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# Limited Usefulness of Microsatellite Markers from the Malaria Vector *Anopheles gambiae* When Applied to the Closely Related Species *Anopheles melas*

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*Anopheles melas* is a brackish water mosquito found in coastal West Africa where it is a dominant malaria vector locally. In order to facilitate genetic studies of this species, 45 microsatellite loci originally developed for *Anopheles gambiae* were sequenced in *An. melas*. Those that were suitable based on repeat number and flanking regions were examined in 2 natural populations from Equatorial Guinea. Only 15 loci were eventually deemed suitable as polymorphic markers in *An. melas* populations. These loci were screened in 4 populations from a wider geographic range. Heterozygosity estimates ranged from 0.18 to 0.79, and 2.5–15 average alleles were observed per locus, yielding 13 highly polymorphic markers and 2 loci with lower variability. To examine the usefulness of microsatellite markers when applied in a sibling species, the original *An. gambiae* specific markers were used to amplify 5 loci in *An. melas*. Null alleles were found for 1 *An. gambiae* marker. We discuss the pitfalls of using microsatellite loci across closely related species and conclude that in addition to the problem of null alleles associated with this practice, many loci may prove to be of very limited use as polymorphic markers even when used in a sibling species.

**Key words:** *Anopheles gambiae, Anopheles melas, cross-amplification, microsatellite markers, null alleles*

Microsatellite markers have been widely used in studies of population genetics, molecular ecology, evolutionary genetics, and genetic mapping. Although recent advances in genomics have made available other methods of population-scale genotyping (Thomas and Klaper 2004; Stinchcombe and Hoekstra 2008; Ekblom and Galindo 2011), microsatellite markers remain a cost-effective and useful tool. Microsatellite markers are developed for specific species, but it is common for such markers to be used across species boundaries in closely related taxa (e.g., Donnelly et al. 1999; Athrey et al. 2007). However, such use is often complicated by the occurrence of null alleles due to failed amplification as a result of a polymorphism in the annealing site of one or both primers (Callen et al. 1993; Paetkau and Strobeck 1995; Pompanon et al. 2005).

Data sets containing null alleles will be deficient in heterozygotes, and such errors can result in incorrect parentage assignment or exclusion (Dakin and Avise 2004) and biases in population genetic data. Null alleles have been shown to lead to an underestimation of intrapopulation variance and overestimation of interpopulation genetic differentiation ($F_{ST}$) and genetic distance (Chapuis and Estoup 2007). These problems can be ameliorated through careful selection and screening of microsatellite loci, identification, and quantification of genotyping errors (Pompanon et al. 2005; Guichoux et al. 2011), screening for the presence of null alleles in the data set, and the subsequent correction of allele frequencies (van Oosterhout et al. 2004).

Despite the availability of methods to detect and correct for null alleles (Chakraborty et al. 1992; Brookfield 1996), Dakin and Avise (2004) found that of 233 reviewed articles, 90% of the studies that identified null alleles merely reported

their presence and failed to take corrective action. This is in part due to the inability to use null-corrected allele frequencies in a variety of analyses that are based on individual multilocus genotypic data; for example, the popular Bayesian assignment tests implemented in the programs STRUCTURE 2.1 (Pritchard et al. 2000; Falush et al. 2003) and BAPS 5.4 (Corander et al. 2006, 2008) and the inference of parentage and kinship (Queller et al. 1993).

Some authors have made an effort to develop microsatellite markers for use in multiple species. For example, Dawson et al. (2010) identified microsatellites conserved across passerine birds by characterizing expressed sequence tag loci with sequence homology between the zebra finch and chicken genomes. The most common strategy, however, is to simply screen a number of nonspecific markers in a target species and to discard those loci that do not consistently amplify, are monomorphic, or deviate from Hardy–Weinberg equilibrium (HWE) (Chambers et al. 2004; Kim et al. 2004; Wilson et al. 2004; Miles et al. 2009).

If microsatellite loci are used across species boundaries, the presence of null alleles is increasingly likely as genetic distances increase (Jarne and Lagoda 1996; Dakin and Avise 2004). Barbará et al. (2007) found that the cross-species utility of microsatellite loci, including the level of polymorphism, was inversely correlated with taxonomic distance in a large number of animals, fungi, and plants. Not surprisingly, Hendrix et al. (2010) showed microsatellite cross-species amplification success was negatively correlated ($r = -0.84$) with increasing mitochondrial DNA (mtDNA) sequence divergence between species within the family Salamandridae. These authors also reported the amplification of a large number of loci over considerable genetic distances. Of 20 tested loci, 12 or 13 amplified in species with mtDNA divergence of 14–16%. However, these authors only tested a few individuals per species to see if the loci amplified and did not test for the presence of null alleles in population samples.

Despite the well-known null allele problem associated with using microsatellite markers across species boundaries (Panova et al. 2008), this practice is common in studies of a wide variety of organisms, including malaria vectors within the *Anopheles gambiae* complex. This complex is comprised of 7 morphologically indistinguishable species (Coluzzi et al. 1979; Favia et al. 1997; Hunt et al. 1998; Coetzee 2004), 2 of which are major malaria vectors and are intensively studied. Insight into the population genetics of these species can inform the potential introduction of transgenes into populations (e.g., Tabachnick 2003; Tripet et al. 2005), help to monitor and predict the spread of insecticide resistance alleles (e.g., Chandre et al. 1999; Weill et al. 2000; Reimer et al. 2005), and gauge the efficacy of malaria control campaigns (e.g., Pinto et al. 2002). Such studies were facilitated by the development of 131 microsatellite markers for *An. gambiae* by Zheng et al. (1996). These loci have since been used in the closely related malaria vector *A. arabiensis* for mapping studies (e.g., Slotman et al. 2004), studies investigating the population structure (e.g., Donnelly et al. 1999; Kamau et al. 1999; Onyabe and Conn 2001; Kent et al. 2007) and effective population size estimates (e.g., Simard et al. 2000; Wondji et al. 2005). Kent et al. (2007) evaluated 20 of the *An. gambiae* loci for use in *A. arabiensis* and found that only 12 amplified well. Furthermore, 2 of the 12 loci that amplified had failed to produce a PCR product in a previous study (Donnelly et al. 1999), demonstrating that cross-amplification success, and by extension, null alleles, can be population specific.

Based on a documented history of introgression between *An. gambiae* and *A. arabiensis* (Besansky et al. 2003; Slotman et al. 2007) as well as evidence from several immune genes (Parmakelis et al. 2008; Parmakelis et al. 2010), the genetic distance between *An. gambiae* and *A. arabiensis* appears to be less than between *An. gambiae* and *An. melas*, another member of the *An. gambiae* complex (Besansky et al. 2003). *Anopheles melas* is a brackish water breeding malaria vector confined to the west coast of Africa (Sinka et al. 2010), where it is an important malaria vector locally (e.g., Bryan 1983; Bryan et al. 1987). On Bioko Island, Equatorial Guinea, *An. melas* is an important and dominant malaria vector locally (Sharp et al. 2007; Slotman MA, unpublished data). Bioko Island has been subject to intensive vector control under the Bioko Island Malaria Control Project (BIMCP) since 2004 (Sharp et al. 2007; Kleinschmidt et al. 2009). Knowledge of the population genetic structure of *An. melas* and estimates of migration rates between Bioko and mainland Africa will better inform current control measures.

In a microsatellite study on 5 of the species of the *An. gambiae* complex, Wang-Sattler et al. (2007) used 42 *An. gambiae*–specific loci in an *An. melas* population from Senegal. The authors found that 4 of the loci did not amplify and that 5 loci were monomorphic in their *An. melas* sample. Unfortunately, this study did not examine the data for the presence of null alleles, so it is not known if the remaining 33 loci are useful for population genetic studies for this species. As part of the operational research component of the BIMCP, we have adapted *An. gambiae* microsatellite loci (Zheng et al. 1996) for use in *An. melas* by resequencing and evaluating 45 loci and examining a subset of these in natural populations of *An. melas* from a wide geographic region. This yielded 15 polymorphic microsatellite markers for use in this species. We also amplified 5 of these loci in 4 populations using the original *An. gambiae* primers and compared the 2 data sets. We discuss the implications for cross-amplification of microsatellite loci across species boundaries.

## Materials and Methods

### Mosquito Collections

Adult female of *An. melas* were collected from 4 countries along the West African coast. Mosquitoes from Equatorial Guinea (E.G.) were collected using CDC light traps and human landing catches from Cacahual (CAC) (lat 3°46′N, long 8°42′E) and Bomé (BOM) (lat 1°53′N, long 9°47′E) in October 2008 and Luba (LUB) (lat 3°27′N, long 8°33′E) in April 2009. Cacahual and Luba are located on Bioko Island, whereas Bomé is on the Equatorial Guinea

mainland. Mosquitoes were collected from Ipono, Cameroon (CAM) (lat 2°22′N, long 9°49′E), using human landing catches in December 2005. Resting female mosquitoes were collected using aspirators inside residences in Ballingho, The Gambia (GAM) (lat 13°30′N, long 15°36′W) in February 2010 and Ponta Abanaca, Guinea Bissau (GUI) (lat 11°18′N, long 16°14′W) in December 2009.

### Molecular Methods

Mosquito DNA was extracted from partial or whole mosquitoes using a Qiagen Biosprint 96 DNA extraction kit (Qiagen Inc., Valencia, CA). Species diagnostics were performed following Scott et al. (1993). A total of 45 *An. gambiae* microsatellite markers (Zheng et al. 1996) were selected based on chromosomal location, distance from each other, and location relative to chromosomal inversions on right arms of the second ($2Rm^1$, 2Rn, and $2Rn^1$) and third (3Re and 3Rc) chromosomes (Bryan et al. 1987). Markers inside polymorphic inversions known to be present in *An. melas* were excluded from this study, as these can create a false impression of population subdivision. Sequences containing the published microsatellite loci were downloaded from the *An. gambiae* genome (Holt et al. 2002) using VectorBase (Lawson et al. 2009). Primer3 0.4.0 (Rozen and Skaletsky 2000) was used to design primers located outside the original *An. gambiae* primer annealing sites. These primers were used to amplify 1–3 *An. melas* individuals from Ipono, Cameroon. Reactions contained 10–20 ng DNA template, with 1× PCR buffer (10 mM Tris–HCl pH 8.5, 50 mM KCl), 2.5 mM $MgCl_2$. Two hundred micromolars of each dNTP, 2.0 μM of each forward (F) and reverse (R) primer, 0.03 U of Promega GoTaq DNA Polymerase (Promega Co., Madison, WI), and $ddH_2O$ to the final 20 μl reaction volume. PCRs were performed with an initial denaturing time of 2 min at 94 °C followed by 5 cycles of 30 s at 94 °C, 30 s at 50 °C, 35 s at 72 °C, 30 cycles of 30 s at 94 °C, 30 s at 52 °C, 35 s at 72 °C, followed by a 15 min extension step at 72 °C.

PCR products were ligated in a pGEM-T vector (Promega Co.) and transformed into *Escherichia coli* competent cells. Colonies were grown in LB media overnight, and DNA was extracted using a Qiagen QIAprep Spin Miniprep Kit (Qiagen Inc.). Plasmid DNA was used as template in 10 μl sequencing reactions, which were performed in forward and reverse directions using BigDye Terminator 3.1 Cycle Sequencing Kit (Life Technologies Corporation, Carlsbad, CA), with a final primer concentration of 250 nM. Sequences have been submitted to GenBank under accession numbers JQ341414–JQ341458.

The *An. melas* sequences were used to design *An. melas*–specific primers using Primer3 0.4.0 (Rozen and Skaletsky 2000). As part of an initial screen, *An. melas*–specific primers were used to amplify 24 loci in 35 individuals from Cacahual and 43 individuals from Bomé, located on Bioko Island and the Equatorial Guinea mainland, respectively. PCR conditions were as above, except that a fluorescently labeled forward primer was used so PCR products could be analyzed on a 96-cappillary Applied Biosystems 3730*xl* DNA Genetic Analyzer (Life Technologies Corporation). To verify levels of polymorphism across a broader geographic scale, a second screen was conducted including 4 additional populations (LUB, CAM, GAM, and GUI).

Five of these 15 microsatellite loci (agxh25, agxh38, ag2h157, ag2h215, and ag3h127) were also amplified in these 4 *An. melas* populations using the original *An. gambiae*–specific primers published by Zheng et al. (1996) rather than the *An. melas*–specific ones. These 5 markers vary in the number of mismatches (1–3 bp) between the *An. gambiae* primer and the *An. melas* sequence (Supplementary Table S1). PCRs were performed and analyzed as outlined above.

### Data Analysis

Sequence data were aligned using Sequencher 4.9 (GeneCodes, Ann Arbor, MI), and genotypes were assigned using GeneMarker 1.85 (SoftGenetics, LLC, State College, PA). Observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), tests for deviation from HWE, and population pairwise $F_{ST}$ values were calculated using Arlequin 3.5.1.2 (Excoffier and Lischer 2010). Locus-specific $F_{ST}$ values were calculated using Genepop 4.0.10 (Raymond and Rousset 1995; Rousset 2008). Micro-Checker 2.2.3 was used to examine the data for the presence of null alleles (van Oosterhout et al. 2004), and allele frequencies were corrected for the presence of null alleles using the method of van Oosterhout et al. (2004). Null-corrected allele frequencies were then used to recalculate population pairwise $F_{ST}$ values.

### Ethical Considerations

Human landing catch protocols in Equatorial Guinea were reviewed and approved by authorities from the National Malaria Control Programme (NMCP) of the Equatoguinean Ministry of Health and Social Welfare. Collections were monitored by the lead entomologist of the NMCP. Malaria screening and treatment was provided under the NMCP. Landing catches in Cameroon were approved by the Ministry of Public Health. A national ethical clearance (No: FWA IRB00001954, dated 05/11/2005) was obtained from the National Ethics Committee (Yaounde, Cameroon) and an institutional ethical approval from OCEAC (No: 0287-05/SG/CAB, dated 03/17/2005) for the collections in Ipono, Cameroon. Volunteers received presumptive malaria treatment throughout the course of the study as recommended by the NMCP.

## Results

### *Anopheles melas*–Specific Microsatellite Markers

A total of 45 *An. gambiae* microsatellite loci were resequenced in 1–3 *An. melas* individuals. Out of 90 *An. gambiae* primers, 48 contained a mismatch with the obtained *An. melas* sequence. The number of mismatches between primer and annealing site ranged from 0 to 5 (Supplementary Table S1), with 21 primers containing more than a single mismatch; 14 primers contained 2 mismatches, 2 primers contained

3 mismatches, 3 primers contained 4 mismatches, and 2 primers contained 5 mismatches. A total of 42 of 90 primers did not contain any mismatches. In 2 loci (agxh100 and agxh678), no microsatellite repeat was present in *An. melas*. Because the mutation rate of microsatellites is positively correlated with repeat number (Weber 1990), only loci containing more than 5 uninterrupted repeats in the sequenced individuals were selected for adaptation to *An. melas*. This was done to obtain markers that are likely to contain a suitable amount of variability for a population genetic study. Nine loci were discarded for this reason. Two loci with only 5 continuous repeats were examined in *An. melas* populations from Bome and/or Cacahual, Equatorial Guinea, but both proved to be monomorphic. Locus agxh810 was discarded because it contained 36 uninterrupted repeats, which would likely have resulted in significant slippage during PCR amplification, making it difficult, if not impossible, to assign alleles to the correct size class. An additional 5 loci were discarded because single- or tri-nucleotide repeats were present in the flanking regions of the microsatellite repeat. Finally, on comparison of *An. melas* sequences, it was realized that ag3h312 and ag3h154 (Zheng et al. 1996) are in fact the same locus.

Therefore, 18 of 45 resequenced *An. gambiae* loci were not considered suitable as genetic markers. If mismatches were present, *An. melas*–specific primers were designed for the remaining 27 suitable loci. Additionally, several primers were redesigned for these loci because Primer3 predicted more optimal priming sites. Three of these *An. melas*–specific markers did not amplify in our test populations using standard conditions and were excluded (Supplementary Table S1). The remaining 24 loci were examined in populations from Cacahual and Bomé, and levels of heterozygosity are reported in Supplementary Table S2. Six of the examined loci contained a limited amount of polymorphism (low $H_o$ and/or low $N_a$); and 2 loci deviated significantly from HWE, in one or both of the test populations. Locus ag2h417 was excluded to prevent overrepresentation of chromosome 2R. Therefore, our resequencing effort yielded 15 microsatellite markers for use in *An. melas* (Table 1).

To better characterize these 15 loci, they were subsequently amplified in an additional 26 samples from Bomé as well as 48 *An. melas* specimens from each of 4 populations representing a wider geographic region covering as much as 1380 km. Table 1 reports average levels of genetic diversity for each locus. Both allelic diversity and heterozygosity levels were higher in mainland versus Bioko Island populations, with 2 loci (am2h603 and am3h753) being less variable than the other loci (Supplementary Table S2).

Out of 60 HWE tests, 4 indicated an excess of homozygotes: am2h793 in Ipono, am3h93 and am2h157 in Luba, and am2h143 in Ballingho (Supplementary Table S3). After Bonferroni correction, only locus am3h93 in Luba showed a significant excess of homozygotes. Microchecker detected the presence of null alleles in this locus/population combination, but also in locus am2h143 in Luba and am3h753 in Ipono, both of which did not deviate significantly from HWE.

### Cross-Amplification Using *An. gambiae* Markers

To examine if the original *An. gambiae* microsatellite markers produce a usable data set when applied to *An. melas* populations, 5 loci were amplified in 48 individuals in each of 4 *An. melas* populations using the original *An. gambiae* primers (ag2h#, ag3h#, or agxh#, respectively). These loci were chosen because they included a variable number of mismatches (1–3) between the *An. gambiae* primers and the corresponding *An. melas* sequence (Supplementary Table S4). The use of *An. melas*–specific primers resulted in slightly higher levels of heterozygosity than the *An. gambiae* primers; 65.92% of *An. melas*–primer individuals and 63.70% of *An. gambiae*–primer individuals amplified as heterozygotes. Most of this difference is due to differences at a single locus, as 21 individuals that were homozygous in the ag3h127 but not the am3h127 data set. Conversely, 3 individuals were heterozygous in the ag3h127 but not the am3h127 data set. In the agxh38 data set, 4 individuals were homozygous that were heterozygous in the respective *An. melas*–primer data set. In the other 2 loci, the respective data sets were in complete congruence with respect to heterozygosity levels. Overall, our data indicate that the use of nonspecific primers caused 2.6% (25/960) of individuals to amplify as false homozygotes using *An. gambiae* primers.

In the *An. gambiae*–primer data set, 2 of 20 tests showed a significant excess of homozygotes before Bonferroni correction: ag2h157 in Luba and ag3h127 in Guinea Bissau. Only the latter was significant after Bonferroni correction (Supplementary Table S4). Microchecker analyses confirmed the presence of null alleles in ag3h127 in Guinea Bissau but not in ag2h157 in Luba. Given that am2h157 also showed a slightly significant deviation from HWE, factors besides null alleles are likely responsible. Microchecker analyses also detected null alleles in ag3h127 in Ballingho, The Gambia, for which the excess of homozygotes was just below significant ($P = 0.0577$). Because no excess of homozygotes (or null alleles; Microchecker) were detected for am3h127 in Ballingho ($P = 0.633$) or Guinea Bissau ($P = 0.967$), these results indicate that the development of *An. melas*–specific primers for this locus circumvented the problem of null alleles in these 2 populations.

The *An. gambiae* loci chosen for cross-amplification in *An. melas* populations showed a varying number of mismatches (1–3) between the primers and the obtained *An. melas* sequences (Supplementary Table S4). Not surprisingly, the *An. gambiae* primers amplifying locus ag2h127, which has a null allele problem in 2 examined populations, had the largest number of mismatches; 2 and 3 in the forward and reverse primer, respectively. Single base pair mismatches in the other primers did not appear to lead to the occurrence of null alleles.

When comparing the $F_{ST}$ values derived from the *An. gambiae*–primer and *An. melas*–primer data sets (Table 2), the former showed somewhat higher levels of genetic differentiation between CAM versus GUI (0.20 vs. 0.18). $F_{ST}$ values calculated from null corrected allele frequencies still showed some disparity between the 2 data sets. The

**Table 1** Description of 15 *Anopheles melas* microsatellite loci

| Locus | *An. melas* primer (5′ → 3′) | *An. melas* repeat motif | $T_m$ (°C) | AS | AR (bp) | Mean $N_a$ | Mean *Ho* | Loc. | GenBank No. |
|---|---|---|---|---|---|---|---|---|---|
| amxh25 | F: AAAAGGGAAGCCGAAAACAT | (GT)3 + (GT)5 | 47.7 | 143 | 134–148 | 4.25 | 0.4688 | X: 2C | JQ341445 |
| | R: CAGTTATGCCGGCATGCTAC | | 53.8 | | | | | | |
| amxh38 | F: TCCAGTGACTACGCTTCTCG | (GT)26 | 53.8 | 236 | 180–244 | 11.25 | 0.7857 | 3R: 32A | JQ341446 |
| | R: TCAGCGCTATCACAGCAAAC | | 51.8 | | | | | | |
| am2h46 | F: GCGCCCATAGACAAAGAGAG | (GT)13 | 53.8 | 124 | 104–136 | 10 | 0.7458 | 2R: 7A | JQ341415 |
| | R: GAGGGTGCAGAACATTTACCA | | 52.4 | | | | | | |
| am3h93 | F: GTTGTCTCGCGCCGTCTA | (GT)26 | 52.6 | 306 | 276–332 | 15 | 0.7176 | 3R: 29A | JQ341431 |
| | R: TCGTCATCGTACATCAATACCC | | 53.0 | | | | | | |
| am3h127 | F: CGCCGGTACGTCATTAAACT | (GT)10 | 51.8 | 129 | 119–131 | 5.25 | 0.6495 | 3L: 41C | JQ341432 |
| | R: CTGGGAGTTCAGGGAATTGA | | 51.8 | | | | | | |
| am2h143 | F: TCTACGCACAAGGTCGTTTC | (CT)6 | 51.8 | 296 | 289–303 | 7.25 | 0.6802 | 2L: 25D | JQ341417 |
| | R: CGCACGTCTCTGTTATGCTC | | 53.8 | | | | | | |
| am2h157 | F: TTAAAGTGTGCACGGGAAATC | (TG)12 | 50.5 | 173 | 158–186 | 7.25 | 0.6326 | 2R: 9A | JQ341419 |
| | R: AGTTCGCGCAACTAGAAACG | | 51.8 | | | | | | |
| am2h215 | F: GGAACTGATTGTGGTGATCAAA | (GT)10 | 51.1 | 125 | 112–132 | 7.25 | 0.7712 | 2L: 24A | JQ341423 |
| | R: ACGGTTTGGTCTGCAAGTGT | | 51.8 | | | | | | |
| amxh293 | F: ACATCTTTCAGCACCACTGG | (GA)7 | 51.8 | 145 | 138–170 | 7.25 | 0.6950 | X: 4C | JQ341453 |
| | R: GGTGCCACATTGTGTTACTGA | | 52.4 | | | | | | |
| am3h555 | F: GTGGAGCAGCTGACCTCATT | (GT)13 | 53.8 | 155 | 140–232 | 10.75 | 0.6946 | 3R: 32C | JQ341437 |
| | R: TTGCCGTCTGATATGAATGC | | 49.7 | | | | | | |
| am2h603 | F: TGCACCGTTGATGCACATGC | (GT)6 | 53.8 | 111 | 107–111 | 2.5 | 0.1789 | 2L: 26D | JQ341426 |
| | R: GTTGGTTGTGGACGATGTGA | | 51.8 | | | | | | |
| am3h753 | F: GGCAAAACAGGATGGTCGT | (AC)6 + (AC)5 | 51.1 | 112 | 106–114 | 2.75 | 0.1952 | 3L: 44C | JQ341440 |
| | R: CAGGCCAATGAGGTATCGAG | | 53.8 | | | | | | |
| amxh755 | F: CAGCAGCAGCTGAACGATATT | (GT)8 | 52.4 | 156 | 149–159 | 4.5 | 0.6605 | X: 4A | JQ341456 |
| | R: AGGCAGCGGGTTAAAAAGAT | | 49.7 | | | | | | |
| am2h793 | F: TTACGACGGAATGCAATGTT | (TG)10 | 47.7 | 198 | 191–249 | 12.75 | 0.7119 | 2R: 8B | JQ341428 |
| | R: GTAATCGGCTCGTTTTCTGC | | 51.8 | | | | | | |
| amxh808 | F: CAGTGTGACCGAAGCTGTTG | (GT)15 | 53.8 | 177 | 152–178 | 7.5 | 0.7226 | X: 3D | JQ341457 |
| | R: AAACGGGTGGACACGATAAG | | 51.8 | | | | | | |

Loci were renamed to indicate *An. melas* specificity. We followed the Zheng et al. (1996) naming convention but replaced *ag* with *am*. $T_m$: *An. melas*–specific primer melting temperature; AS: allele size of original *An. melas* sequence; AR: allele range in test populations. Mean $N_a$: Mean allelic richness calculated across 4 test populations; Mean $H_o$: Mean observed heterozygosity calculated across 4 test populations; Loc.: Location on *Anopheles gambiae* chromosome. GeneBank No.: GenBank accession number.

other $F_{ST}$ values were very similar between the 2 data sets (Table 2). A comparison of locus-specific $F_{ST}$ values for marker am3h127 versus ag3h127 is presented in Table 3 (for other loci, see Supplementary Table S5.1–S5.4). Micro-checker analyses detected null alleles only in the population from Guinea Bissau (0.22) and The Gambia (0.11). The presence of these null alleles did have a substantial impact on $F_{ST}$ estimates in 3 pairwise comparisons. The presence of null alleles in the Guinea Bissau sample led to an overestimation of $F_{ST}$ values for IPO-GUI ($F_{ST}$ = 0.190 vs. 0.110) and GUI-LUB ($F_{ST}$ = 0.178 vs. 0.150). No differentiation was detected between GUI-GAM using either am3h127 or ag3h127. Additionally, the presence of null alleles in The Gambia population resulted in an overestimation of $F_{ST}$ in the IPO-GAM comparison ($F_{ST}$ = 0.184 vs. 0.159). As expected, the other 2 pairwise $F_{ST}$ estimates were very similar.

## Discussion

The usefulness of 45 microsatellite loci developed for the malaria mosquito *An. gambiae* was evaluated based on a set of criteria designed to determine their potential as polymorphic markers for *An. melas*. Out of 45 loci, 17 were not considered promising markers for use in *An. melas*. Of the

**Table 2** *Anopheles melas*–primer and *Anopheles gambiae*–primer amplified pairwise $F_{ST}$ and null-corrected pairwise $F_{ST}$ values

| | Ipono, Cameroon | Luba, Bioko | The Gambia | Guinea Bissau |
|---|---|---|---|---|
| Ipono, Cameroon | — | 0.332 (0.332) | 0.176 (0.176) | 0.202 (0.201) |
| Luba, Bioko | 0.338 (0.320) | — | 0.230 (0.230) | 0.247 (0.250) |
| The Gambia | 0.171 (0.171) | 0.236 (0.220) | — | 0.009 (*0.005*) |
| Guinea Bissau | 0.185 (0.185) | 0.251 (0.239) | 0.010 (0.010) | — |

Italicized value is nonsignificant with *P* value > 0.05. Lower diagonal: *An. melas*–specific primer $F_{ST}$. Upper diagonal: *An. gambiae* primer $F_{ST}$. Null-corrected $F_{ST}$ values in parentheses.

**Table 3** Comparison of $F_{ST}$ values for marker am3h127 versus ag3h127

| | Ipono, Cameroon | Luba, Bioko | The Gambia | Guinea Bissau |
|---|---|---|---|---|
| Ipono, Cameroon | — | 0.256 | 0.184 | 0.190 |
| Luba, Bioko | 0.255 | — | 0.175 | 0.178 |
| The Gambia | 0.159 | 0.170 | — | 0.000 |
| Guinea Bissau | 0.110 | 0.150 | 0.000 | — |

am3h127 $F_{ST}$ values are depicted below the diagonal. ag3h127 $F_{ST}$ values are depicted above the diagonal.

remaining loci, 6 contained very low polymorphism and 2 deviated significantly from HWE in 1 or both of the 2 *An. melas* populations from Equatorial Guinea. Additionally, 3 loci failed to amplify consistently. The result is 15 loci with varying levels of polymorphism in populations from a wider geographic area. The availability of these markers will facilitate genetic studies of this locally important vector along the West African coast.

Excluding the duplicate locus and locus ag2h417, 23 of 43 (53.48%) of the *An. gambiae* loci examined in this study are not readily suitable for use as polymorphic markers in its sibling species *An. melas* due to either a very low/high repeat number, low variability, or single- or trinucleotide repeats in the flanking sequence. This also does not include the 3 loci that failed to amplify or the 2 loci that are in HW disequilibrium even when using *An. melas*–specific primers, as additional effort presumably could have led to the development of better working primers. Besides null alleles, deviations from HWE can also be caused by the dropout of large alleles, poor sample quality, and population subdivision. The latter 2 are expected to affect a large number of loci, and we paid particular attention to detecting the very low multiple peaks typical of alleles with a very high number of repeats. Therefore, the fact that 2 loci showed a deviation from HWE even after designing species-specific primers, illustrates that null alleles are a potential problem even within a single species.

Based on 4 protein-coding genes examined by Besansky et al. (2003), the level of DNA divergence between *An. gambiae* and *An. melas* ranges from 1.8% to 6.8% (*Dxy*). Based on 32 protein-coding genes, Obbard et al. (2009) calculated an average divergence of 6.4% (*Ks*). The high number of microsatellite loci that had to be screened to yield sufficient polymorphic markers for a population genetic study demonstrates the difficulty of using microsatellite loci across species boundaries besides the occurrence of null alleles and the necessity of extensive screening of loci even between closely related species.

Of the loci that were amplified in *An. melas* populations with *An. gambiae*–specific primers, 4 of 5 yielded reliable data sets without the presence of null alleles. In all of these cases, the number of mismatches between the *An. gambiae* primer and the *An. melas* sequence was small, with single base pair mismatches present in one (agxh25, agxh38, and ag2h157) or in both primers (ag2h215). In locus ag3h127, 2 and 3 mismatches were present in the forward and reverse primers, respectively, and null alleles were detected when this locus was amplified in *An. melas* populations from The Gambia and Guinea Bissau. The null alleles at this locus resulted in an overestimation of the $F_{ST}$ values between some populations. Our limited data are consistent with a report based on a simulation study showing that null alleles lead to an overestimation of $F_{ST}$ values (Chapuis and Estoup 2007).

We also found that even between these very closely related species, 21 of a total of 90 primers contained more than a single base pair mismatch (2–5 bp). Although we did not examine the amplification success of all of these primers, we expect that the probability of null alleles occurring will increase with a higher number of mismatches. A single base pair mismatch between primers away from the 3′ region did not cause any null allele problems in our data set. Other studies have examined the amplification success of microsatellite markers in species other than the one they were designed for. Carreras-Carbonell et al. (2008), Hendrix et al. (2010), and Primmer et al. (2005) assessed the cross-species amplification efficiency of microsatellites in fish, true salamanders, and birds, respectively, with an emphasis on the correlation between mtDNA divergence and amplification success. All of these authors concluded, not surprisingly, that amplification success decreased with increased mtDNA genetic divergence. Both Primmer et al. (2005) and Carreras-Carbonell et al. (2008) report that microsatellite polymorphism also decreases with increased genetic distance from the target species, though only Primmer et al. (2005) warn against the potential for null alleles.

In their paper, Chambers et al. (2004) screened 47 human microsatellite markers for use in *Hylobates lar*, the white-handed gibbon. Of the screened loci, only 8 amplified well and were polymorphic when tested in 49 individuals from 12 social groups. Of the other 39 loci, 23 were excluded because they failed to amplify or amplified poorly, and the remaining 16 were mono- or dimorphic. These data also indicate that although a high proportion of loci may amplify across more distantly related species boundaries, this is not a good measure of their usefulness as many of such loci will not have the qualities that make them suitable genetic markers, such as lack of null alleles, high genetic variability, lack of flanking repeats with different repeat length, or lack of high repeat number.

A number of *An. gambiae* microsatellite loci (Zheng et al. 1996) have been used successfully in various studies of another sibling species in the complex, *A. arabiensis* (e.g., Donnelly et al. 1999, Kamau et al. 1999, Simard et al. 2000, Onyabe and Conn 2001, Wondji et al. 2005), even though Kent et al. (2007) found that only 12 of 20 *An. gambiae* loci amplified well in this species. The level of protein-coding gene divergence between *An. gambiae* and *A. arabiensis* is about half of that between *An. gambiae* and *An. melas* (Obbard et al. 2009). In addition, a history of introgression is well documented between the 2 former species (della Torre et al. 1997; Besansky et al. 2003; Slotman et al. 2005). This may account for some of the success in using *An.*

*gambiae* microsatellite loci in *A. arabiensis*, although numerous *An. gambiae* markers are available and the number screened before the start of a study is not always reported.

Although a common practice, the main hazard of using microsatellite markers across species boundaries is generally considered the occurrence of null alleles in the data set. Our results highlight another substantial problem; the rapid evolution of these markers renders many of them useless even in closely related species due to the absence of the repeat or low repeat number/low polymorphism. Researchers planning a study reliant on the amplification of markers across species boundaries are well advised to plan for the inclusion of 2–3 times the number of markers needed to produce the desired data set. This is particularly problematic for taxa for which only a limited number of microsatellite markers are available, and researchers should evaluate the need to develop species-specific microsatellite markers for their research subject.

## Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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