**Supplementary file 1 - Microsatellite marker development**

Total genomic DNA was extracted from *U. lobata* material collected from Efate, Vanuatu (GPS coordinates: latitude -17.7934 longitude 168.449), using the NucleoSpin Plant II extraction kit with PL2/PL3 buffers (Macherey-Nagel, Duren, Germany). DNA was quantified with the Qubit Fluorometer (Thermo Fisher Scientific, Waltham, United States) and NanoDrop Spectrophotometer (Thermo Fivansher Scientific) to ensure a concentration of at least 20ng/ul and an A260/A280 quality ratio of at least 1.7. DNA was sent to Macrogen (Seoul, Republic of Korea), where a shotgun library was prepared with the TruSeq Nano DNA kit (Illumina, San Diego, United States) and run through a HiSeq2500 sequencer (Illumina) using 100 base-pair paired-end reads. The resulting library (NCBI SAMN33906725) produced 163,168,306 total reads.

The sequencing library was uploaded to the Palfinder Galaxy service for implementing the microsatellite development pipeline as described in Griffiths et al. (2016). The pipeline consisted of initially checking and trimming poor quality reads with FastQC. Reads were then removed with Trimmomatic that did not have both the forward and the complementary reverse read. Palfinder and the programs within (Palfilter, Primer3, PANDAseq) was then used to identify microsatellites of all repeat unit types with a minimum number of 6 repeats, identify appropriate primers that flank the microsatellite using the default settings, and finally to merge the forward and reverse reads. All additional default filtering options were utilised to identify the highest quality microsatellites and primers. Primers were initially searched for within the merged reads output, however as no assembled reads appeared, primers were obtained from the output containing filtered microsatellites and their flanking primers. The number of microsatellites identified with appropriate flanking primers was 8644.

Forty-eight primer pairs were chosen from the above output for screening. One of four M13 tags, as described by (Culley et al., 2013), were attached to the 5’ end of the forward primer for subsequent fluorescent labelling and efficient multiplexing. The primers were screened with six samples; two from Vanuatu (Efate, latitude -17.8019237 longitude 168.5069355, and Santo, latitude -15.44816667 longitude 167.2127222), plus four from specimens held in the Allan Herbarium at MWLR Lincoln – one from China (CHR459977A), one from Sri Lanka (CHR304497) and two from Thailand (CHR408504, CHR408543). DNA was extracted from these specimens as described above. PCR was performed in 20ul reactions, using the KAPA3G Plant PCR Kit (Kapa Biosystems, Wilmington, United States), with each reaction consisting of final concentrations of 1x KAPA3G 2x Buffer, 0.05uM forward primer, 0.2uM reverse primer, 0.2uM M13 fluorescent labelled primer, 0.5U KAPA3G Plant Polymerase. Cycling parameters were as follows: initial denaturation of 95oC for 3 min followed first by 30 cycles of 95oC for 20 sec, 55oC for 15 sec, and 72oC for 30 sec, then by 15 cycles of 95oC for 20 sec, 51oC for 15 sec, and 72oC for 30 sec, and a final extension at 72oC for 1 min. For each PCR product, 1ul was added to 9ul of Hi-Di formamide (Applied Biosystems, Carlsbad, United States) and 1ul of LIZ-labelled size standard (Applied Biosystems), before being separated on an ABI 3130xl Genetic Analyser (Applied Biosystems) at the MWLR sequencing laboratory (Auckland, New Zealand). Fragments were sized and scored with Geneious v10.2.6 (Biomatters, Auckland, New Zealand), with polymorphism and repeatability for each loci assessed.

Out of the 48 primer pairs, 27 produced polymorphic fragments and no more than two alleles per individual. The remaining primers either did not amplify the loci with all samples (likely due to DNA quality from Herbarium material), did not amplify at all with any sample, or produced multiple bands. 17 primers that identified the most polymorphic loci were selected for repeatability checking and arranging in multiplexes for efficient downstream processing. All 17 were repeatable and performed well in multiplexes. Only 14 were required for processing further samples from a number of Vanuatu islands, from Malaysian field collected material and from various herbaria samples throughout the native range. Four primers were omitted following further sample processing, due to allelic dropout or providing limited polymorphic information. The presence of null alleles were checked with MicroChecker v2.2.3 (Van Oosterhout et al., 2004), which identified null alleles in all but one loci (U.lob\_7).

Summary statistics were calculated per locus for the Vanuatuan and Malaysian material (herbaria material was omitted due to variable sample sizes per population). Numbers of alleles (*Na*) and observed (*Ho*) and expected (*He*) heterozygosities were calculated per locus for each population with GenAlEx v6.5 (Peakall and Smouse 2006). *Ho* and *He* ranged from 0.000-1.000 and 0.000-0.796, respectively, while average *Ho* and *He* across all loci and populations were 0.210 and 0.393. Alleles per locus ranged from 1-9, with an average across all loci and populations of 6.3 (Table 1). Table 2 provides characteristics of the final 10 polymorphic primers developed.

The populations with the least diversity (lowest numbers of alleles and heterozygosities) were Malekula, Santo and Epi. This is not surprising for introduced populations as they typically only receive a subset of what was present in the origin range. However, the diversity seen in Efate and Tanna is comparable to the Malaysian populations, suggesting multiple introduction events or one event containing diverse material. All populations appear quite inbred, as the observed heterozygosities are generally much lower than the expected and the high rates of homozygosity identified by Microchecker at 9 of the loci. This could be attributed to the reproductive strategy as *U. lobata* has a mixed mating mode of both allogamy and autogamy, with the latter being the predominant strategy (Clément et al., 2022).

**References**

Clément, D.W., Mogho, N.M.T., Atibita, E.N.O., Fernand-Nestor, T.F., 2022. Evaluation of the foraging activity and pollination efficiency of *Apis mellifera* L.(Hymenoptera: Apidae) on *Urena lobata* L.(Malvaceae) flowers at Dang (Ngaoundere, Cameroon). Int. J. Sci. Res. 11**,** 1219-1226.

Culley, T.M., Stamper, T.I., Stokes, R.L., Brzyski, J.R., Hardiman, N.A., Klooster, M.R., Merritt, B.J., 2013. An efficient technique for primer development and application that integrates fluorescent labeling and multiplex PCR. Appl. Plant Sci. 1**,** 1300027.

Griffiths, S.M., Fox, G., Briggs, P.J., Donaldson, I.J., Hood, S., Richardson, P., Leaver, G.W., Truelove, N.K., Preziosi, R.F., 2016. A Galaxy-based bioinformatics pipeline for optimised, streamlined microsatellite development from Illumina next-generation sequencing data. Conserv. Genet. Resour. 8**,** 481-486.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P., Shipley, P., 2004. MICRO‐CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4**,** 535-538.

Table 1. Results of *U. lobata* primer screening in five populations from Vanuatu and five populations from Malaysia.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Locus | Vanuatu (Santo)  *N*=35 | | | Vanuatu (Malekula)  *N*=20 | | | Vanuatu (Epi)  *N*=17 | | | Vanuatu (Efate)  *N*=24 | | | Vanuatu (Tanna)  *N*=21 | | |  |
| *Na* | *Ho* | *He* | *Na* | *Ho* | *He* | *Na* | *Ho* | *He* | *Na* | *Ho* | *He* | *Na* | *Ho* | *He* |  |
| U.lob\_1 | 2 | 0.029 | 0.302 | 2 | 0.000 | 0.180 | 2 | 0.059 | 0.161 | 3 | 0.125 | 0.430 | 3 | 0.333 | 0.618 |  |
| U.lob\_2 | 3 | 0.114 | 0.372 | 2 | 0.000 | 0.180 | 2 | 0.059 | 0.161 | 3 | 0.167 | 0.497 | 2 | 0.143 | 0.278 |  |
| U.lob\_3 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 2 | 0.059 | 0.161 | 2 | 0.042 | 0.395 | 2 | 0.190 | 0.308 |  |
| U.lob\_4 | 2 | 0.029 | 0.302 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 3 | 0.000 | 0.314 | 3 | 0.077 | 0.565 |  |
| U.lob\_5 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 2 | 0.000 | 0.111 | 2 | 0.000 | 0.278 | 2 | 0.048 | 0.337 |  |
| U.lob\_6 | 2 | 0.000 | 0.284 | 2 | 0.000 | 0.180 | 2 | 0.059 | 0.161 | 4 | 0.167 | 0.661 | 4 | 0.476 | 0.507 |  |
| U.lob\_7 | 2 | 1.000 | 0.500 | 2 | 1.000 | 0.500 | 4 | 1.000 | 0.555 | 4 | 1.000 | 0.652 | 4 | 1.000 | 0.713 |  |
| U.lob\_8 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 2 | 0.059 | 0.161 | 3 | 0.125 | 0.369 | 3 | 0.238 | 0.636 |  |
| U.lob\_9 | 2 | 0.086 | 0.180 | 2 | 0.000 | 0.188 | 2 | 0.000 | 0.208 | 3 | 0.083 | 0.601 | 3 | 0.048 | 0.285 |  |
| U.lob\_10 | 1 | 0.000 | 0.000 | 2 | 0.000 | 0.100 | 2 | 0.059 | 0.161 | 4 | 0.042 | 0.326 | 3 | 0.190 | 0.652 |  |
| Average | 1.70 | 0.126 | 0.194 | 1.60 | 0.100 | 0.133 | 2.10 | 0.135 | 0.184 | 3.10 | 0.175 | 0.452 | 2.90 | 0.274 | 0.490 |  |
| Locus | Malaysia (Selangor)  *N*=51 | | | Malaysia (Melaka)  *N*=10 | | | Malaysia (Johor)  *N*=19 | | | Malaysia (Perak)  *N*=9 | | | Malaysia (Pulau Pinang)  *N*=10 | | | Total  *N*=216 |
| *Na* | *Ho* | *He* | *Na* | *Ho* | *He* | *Na* | *Ho* | *He* | *Na* | *Ho* | *He* | *Na* | *Ho* | *He* | *Na* |
| U.lob\_1 | 3 | 0.725 | 0.488 | 2 | 1.000 | 0.500 | 4 | 1.000 | 0.569 | 2 | 0.778 | 0.475 | 2 | 1.000 | 0.500 | 5 |
| U.lob\_2 | 4 | 0.000 | 0.676 | 4 | 0.300 | 0.575 | 3 | 0.053 | 0.636 | 2 | 0.222 | 0.444 | 3 | 0.100 | 0.645 | 5 |
| U.lob\_3 | 2 | 0.059 | 0.438 | 1 | 0.000 | 0.000 | 2 | 0.000 | 0.100 | 2 | 0.111 | 0.401 | 1 | 0.000 | 0.000 | 2 |
| U.lob\_4 | 6 | 0.000 | 0.771 | 4 | 0.100 | 0.625 | 6 | 0.000 | 0.796 | 5 | 0.111 | 0.747 | 5 | 0.300 | 0.585 | 11 |
| U.lob\_5 | 3 | 0.000 | 0.541 | 2 | 0.000 | 0.480 | 2 | 0.000 | 0.100 | 3 | 0.111 | 0.623 | 1 | 0.000 | 0.000 | 3 |
| U.lob\_6 | 5 | 0.196 | 0.647 | 2 | 0.200 | 0.480 | 4 | 0.000 | 0.649 | 4 | 0.222 | 0.691 | 3 | 0.167 | 0.403 | 9 |
| U.lob\_7 | 4 | 0.980 | 0.728 | 2 | 0.700 | 0.455 | 4 | 0.947 | 0.548 | 4 | 1.000 | 0.698 | 2 | 1.000 | 0.500 | 4 |
| U.lob\_8 | 3 | 0.041 | 0.659 | 2 | 0.000 | 0.180 | 3 | 0.000 | 0.277 | 4 | 0.111 | 0.685 | 3 | 0.200 | 0.645 | 6 |
| U.lob\_9 | 3 | 0.000 | 0.462 | 3 | 0.100 | 0.605 | 5 | 0.000 | 0.759 | 1 | 0.000 | 0.000 | 3 | 0.300 | 0.585 | 7 |
| U.lob\_10 | 9 | 0.317 | 0.709 | 3 | 0.300 | 0.460 | 4 | 0.053 | 0.691 | 1 | 0.000 | 0.000 | 3 | 0.111 | 0.549 | 11 |
| Average | 4.20 | 0.232 | 0.612 | 2.50 | 0.270 | 0.436 | 3.70 | 0.205 | 0.512 | 2.80 | 0.267 | 0.477 | 2.60 | 0.318 | 0.441 | 6.3 |

*N* = number of samples, *Na* = number of alleles, *Ho* = observed heterozygosity, *He* = expected heterozygosity.

Note: Melaka, Perak and Pulau Pinang consist of fewer sites across the region compared to Selangor and Johor.

Table 2. Characteristics of 10 polymorphic microsatellite loci developed for *U. lobata*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Locus | Primer sequence (5’-3’) | Repeat motif | Allele Size Range (bp) | Multiplex | Fluorescent Dye |
| U.lob\_1 | F: CAAGCAATAGAACAACGCCG  R: CCACCATCTCCACCTTCTCC | ACC(18) | 358-383 | 3 | 6Fam |
| U.lob\_2 | F: TTTTCAAATCGTCAGCGG  R: TGAGCCCAAGTAGATACCCG | AT(14) | 589-595 | 3 | 6Fam |
| U.lob\_3 | F: TCCAAATTAGGAAAAGGTGGC  R: TCCAAATTAGGAAAAGGTGGC | TC(12) | 510-512 | 4 | Ned |
| U.lob\_4 | F: ATGTGTGACACGGGTCGG  R: GGAATATGTGATGACAATGTTTGG | AT(16) | 379-406 | 2 | Ned |
| U.lob\_5 | F: GGTATGAGGGTCATTTGAGGC  R: CGTGTATCAAATTACTTCTGTCTATTGC | AAG(24) | 307-316 | 2 | Vic |
| U.lob\_6 | F: TTTATGCATGAATTTGGGAGC  R: CTCTTACTAATAAACCAACCGCC | AT(20) | 221-233 | 1 | Vic |
| U.lob\_7 | F: CACCATTAACTCAACATTTAACCCC  R: GCTAAACGAAAATGCCAGGG | TG(12) | 429-437 | 4 | Vic |
| U.lob\_8 | F: AGAAACAGCATGTCAAACGG  R: CACACACATGTTACACGGGG | AATT(24) | 264-277 | 1 | Ned |
| U.lob\_9 | F: TGGTTTTGCTGTTGAGACGG  R: AACTGCCTTATCTTTGGTTCTTGG | AGC(21) | 378-390 | 1 | Ned |
| U.lob\_10 | F: TTGCAAACCCTTTAAACGCC  R: GAGTCCGATACCCTCATCGC | ATA(24) | 305-367 | 4 | Ned |