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PRIMER NOTE

# Isolation and characterization of polymorphic microsatellite loci in the plural cooperatively breeding superb starling, *Lamprotornis superbus*

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## Abstract

**Superb starlings (*Lamprotornis superbus*) are separate nesting plural cooperative breeders endemic to East Africa that show high levels of reproductive conflict and low reproductive skew. I isolated 31 polymorphic microsatellite loci from the superb starling genome and characterized them in approximately 31 individuals. The numbers of alleles per locus ranged from two to 16 and observed heterozygosities ranged from 0.03 to 0.88. Many of these loci amplified in other passerine species including the European starling, *Sturnus vulgaris*, and a variety of other starlings and mockingbirds.**

*Keywords*: cooperative breeding, microsatellite, paternity, starling, Sturnidae, *Sturnus vulgaris*

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The superb starling (*Lamprotornis superbus*) is a separate nesting plural cooperative breeder endemic to East Africa. Superb starlings live in social groups of 10–35 individuals with two to six breeding pairs (Fry *et al*. 2000). Birds defend group territories year-round and breed biannually during both the long and short rainy seasons. Here, I report the development of 31 polymorphic microsatellite loci for the superb starling that will be useful for studying sexual conflict, parentage and relatedness as part of a larger study examining the evolution of cooperative breeding in African starlings.

To isolate microsatellite markers, I used a modified enrichment protocol (Hamilton *et al*. 1999) that incorporated magnetic capture of streptavidin beads with biotinylated probes bound to microsatellite-containing genomic fragments (Kijas *et al*. 1994). Blood was collected from superb starlings in Queens lysis buffer (Seutin *et al*. 1991) at study sites at the Mpala Research Centre, Kenya. Using a DNeasy Tissue Kit (QIAGEN), I extracted genomic DNA from three males and three females. Genomic DNA was pooled and digested with two restriction enzymes (*Hinc*II, *Rsa*I) and simultaneously ligated to SNX linker sequences (Hamilton *et al*. 1999). I enriched for dinucleotide repeats with synthetic, single-stranded biotintylated repeats of $(CT)_{15}$ and $(GT)_{15}$ and then combined the digests for capture of microsatellite-enriched fragments by binding to streptavidin beads (Dynabeads, Dynal) (Kijas *et al*. 1994). Enriched fragments were made double-stranded by polymerase chain reaction (PCR), digested with *Nhe*I and ligated into *Xba*I-digested, dephosphorylated pUC19 (New England BioLabs). I then transformed the clones into MAX Efficiency DH5α competent *Escherichia coli* (Invitrogen) and grew them on Luria–Bertani agar plates with ampicillin. Bacterial colonies were transferred to nylon membranes (MagnaLift; GE Osmonics), hybridized with $(CT)_{12}$ and $(GT)_{12}$ oligonucleotides end-labelled with [$\gamma^{33}$P] dATP (T4 Polynucleotide kinase RTG kit; Pharmacia) and washed at 52 °C with a 3M tetramethylammonium chloride buffer.

I produced minipreps (Sambrook *et al*. 1989) for 133 positive clones, amplified the inserts with M13 forward and reverse primers and sequenced them on a 3100 Genetic Analyser (Applied Biosystems) using BigDye™ Terminator cycle sequencing system version 3.1 (Applied Biosystems). Of the 133 sequenced inserts, 96 of 109 (88%) with good sequence contained a microsatellite repeat and of those, 67 (62%) contained unique microsatellite repeats. I designed primers for 47 of the 67 unique microsatellite-containing sequences using PrimerSelect (DNAStar) and

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ultimately developed 45 microsatellite loci for the superb starling. Forward primers were labelled using the fluorescent dyes 6-FAM™, NED™, PET™ and VIC™ (Applied Biosystems). To test for variability, I screened 17–32 adult individuals (mean 31) chosen randomly from a population of > 400 individuals from 10 social groups (Table 1). Each 10 µL PCR contained: 0.5–1 U JumpStart™ *Taq* DNA polymerase (Sigma), 0.2 mm dNTPs (Invitrogen), 10 mm Tris-HCL (pH 8.3), 50 mm KCl, 1–3 mm $MgCl_2$, 0.15 µm of forward and reverse primers and 1–50 ng of genomic DNA. PCRs consisted of 35 cycles at 94 °C for 50 s, $T_a$ for 1 min, 72 °C for 1 min and a final extension at 72 °C for 4 min 30 s. I also tested six markers — four of which were polymorphic in the European starling (Loyau *et al.* 2005) — developed for other passerine species using the same methods (Appendix 1).

Genotyping was performed on a 3100 Genetic Analyser (Applied Biosystems) and fragment sizes were scored using GENEMAPPER™ 3.0 software (Applied Biosystems). Of the 45 superb starling loci screened, 31 (69%) were polymorphic and had between two and 16 alleles (mean 4.6) (Table 1). Observed heterozygosities ($H_O$) ranged from 0.03 to 0.88, whereas expected ($H_E$) heterozygosities ranged from 0.03 to 0.90 (Table 1) (CERVUS 2.0; Marshall *et al*. 1998). I did not conduct tests of Hardy–Weinberg equilibrium because individuals were sampled from multiple small social groups with a high likelihood of inbreeding. None of the markers showed strong evidence of linkage disequilibrium (all $P > 0.001$ after sequential Bonferroni correction) (GENEPOP 3.4; Raymond & Rousset 1995). I tested all 31 markers for cross-species amplification on other species of Sturnidae, as well as species in the sister taxa Mimidae and other species of Passerines, and found that 28 (90%) of the markers amplified product for at least one species (Table 2). Since these 31 markers had a mean expected heterozygosity of 0.42 and yielded a combined parental exclusionary power of 0.99998, they will be useful for examining family relationships and the social structure of the plural cooperatively breeding superb starling.

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**Table 1** Characterization of 31 polymorphic microsatellite loci from the superb starling (*Lamprotornis superbus*). Recommended annealing temperatures ($T_a$), $MgCl_2$ concentrations and amounts of *Taq* are listed for each locus. The product size range and number of alleles for each locus are given for sample sizes of *N* individuals. Observed ($H_O$) and expected ($H_E$) heterozygosities were calculated using the program CERVUS 2.0 (Marshall *et al*. 1998)

| Locus | GenBank Accession no. | Repeat motif | Primer sequence (5′–3′) | $T_a$ (°C) | $MgCl_2$ (mm) | *Taq* (U) | Product size range (bp) | No. of alleles | *N* | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SS1-6 | AY906875 | $(CA)_{15}$ | F: TTTCACTGGCTGGATCTGGTAAACC<br>R: CTAGCAACATATAGCCCAAGCTGTATTGAT | 60 | 2.0 | 0.5 | 196–212 | 8 | 32 | 0.88 | 0.80 |
| SS2-10 | AY906876 | $(GT)_{10}(CTGT)_3(GT)_5CACT(GT)_4CT(GT)_5CAGTAT(GT)_2GGGTCT(GT)_2$ $GGGTGTATCT(GT)_2CT(GT)_2(CT)_2(GT)_5CT(GT)_6CT(GT)_6(CTGT)_2(GT)_4$ $CT(GT)_6CT(GT)_5CAGTAT(GT)_{17}$ | F: ATTCAGAAGTGGGATGTTGTGACAGTG<br>R: CTGACAAGAGATTCTCAAGAAGCAACAC | 59 | 1.0 | 1.0 | 314– 513 | 14 | 32 | 0.44 | 0.89 |
| SS1-11 | AY906870 | $(AG)_{20}AC(AG)_3(GGA)_2GTGC(GT)_4GA(GT)_{10}$ | F: AAATTTGAACCGATCCAGCCTGTTTA<br>R: CTCGCTCCCTCTCCCTCTTTCAC | 61 | 2.0 | 1.0 | 180–192 | 8 | 31 | 0.52 | 0.83 |
| SS1-12 | AY906871 | $(AC)_{11}$ | F: TATTTTCCCTTTTCTTCCCTTAGCAG<br>R: GAAGCCAACAATATGTACAGAATGTGC | 60 | 2.0 | 1.0 | 207– 213 | 4 | 31 | 0.13 | 0.13 |
| SS1-15 | AY906872 | $(GA)_8(GT)_{10}$ | F: ATCCCCTTCTGCATTGATCTGTGT<br>R: CACCCGACTCTGATGGCAAGTAG | 58 | 2.0 | 0.5 | 308–316 | 4 | 31 | 0.48 | 0.45 |
| SS2-16 | AY906886 | $(AT)_2(GT)_{11}$ | F: CTGTATTGCCACCTTCCCTTGTAGTTC<br>R: TTAGGTGGCTTTCTGAGCAGGTTATGT | 62 | 2.0 | 0.5 | 196–198 | 2 | 31 | 0.16 | 0.20 |
| SS2-28 | AY906887 | $(AC)_6AT(AC)_5$ | F: AGACCGCTACCTTCTTTTCCTA<br>R: TCTTCACAATTAAGAATATTTAATTAAAAA | 58 | 2.5 | 1.0 | 168–170 | 2 | 30 | 0.07 | 0.07 |
| SS2-29 | AY906888 | $(TA)_2TG(TA)_4TG(CA)_4CT(CA)_4$ | F: AAATAAAGCAGAAATCAGATAAAAGTCC<br>R: TCTGTTTTGTTTCTCATAAAATTTTAACA | 58 | 2.5 | 1.0 | 171–173 | 2 | 30 | 0.03 | 0.03 |

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**Table 1** *Continued*

| Locus | GenBank Accession no. | Repeat motif | Primer sequence (5′–3′) | $T_a$ (°C) | $MgCl_2$ (mM) | *Taq* (U) | Product size range (bp) | No. of alleles | *N* | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SS2-32 | AY906889 | (TG)$_{13}$ | F: GGTATCACCATATCTGCTGCCAGTA<br>R: CAGGCTTTTGCTGACAATTATTTTG | 58 | 2.0 | 0.5 | 246–252 | 4 | 31 | 0.36 | 0.34 |
| SS2-40 | AY906892 | (CA)$_{11}$ | F: AACACTAAAGCACTGTTAATTCACAC<br>R: GATTCTGGAGTTCTAATCCTGAGAA | 62 | 2.0 | 1.0 | 180–184 | 2 | 31 | 0.29 | 0.25 |
| SS3-42C | AY906917 | (CA)$_2$TACG(CA)$_{20}$CTCG(TG)$_2$ | F: TATATCCCAGGGAGGGTTGTGGTGTG<br>R: ATCAAACTGCAGCAGGACTCTGACTGTG | 60 | 2.0 | 0.5 | 150–184 | 16 | 32 | 0.84 | 0.90 |
| SS2-45 | AY906894 | (AC)$_9$AACT(AC)$_5$TC(TA)$_2$(TG)$_2$ | F: CAGTGATGAACGCCTCCTATAACAGCT<br>R: GTAGGCACCATTGAATGCTGAGTGAC | 58 | 2.0 | 0.5 | 263–269 | 3 | 30 | 0.20 | 0.57 |
| SS2-46 | AY906895 | (CA)$_9$TG(CA)$_2$ | F: TTATGGGTAAACTTTCTGTACACTGG<br>R: ATTCCAAATCTAAACACTCTGAGGTT | 62 | 2.0 | 1.0 | 183–189 | 3 | 31 | 0.23 | 0.26 |
| SS2-49 | AY906896 | (AC)$_8$ | F: TGCCACTGCATTCAATCCA<br>R: ACACTTCAGTTTAATAGCTATCTTGGTG | 60 | 2.0 | 0.5 | 114–118 | 3 | 31 | 0.45 | 0.50 |
| SS2-52 | AY906898 | (CA)$_5$TA(CA)$_4$(TA)$_2$CAAA(CA)$_3$ | F: CTTCGGGCGATGCAATAGATGAG<br>R: CATTGAGCTGGCACTGGAATCAAA | 60 | 2.0 | 0.5 | 356–358 | 3 | 31 | 0.16 | 0.26 |
| SS2-53 | AY906899 | (TA)$_3$(TG)$_6$ | F: ACAAAATTAATTCTCATTCTTCCAGAG<br>R: ACACTCCTAAAAATCACAAACTGAAA | 56 | 2.5 | 0.5 | 163–167 | 2 | 30 | 0.03 | 0.03 |
| SS2-56 | AY906901 | (TC)$_5$TG(TC)$_5$ | F: AACCACAAAGCTCACGCTGGTACA<br>R: TGGAAACTGCAGCTGGTCCTCAA | 60 | 2.5 | 0.5 | 82–83 | 2 | 32 | 0.03 | 0.03 |
| SS2-68 | AY906905 | (GT)$_6$AT(GT)$_7$ | F: AACTTGCTGGTTGAAAATTTTAATG<br>R: TGTTCTTTAATTGTTACTCAGAAGTGAA | 60 | 2.0 | 1.0 | 142–148 | 4 | 31 | 0.52 | 0.54 |
| SS2-71B | AY906906 | (TG)$_{18}$ | F: CACACCCAACATGTAACAAATCTTACA<br>R: CTTTGAGCCTCTGCTTTTAGAAATTG | 62 | 2.0 | 0.5 | 332–338 | 5 | 31 | 0.74 | 0.67 |
| SS2-76 | AY906908 | (TG)$_7$TC(TG)$_2$ | F: TCCTCTTGTACTGCCTCTATTTTCTTGT<br>R: AGCATGGCAGTCACTTTTTCTCC | 60 | 2.0 | 0.5 | 154–171 | 6 | 32 | 0.22 | 0.24 |
| SS2-78 | AY906909 | (AG)$_{10}$ | F: TGAACTTTCTTACGCACTTATATCACAACA<br>R: ATTACCCAATGAGCTACTGTCCTGTCTT | 58 | 2.0 | 1.0 | 277–281 | 3 | 30 | 0.77 | 0.49 |
| SS2-80 | AY906910 | (CA)$_8$AAA(CA)$_3$TA(CA)$_2$ | F: ACCCACTTTTACCTACCTAGCATGTTCTGT<br>R: ATTAGAGTGCCCAAGGACTTGTTCTCA | 58 | 2.0 | 0.5 | 320–326 | 4 | 31 | 0.19 | 0.29 |
| SS2-82 | AY906911 | (AC)$_3$AA(AC)$_5$AT(AC)$_3$ | F: CTGGGGCAAACCCTAACTAATTTCTG<br>R: GGTGCATGAAAAATATGGGTCAAAATAC | 60 | 2.0 | 0.5 | 285–289 | 2 | 28 | 0.32 | 0.43 |
| SS2-83 | AY906912 | (AC)$_{13}$ | F: TGTCCCAGTTTTACATTTTTGTTAA<br>R: GACTTTTAATATGGAGCCTGCTCT | 58 | 2.0 | 1.0 | 162–176 | 7 | 32 | 0.72 | 0.73 |
| SS2-88 | AY906913 | (CT)$_2$(GT)$_6$ | F: GTCTAGAGTAGTGTTTAAACCCATATACTT<br>R: AACAGCTAACACCCCACCTC | 52 | 1.5 | 1.0 | 101–105 | 2 | 32 | 0.34 | 0.40 |
| SS2-106 | AY906877 | (GT)$_9$ | F: TGTGTTATCCCATTGTAAGGGCTCTTT<br>R: GACTCTAGGTGGAAACCCCCATTTT | 56 | 3.0 | 1.0 | 292–301 | 5 | 32 | 0.25 | 0.36 |
| SS2-114 | AY906878 | (GT)$_{16}$(AT)$_2$(GTAT)$_2$(GT)$_3$ | F: TGAGGCCTGCTTCAGATTTTTCAT<br>R: CCATATTGAGTATTGGGACATGTGAGTTAT | 60 | 2.0 | 1.0 | 410–414 | 3 | 31 | 0.13 | 0.15 |
| SS2-119* | AY906879 | (TG)$_4$CA(TG)$_{12}$ | F: CGGTTAAGTATGCAATGCACCTTTTG<br>R: GCCCTGTCCCAACCCCTCAT | 61 | 2.0 | 0.5 | 273–275 | 2 | 30 | 0.30 | 0.46 |
| SS2-121 | AY906880 | (AT)$_2$GTCT(AT)$_5$(AC)$_7$ | F: AGGTGCACAAGTTCTGCTCCTCAGTAT<br>R: AACTTTCCTTTTCAACCCGCTATCC | 61 | 2.0 | 1.0 | 167–172 | 6 | 32 | 0.78 | 0.79 |
| SS2-130 | AY906884 | (CT)$_3$C(CA)$_7$CCAGTGAG(CA)$_2$TG(CA)$_5$ | F: CTGAAGGCACCCAGCAGGTTCT<br>R: AGACCCACTGTGATAATTACCACTTCTCTG | 58 | 2.0 | 0.5 | 261–267 | 4 | 30 | 0.50 | 0.61 |
| SS2-132 | AY906885 | (AC)$_6$CC(AC)$_8$AA(TC)$_8$ | F: TATATTGACACCCTCGCTGGAATCC<br>R: TTTCGTAAGGTGGCAAACTTCTGTGT | 60 | 1.5 | 1.0 | 224–252 | 6 | 32 | 0.38 | 0.36 |

*high flanking sequence similarity with microsatellite sequence from the mallard (*Anas platyrhynchos*) (GenBank Accession no. AY493257).

**Table 2** Cross-species amplification of 31 polymorphic superb starling microsatellite markers. I tested one individual of each species for each microsatellite marker and visualized them on a 2% agarose gel. Markers that did not amplify are indicated by (–), whereas markers that amplified brightly are indicated by (+) and those that only weakly amplified are indicated by (W)

| | Sturnidae | | | Mimidae | | Other species | | |
|---|---|---|---|---|---|---|---|---|
| Locus | European starling (*Sturnus vulgaris*) | Spotless starling (*Sturnus unicolor*) | Common myna (*Acridotheres tristis*) | Northern mockingbird (*Mimus polyglottos*) | Tropical mockingbird (*Mimus gilvus*) | House finch (*Carpodacus mexicanus*) | Banded wren (*Thryothorus pleurostictus*) | Bicknell's thrush (*Catharus bicknelli*) |
| SS1-6 | + | + | + | + | + | + | – | + |
| SS2-10 | – | – | – | – | – | – | – | – |
| SS1-11 | + | + | + | + | + | + | + | + |
| SS1-12 | + | + | – | + | + | + | + | + |
| SS1-15 | – | – | – | + | + | – | – | – |
| SS2-16 | + | + | + | + | + | + | + | + |
| SS2-28 | – | – | W | – | – | – | – | – |
| SS2-29 | + | + | + | + | + | – | – | – |
| SS2-32 | + | + | + | + | + | + | + | + |
| SS2-40 | + | + | + | + | + | – | – | – |
| SS3-42C | + | + | + | + | + | +* | + | + |
| SS2-45 | + | + | + | – | – | – | – | – |
| SS2-46 | + | + | + | – | – | – | – | – |
| SS2-49 | – | – | W | + | + | – | – | – |
| SS2-52 | + | + | + | + | + | + | + | + |
| SS2-53 | – | – | + | +† | + | – | – | +* |
| SS2-56 | + | + | + | + | + | + | – | + |
| SS2-63 | + | + | – | + | + | – | – | + |
| SS2-68 | + | + | – | + | + | + | – | + |
| SS2-71B | + | + | + | – | – | – | W | – |
| SS2-76 | W | W | W | W | W | – | – | – |
| SS2-78 | – | – | – | – | – | – | – | – |
| SS2-80 | + | + | + | + | + | – | + | + |
| SS2-82 | – | – | – | + | + | + | – | + |
| SS2-83 | + | + | + | + | + | + | – | + |
| SS2-106 | + | + | + | + | + | + | + | + |
| SS2-114 | + | + | + | + | + | – | – | + |
| SS2-119 | + | + | + | + | + | + | + | + |
| SS2-121 | – | – | – | – | – | – | – | – |
| SS2-130 | + | + | + | + | + | + | + | + |
| SS2-132 | – | – | – | W | – | – | – | – |

*bands of an unexpected size; †double bands.

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**Appendix 1** Six microsatellites developed for other species cross-amplified on the superb starling (*Lamprotornis superbus*) and were polymorphic. Recommended annealing temperatures ($T_a$), $MgCl_2$ concentrations and amounts of *Taq* are listed for each locus. The product size range and number of alleles for each locus are given for sample sizes of *N* individuals. Observed ($H_O$) and expected ($H_E$) heterozygosities were calculated using the program CERVUS 2.0 (Marshall *et al*. 1998)

| Locus | Initial species | Citation | Repeat motif | Primer sequence (5′–3′) | $T_a$ (°C) | $MgCl_2$ (mM) | *Taq* (U) | Product size range (bp) | No. of alleles | *N* | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ase18 | Seychelles warbler (*Acroephalus sechellensis*) | Richardson *et al*. (2000) | $(GT)_{12}$ | F: ATCCAGTCTTCGCAAAAGCC<br>R: TGCCCCAGAGGGAAGAAG | 51 | 1.5 | 0.5 | 187–189 | 2 | 28 | 0.29 | 0.25 |
| FhU3 | Pied flycatcher (*Ficedula hypoleuca*) | Primmer *et al*. (1996) | $(GT)_8A(TG)_{12}$ | F: ATATTCCCCATAAGATAATGG<br>R: ATAGTGTTGTCTTAAGGTCTCT | 48 | 3.0 | 0.5 | 172–176 | 5* | 29 | 1.00 | 0.74 |
| Mjg1 | Mexican jay (*Aphelocoma ultramarina*) | Li *et al*. (1997) | $(AAAG)_{>20}$ | F: CCCGGGAAAGGCTTCGTCTTC<br>R: GGAGATTTTATATCGGTGGC | 58 | 2.5 | 0.5 | 146–153 | 8* | 29 | 0.76 | 0.76 |
| MpAAT 24 | Northern mockingbird (*Mimus polyglottos*) | Hughes & Deloach (1997) | $(AAT)_6 \ldots (AAT)_6$ | F: CATGGACAGGCAGGATAGAC<br>R: TGGCAGAGAGGAACAAAGATA | 55 | 2.0 | 0.5 | 121–133 | 3 | 30 | 0.40 | 0.51 |
| MpAAT 25 | Northern mockingbird (*Mimus polyglottos*) | Hughes & Deloach (1997) | $(AAT)_9$ | F: ACCAAATTCCCCACAGAGAC<br>R: GCTGGGGAGCACTGGTGTA | 55 | 2.0 | 0.5 | 94–98 | 2 | 20 | 0.25 | 0.22 |
| Pca7 | Blue tit (*Parus caeruleus*) | Dawson *et al*. (2000) | $(TG)_{24}$ | F: TGAGCATCGTAGCCCAGCAG<br>R: GGTTCAGGACACCTGCACAATG | 57 | 2.0 | 0.5 | 90–94 | 2 | 29 | 0.00 | 0.07 |

*confidence in marker is low since alleles show many stutter bands and are only separated by one bp.