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# CHLOROPLAST MICROSATELLITE MARKERS FOR *ARTOCARPUS* (MORACEAE) DEVELOPED FROM TRANSCRIPTOME SEQUENCES<sup>1</sup>

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- **Premise of the study:** Chloroplast microsatellite loci were characterized from transcriptomes of *Artocarpus altilis* (breadfruit) and *A. camansi* (breadnut). They were tested in *A. odoratissimus* (terap) and *A. altilis* and evaluated in silico for two congeners.
- **Methods and Results:** Fifteen simple sequence repeats (SSRs) were identified in chloroplast sequences from four *Artocarpus* transcriptome assemblies. The markers were evaluated using capillary electrophoresis in *A. odoratissimus* (105 accessions) and *A. altilis* (73). They were also evaluated in silico in *A. altilis* (10), *A. camansi* (6), and *A. altilis* × *A. mariannensis* (7) transcriptomes. All loci were polymorphic in at least one species, with all 15 polymorphic in *A. camansi*. Per species, average alleles per locus ranged between 2.2 and 2.5. Three loci had evidence of fragment-length homoplasy.
- **Conclusions:** These markers will complement existing nuclear markers by enabling confident identification of maternal and clone lines, which are often important in vegetatively propagated crops such as breadfruit.

**Key words:** *Artocarpus altilis*; *Artocarpus camansi*; *Artocarpus mariannensis*; *Artocarpus odoratissimus*; breadfruit; Moraceae.

*Artocarpus* J. R. Forst. & G. Forst. (Moraceae) contains approximately 70 species of monoecious trees with a center of diversity in Malesia (Zerega et al., 2010). Species include several underutilized crops that can improve food security (Jones et al., 2011). In addition to breadfruit (*A. altilis* (Parkinson) Fosberg) and jackfruit (*A. heterophyllus* Lam.), *Artocarpus* contains lesser-known crops like cempedak (*A. integer* (Thunb.) Merr.) and terap (*A. odoratissimus* Blanco), and more than a dozen other species with edible fruits whose potential remains largely unexplored (Zerega et al., 2010).

Nuclear microsatellites developed for *Artocarpus* (Witherup et al., 2013) have been used in characterizing genetic diversity of breadfruit germplasm (Zerega et al., 2015). We present primers for 15 chloroplast simple sequence repeat (SSR) loci from transcriptomes of *A. altilis* and *A. camansi* that will complement the nuclear markers in analyzing genetic diversity and population

structure. Chloroplast SSRs are usually mononucleotide repeats, and as nonrecombinant, maternally inherited loci (Wheeler et al., 2014), they allow confident identification of maternal and clone lines—often important in vegetatively propagated crops such as breadfruit. These markers were developed from next-generation sequencing (NGS) transcriptome data. This approach enables rapid marker development directly from sequences in the target organisms. Primers were tested in *A. altilis* (diploid and triploid) and *A. odoratissimus*. We also constructed an in silico data set from additional transcriptomes of *A. altilis*, its wild progenitor (*A. camansi* Blanco), and *A. altilis* × *A. mariannensis* hybrids to test for fragment size homoplasy, a common problem with chloroplast SSRs that can overestimate relatedness by masking sequence variations that do not change allele sizes (Wheeler et al., 2014).

## METHODS AND RESULTS

Total RNA from two *A. altilis* accessions and one *A. camansi* accession (Appendix 1) was extracted using the QIAGEN RNeasy Universal Mini Kit (QIAGEN, Valencia, California, USA). Illumina TruSeq library preparation and sequencing in one lane of HiSeq 2000 (2 × 100, paired-end; Illumina, San Diego, California, USA) took place at Argonne National Laboratory (Lemont, Illinois, USA). Reads were de-multiplexed, quality-trimmed (>Q20 in a 5-bp window), and assembled using Trinity (Grabherr et al., 2011; Bolger et al., 2014). Chloroplast contigs were extracted using a BLAST search seeded with the *Morus indica* L. (Moraceae) chloroplast genome (GI: 89,574,460). Mono- and dinucleotide repeats were identified, aligned using BLAST, and screened for variability. Initially, primers for 16 chloroplast SSR loci were designed

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using Primer3 (Rozen and Skaletsky, 1999) (Table 1). Fifteen loci reliably amplified and were subjected to further testing.

To test for variability in *A. odoratissimus*, all loci were amplified in 105 accessions collected from four districts in Sabah, Malaysia (Appendix 2). PCR reactions were performed in two steps (Schuelke, 2000). For the first step, 10-μL reactions contained 5 μL of MyTaq Master Mix (Bioline USA, Taunton, Massachusetts, USA), 0.5 μL of 10 mg/mL bovine serum albumin (BSA), 0.25 μL of 10 μM forward primer with the M13 tail (5'-CAGGAAACAGCTATGAC-3'), 0.25 μL of 10 μM reverse primer, 3 μL of H<sub>2</sub>O, and 1 μL of template DNA. PCR conditions for the first step were 94°C for 3 min; 13 cycles at 94°C for 30 s, 59.8°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. The following were then immediately added: 2.5 μL MyTaq Master Mix, 0.25 μL of 10 mg/mL BSA, 0.125 μL of 2.5 μM MgCl<sub>2</sub>, 0.25 μL of 10 μM labeled M13 primer (WellRED Dye D2, D3, or D4 [Beckman Coulter, Brea, California, USA]), and 1.875 μL of H<sub>2</sub>O. PCR conditions for the second step were 94°C for 3 min; 27 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. Product was pooled as follows: 2 μL of D4-labeled product, 1 μL of D3, and 0.5 μL of D2. Pooled products were added to 30 μL of HiDi formamide (Azco Biotech, San Diego, California, USA) and 3.3 μL of 400-bp size standard (Beckman Coulter) and analyzed on a Beckman Coulter CEQ 8000 Genetic Analysis System. Alleles were scored using the CEQ 8000 software version 9.0 (Beckman Coulter).

To test for variability in *A. altalis*, all loci except AALTCP04, AALTCP07, AALTCP11, and AALTCP12 (which were less variable in transcriptomes) were amplified in 73 accessions of *A. altalis* from Vanuatu (Navarro et al., 2005, 2007), the Caribbean, and India (Appendix 1). Locus AALTCP14 followed the protocol described above. Other loci were amplified at the USDA in reduced PCR reaction volumes (step 1: 5 μL, step 2: 3 μL) without BSA using QIAGEN Multiplex PCR Master Mix (QIAGEN) and analyzed using ABI reagents on a 3730xl DNA Analyzer and GeneMapper 5 (Applied Biosystems, Foster City, California, USA).

To test for variability in *A. camansi* and *A. altalis* × *A. mariannensis* hybrids and to explore the presence of homoplasy in these markers, loci were amplified

in silico from the draft genome of *A. camansi*, the original four transcriptomes used for developing primers, and 18 additional transcriptome assemblies (Laricchia, 2014) (Appendix 1). Chloroplast contigs were extracted using the BLAST method described above, and amplification in silico took place following Bikandi et al. (2004). Some loci that failed to amplify because the region was split between two contigs or because a priming site was truncated were recovered using BLAST. Sequences were aligned using MUSCLE (Edgar, 2004), and a fragment-length data set was constructed. For both data sets, the number of alleles and a haplotype diversity index for each locus were calculated using GenAlEx (Table 2) (Peakall and Smouse, 2012).

Allele sizes were recovered from >60 individuals of *A. odoratissimus* for all loci but one (37 individuals for AALTCP05), and from >60 individuals of *A. altalis* for all 11 tested loci (Table 2). In silico capture recovered sequences and fragment sizes from most transcriptomes for all loci except AALTCP13, which tended to be absent from transcriptomes (Table 2, Appendix 3). All loci were polymorphic in the breadfruit complex (*A. altalis*, *A. camansi*, and *A. altalis* × *mariannensis* hybrids), with *A. camansi* showing the greatest unbiased haplotype diversity. Although the in silico sample size was small, this finding is consistent with a domestication bottleneck in *A. altalis* with respect to its wild progenitor, *A. camansi* (Zerega et al., 2005). The polymorphism in AALTCP04 in *A. camansi* was not in the repeat motif, but in a 22-bp indel. Six loci (AALTCP03, AALTCP05, AALTCP08, AALTCP10, AALTCP11, and AALTCP12) were monomorphic in *A. odoratissimus*. Average alleles per locus was 2.5 in *A. altalis*, 2.3 in hybrids and *A. odoratissimus*, and 2.2 in *A. camansi*. For comparison, average alleles per locus in the previously described nuclear markers using the same individuals as our in silico data set (with one parent-sibling substitution in *A. camansi*) were 2.1 in *A. camansi*, 5.0 in *A. altalis*, and 4.6 in hybrids (Zerega et al., 2015).

The in silico data revealed within-species homoplasy due to multiple SSRs in the same amplified fragment in loci AALTCP01, AALTCP09, and AALTCP10. All other loci showed no evidence of fragment-length homoplasy. We also identified single-nucleotide polymorphisms in flanking regions outside the target repeats in loci AALTCP01, AALTCP02, AALTCP07, AALTCP09,

TABLE 1. Chloroplast SSRs developed in this study, showing region, primers, motif, melting temperature, suggested pool and dye color for multiplexing, and GenBank accession number for sequences from *Artocarpus camansi* (NTBG 960,576,001).

| Locus    | Region             | Primer sequences (5'–3')                                    | Repeat motif                                         | T <sub>a</sub> (°C) <sup>a</sup> | Pool/Dye | GenBank accession no. |
|----------|--------------------|-------------------------------------------------------------|------------------------------------------------------|----------------------------------|----------|-----------------------|
| AALTCP01 | <i>ndhA</i>        | F: TTGGGGCTTTACGTTGGTAG<br>R: CGTTTCTATTCTTCTTCTTCTG        | (T) <sub>9</sub> , (C) <sub>7</sub> (T) <sub>7</sub> | 60.0<br>58.5                     | 1/D4     | KR185519              |
| AALTCP02 | <i>ndhA</i>        | F: CAGAAAGAGAAAGAAGATAGAAACG<br>R: AAACCTCGTCCTTTTCACTTACGA | (A) <sub>10</sub> , (TA) <sub>7</sub>                | 58.5<br>59.4                     | 2/D4     | KR185520              |
| AALTCP03 | <i>petB</i>        | F: ACCTCGTGGCCGGACTTT<br>R: TCCTTGAGTAAGAACCCTTGG           | (T) <sub>14</sub>                                    | 63.0<br>59.2                     | 4/D4     | KR185521              |
| AALTCP04 | <i>petB–petD</i>   | F: TCACTTGGGGTAGGAACAATAG<br>R: TTCTGCATAGCCCAATCAAT        | (TA) <sub>6</sub> , 22-bp indel                      | 58.1<br>58.1                     | 1/D3     | KR185522              |
| AALTCP05 | <i>psbE–petL</i>   | F: TTCCAAGGATAGGGCTTTGT<br>R: TTTTATTGTATCGCCGAATCC         | (A) <sub>11</sub>                                    | 58.7<br>59.0                     | 4/D3     | KR185523              |
| AALTCP06 | <i>rpl16</i>       | F: TGAATCATCCACCTTACCTTACA<br>R: CATCGTTTCGCATTATCTGG       | (T) <sub>7</sub> A(T) <sub>9</sub>                   | 58.5<br>59.1                     | 1/D3     | KR185524              |
| AALTCP07 | <i>rps8–rpl14</i>  | F: TTTTATTTCATGTCAGCATTTTCG<br>R: AGGAAATTGTTGTGTCTCACG     | (T) <sub>10</sub>                                    | 59.1<br>59.1                     | 5/D4     | KR185525              |
| AALTCP08 | <i>rpl14–rpl16</i> | F: TCAAAATGGGTTTGGAGTTGA<br>R: AGCGGTATCCAAAATGCCTA         | (A) <sub>11</sub> , (T) <sub>9</sub>                 | 59.0<br>59.6                     | 3/D4     | KR185526              |
| AALTCP09 | <i>trnS–trnG</i>   | F: TCCGACGCTTTAGTCCACTC<br>R: GCCAAGCCGTGTAAGAAAAA          | (T) <sub>13</sub> , 5-bp indel                       | 60.4<br>60.2                     | 2/D3     | KR185527              |
| AALTCP10 | <i>trnS–trnG</i>   | F: GGGCCTCTTTTGTCTTAACG<br>R: TGTTCAAAAATTCATAGTTCTTTTGT    | (T) <sub>9</sub> , (A) <sub>9</sub>                  | 58.8<br>58.7                     | 3/D2     | KR185528              |
| AALTCP11 | <i>rps16</i>       | F: GCCGTACGAGGAGAAAACTTC<br>R: GCGCCCTTTTCAAGGAAATA         | (G) <sub>5</sub> A(G) <sub>9</sub>                   | 60.3<br>61.4                     | 4/D2     | KR185529              |
| AALTCP12 | <i>rps16</i>       | F: GCTCTTCGGAAGTGGGTTT<br>R: TCATTACACCTTAAACGCTCT          | (AT) <sub>6</sub>                                    | 60.6<br>57.5                     | 5/D4     | KR185530              |
| AALTCP13 | <i>rps16</i>       | F: GAAAGTGCTTTTGGCTCGAC<br>R: AGATTCTTGCTCCGAAAAA           | (T) <sub>12</sub> (G) <sub>10</sub>                  | 60.0<br>58.9                     | 3/D3     | KR185531              |
| AALTCP14 | <i>trnT–trnE</i>   | F: CGGATTTGAACCGATGACTT<br>R: TCGTTCCTGAGTGAACCACTA         | (TA) <sub>5</sub>                                    | 59.9<br>58.3                     | 2/D2     | KR185532              |
| AALTCP15 | <i>trnT–trnE</i>   | F: TGGTTCCTCAGGAACGATAAA<br>R: TGGATCTAGGTTGAATTGGTAGG      | (A) <sub>8</sub>                                     | 59.6<br>59.4                     | 1/D2     | KR185533              |

Note: T<sub>a</sub> = annealing temperature.

<sup>a</sup>All primers amplified with an annealing temperature of 59.8°C (step 1) and 55°C (step 2).

TABLE 2. Summary of allele size data for species in the breadfruit complex.

| Locus    | <i>A. odoratissimus</i> |          |          |          | <i>A. camansi</i> <sup>a</sup> |          |          |          | <i>A. altilis</i> |          |          |          | <i>A. altilis</i> × <i>A. mariannensis</i> <sup>a</sup> |          |          |          |
|----------|-------------------------|----------|----------|----------|--------------------------------|----------|----------|----------|-------------------|----------|----------|----------|---------------------------------------------------------|----------|----------|----------|
|          | <i>N</i>                | <i>A</i> | ASR (bp) | <i>h</i> | <i>N</i>                       | <i>A</i> | ASR (bp) | <i>h</i> | <i>N</i>          | <i>A</i> | ASR (bp) | <i>h</i> | <i>N</i>                                                | <i>A</i> | ASR (bp) | <i>h</i> |
| AALTCP01 | 106                     | 4        | 187–190  | 0.532    | 6                              | 3        | 171–174  | 0.733    | 73                | 4        | 190–193  | 0.648    | 7                                                       | 2        | 171–172  | 0.286    |
| AALTCP02 | 105                     | 2        | 186–187  | 0.074    | 6                              | 2        | 175–178  | 0.600    | 73                | 2        | 193–194  | 0.027    | 7                                                       | 3        | 174–176  | 0.667    |
| AALTCP03 | 93                      | 1        | 206      | 0.000    | 6                              | 2        | 193–194  | 0.533    | 73                | 4        | 209–213  | 0.549    | 7                                                       | 4        | 191–196  | 0.810    |
| AALTCP04 | 98                      | 3        | 229–233  | 0.099    | 6                              | 2        | 210–232  | 0.600    | 10 <sup>a</sup>   | 1        | 210      | 0.000    | 7                                                       | 1        | 210      | 0.000    |
| AALTCP05 | 61                      | 1        | 247      | 0.000    | 6                              | 3        | 231–233  | 0.600    | 66                | 2        | 248–249  | 0.142    | 7                                                       | 2        | 231–232  | 0.286    |
| AALTCP06 | 105                     | 2        | 252–253  | 0.038    | 6                              | 2        | 229–232  | 0.600    | 73                | 3        | 248–252  | 0.475    | 6                                                       | 3        | 229–233  | 0.733    |
| AALTCP07 | 104                     | 2        | 204–208  | 0.019    | 6                              | 2        | 183–184  | 0.533    | 11 <sup>a</sup>   | 2        | 183–184  | 0.436    | 7                                                       | 2        | 183–184  | 0.286    |
| AALTCP08 | 105                     | 1        | 232      | 0.000    | 6                              | 2        | 216–218  | 0.600    | 73                | 2        | 233–234  | 0.465    | 7                                                       | 3        | 214–217  | 0.667    |
| AALTCP09 | 106                     | 6        | 228–234  | 0.641    | 6                              | 3        | 203–206  | 0.733    | 69                | 3        | 220–225  | 0.470    | 6                                                       | 4        | 202–207  | 0.800    |
| AALTCP10 | 89                      | 1        | 278      | 0.000    | 5                              | 2        | 280–281  | 0.600    | 71                | 3        | 296–299  | 0.481    | 7                                                       | 2        | 278–281  | 0.286    |
| AALTCP11 | 91                      | 1        | 228      | 0.000    | 6                              | 2        | 209–211  | 0.600    | 10 <sup>a</sup>   | 1        | 211      | 0.000    | 5                                                       | 1        | 211      | 0.000    |
| AALTCP12 | 37                      | 1        | 236      | 0.000    | 6                              | 2        | 221–223  | 0.600    | 9 <sup>a</sup>    | 1        | 221      | 0.000    | 7                                                       | 2        | 221–223  | 0.476    |
| AALTCP13 | 104                     | 2        | 163–167  | 0.379    | 2                              | 2        | 148–151  | —        | 72                | 5        | 169–173  | 0.559    | 2                                                       | 2        | 149–152  | —        |
| AALTCP14 | 103                     | 3        | 215–219  | 0.246    | 4                              | 2        | 198–200  | 0.500    | 65                | 2        | 218–220  | 0.031    | 5                                                       | 1        | 198      | 0.000    |
| AALTCP15 | 103                     | 4        | 220–223  | 0.182    | 5                              | 2        | 202–203  | 0.600    | 73                | 3        | 219–221  | 0.475    | 5                                                       | 3        | 202–204  | 0.700    |

Note: *A* = number of alleles; ASR = allele size range; *h* = unbiased haplotype diversity; *N* = number of individuals.  
<sup>a</sup>In silico data, without the 17-bp M13 tail.

AALTCP12, and AALTCP14 (in *A. camansi* only for AALTCP02, AALTCP09, AALTCP12, and AALTCP14). These loci thus may provide additional resolution when a sequencing approach is used as opposed to a fragment-size approach.

CONCLUSIONS

These chloroplast SSR loci will be useful for rapid and low-cost genotyping in *Artocarpus* and possibly in other Moraceae species, given the level of conservation typical in chloroplast genomes. By enabling the isolation of maternal lineages, these markers can be applied to characterizing genetic diversity, tracing seed and vegetative dispersal history, and assessing relatedness of germplasm accessions. Even as NGS tools become more widespread, SSRs remain important, as they enable efficient genotyping with common laboratory equipment. This is particularly relevant for nonmodel, underutilized crops, which are often grown in less developed areas where only basic genotyping equipment is available.

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APPENDIX 1. Accession and locality information for *Artocarpus altilis*, *A. camansi*, and *A. altilis* × *A. mariannensis*. Individuals labeled “NTBG” are part of a living germplasm collection at the National Tropical Botanical Garden’s Breadfruit Institute (Kalaheo, Hawai‘i, USA). Germplasm source localities appear in parentheses. Individuals labeled “VUT” were collected as part of the Vanuatu Breadfruit Project; detailed accession information appears in Navarro et al. (2005), and additional information about 36 accessions comprising a living collection at the Vanuatu Agricultural Research and Technical Center appears in Navarro et al. (2007). Individuals labeled “CHIC” refer to vouchers deposited at the Chicago Botanic Garden Nancy Poole Rich Herbarium (CHIC). Asterisks denote the individuals used for the initial marker development. FSM = Federated States of Micronesia.

***Artocarpus altilis*** \*NTBG 030042.001 (Society Islands), \*NTBG 040063.001 (Samoa), NTBG 900261.001 (Fiji), NTBG 790487.001 (Society Islands), NTBG 890479.002 (Pohnpei, FSM), NTBG 890167.002 (Pohnpei, FSM), NTBG 880690.001 (Tonga), NTBG 790485.001 (Society Islands), NTBG 900265.001 (Fiji), NTBG 890455.001 (Samoa), VUT001, VUT002, VUT003, VUT004, VUT005, VUT006, VUT007, VUT008, VUT009, VUT010, VUT011, VUT012, VUT013, VUT014, VUT015, VUT016, VUT017, VUT018, VUT019, VUT020, VUT021, VUT022, VUT023, VUT024, VUT025, VUT026, VUT027, VUT028, VUT029, VUT030, VUT031, VUT032, VUT033, VUT034, VUT035, VUT036, VUT037, VUT038, VUT039, VUT040, VUT041, VUT042, VUT043, VUT044, VUT045, VUT046, VUT047, VUT048, VUT049, VUT050, VUT051, VUT052, VUT053, VUT054, VUT055, VUT056, VUT057, VUT058, VUT059, VUT060, VUT061, VUT062, VUT063, VUT064, VUT065,

VUT066, VUT067, VUT068, VUT069, *N. Zerega* 955 (India, photo voucher at CHIC), *N. Zerega* 958 (India, photo voucher at CHIC), *N. Zerega* 959 (India, photo voucher at CHIC), *N. Zerega* 960 (Caribbean, CHIC), *N. Zerega* 961 (Caribbean, CHIC), *N. Zerega* 962 (Caribbean, CHIC).

***Artocarpus camansi*** \*EG 140 (CHIC), seed offspring of NTBG 000501.005 (Papua New Guinea), NTBG 910280.001 (Pohnpei, FSM), NTBG 000389.001 (Papua New Guinea), NTBG 980212.001 (Palau), NTBG 770444.001 (Tahiti), NTBG 960576.001 (Honduras).

***Artocarpus altilis* × *A. mariannensis*** NTBG 890174.001 (Tokelau), NTBG 890173.002 (Tokelau), NTBG 890184.001 (Yap, FSM), NTBG 790490.001 (Society Islands), NTBG 890183.001 (Palau), NTBG 910269.001 (Chuuk, FSM), NTBG 910265.001 (Society Islands).

APPENDIX 2. Voucher and locality information for *Artocarpus odoratissimus* collected in Sabah, Malaysia. At least one voucher was made per site, with the exception of two sites in Sandakan District for which only photographic vouchers were taken. All voucher specimens were deposited at the Chicago Botanic Garden Nancy Poole Rich Herbarium (CHIC).

| District | Locality                             | N  | Geographic coordinates             | Collection no.                                   | Collection date                    | Voucher no.            |
|----------|--------------------------------------|----|------------------------------------|--------------------------------------------------|------------------------------------|------------------------|
| Beaufort | Beaufort Hill                        | 3  | 5°20'48"N, 115°44'59.82"E          | NZ 839, 841<br>SAN 156751                        | June 23, 2013<br>May 20, 2014      | NZ 839<br>SAN 155751   |
| Beaufort | Ganui Forest Reserve                 | 7  | 4°59'42.96"N, 115°41'19.86"E       | NZ 879, 884–886, 888, 892, 893                   | June 25, 2013                      | NZ 884–886             |
| Beaufort | Near Binsuluk Forest Reserve         | 7  | 5°29'36"N, 115°38'21"E (estimated) | NZ 895–901                                       | June 26, 2013                      | NZ 895                 |
| Beaufort | Sianggau Forest Reserve              | 11 | 5°10'44.4"N, 115°36'26.46"E        | NZ 855–857, 862, 866, 867, 870–873, 876          | June 24, 2013                      | NZ 855, 866, 867       |
| Beluran  | Along Sungai Telupid                 | 4  | 5°37'14.58"N, 117°6'12.42"E        | NZ 735, 741, 742, 744                            | June 18, 2013                      | NZ 735                 |
| Papar    | Kampung Kopozon                      | 10 | 5°42'30"N, 116°00'59.94"E          | NZ 789–791, 797, 802, 805–809                    | June 21, 2013                      | NZ 789                 |
| Ranau    | Kinabalu Park, Poring Springs        | 14 | 6°24'42.48"N, 116°42'10.86"E       | NZ 749–752, 755, 760, 764, 765, 768–770, 772–774 | June 19, 2013                      | NZ 755, 769            |
| Sandakan | Kampung Sungai Batang                | 1  | 5°56'7.9"N, 118°0'41.5"E           | NZ 706                                           | June 17, 2013                      | Photo only             |
| Sandakan | Kinabatangan                         | 1  | 5°30'13.2"N, 118°13'9.24"E         | NZ 951                                           | June 29, 2013                      | Photo only             |
| Sandakan | Sepilok                              | 4  | 117°56'27.7"N, 117°56'27.7"E       | NZ 614, 704, 706, 714, 720                       | June 13 & 17, 2013                 | NZ 614, 714            |
| Sandakan | Ulu Dusun ARS                        | 24 | 5°47'25.96"N, 117°46'31.56"E       | NZ 618–631, 678–685<br>EG 94, 131                | June 14, 2013<br>May 15 & 29, 2014 | NZ 618, EG 94          |
| Tambunan | Kipundu Butterfly Park               | 8  | 5°52'16.2"N, 116°15'1.44"E         | NZ 810, 811, 816, 817, 819–822                   | June 21, 2013                      | NZ 810                 |
| Tenom    | Sabah Agriculture Park and ARS Tenom | 16 | 5°11'11.4"N, 116°00'1.6"E          | NZ 912, NZ 935–937<br>EG 60, 62, 102–111         | June 27, 2013<br>May 6 & 19, 2014  | NZ 912, EG 102, EG 106 |

Note: ARS = Agriculture Research Station; N = number of individuals.

APPENDIX 3. GenBank accession numbers for sequences from the in silico data set.

| Accession no.    | Species                             | AALTCP01 | AALTCP02 | AALTCP03 | AALTCP04 | AALTCP05 | AALTCP06 | AALTCP07 | AALTCP08 | AALTCP09 | AALTCP10 | AALTCP11 | AALTCP12 | AALTCP13 | AALTCP14 | AALTCP15 |
|------------------|-------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| NTBG 030,042.001 | <i>altilis</i>                      | KR185384 | KR185385 | KR185386 | KR185387 | KR185388 | KR185389 | KR185390 | KR185391 | KR185392 | KR185393 | KR185394 | —        | —        | KR185395 | KR185396 |
| NTBG 040,063.001 | <i>altilis</i>                      | KR185397 | KR185398 | KR185399 | KR185400 | KR185401 | KR185402 | KR185403 | KR185404 | KR185405 | KR185406 | KR185407 | KR185408 | —        | KR185409 | KR185410 |
| NTBG 790,485.001 | <i>altilis</i>                      | KR185411 | KR185412 | KR185413 | KR185414 | KR185415 | —        | KR185416 | KR185417 | KR185418 | KR185419 | KR185420 | —        | —        | KR185421 | KR185422 |
| NTBG 790,487.001 | <i>altilis</i>                      | KR185423 | KR185424 | KR185425 | KR185426 | KR185427 | KR185428 | KR185429 | KR185430 | KR185431 | KR185432 | KR185433 | —        | —        | KR185434 | KR185435 |
| NTBG 880,690.001 | <i>altilis</i>                      | KR185436 | KR185437 | KR185438 | KR185439 | KR185440 | KR185441 | KR185442 | KR185443 | KR185444 | KR185445 | KR185446 | KR185447 | KR185448 | KR185449 | KR185450 |
| NTBG 890,167.002 | <i>altilis</i>                      | KR185451 | KR185452 | KR185453 | KR185454 | KR185455 | KR185456 | KR185457 | KR185458 | KR185459 | KR185460 | KR185461 | —        | —        | KR185462 | KR185463 |
| NTBG 890,455.001 | <i>altilis</i>                      | KR185464 | KR185465 | KR185466 | KR185467 | KR185468 | KR185469 | KR185470 | KR185471 | KR185472 | KR185473 | KR185474 | KR185475 | —        | KR185476 | KR185477 |
| NTBG 890,479.002 | <i>altilis</i>                      | KR185478 | KR185479 | KR185480 | KR185481 | KR185482 | KR185483 | KR185484 | KR185485 | KR185486 | KR185487 | KR185488 | —        | —        | KR185489 | KR185490 |
| NTBG 900,261.001 | <i>altilis</i>                      | KR185491 | KR185492 | KR185493 | KR185494 | KR185495 | KR185496 | KR185497 | KR185498 | KR185499 | KR185500 | KR185501 | KR185502 | —        | KR185503 | KR185504 |
| NTBG 900,265.001 | <i>altilis</i>                      | KR185505 | KR185506 | KR185507 | KR185508 | KR185509 | KR185510 | KR185511 | KR185512 | KR185513 | KR185514 | KR185515 | KR185516 | —        | KR185517 | KR185518 |
| NTBG 000,389.001 | <i>camansi</i>                      | KR185534 | KR185535 | KR185536 | KR185537 | KR185538 | KR185539 | KR185540 | KR185541 | KR185542 | KR185543 | KR185544 | KR185545 | —        | —        | KR185546 |
| NTBG 770,444.001 | <i>camansi</i>                      | KR185547 | KR185548 | KR185549 | KR185550 | KR185551 | KR185552 | KR185553 | KR185554 | KR185555 | —        | KR185556 | KR185557 | —        | KR185558 | KR185559 |
| NTBG 910,280.001 | <i>camansi</i>                      | KR185560 | KR185561 | KR185562 | KR185563 | KR185564 | KR185565 | KR185566 | KR185567 | KR185568 | KR185569 | KR185570 | KR185571 | KR185572 | —        | —        |
| NTBG 960,576.001 | <i>camansi</i>                      | KR185519 | KR185520 | KR185521 | KR185522 | KR185523 | KR185524 | KR185525 | KR185526 | KR185527 | KR185528 | KR185529 | KR185530 | KR185531 | KR185532 | KR185533 |
| NTBG EG 140      | <i>camansi</i>                      | KR185573 | KR185574 | KR185575 | KR185576 | KR185577 | KR185578 | KR185579 | KR185580 | KR185581 | KR185582 | KR185583 | KR185584 | —        | KR185585 | KR185586 |
| NTBG 790,490.001 | <i>altilis</i> × <i>marianensis</i> | KR185587 | KR185588 | KR185589 | KR185590 | KR185591 | KR185592 | KR185593 | KR185594 | KR185595 | KR185596 | KR185597 | KR185598 | —        | KR185599 | KR185600 |
| NTBG 890,173.002 | <i>altilis</i> × <i>marianensis</i> | KR185601 | KR185602 | KR185603 | KR185604 | KR185605 | KR185606 | KR185607 | KR185608 | KR185609 | KR185610 | KR185611 | KR185612 | KR185613 | KR185614 | KR185615 |
| NTBG 890,174.001 | <i>altilis</i> × <i>marianensis</i> | KR185616 | KR185617 | KR185618 | KR185619 | KR185620 | KR185621 | KR185622 | KR185623 | —        | KR185624 | —        | KR185625 | —        | —        | —        |
| NTBG 890,183.001 | <i>altilis</i> × <i>marianensis</i> | KR185626 | KR185627 | KR185628 | KR185629 | KR185630 | KR185631 | KR185632 | KR185633 | KR185634 | KR185635 | —        | KR185636 | KR185637 | —        | —        |
| NTBG 890,184.001 | <i>altilis</i> × <i>marianensis</i> | KR185638 | KR185639 | KR185640 | KR185641 | KR185642 | KR185643 | KR185644 | KR185645 | KR185646 | KR185647 | KR185648 | KR185649 | —        | KR185650 | KR185651 |
| NTBG 910,265.001 | <i>altilis</i> × <i>marianensis</i> | KR185652 | KR185653 | KR185654 | KR185655 | KR185656 | KR185657 | KR185658 | KR185659 | KR185660 | KR185661 | KR185662 | KR185663 | —        | KR185664 | KR185665 |
| NTBG 910,269.001 | <i>altilis</i> × <i>marianensis</i> | KR185666 | KR185667 | KR185668 | KR185669 | KR185670 | —        | KR185671 | KR185672 | KR185673 | KR185674 | KR185675 | KR185676 | —        | KR185677 | KR185678 |
|                  | <i>altilis</i> × <i>marianensis</i> | KR185679 | KR185680 | KR185681 | KR185682 | KR185683 | KR185684 | KR185685 | KR185686 | KR185687 | KR185688 | KR185689 | KR185690 | —        | KR185691 | KR185692 |