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Approaches to genotyping individual miracidia of *Schistosoma japonicum*

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Abstract

Molecular genetic tools are needed to address questions as to the source and dynamics of transmission of the human blood fluke *Schistosoma japonicum* in regions where human infections have re-emerged, and to characterize infrapopulations in individual hosts. The life-stage that interests us as a target for collecting genotypic data is the miracidium, a very small larval stage that consequently yields very little DNA for analysis. Here, we report the successful development of a multiplex format permitting genotyping of 17 microsatellite loci in four sequential multiplex reactions using a single miracidium held on a Whatman Classic FTA indicating card. This approach was successful after short storage periods, but after long storage (>4 years) considerable difficulty was encountered in multiplex genotyping, necessitating the use of whole genome amplification (WGA) methods. WGA applied to cards stored for long periods of time resulted in sufficient DNA for accurate and repeatable genotyping. Trials and tests of these methods, as well as application to some field-collected samples, are reported, along with discussion of the potential insights to be gained from such techniques. These include recognition of sibships among miracidia

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Author Contributions

N. Xiao, J.V. Remais, R.Z. Li and D. Blair equally contributed to this work. Conceived and designed the experiments: N. Xiao, J.V. Remais, P.J. Brindley, D.C. Qiu, E.J. Carlton and D. Blair. Performed the experiments: N. Xiao, R.Z. Li, Y. Lei. Analyzed the data: D. Blair, N. Xiao, J.V. Remais and R.Z. Li. Contributed reagents/materials/analysis tools: N. Xiao, J.V. Remais, D. Blair. Wrote and edited the paper: D. Blair, N. Xiao, J.V. Remais and E.J. Carlton.

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from a single host, and inference of the minimum number of worm pairs that might be present in a host.

Keywords

China; miracidia; FTA; multiplexing; microsatellites; *Schistosoma japonicum*

Introduction

Our interest is in tracing the movement of pathogens through landscapes, identifying specific pathways of dispersal with particular emphasis on the blood fluke, *Schistosoma japonicum*, in China (Remais et al. 2010; Remais et al. 2011; Akullian et al. 2012). After nearly 60 years of control activities, Sichuan, a province in southwest China, had successfully controlled schistosomiasis transmission to a low level. However, re-emergence of the disease has been documented in the province, highlighting the challenge of achieving local elimination and raising questions as to the pathways by which the pathogen is returning to previously controlled areas (Liang et al. 2006; Liu et al. 2010; Carlton et al. 2011). Models suggest even modest parasite inputs may be sufficient to re-establish the parasite life cycle and cause new human schistosomiasis cases (Spear et al. 2011). What are the sources of these inputs – are pathogens transported across water ways, social networks, or by non-human definitive hosts such as bovines? Moreover, are the human infections found in post-control environments truly new infections, or residual infections that persisted due to lack of treatment or treatment failure? Global initiatives to control schistosomiasis have made these questions relevant not just to Sichuan, but to many areas where efforts are underway to greatly reduce parasite populations (WHO 2012). Our ability to describe the dynamics of schistosomiasis re-emergence can assist control and surveillance programs as progress is made towards elimination. We have pursued a population-genetic approach that exploits parasite microsatellites (Xiao et al. 2011) as markers as part of a multidisciplinary effort to characterize the dynamics of schistosomiasis re-emergence in Sichuan.

The ideal parasite stage to genotype is the adult, the stage causing disease in mammals. Adult schistosomes are large enough to yield sufficient DNA for many analyses. However, this stage occurs in the circulatory system of the human host and is therefore not available for analysis. Eggs passed from the human host, and especially the miracidial stage emerging from the egg, are the immediate products of adult worms, carrying schistosome genetic material into the environment. Miracidia are therefore suitable for landscape genetic studies (Steinauer et al. 2010), which we intend to use for understanding transmission dynamics. Genetic data from miracidia can also be used to explore sexual interactions between schistosomes within an infrapopulation (Beltran and Boissier 2009) and, by extension, can provide a means of estimating numbers and persistence through time of reproducing worm pairs in that infrapopulation.

Many efforts have been made to genotype miracidia. Given their tiny size (Eklu-Natey et al. 1985), some workers have opted to passage harvested miracidia through to adults in laboratory hosts in order to obtain enough DNA for genotyping (Stohler et al. 2004; Thiele et al. 2008; Stothard et al. 2009). This has ethical implications, is expensive, time consuming and subject to genetic bottlenecking (Stohler et al. 2005; Gower et al. 2007). An alternative is to genotype directly from miracidia themselves. Freshly hatched miracidia can be held briefly in water before being taken directly on to the genotyping stage if laboratory facilities are immediately available (Beltran et al. 2008; Steinauer et al. 2008; Valentim et al. 2009). However, some sort of storage method is generally required since most samples are field-collected. Whatman FTA indicating cards have been used by a number of groups. A

miracidium (or other larval stage) can be added directly to the card in a small amount of water or saline, and the card dried and used later as a source of DNA (Rudge et al. 2008; Standley et al. 2010; Gower et al. 2007, 2011). Alternatively, preservation in RNAlater appears to have been successful, but only one example of this approach has been published (Webster 2009). Similarly, preservation of miracidia in ethanol seems only to have been reported once (Van den Broek et al. 2011) and was said to be the most cost-effective method. This last study found relatively high levels of failure and genotyping errors when FTA Classic cards were used for storage of miracidia, a finding at odds with other studies.

Workers reporting genotyping of miracidia have always cited the small quantity of DNA available as a major consideration in their planning. Some have PCR-amplified only a single locus for each miracidium (Shrivastava et al. 2005), while others have multiplexed several loci within a single reaction per miracidium, and these studies have all used FTA cards (Rudge et al. 2008; Gower et al. 2007, 2011). A few studies, extracting DNA from freshly hatched miracidia in water or saline, have reported successful amplification of up to 21 loci in four reactions (Steinauer et al. 2008; Valentim et al. 2009).

Given the small amount of genetic material in a single miracidium, it is surprising that use of whole genome amplification (WGA) methods has not been more widespread. WGA provides a means of near-uniform amplification of any genome from very small starting quantities (Dean et al. 2002). Low uptake of this approach for schistosomes has likely been due to concerns about bias that might be introduced during the WGA process, and also cost factors. Valentim et al. (2009) appears to be the only study to have used WGA to generate sufficient schistosome DNA for genotyping (Valentim et al. 2009). They genotyped 28 F1 miracidia from a known parental cross at 56 loci, apparently locus-by-locus, and found an error rate of only 0.45%.

After reviewing published options, we decided to use the Whatman FTA Classic indicating cards to immobilise and preserve miracidia obtained from human and animal feces in the field. Initially preferring not to use WGA methods because of concerns about uniformity of amplification across the genome and cost, we tested the possibility of using a single FTA disk for several sequential PCRs. We were able to do this successfully with miracidia, which had been held on FTA cards for 11 months (see below). However, we later encountered difficulties when using miracidial DNA on disks as template for multiplex genotyping reactions, especially after long storage (>4 years). This was assumed to be due to the small amount of DNA available in a single miracidium and the effect that even slight degradation over time might have on success of amplification. Consequently, we moved to the use of whole-genome amplification (WGA) methods to increase the yield of DNA. Here, we describe the materials and methods used to successfully genotype *Schistosoma japonicum* miracidia directly sampled from human and animal stool using WGA and a multiplexing procedure.

Methods

Field collection and preservation of miracidia

A field survey of human schistosomiasis was initiated in 2007 in 53 villages in three counties where schistosomiasis had re-emerged following reduction of human *S. japonicum* infection prevalence below 1% (Liu et al. 2010; Carlton et al. 2011). Residents were asked to submit fecal samples on three consecutive days which were examined using the Kato-Katz method and the miracidia hatching tests. Additionally stool samples were collected from bovines in study villages over three consecutive days and examined using the miracidia hatching tests. Each hatching test was conducted according to national standards described in detail elsewhere (Chinese MoH 2001). Due to the May 2008 magnitude 8.0 earthquake,

which severely impacted one of the three counties surveyed, follow-up infection surveys were conducted in 2008 and 2010 in the 36 study villages in the two other counties (Carlton et al. 2013).

During each survey, miracidia were harvested from positive hatching test flasks for use in experiments to optimize preservation and DNA extraction techniques. Miracidia observed on the water surface of the incubation flask during the hatch test were isolated individually using a hematocrit tube or a Pasteur pipette drawn to a narrow bore in a flame. After three washes with autoclaved deionized water, one miracidium together with 2-5μl water were loaded onto the FTA indicating card, producing a 2-3 mm diameter white circle contrasting with the FTA card background color. After being dried at room temperature for at least 1 hour, the cards were stored in a desiccator in a cool and dry environment for up to 5 years.

Isolation of Genomic DNA from adult worms

The Sichuan Center for Disease Control and Prevention (SCDC) maintains *S. japonicum* in the laboratory using cercariae from infected snails sourced from Hubei and Anhui Provinces and passed through rabbit hosts. For this study, existing samples were obtained from SCDC of adult schistosomes that were derived from a single passage of cercariae through a definitive host as described elsewhere (Xiao et al. 2011). DNA from these was used for development of multiplex genotyping reactions, as controls in various experiments and for testing repeatability of WGA methods.

Genomic DNA was extracted from individual male or female worms by incubation in hot sodium hydroxide (20μl) with pH adjustment using a Tris solution (20μl) (HotSHOT) (Truett et al. 2000). The lysates were diluted in 1:8 and 1μl of each used as templates for PCR directly.

Use of DNA from miracidia on disks, and repetitive use of disks

When needed, the white disk indicating the location of a miracidium was excised from the Whatman card using a 2 mm Harris Uni-Core punch (available from Whatman) and washed following the recommended protocol (Whatman Inc. NJ, USA). As DNA remains bound to the disk, the disk itself was used as a template for either direct PCR or whole genome amplification.

Statements in the literature suggest that each disk can only be used in a single reaction (Gower et al. 2007). We tested this assertion using miracidia held on the FTA cards for periods of up to 11 months. Each disk was first used in a PCR with a pair of primers, forward 5'-GGTACCGGTGGATCACTCGGCTCGTG-3', reverse 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (3S/A28) (Bowles et al. 1993; Blair et al. 1997) designed to amplify about 500 bp of a repetitive region in the schistosome genome, specifically a portion of the 5.8S ribosomal RNA gene, the complete internal transcribed spacer 2 (ITS2) and the 5' end of the 28S ribosomal RNA gene. The purpose of this was to confirm that miracidial DNA was present before using disks in subsequent reactions. PCR conditions were as follows: denaturation for 5 min at 94 C followed by 25 cycles of 30s at 94 C, 30s at 55 C and 30s at 72 C, with a final extension at 72 C for 5min. Subsequently, the disk was used as substrate in a series of PCRs, each using primers designed to amplify a different microsatellite locus (Xiao et al. 2011). After each PCR, the disk was washed following the Whatman protocol mentioned above. Thermal cycling was performed under the following conditions: 10min at 94 C; 30 cycles of 30s at 94 C, 30s at 55 C and 60s at 72 C, with a final 10 min extension at 72 C. Five μl of PCR product were run on a 1.5% agarose gel. TE buffer was used as "template" in negative controls. A 100 bp DNA ladder (GENEray™ Biotechnology, Shanghai, China) was used to provide size markers.

Final multiplex format used and PCR conditions

Multiplex Manager (http://www.multiplexmanager.com/) (Holleley and Geerts 2009) was used to design a multiplex format for genotyping of 17 loci. The final design contained four groups (Table 1). We found it necessary to modify the grouping of loci several times to arrive empirically at a set that worked most reliably. For some loci, the primers from Xiao et al (2011) (Xiao et al. 2011) were not used and new primers were designed, usually to generate shorter amplicons for more efficient multiplexing (Table 1). To save costs of dye-labelling each primer pair separately, labelled tails were used (Schuelke 2000). In this approach, each forward primer is present in limited amounts and has a 20bp "tail" at its 5' end corresponding to a 20bp labelled oligonucleotide which then "takes over" as the forward primer, incorporating the label into the PCR products. To permit multiplexing, we used three different tails, each with a separate dye label (Table 2) (Real et al. 2009). A fourth dye, NED, gave poor results, as not only did it display low signal of its incorporated PCR products but it also interfered with signal from other dyes, so we did not use it. Within a single multiplex, loci could be distinguished by a combination of "tail" used and expected amplicon sizes.

The multiplex PCR thermocycler conditions are as follows: 94 C for 5 min, then 35 cycles at 94 C (30s), 57 C (90s), 72 C (60s) and a final extension at60 C for 30 min.

All data were checked by eye. All loci have trimer repeats, making recognition of out-of-phase alleles relatively easy. Individuals exhibiting any such alleles were re-genotyped at the offending loci as often as necessary to resolve the point (a very few apparently real phase shifts were found).

Whole-genome amplification

Whole-genome amplification (WGA) was done using the GenomiPhi V2 amplification kit (GE Healthcare Bio-Sciences, USA) (Kumar et al. 2008). Individual disks punched from a Whatman card and processed as above were placed singly in tubes along with the reagents for WGA. The protocol recommended by the manufacturer was followed with slight modification. A disk was rinsed five times (5 min each time). The first three washes used 200μl of the FTA Purification Reagent and the last two used TE buffer. The disk was then dried for one hour at room temperature. Nine μl of the GenomiPhi Sample Buffer were subsequently added to each tube for incubation at 95 C for 3 min. Then the tube was cooled to 4 C on ice and centrifuged briefly. A prepared mixture of 9 μl of the GenomiPhi Reaction Buffer and 1 μl of the Enzyme Mix was added into the tube for incubation at 30 C for 90 min. Finally, the tube was held at 65 C for 10 min to inactivate the enzyme. Subsequently, aliquots of 0.5 μl of reaction product were used in each of four multiplex PCR reactions as outlined in the previous section.

In some cases, the white circle on the card, within which a miracidium was located, was too large to be completely excised with the 2 mm punch. Instead, as many punches as required to excise the entire circle were taken and combined in a tube for the WGA reaction. As many as 6 disks were added to a reaction with generally good results (data not shown).

We evaluated the effects of multiple displacement amplification on repeatability of subsequent genotyping. To do this, we first multiplex-genotyped DNAs (1:8 diluted) extracted from two adult worms raised in the laboratory. Then we used a small aliquot (one μl of a 1:1000 dilution) of DNA from each adult worm, added to an FTA card, as the substrate for genotyping directly (without WGA). Additionally, DNA, diluted as above, from each adult worm on an FTA card was used in a WGA reaction and the resulting products multiplex-genotyped.

To further understand the potential error after WGA processing, additional tests were carried out using five randomly selected loci. Each of three miracidial DNAs on FTA cards (and after WGA) was used as a template at least twice for PCR and genotyping of each locus using tailed primers. Further, the forward primer for each of the five loci was labeled with dye directly and genotyping results compared with those obtained using the multiple tailing method. Finally, representative amplicons were sequenced to check for sequence fidelity relative to the original genomic sequence.

Application to field-collected material

Genotyping of miracidia collected is ongoing and full results will be published elsewhere in due course. Data from two human cases are presented here as examples. We need to stress that the statistical analytical issues surrounding genotypic data from miracidia are not under discussion here. These issues have been reviewed elsewhere (Steinauer et al. 2010). We wish only to present some data to demonstrate the utility of the method. The two cases, both farmers aged 50-60 years, were found to be infected during each of the three infection surveys. From the first individual ("A"), 15 miracidia collected in 2007, 15 in 2008 and 8 in 2010 (total 38) were genotyped following WGA of miracidia on the FTA disks. From the second individual ("B"), the corresponding numbers genotyped are 15, 10 and 4 from the 2007, 2008 and 2010 infection surveys, respectively. Data were analysed using GenAlEx v6.x (Peakall and Smouse 2006) and Colony v2.0 (Jones and Wang 2010).

Ethics statement

The research was approved by the Sichuan Institutional Review Board and the University of California, Berkeley, Committee for the Protection of Human Subjects. Participants provided written, informed consent before participating in this study. All children provided assent and their parents or guardians provided written, informed permission for them to participate in this study. After each infection survey, infected individuals were notified and provided praziquantel tablets (40mg per kg body weight) by the county Anti-Schistosomiasis Control Station with instructions for taking these as a single dose to clear the infection. Because bovine stool samples were collected after they were excreted, the Animal Care and Use Committee at the University of California, Berkeley determined the protocol was exempt from review. All bovines testing positive for schistosomiasis were provided treatment with praziquantel by the county veterinary station.

Results and Discussion

Collection and preservation of miracidia

Miracidia (a total of 4199) were obtained from 365 human and 38 bovine schistosomiasis cases during the surveys in 2007, 2008 and 2010 and stored on the Whatman Classic FTA indicating cards. The number of miracidia collected per case ranged from 1 to 72 (median: 7).

Repetitive use of disks

Amplification of the ITS2 and portions of the flanking nuclear 5.8S and 28S rRNA was routinely done prior to WGA. Presence of a clear band of the expected length of around 500 bp was proof that the disk punch held DNA from a miracidium (Fig 1A).

Having demonstrated that a miracidium was present on a disk, one preliminary experiment (two replicates) was to use the same disk in a series of single-plex PCRs, using a different primer pair each time. Bands of the expected sizes were evidence of successful amplification. Results of this for one replicate are in Fig. 1B-F. This single disk was re-used

11 times, yielding a band in each case, although the last two loci amplified were visible only as very faint bands.

Whole-genome amplification

Although multiplexing worked well with DNA from adult worms and using miracidia recently preserved on FTA cards, poor results (failure to amplify loci and appearance of spurious bands) after long storage (>4 years) of miracidia on the FTA cards led us to use WGA methods to increase the amount of template. The results indicated that WGA-amplification of genomic DNA from a miracidium on a disk could yield as much as 1 mg/μl of DNA for subsequent work.

Repeated testing and quality-control steps in WGA convinced us that error rates are low. We used two approaches to satisfy ourselves of this. One was to test the repeatability of multiplex genotyping (17 loci) from the lysates of two different adult worms, each treated in three different ways prior to genotyping: a) direct use of lysate for genotyping; b) use of lysate added to FTA disks without WGA prior to genotyping; and c) use of lysate added to FTA disks followed by WGA and genotyping. With a single exception, allele sizes were identical across the three treatments. The exception was a single base-pair insertion in the region flanking the microsatellite repeats at only one locus after WGA, a result confirmed by sequencing.

The second approach was to test stability and repeatability on PCR and genotyping of five randomly selected loci. The results were completely consistent across methods, providing evidence to confirm the stability and repeatability of the loci used for population genetic analysis.

Application to field-collected material

The method was applied to field-collected samples, and after repeating any PCRs where the results were questionable, only five loci (out of a total of 646) could not be scored for the 38 miracidia from subject A. This equates to a failure rate of 0.8%. The failure rate for individual B was 3.4%.

Assignment tests in GenAlEx assigned all miracidia from A to that host. One miracidium from B was assigned to A. Between populations of free-living animals, such a result might imply absence of gene flow. However, between schistosome infrapopulations, this result might be due to the presence of sibling miracidia within each host, or inbreeding within each host (Steinauer et al. 2010). Full exploration of these possibilities is beyond the scope of this paper. However, we did examine the possibility of siblings being present within either host, using the program Colony v2.0. There was support, with probabilities >0.9 in most cases, for siblings among miracidia collected in the same year. For individual A, 2 pairs of miracidia collected in 2007 were likely full sibs (i.e. 2 sibships, each with 2 members). For 2008, there were 3 likely pairs of siblings. In 2010, one sibship with 3 members and one with 2 were likely present. Host B, for whom fewer miracidia were genotyped, had two pairs of sibs in 2007 and one in 2008.

This suggests that individual A was infected with at least 13 reproducing adult worm pairs in 2007 and 12 in 2008 and, more generally, demonstrates the use of multilocus genotyping to infer at least the minimum worm burden. The number of reproducing adult worm pairs has important implications for morbidity, as pathology is generally proportional to egg exposure. Furthermore, worm burden is a key variable of interest when studying transmission dynamics, and is particularly essential when characterizing dynamics at low transmission levels. To date, inference about the number of reproducing adult worm pairs has largely been based on repeated, quantitative egg counts from the Kato-Katz thick smear method

(Hubbard et al. 2002). However, the Kato-Katz method performs poorly at low infection intensities. In our study region, where infection intensities are low, we found that many infected individuals tested positive for *S. japonicum* using the miracidia hatching test, but negative using the Kato-Katz method, likely due to the very different amounts of stool used in the two tests (41.7 mg per slide for the Kato-Katz test vs. 30 g used for the hatching test) (Carlton et al. 2013). In fact, individual A had no detectable *S. japonicum* eggs upon Kato-Katz examination in 2007 and individual B had no detectable *S. japonicum* eggs in 2010. Identifying parasite siblings within a host using multilocus genotypes offers a new technique for estimating worm burdens, one that may be particularly useful in low transmission environments.

Interestingly, strong support was given for several pairs of miracidia being siblings despite being collected in different years. There were 2 such pairs in A and 5 in B. Such a result might indicate that the parental worm pairs had persisted between years. We have previously found that some individuals are repeatedly infected with *S. japonicum*, a phenomenon not fully explained by host exposure (Carlton et al. 2013). However, to date we have been unable to determine whether infections detected in the same host over time represent new infections, suggesting differential host susceptibility, or whether such infections are, in fact residual infections, as adult worms are known to be able to survive for 4.5 year (Li and Guan 2010). This longitudinal sibship analysis suggests some adult worm pairs may have persisted in the two case individuals over time, despite provision of treatment. While it is unclear whether the adult worms survived due to non-compliance with treatment regimens or the occasional failure of praziquantel to kill adult worms (Seto et al. 2011), the finding demonstrates that *S. japonicum* infections may persist in some individuals despite targeted treatment campaigns. Our multilocus genotypic method therefore provides a much-needed means of distinguishing new from residual infections, a phenomenon that we will investigate further with a larger sample size.

We have demonstrated that it is possible to re-use FTA disks carrying *Schistosoma japonicum* miracidia as template in several sequential PCRs. However, genotyping is easier using a multiplex PCR format following WGA of miracidial DNA. These methods provide an array of potential applications – from analysing parasite dispersal and connectivity over landscapes to characterizing within-host parasite populations over time (Seto et al. 2011). Our future work will utilize these methods with the long-term goal of generating knowledge to halt schistosomiasis transmission in re-emerging endemic foci in Sichuan Province.

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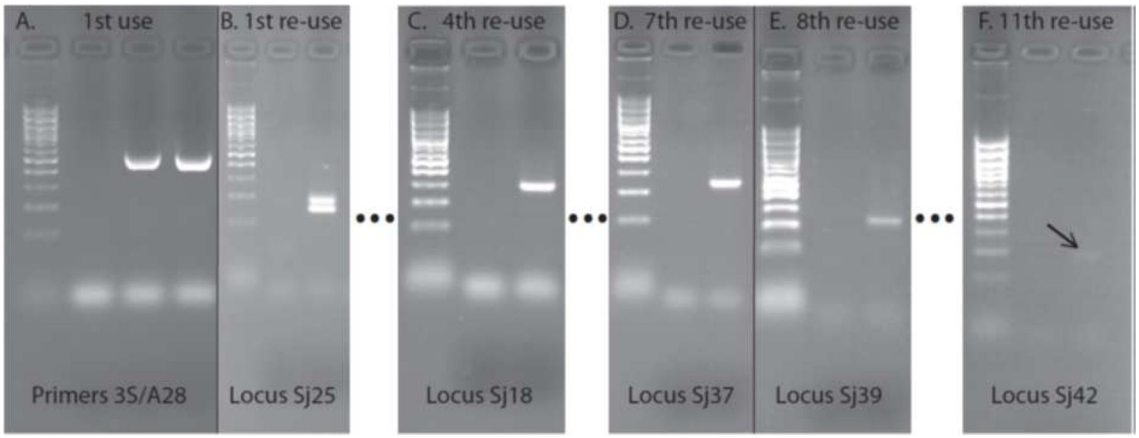

Figure 1.

Demonstration of repetitive use for PCR of punched the Whatman FTA card disks holding a single miracidium stored for 11 months. A-F: Images of 1.5% agarose gels on which 5 ml PCR product was run after each successive PCR using a different primer pair. An arrow shows the location of the faint band in F. Image A shows the results (for two disks) amplified using primers 3S/A28 that span the nuclear ribosomal ITS2 region. Remaining images (B-F) show results of re-use of the disk represented in the right-hand lane of A up to 11 times, each time using primers for a different locus. Note that one primer pair (for locus Sj25) used in this test was not used in the final multiplex format, so is not listed in Table 1. 100bp DNA ladder was used as molecular size marker. The blank lane to the right of the molecular size marker is the negative control in each case.

Table 1

Primers used for genotyping at 17 microsatellite loci. Their distribution into four multiplex groups is indicated, along with the labelled tail (Real et al. 2009) used. The 5' tail sequence for each forward primer is not shown (see Table 2). Where primer sequences have been altered from those reported in (Xiao et al. 2011), this is indicated by the word "redesigned".

Locus	Primers (without tails)	Multiplex Group	Length Range*	Tail used	Notes
Sj4	F: ACAAGCTCCAATCGTCTCTGA R: GAATACTGCCGCCCTTGTAA	G2	193-258	T1	
Sj8	F: ATGCACGTAAAGAAAAGGGTAAA R: TGATCTCCTACTGCGTTTCTGA	G1	194-287	T4	
Sj18	F: TCCTTTATCTGGGCTGTGGA R:TTTCAGCAGGATAACATGACG	G3	261-298	T2	
Sj22	F: CAAAGCCTAAACGTCATAGACAG R:CAACCACCGATAAGTAGAGTGGA	G3	105-167	T2	
Sj23	F: GTACGATATGAGGGAAAGTTCA R:CTCTCCTTCAGACGAATTGAG	G4	192-253	T2	
Sj28	F: TAACGCCTTTTCCCACATTC R:ATAACCACGATGGGAACCAA	G3	232-269	T1	
Sj32	F: TGTCACCGAGTCTTCATTAGC R:ACAGTCAGTAGACCTGGATAAAC	G3	142-193	T4	
Sj34	F: GGCGACCATACATAAGGAGAAT R:GCAAAGGCGTTACCTTCGTCT	G1	265-314	T2	Redesigned Redesigned
Sj37	F: TCCTTGACACGAGGTACATGT R:ATTACGTAACAGAAGGCTGGA	G4	149-223	T4	
Sj39	F: GACGACTGTTAAGTCCATCTGA R:ATAACCAATCTCCACGAAAGC	G3	316-344	T4	
Sj41	F: ACTGTACCATTGACACCTTGA R:GGACAACCATCAAAATCAAC	G4	281-310	T2	
Sj42	F: GCTGCAGCTTCTGTGTAGTAA R:GTCTTGCTCAGATCAGTTCGT	G2	199-234	T4	
Sj45	F: ATAACACCGAATCTGTTCAGC R:TAATCCGGTCAGGATGTATGT	G2	150-244	T2	
Sj48	F: TTGTTGGGTAGTGATGGTAGG R:TAGTTCATTCCACCTCTTGGA	G1	246-273	T1	
Sj58	F: CCTCTCAACGTATCAAACTTC R:CTAATAAAGTCGTCAAGGAGCA	G1	138-198	T2	Redesigned
Sj60	F: CGATTCATTCATAGCCTGACT R:GAATCCCATCACAGATTAACG	G2	134-165	T1	
Sj63a	F: CCGCCACTACCACTAACCTC R: GGAACCAAATGACGTATCTAAAGC	G4	100-156	T1	Redesigned Redesigned

*Length range, excluding tails, from[Xiao et al. 2011], and as modified subsequently.

Table 2

Sequences of the three tailing oligonucleotides used (Real et al. 2009), and the dye with which each was labelled.

Name	Sequence 5’ – 3’	Label
T1	CTCTTCGCTATTACGCCAGC	FAM
T2	GCTGCAAGGCGATTAAGTTG	VIC
T4	TAGAGTCGACCTGCAGGCAT	PET