At the APMEN Vivax working group’s (VxWG) inaugural meeting in February 2011, Sri Lanka, the development of *P. vivax* genotyping methods supporting malaria elimination efforts in the Asia-Pacific region was identified as a major research priority. The genetic diversity in *P. vivax* populations is a major obstacle to the control and elimination of the parasite. This diversity influences the stability of transmission, the development of immunity and has consequences for the emergence and spread of virulent or drug-resistant mutations. Comparative studies across populations with differing epidemiologies enable a strong framework to elucidate the key factors which govern parasite diversity and structure.

The Vivax genotyping workshop in Kota Kinabalu provided an important opportunity to address standardisation of genotyping approaches to facilitate multicenter *P. vivax* genetic studies across the Asia-Pacific region. As well as standardisation of existing genotyping methodologies, new genotyping approaches to facilitate key gaps in our knowledge of the basic biology of *P. vivax* such as the interpretation of clinical trials, drug resistance mechanisms and molecular markers, were major foci of the workshop.
1) Welcome and Introductions

Dr Lorenz von Seidlein welcomed the group and reviewed the rationale for the workshop. A more comprehensive understanding of the genetic diversity in *P. vivax* parasites is a major research priority of the Vivax working group. 6 of the 12 proposals received by the Vivax working group small grants scheme included a component on *P. vivax* genotyping, largely addressing parasite diversity and gene flow. The coordinating team of the VxWG group acknowledged that a multicentre approach to address the genetic diversity of *P. vivax* parasites in the Asia-Pacific region would add substantial value to the individual studies. Comparative studies across populations provide a critical opportunity to inform on the impact of different control and intervention strategies as well as epidemiological factors such as transmission intensity and relapse rate on parasite diversity and structure. However, standardised methodologies across sites are essential for effective comparisons.

The main objectives of the workshop were:

1) identify the genotyping applications of greatest importance to the APMEN country members

2) establish consensus genotyping methodologies for these applications.

2) Genotyping Applications

Prof Georges Snounou, Dr Qin Cheng and Prof John Reeder discussed applications of *P. vivax* genotyping studies.

Georges Snounou reviewed the informative value of genetic studies to our understanding of population genetic dynamics, vaccine and drug development, and intervention and drug resistance monitoring. Genotyping to “finger-print” *Plasmodium* isolates in clinical drug trials was the main focus of the talk. The utility of this application was highlighted with reference to the standardised genotyping approach for distinction of recrudescence from re-infection events in *P. falciparum*. The complexity in *P. vivax*, where interpretation is challenged by relapse events, was discussed. Some effective drug treatments may be misclassified as recrudescence events if the genotype of the relapse is identical to the primary/incident infection. However, the presence of heterologous genotypes between the primary and recurrent infection can confidently be classified as an effective drug treatment (relapse or re-infection).

Qin Cheng summarised the feature and use of two different types of genotyping markers: polymorphic antigen gene markers and microsatellite markers. She illustrated genotyping using polymorphic antigen gene markers to distinguish different types of relapsing *P. vivax* infections in returning soldiers. Three highly diverse antigen markers (pvmsp1, pvama1 and pvcsp) enabled confident resolution of heterologous and homologous infections. A high rate of heterologous relapse events (~75%), and a predominance of single clones (99%) were observed supporting the role of a parasite-determined reactivation signal. This insight into the dynamics of relapsing infections highlighted the utility of genotyping studies to inform on the basic biology of *P. vivax* parasites as well as facilitating clinical research. Cheng also demonstrated the use of microsatellite markers MS16, Pv3.27, msp1F3 to study *P. vivax* allele frequency and distributions at the baseline survey in Tanna Island, Vanuatu and Temotu province, Solomon islands. The baseline spatial distribution and parasite population structure may be important to interpreting the impact of interventions.
John Reeder focused on the applications of population genetic studies, describing the dynamics of parasite gene flow and its implications for the reintroduction of parasites into a population, and the emergence and spread of drug resistance. The baseline spatial and temporal patterns of genotypic diversity in a population are important to interpret the impact of interventions. The utility of candidate genes was addressed, with reference to the identification of surface antigens under selective pressure and prediction of their efficacy as vaccine targets in different populations.

In summary, the session highlighted a broad range of applications for *P. vivax* genotyping with regard to malaria control and elimination.

3) Proposed Genotyping Studies of the Country Partners

Country partners who had received APMEN grants for *P. vivax* genotyping studies were invited to present their proposal for group discussion and feedback from the expert panel. The projects presented included those led by Dr Noor Rain Abdullah (Malaysia), Dr Gawrie Gallapaththty (Sri Lanka), Dr Rintis Noviyanti (Indonesia), Prof Gao Qi (China) and Prof Preethi Udagama-Randeniya (Sri Lanka). Dr Jung Yeon Kim (Republic of Korea), was invited but unable to attend. Each investigator presented the *P. vivax* elimination challenges in their country and the objectives of their proposals to address these challenges. Study proposals included genotyping to describe *P. vivax* population genetic structure, parasite finger-printing, and elucidation of the geographic origin of an infection (local transmission or imported). Population genetics was a recurring theme. There was also interest in molecular monitoring of *P. vivax* drug resistance but the lack of effective candidate genes presented a major hurdle to these studies. The opportunity for cross-population comparative studies on recurring themes was acknowledged and it was agreed that a standardised genotyping approaches was essential.

4) Practical Aspects of Genotyping

Dr Sarah Auburn addressed the utility of different genotyping approaches to the research questions raised in the country member’s proposals. The practical, analytical and cost-based pros and cons of several commonly used genotyping methods were discussed. The group agreed that markers in candidate genes under selective pressure are not suitable for addressing the objectives of the country member’s studies. It was agreed that neutral microsatellites would be the most effective markers for population genetics. Six to nine markers are recommended for this purpose. A consensus set of six markers were identified as the **minimal required markers** for population genetic studies, and an additional 3 markers were added as “highly recommended” (see Appendix 1). The consensus set of six markers included 3 markers (MS16, Pv3.27, msp1F3) which have been observed to have the highest diversity in Papua New Guinea (Koepfli et al. JID, 2009 ) and have been validated in Vanuatu and the Solomon Islands by Cheng’s group, and by several other groups. These markers alone should be sufficient for finger-printing. For effective characterisation of population structure, the additional 3 consensus markers (MS1, MS8, MS10), are required (a total of 6 markers). For groups who wish to type 9 markers, an additional 3 markers (MS5, MS12 and MS20) are highly recommended. These additional markers are highly diverse, neutral microsatellite markers which have been described and validated in several *P. vivax* populations (see Appendix 1). Capillary
electrophoresis was agreed upon as the consensus genotyping platform owing to its high allele resolution. It was acknowledged that this platform is generally not available in-house. The country members expressed approval to outsource the capillary electrophoresis component of their study, preferring to focus capacity building on the data analysis. Regarding sample quality, consensus was reached to avoid filter spot sampling where possible due to its low sensitivity; venous blood or capillary blood in EDTA-containing tubes were considered preferable. The consensus genotyping methods are presented in Appendix 1.

Emerging technologies which are increasingly employed in *P. falciparum* studies, such as high-throughput SNP genotyping and next-generation sequencing were discussed. The former offers a cost effective approach to derive a large amount of data from a small quantity of DNA, but is currently limited by the sparse *P. vivax* SNP catalogue. Genome sequencing provides the most comprehensive assessment of genetic diversity and a valuable tool to identity novel variants. However, low parasitaemias and abundant human DNA challenge *P. vivax* genome sequencing. Prof Ric Price’s group at the Menzies School of Health Research are developing simple and effective methods to prepare high quantity and quality *P. vivax* samples for genome sequencing with promising results. Menzies offered support to interested groups.

5) Whole Genome Sequencing

Dr Olivo Miotto described the potential of genome sequencing for high resolution characterisation of the diversity in the *P. vivax* genome, and to elucidate the parasite genetic basis of critical disease-causing, immune evasion and drug resistance mechanisms. The utility of genome sequencing to identify drug resistance candidates using the phenotype-free detection of “signatures of selection” was the highlight of the talk. The power of this approach was demonstrated with well-described and validated *P. falciparum* drug resistance loci (*pfcrt, pfmdr1, pfddhfr and pfddhps*) in a large, multi-centre dataset. Exciting new candidates exhibiting signatures of selection of similar strength to the known candidates were also identified.

Preliminary sequence quality data was presented on twelve *P. vivax* samples. Despite moderate levels of human DNA contamination (up to 40% of the total DNA), the samples exhibited high sequence depth (many sequence reads covering a given position) and breadth (most of the genome was covered by sequence reads). Indeed, in general, a greater breadth of the genome is covered in *P. vivax* than in *P. falciparum*. These results are highly promising for *P. vivax* genome studies. Particularly given the current lack of effective drug-resistance candidates, the potential for a multicentre genome sequencing study on *P. vivax* was of considerable interest. The participation of partner countries in whole genome sequencing was encouraged.

6) Genotyping Summary

Prof Ric Price closed the day’s session with a summary of the proceedings. Several key outputs were achieved.

**Objective 1.** Parasite finger-printing for clinical studies, population genetic characterisation, and elucidation of parasite geographic origin were identified as immediate *P. vivax* genotyping priorities
for the APMEN countries. Molecular monitoring of *P. vivax* drug resistance was also a major interest but not included in current proposals owing to the lack of a reliable marker.

**Objective 2.** A consensus genotyping approach was established to provide a strong framework for collaborative, multicentre studies across the Asia-Pacific region. Each country was invited to participate in this collaboration. Ideally a minimum of 30 *P. vivax* isolates from each site will be genotyped to achieve a better understanding of *P. vivax* genetic diversity and structure in the Asia-Pacific region.

The planned multicentre study may be an appropriate framework to organise *P. vivax* whole genome sequencing across the APMEN countries. Rising multidrug resistance in *P. vivax* poses a major threat to elimination. A multicentre genome sequencing study comparable to that undertaken in *P. falciparum* should prove invaluable for the identification of effective drug resistance markers.
Appendix 1: Consensus Genotyping Methods

Sample details

Consensus: Essential sample details to record for technical and analytical purposes:

- Patient name and study ID
- Site of collection
- Date of collection
- Volume of whole blood
- Blood withdrawal method (venous/capillary)
- Parasite density (parasites/ul blood)
- Species present (PV pure or PV mix)
- Species determination method (e.g. microscopy, PCR details)
- Patient clinical status (asymptomatic, uncomplicated, severe)
- Patient age
- Patient ethnicity

Sample preparation

Consensus: Preference not to use filter spot samples as this may reduce the sensitivity of detection. Where possible, either venous blood or capillary blood will be drawn into an EDTA-coated container.

Venous blood samples

- EDTA-coated tubes
- Storage ≤4°C as soon as possible (avoid frequent freeze-thaw)
- DNA extraction on 200ul whole blood
- DNA extraction using Qiagen QIAamp blood mini DNA kits as per the manufacturer’s protocol for high DNA concentration eluting in 200ul AE

OR

Capillary blood sampling

Sampling method a or b:
- a) Capillary blood drawn into EDTA microtainer
- b) Or capillary blood drawn into EDTA-containing Eppendorf tubes prepared in-house (SOP-1)

Sample storage/preparation:
- Storage ≤4°C as soon as possible (avoid frequent freeze-thaw)
- DNA extraction on 200ul whole blood

---

1 Where collaborators can spare 3-5mls (or more) venous blood, they may wish to undertake CF11 filtration to deplete the human white blood cells for whole genome sequencing of *P. vivax*. If you are interested in participating in a collaborative, multi-centre *P. vivax* genome sequencing study, please contact Ric Price (ric.price@menzies.edu.au) or Sarah Auburn (sarah.auburn@menzies.edu.au) for further information on SOPs.
DNA extraction using Qiagen QIAamp blood mini DNA kits as per the manufacturer’s protocol for high DNA concentration eluting in 200ul AE

Markers

Consensus: APMEN country members expressed interests in genotyping to describe *P. vivax* population genetic diversity and structure (spatially and temporally), to characterise intra-host diversity (multiplicity of infection), to distinguish different isolates within a population (finger-printing), and to determine the geographic origin of an infection (local transmission or imported). The latter application remains a challenge owing to the current lack of effective markers. The other applications may be addressed by genotyping the consensus markers defined below.

The six **minimum required markers** are defined below. The first three markers (MS16, Pv3.27, msp1F3) are particularly effective for finger-printing (e.g. for clinical drug trials) and assessment of multiplicity of infection owing to their high sensitivity of detection (nested PCR assays). For effective characterisation of population genetic diversity and structure, 6-9 neutral (non-surface antigen) highly diverse markers are required. Therefore, a further 3 highly diverse, neutral microsatellite markers (MS1, MS8, MS10) are required. See Table 1 for further details.

- MS16
- Pv3.27
- msp1F3
- MS1
- MS8
- MS10

Please note: the above 6 markers are the minimum required markers - additional markers of the researcher’s choice may be genotyped.

**Additional highly recommended markers (for typing 9 markers)** for analysis of population structure are the 3 highly diverse, neutral microsatellite markers defined below (see Table 1 for further details).

- MS5
- MS12
- MS20

Genotyping Method

Consensus: Capillary electrophoresis was agreed upon as the genotyping approach offering the highest allele resolution.

- **Koepfli markers**: nested PCR with size separation by capillary electrophoresis (Details of PCR programs in SOP-2).
- **Karunaweera markers**: single round of PCR with size separation by capillary electrophoresis (Details of PCR programs in SOP-3).
**Allele Calling**

- Threshold minimum allele intensity of 100 Fluorescence Units (FU)
- Check for “bleed” (also known as “pull up” peaks) from other high intensity markers in the multiplex
- Check for stutter peaks (examples in Figures 1-3: courtesy of Dr Jenny Kozlovski)
- For finger-printing, sensitivity of detection is critical, and no major/minor allele intensity ratio threshold is set. Using the nested PCR Koepflı assays, experiments undertaken at the Menzies School of Health Research demonstrated consistent detection of single clone infections between 5 and 50 parasites/ul. In mixed clone infections, the minor allele was consistently detected between 50 and 100 parasites/ul.
- For population genetics, to ensure comparability between samples and populations, a threshold minor allele intensity of 33% (minor alleles must be a minimum of 33% peak intensity of major allele) is set, as described and validated elsewhere (Imwong et al., Int J Parasitol (2007); Karunaweera et al., Gene (2008)).

**Figure 1.** MS16 peaks. Two real peaks, al 376 and al 403, are present.

**Figure 2.** Pv3.27 peaks. Two real peaks, al 351 and al 368, are present.

**Figure 3.** Msp1F3 peaks. Two real peaks, al 234 and al 270, are present.
Table 1. Marker Details

<table>
<thead>
<tr>
<th>Marker</th>
<th>Literature*</th>
<th>Marker Type</th>
<th>Main Application</th>
<th>Chr</th>
<th>Nest PCR?</th>
<th>Primers (5’ to 3’)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS16</td>
<td>1-9</td>
<td>Microsatellite</td>
<td>Finger-printing, MOI</td>
<td>9</td>
<td>Yes</td>
<td>F-TTCTGGATGACATTTCTGCACGG</td>
<td>153–308</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R-TCCTCCATTTGGAAGCATGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest-F: <strong>NED</strong>-CTTGGTTGAGTATGTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest-R: aaaaaAGTAGTACAACTGTGTTGAGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest:F: 6FAM-GGAGACATCTACCTTGCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest-R: aaaaaAAAAGAAGAGAGGAGGAAAAC</td>
<td></td>
</tr>
<tr>
<td>Pv3.27</td>
<td>1, 6, 8</td>
<td>Microsatellite</td>
<td>Finger-printing, MOI</td>
<td>3</td>
<td>Yes</td>
<td>F-TTTTTCAACTGTGGCCCCCTGT</td>
<td>85–240</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R-aaaaaaaCGTCATCTACCTTGCGAGGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest:F: 6FAM-GGACATTCCAAATGTATGTCAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest-R: aaaaaAAAAGAAGAGAGGAGGAAAAC</td>
<td></td>
</tr>
<tr>
<td>msp1F3</td>
<td>6, 8</td>
<td>Surface antigen</td>
<td>Finger-printing, MOI</td>
<td>7</td>
<td>Yes</td>
<td>F-GGAGAACATAAGCTACCTGTCC</td>
<td>226–372</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R-GTTGTACCTGTGCTTCTCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest:F: VIC-CAAGCCTACCAAGAATTGATTCCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nest-R: aaaaaAAAAGAAGAGAGGAGGAAAAC</td>
<td></td>
</tr>
<tr>
<td>MS1</td>
<td>2, 3, 4, 5, 7, 9</td>
<td>Microsatellite</td>
<td>Population structure</td>
<td>3</td>
<td>No</td>
<td>F: <strong>6FAM</strong>-TCAACTGTGGAAAGGCAAT</td>
<td>228–246</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ctgctctTGTGCGTTTTTCTTCTG</td>
<td></td>
</tr>
<tr>
<td>MS5</td>
<td>2, 3, 4, 5, 7</td>
<td>Microsatellite</td>
<td>Population structure</td>
<td>6</td>
<td>No</td>
<td>F: <strong>NED</strong>-CTCTCTCATCGCGTACACA</td>
<td>163–187</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ctgctctAAGAGAGAGAGGAGGAAAC</td>
<td></td>
</tr>
<tr>
<td>MS8</td>
<td>2, 3, 4, 5, 7, 9</td>
<td>Microsatellite</td>
<td>Population structure</td>
<td>12</td>
<td>No</td>
<td>F: <strong>VIC</strong>-AGAGAGAGCACGAAATGCAGA</td>
<td>222–306</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ctgctctAGGCCCTTTTGGGTCTTCTAT</td>
<td></td>
</tr>
<tr>
<td>MS10</td>
<td>2, 3, 4, 5, 7</td>
<td>Microsatellite</td>
<td>Population structure</td>
<td>13</td>
<td>No</td>
<td>F: <strong>PET</strong>-TTATCTCAGCGGATAGTGA</td>
<td>180–306</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ctgctctCTTAGCGGGAGACTGTTT</td>
<td></td>
</tr>
<tr>
<td>MS12</td>
<td>2, 3, 4, 5, 7</td>
<td>Microsatellite</td>
<td>Population structure</td>
<td>5</td>
<td>No</td>
<td>F: <strong>6FAM</strong>-AATGCACATCATCTGTCCTC</td>
<td>209–333</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ctgctctCTGCTTGTTGCTGCT</td>
<td></td>
</tr>
<tr>
<td>MS20</td>
<td>2, 3, 4, 5, 7</td>
<td>Microsatellite</td>
<td>Population structure</td>
<td>10</td>
<td>No</td>
<td>F: <strong>VIC</strong>-GCACAAACATGCAAGATCC</td>
<td>195–237</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ctgctctGTCCTAGTGCTACTTCT</td>
<td></td>
</tr>
</tbody>
</table>

*Reference
8. Currently in use at MSHR (Ric Price), QIMR (Qin Cheng), Burnett Institute (John Reeder) Indonesia, Solomon Islands, Vanuatu, Papua New Guinea
9. Currently in use at Korea CDC (Jung-Yeon Kim) Korea
**SOP-1 courtesy of Prof Georges Snounou**

“Home-made” EDTA-containing eppendorf tube

- For 200ul whole blood, add 15-20ul 0.5M EDTA to a 1.5ml eppendorf tube (final EDTA concentration 37.5 - 50mM).
- Store tubes at 2-8°C until use and ensure the lids of the tubes are on firmly to avoid drying out the EDTA.
- Once blood is added, immediately invert the tube 8-10 times to mix the blood with the EDTA.
- It is important to ensure that the EDTA is removed during DNA extraction – to avoid PCR inhibition, there must be no EDTA present in the final template.

**SOP-2 Genotyping Koepfli Markers**

Protocol derived from Koepfli et al., JID, 2009: courtesy of Dr Jenny Kozlovski and Dr Jutta Marfurt

**Preparation of PCR products for capillary electrophoresis**

PCRs were performed in 20 ul reactions containing 0.25 uM of each primer, 2 ul of 10 x buffer, 0.2 mM each dNTP, 2 mM MgCl₂, 1.5 U Taq DNA polymerase and 0.5 ul genomic DNA. One ul of diluted primary PCR product was used as template for the nested (MS16, msp1F3) or semi-nested (Pv3.27) reaction. PCRs were performed in a thermocycler with conditions as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles (primary PCR) or 25 cycles (nested PCR) of denaturation at 95°C for 1 min, annealing at 56-60°C (see Table 2 for temperature) for 1 min, elongation at 72°C for 1 min, followed by a final elongation at 72°C for 5 min. PCR products were stored in the dark (plates wrapped in foil) at 4°C until capillary electrophoresis run.

**Table 2. PCR cycling conditions**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Annealing temperature (°C)</th>
<th>Dilution</th>
<th>Nested PCR product for capillary electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary PCR</td>
<td>Nested PCR</td>
<td>Primary PCR product</td>
</tr>
<tr>
<td>MS16</td>
<td>57</td>
<td>56</td>
<td>1/10</td>
</tr>
<tr>
<td>Pv3.27</td>
<td>58</td>
<td>58</td>
<td>1/10</td>
</tr>
<tr>
<td>msp1F3</td>
<td>59</td>
<td>60</td>
<td>1/100</td>
</tr>
</tbody>
</table>

**Capillary electrophoresis**

For capillary electrophoresis, equal quantities of the diluted nested PCR products were pooled into a single multiplex. One ul of this pool was added to 10 ul Hi-Di Formamide
containing 0.06 ul size standard LIZ-600 and run on the Genetic Analyser 3130xl (Applied Biosystems). Data was analysed using GeneMapper software v4.0 (Applied Biosystems) and alleles were grouped into bins according to their repeat length.

If you use a commercial option for the capillary electrophoresis, check details on the procedure with the assisting laboratory.

**SOP-3 Genotyping Karunaweera Markers**


**Preparation of PCR products for capillary electrophoresis**

PCRs were performed in 20 ul reactions containing 6.4 pmol of each primer, 2 ul of 10 x buffer, 1 mM each dNTP, 0.7ul 50 mM MgCl₂, 1 U Taq DNA polymerase and 2 ul genomic DNA. PCRs were performed in a thermocycler with conditions as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 40 sec, elongation at 72°C for 30 sec, followed by a final elongation at 72°C for 5 min. PCR products were stored in the dark (plates wrapped in foil) at 4°C until capillary electrophoresis run.

**Capillary electrophoresis**

For capillary electrophoresis, equal quantities of the diluted nested PCR products were pooled into two separate multiplexes. MS1, MS5 and MS8 were pooled in one multiplex, and MS10, MS12 and MS20 were pooled in a separate multiplex. One ul of this pool was added to 10 ul Hi-Di Formamide containing 0.06 ul size standard LIZ-600 and run on the Genetic Analyser 3130xl (Applied Biosystems). Data was analysed using GeneMapper software v4.0 (Applied Biosystems) and alleles were grouped into bins according to their repeat length.

If you use a commercial option for the capillary electrophoresis, check details on the procedure with the assisting laboratory.