiguously by the nucleotide sequence of the repeat unit and flanking regions, instead than solely by length polymorphism as with CE. In this study, we revealed substantial allele sequence variation at some loci. Uncovering such variation also increases statistical power for individual identification, relatedness and population genetic analyses (i.e. see Darby *et al.* 2016 for inferences of genetic structure), as well as the ability to identify and resolve mixed samples, which can be encountered in forensic cases and in wildlife studies utilizing for example hair snaring methods and scat sampling. Characterization of sequence variants will also benefit evolutionary inferences through identification of size homoplasy at STR loci, improving understanding of microsatellite mutation models and estimate of mutation rates (Putman & Carbone 2014).

We validated the ability of the new markers to provide accurate genotyping on samples from two European brown bear populations. The diversity metrics we obtained are consistent with previous analyses of dinucleotide markers and with the known demographic histories of these populations. The observed deviations from equilibrium in both data sets and apparent evidence for null alleles in the Scandinavian samples when analysed as a whole are most likely due to the small size and re-introduction history of the Pyrenean population and to the dynamic of population expansion and structure of the Scandinavian population (Waits *et al.* 2000). In addition, all markers were mapped on different scaffolds of the reference polar bear genome, which excludes physical proximity.

In this study, sex determination was performed using a marker amplifying homologous short fragments of equal size on the X and Y chromosomes, but with a polymorphic site in the sequence that was visualized using HTS. This system permits to eliminate amplification bias encountered with CE-based sexing methods targeting regions of different size on the two chromosomes (i.e. Ennis & Gallagher 1994), but which may result in increased amplification failure or sex misidentification. In addition, a sex determination method based on the sequence has the great advantage of providing certainty that the sex of the target species is being determined through comparison of the amplified sequence to reference sequences; it also allows verifying sources of contamination by comparison against public sequence databases. This is particularly important for the analysis of poor-quantity/quality DNA samples, because amplification of DNA from contaminating sources (i.e. humans or other animals) or from prey remains in faecal samples cannot be ruled out with most existing markers.

Efficiency and automation of genotyping

Based on the experimental strategy adopted in this study, we processed 96 samples for 14 markers and eight PCR replicates (i.e. 8 96-well PCR plates representing 96 samples amplified with 14 markers replicated eight times) on the same Illumina Miseq run. Markers targeted short and similar fragment sizes and were multiplexed in a single PCR reaction using unique combinations of tagged primers in each PCR. We could have processed six times the number of samples on a single Illumina Hiseq 2500 rapid run (2×150 bp). This is possible due to unprecedented abilities for parallel sequencing offered by HTS that opens to wide prospects for efficient genotyping of large projects.

Several microsatellite ecological applications involve the analysis of many samples that usually are processed in bulk for minimizing genotyping effort. Compared to the limited capacity of a single CE run, the use of unique combinations of tagged primers in each amplification gives enormous flexibility in the number of PCR products that can be sequenced in the same HTS run and for which data can be independently retrieved. In addition, combining several markers on the same CE run is constrained by the use of fluorescently labelled primers, which has so far prevented the use of panels of short, and therefore overlapping, STRs, despite this being key for successful genotyping of degraded DNA. With HTS, amplicons are not separated by electrophoresis, rendering feasible concurrent sequencing of many markers of equal size. Combined with the current ease of microsatellite development (Gardner *et al.* 2011) and PCR multiplexing (i.e. Hill *et al.* 2009), this facilitates genotyping for applications demanding a larger number of STRs than for individual identification. Other marker types also may be combined in the same run, provided their length is compatible with the sequencing

technology employed. For example, autosomal and Y-chromosome markers (including SNPs), mtDNA markers and phenotypical markers could be added with the microsatellites in the same optimized multiplex PCR, or alternatively amplified separately, and genotyped in a single HTS run (Børsting & Morling 2015). This would increase the information content per experiment while saving time and costs for analyses requiring data from different marker types, as it might be desirable for some population genetic studies, and also when species ID, individual ID, sex ID, ancestry and phenotypic information may all be needed to support timing wildlife conservation and management actions or to solve forensic cases.

The number of samples and markers that can be analysed in the same HTS run depends on the sequencing depth necessary for ensuring reliable allele detection. According to the strategy adopted in this first study, we planned for 2000 reads/locus/PCR to detect alleles based on a conservative estimate from preliminary testing. The observed counts of hair and scat samples were on average lower indicating that alleles can be detected reliably with fewer reads and suggesting that it could be possible to further increase multiplexing capacities of samples and/or markers and therefore the cost-effectiveness of the analysis. The adequate sequencing depth for a given application should be evaluated experimentally, as it will also be contingent upon the sequencing technology employed and the type of samples. In addition, variation in sequencing performance between HTS runs could determine deviations from theoretical expectations in the number of reads and should be considered when planning an experiment.

A key aspect of the efficiency of HTS-based genotyping approaches relates to the fact that the genotyping process becomes amenable to full automation through bioinformatics, which allows treatment of the generated sequences based on their read counts. The bioinformatic pipeline developed for this study implements quantitative relative thresholds derived from the data to sort and filter sequences and to determine consensus genotypes from independent PCR replicates. Alternative approaches can also be used, for example Suez *et al.* (2016). Whatever the pipeline, appropriate loci selection is fundamental to favour complete bioinformatic automation; for example, loci producing high levels of artefactual sequences may require significant manual verifications and should be avoided. This is also the reason why we did not favour dinucleotides in this study, even though dinucleotides can be genotyped with HTS (Darby *et al.* 2016; Suez *et al.* 2016; Zhan *et al.* 2016). Using our current pipeline, processing data for 96 samples amplified with 14 markers replicated 8 times took about 12 h on a 8-core machine. The combination of genotyping efficiency and automation will be advantageous for noninvasive genetic studies usually entailing DNA profiling with high levels of PCR replication. For instance, for some projects, performing a full multitube approach (Taberlet *et al.* 1996) in a single HTS run might be more convenient than initially screening sample quality or selectively replicating samples/loci at different stages, as often is necessary with CE, to minimize costs of analysis. In our laboratory, application of HTS STR genotyping to noninvasive genetic projects provided a >40% cost (consumable and labour) reduction compared to the CE-based approach.

Standardization of the genotype data

One of the greatest criticisms of microsatellite genotyping based on CE is that genotype data are platform dependent. Such a limitation is avoided with a genotyping method that provides access to microsatellite sequences, as genotypes are constructed from discrete data that are not subject to variation in platform-specific conditions. This means that genotypes generated in different laboratories and by different platforms can be compared directly without the need of calibration, facilitating data exchange for transboundary genetic monitoring programs and the construction of common genetic databases at large geographic scales and across time. This is in fact critical for the success of conservation efforts relying on transnational coordination for population level management that requires merging data produced by different institutions. It would also benefit long-term studies, because genetic databases comprised of microsatellite sequences can be expanded unlimitedly as new data are collected from additional sampling. As HTS adds another dimension to the characterization of STR polymorphism by uncovering allele variants containing SNPs, indels and complex sequence structure, an important aspect of standardization regards adoption of appropriate allele annotation for data storage and nomenclature for describing STR sequence variation for reporting, searching and analysis purposes. It will be important to conform to a uniform and practical system based on universally accepted criteria that should be the result of a discussion within the scientific community (Parson *et al.* 2016).

Conclusions and perspectives

Many questions in ecology and conservation can be addressed with a limited number of polymorphic microsatellites (Guichoux *et al.* 2011). The constraints mostly of a technical nature and a technological revolution that in recent years has impacted other marker

types have brought to question the continued use of microsatellites for certain applications. HTS-based genotyping allows overcoming many of the limitations traditionally attributed to STRs and holds enormous potential for improving several aspects of the genotyping process. We anticipate that this approach will primarily impact ecological and conservation studies relying on DNA profiling of many samples/markers and low-quantity/quality DNA, such as typical of wildlife genetic monitoring programs, and noninvasive genetic and forensic studies.

Shifting to HTS STR genotyping will require laboratories to reorganize some components of the workflow and acquire new analytical expertise (Fig. 1). Continued technical and computational developments are expected to assist in this transition by improving and widening the applicability of the method. One of the main challenges at present relates to bioinformatic expertise that becomes indispensable for the analysis of massive sequence data. In future, bioinformatic pipelines could be implemented in user-friendly software validated for wider utilization (Fordyce *et al.* 2015). Following suggestions for human forensics, software should allow data evaluation and validation without visually accessing sequence data, should accommodate enough flexibility for defining locus specific detection limits and threshold values to account for marker specific behaviour, should handle STRs as well as other marker types (SNPs, mtDNA, etc.) and implement automatically generated allele databases and a standardized allele nomenclature based on sequence structure, length and variation (Børsting & Morling 2015; Parson *et al.* 2016). Efforts should also focus on improving automation of the workflow for sample preparation prior to sequencing. This is necessary because many primers with different tags are used in the amplification, which may complicate PCR set-up in large projects. For example, once a panel of loci has been selected, PCR set-up with tagged primers could be facilitated using commercially designed genotyping kits and pipetting robots for minimizing handling steps. In addition, strict compliance to good laboratory practices and quality control, as those required when working with degraded and low-quantity DNA sources (Waits & Paetkau 2005), must become the standard in the sample preparation phases, to avoid contamination risks with HTS. Laboratories not equipped with HTS technologies can outsource the sequencing to numerous commercial services; however, library preparation and sequencing parameters need to be carefully optimized for the analysis of STRs. HTS technologies are currently less efficient for analysing few or single samples (Darby *et al.* 2016) as may be necessary for solving wildlife forensic and management cases. Ongoing technological advances and a trend towards reduced costs of HTS will soon change this situation. In the meanwhile, costs also can be reduced by pooling samples from different projects or from multiple laboratories for efficient processing, or by separately running the few samples with CE. Existing genetic monitoring programs already disposing of collections of genotypes and transitioning to the HTS method would require some initial regenotyping investment. Although this could appear as an issue when large genotype data have already been produced and original samples no longer are available, a system based on allele sequences will ensure that the newly generated data will always be usable thereafter.

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C.M. and P.T. conceived the methods; M.D.B., S.L., C.M. and P.T. developed methods; S.L. performed bioinformatic analysis; P.Q.Y. and J.S. provided samples; C.M.

performed experiments; M.D.B., S.L. and C.M. analysed data; and M.D.B. and S.L. wrote the manuscript.

Data accessibility

Sequence data containing primer sequences deposited to NCBI SRA: SRP078422. R and python scripts of the bioinformatic pipeline and HTS unfiltered data: Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.18tg7.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Marker position mapped on the polar bear (*Ursus maritimus*) genome. Description of the bioinformatic pipeline used to generate genotypes

Table S2. Proportions of reads assigned to alleles, stutters, and other sequences

Table S3. Brown bear STR alleles.