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***Title of the Article***

**A population genetics study of Pale-winged Starlings, *Onychognathus nabouroup,* using novel microsatellite markers.**

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**Cover letter**

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Kristen McNealy
Managing editor
*Ecology and Evolution*

April 21, 2021

Dear Editor

We wish to submit an original research article entitled '' **A population genetics study of Pale-winged Starlings, *Onychognathus nabouroup,* using novel microsatellite markers**'' for consideration by *Ecology and Evolution.*

We confirm that this article is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

In this paper we describe the development of novel microsatellite markers in Pale-winged Starlings, *Onychognathus nabouroup*; a songbird commonly found in southern Africa. Starlings have recently received much attention with regards to vocalisation studies, with many parallels between human speech and birdsong being explored. The developed markers were used to further link the effects of genetic diversity to social structure, behaviour and relatedness in Pale-winged Starlings from two geographic regions in southern Africa.
We have no conflicts of interest to disclose.

Please address all correspondence concerning this manuscript to me at naadhirah.munshi@gmail.com

Thank you for your consideration of this manuscript.

Sincerely,

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# A population genetics study of Pale-winged Starlings, *Onychognathus nabouroup,* using novel microsatellite markers

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## Abstract

Recent research into starling species has revealed the existence of vocal social markers and a link between song temporal structuring and social organisation. The aim of the present study was to develop a genetic tool for understanding the population structuring and behaviour (social/parental transmission) and mating in Pale-winged Starlings (*Onychognathus nabouroup*), a songbird which is found in arid areas of southern Africa. Using next-generation sequencing, microsatellite markers comprising six dinucleotides, eighteen trinucleotides and twenty-four tetra-nucleotides specific to the Pale-winged Starling were isolated and developed. A total of 77 birds were sampled from the Augrabies Falls Nature Reserve in South Africa (n=53) and the Ai Ais-Richtersveld Transfrontier Park resort in Namibia (n=24), respectively. Fifteen polymorphic microsatellite markers were genotyped. The statistical programme STRUCTURE revealed four different genetic clusters within the two populations. There is low genetic divergence (mean Fst value of 0.01) between the two populations, which is supported by the mean number of effective migrants (22.45) between the populations. ML-Relate data analysis indicated that all individuals sampled from both populations have relatives within and across the two populations with three exceptions in the Augrabies Falls Nature Reserve region. Birds from either population migrate and join the other population maintaining gene flow between the two populations. Each population has a high degree of genetic diversity present between individuals. There is little inbreeding and high allelic richness in both sampled populations, which will allow them to adapt to future environmental changes. The developed microsatellites have inferred information for the success of this species. Social structure, relatedness and behaviour were inferred and regardless of genetic relationships these birds maintain a stable social environment and harbour strong social bonds between same and opposite sex group members as well as mates.

*Keywords*: Pale-winged Starling, *Onychognathus nabouroup*, microsatellites, population genetics, genetic diversity, polymorphism.

## Introduction

The study of sociogenomics encompasses a comprehensive understanding of social life in molecular terms (Robinson *et al*., 2005). Analysing social behaviour at the molecular level can help to understand how complex and highly derived patterns of social behaviour have evolved from simpler ancestral behaviour. Both solitary and social animals need to perform many activities during their lifetime for survival and reproduction (Alcock, 1998). Most animals need to mate to reproduce, which requires a complex repertoire of behaviours such as mate recognition or courtship (Robinson *et al*., 2005). Social animals often use various mechanisms to achieve coordination in their populations with communication between individuals and especially pair members being the most important (Alcock, 1998, Hausberger *et al*., in rev.).

Microsatellites have enhanced the way we view mating systems, genetic variation and gene flow among species and populations. They have also become common practice in studies investigating parentage or species relatedness (Germain-Aubrey *et al*. 2016; Crochet *et al*. 2003). Relatedness refers to the proportion of shared genes between individuals (Koenig & Dickinson, 2016). It is a determining factor in understanding how altruism works in nature and influences the behaviour of populations, communities, or species (Hamilton, 1982). Studies of social birds have found that strong and stable social bonds are correlated with increased longevity, offspring survival and territorial establishment (van Overveld, 2020). The relatedness of individuals in a population affects the social structures and behaviour of the populations. Kin recognition is the ability to recognize the degree of relatedness with other individuals (Hepper, 1991). Identifying the level of relatedness has a direct effect on the level of fitness of individuals present in the populations (Riehl & Strong, 2015). In cases where males do not recognize nestlings as their own, they withdraw care and, in some cases, kill the nestling. A study on house sparrows (*Passer domesticus*) found that the relatedness of individuals affected the level of aggression between individuals while scrounging and foraging (Toth *et al*., 2009). The aggressive form of scrounging was implemented less against related birds when compared to unrelated birds (Toth *et al*., 2009). However, in some birds such as the greater ani (*Crotophaga major*), males and females are unable to recognize their own nestlings which results in all group members participating in nest defence and food delivery (Riehl & Strong, 2015). The stable social relationships between unrelated females increased individual fitness in this bird species (Riehl & Strong, 2015).

Heterozygosity or genetic diversity of individuals reflects across multiple traits which are most likely used by females during mate choice decisions (Ferrer *et al*., 2015). In addition, a study on Golden Whistlers (*Pachycephala pectoralis*) used microsatellites to demonstrate the positive influence of genetic diversity on mating success and ornamentation by determining the amount of genetic diversity present in this species (van Dongen & Mulder, 2009).

Southern Africa is home to fifteen starling species (Craig & Feare, 2009). The colonial monogamous Pale-winged Starlings have the ability to produce both whistles and warbling songs, like other Sturnid species (Hausberger, 1997, Houdelier *et al*., 2012). Previous research into starling species has focused on understanding vocalisations and social structures of these bird species based on ecology and morphology (Henry *et al*., 2015a,b). In the context of a long term project by LIA Vocom (now called IRP Vocom), populations of Pale-winged Starlings have been ringed and followed over years, revealing long-term pairing and atypical patterns of breeding such as an overlap of moulting and breeding, but with sex differences (Craig *et al*., 2015). In order to disentangle the link between population structure, social dynamics and vocal changes, a thorough knowledge of its genetic patterning is needed. Molecular research to date in starlings comprises of microsatellite markers isolated from Spotless Starlings (*Sturnus unicolor*) which were used for comparative studies between the Spotless and European Starling (*Sturnus vulgaris*) (Celis *et al*., 2007) and microsatellite markers isolated form in the Superb Starling (*Lamprotornis superbus*) (Rubenstein, 2005). Microsatellite markers developed from Spotless Starlings and Superb Starlings could be used for comparative studies however, to understand a species at a population or intraspecific level, markers would need to be sensitive and polymorphic and from the sample population.

The ability of microsatellite markers to cross amplify in different species of the same genus is based on the conserved flanking regions of these species (Ellegren *et al*., 1995). However, the success rate of amplification decreases as the genetic distance increases, with birds exhibiting only a 50% success rate of transferability of these markers (Ellegren *et al*., 1995; Lillandt *et al*., 2003). Furthermore, intraspecific and population genetic variation would be best detected by markers isolated from their host species. In the present study we isolated 48 microsatellite markers from genomic DNA of *Onychognathus nabouroup*. Fifteen out of the 48 developed microsatellite markers were further selected based on their polymorphic strength and used to gain insight into the social structure and behaviour of Pale-winged Starling populations and to determine the level of genetic diversity present within and between them.

## Methods and materials

*Collection of blood samples*

Blood and feather samples from two populations of Pale-winged Starlings (N=77) were collected: Augrabies Falls Nature Reserve, Northern Cape Province, South Africa (n=53) and Ai Ais-Richtersveld Transfrontier Park, Namibia (n=24). The sample sites are located 400 km from each other. Qualified ringers recognised by SAFRING (Permit 296) captured and ringed the birds and feather samples were collected under the license number R-2012MH01. Blood samples were collected by the VOCOM (Evolution of vocal communication: testing the impact of social systems, phylogeny and conditions of life) team in October 2016 and November 2017, under the ethics approval code RU-LAD-15-09-0001 and agreement number HAUM1381.

*Genomic DNA extraction*

Total genomic DNA extractions were performed according to the method of Blin and Stafford (1976). In brief, the blood samples were stored in ethanol at 4°C and evaporated prior to extraction. The samples were resuspended in 570µl Queen's (Tris-EDTA, Sodium Chloride) buffer (Loparev *et al*., 1991). Samples were vortexed and centrifuged (13 000 rpm for 6 minutes) and the supernatant was discarded. The pellet was resuspended in 570µl STE (Sodium Chloride, Tris-Cl, EDTA) buffer, 30µl 10% SDS, 2µl RNase and 3µl proteinase K (Wiegers & Hilz, 1971; Loparev *et al*., 1991) and incubated at 50°C for 2 hours. The lysate was treated with phenol:chloroform:isoamylalcohol (25:24:1, v/v) and centrifuged (13 000 rpm for 8 minutes). The aqueous phase containing the nucleic acids was recovered and the phenol:chloroform:isoamylalcohol (25:24:1, v/v) step repeated on the remaining solution to maximise DNA yield (Cler *et al*., 2006). DNA was then precipitated in 95% ethanol (v/v) and 3M sodium acetate (v/v) for 30 minutes at -20°C (Cler *et al*., 2006). The precipitated DNA was collected by centrifugation (13 000 rpm for 10 minutes) and subsequently washed in 70% ethanol (v/v) before resuspension in 100ul TE buffer (Loparev *et al*., 1991).

*Development of microsatellite markers*

A library was prepared using the genomic DNA extracted from blood of seven individuals (four from Augrabies Falls Nature Reserve and three from Ai Ais-Richtersveld Transfrontier Park) using the Illumina TruSeq Nano library preparation kit and analysed on the Illumina MiSeq sequencing platform using a nano v2 500 cycles sequencing chip at Ecogenics (Balgach, Switzerland). The resulting paired-end reads which passed Illumina's quality filters were subjected to de-multiplexing and adapter trimming. Read quality was analysed using FastQC v0.117 (Andrews, 2010). High quality paired-end reads were merged using USEARCH v10.0.240 (Edgar, 2010). The resulting 99,009 merged reads were screened with the software Tandem Repeats Finder, v4.09 (Benson, 1999). After this process, 4,811 merged reads contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. Primer design was performed with

primer 3 (Untergasser *et al*., 2012). Suitable primer design was possible in 2,937 microsatellite candidates.

*Microsatellite Analysis*

Fifteen polymorphic loci out of the total 48 microsatellite loci isolated and developed from the genomic library of *O. nabouroup* were used for the analysis of 77 genomic DNA samples. PCR was performed in a 25µl volume containing 1ng of genomic DNA, 0.2µM of each fluorescently labelled primer and 12.5µl of Taq 2x Master mix (OneTaq DNA Polymerase, NEB). The following thermocycling profile was followed: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute, with a final extension at 72°C for 2 minutes. The markers were set up into three multiplex panels with five microsatellite markers in each panel, according to their fluorescent labels and allele sizes (Table 2). Microsatellite PCR product sizes were determined using an ABI3100 Genetic Analyzer (Central Analytical Facility, Stellenbosch University), with a Genescan™ 500 Liz™ (Applied Biosystem Inc., Central Analytical Facility, Stellenbosch University) internal size standard. Peak Scanner Software™ v1.0 was used to visualise and determine allele sizes.

**Table 1**: Markers with primers developed and tested for amplification of microsatellites in *O. nabouroup.*

| Locus | Primer sequences 5′- 3′ | Repeat type | Fluourescent dye | Size bp | No. Of Alleles | Multiplex panel | Genbank accession No. |
|---|---|---|---|---|---|---|---|
| On_1142255 | F - AGCATCACCGTCAGTCCTAC<br>R – AGCCATTTGCTGCACCTATC | (TTA)8 | FAM | 163-188 | 7 | 1 | MT446464 |
| On_507360 | F – GGCAGAACGGGGATGTTTG<br>R – AGATGCTCCCATGTCCACTC | (GGAT)7 | ATTO565 | 162-203 | 5 | 1 | MT446466 |
| On_1106367 | F - GGGCAGTTATCAGTCCTTGG<br>R – AAGCCATGACTGTCCACCAG | (ACAT)7 | ATTO532 | 104-115 | 4 | 1 | MT446463 |
| On_843840 | F – TGCAGATGCCCCACTTTTTC<br>R – TGGGCAAAACATTGAGTGAATAC | (ATGA)14 | ATTO550 | 181-201 | 6 | 1 | MT446465 |
| On_589878 | F – GAGGCTCCATATCCCACCAG<br>R – ATCTGCCAGCCAGGATTGTC | (TAA)14 | ATTO532 | 224-255 | 7 | 1 | MT446467 |
| On_324078 | F - ACTGACAAAATTCAAAGCAAAAGTG<br>R - ACTTAGCAGTAAAACAATTGACATC | (ATA)10 | ATTO532 | 208-229 | 6 | 2 | MT446474 |
| On_852924 | F - ACTTTTGGAGGTCATTGGCTG<br>R – GCAGAAAGGCTGGTTAGGTC | (GATA)15 | FAM | 212-255 | 6 | 2 | MT446475 |
| On_864997 | F - ATTGTTCAGCTGCTTCACGG<br>R – GCTATGAAAGCCAGTGGTGG | (GGA)10 | FAM | 141-148 | 3 | 2 | MT446471 |
| On_877333 | F – TGCCTCCTTCGTACCCATTC<br>R – GGGCGTCTGGATGCAAATAG | (TCCA)15 | ATTO565 | 159-206 | 8 | 2 | MT446472 |
| On_883556 | F – TATGCAGAAGTGGCTGAGGG<br>R – GTGAACTTGTCAGTGGGCAG | (TATC)18 | ATTO550 | 189-206 | 5 | 2 | MT446473 |
| On_23489 | F - CTTCAAGGATGCACAGGCAG<br>R – TCACTTTCCAGTGAGAAGCC | (ATAG)21 | ATTO565 | 178-233 | 8 | 3 | MT446477 |

| Locus | Primer sequences 5′- 3′ | Repeat type | Fluourescent dye | Size bp | No. Of Alleles | Multiplex panel | Genbank accession No. |
|---|---|---|---|---|---|---|---|
| On_290548 | F – GGTGACATCAGTACCTGGGG<br>R – GGGCACAGTGAGGGAATAAC | (TTA)26 | ATTO590 | 178-231 | 11 | 3 | MT446478 |
| On_402628 | F – AGAGGCTTTCAGGGGATGTG<br>R – ATCCAGAGCTGGTTCTCCTC | (AGG)8 | ATTO532 | 210-233 | 4 | 3 | MT446479 |
| On_787859 | F - GCTTCCTTGCACAGATAGCAC<br>R – TGGGGATCTGAGTGCATTTTC | (ATCT)15 | FAM | 171-187 | 5 | 3 | MT446476 |
| On_968476 | F - ACGTGCAAGAAAAGAGCTGG<br>R – AGAGGTTCCTTTACGTGGGC | (TCA)8 | FAM | 224-233 | 3 | 3 | MT446480 |

The mean number of alleles (as determined on basis of fragment lengths from the multiplex PCR) per locus, observed heterozygosities, expected heterozygosities, deviations from Hardy-Weinberg proportions, fixation index (Fst) and AMOVA tests were calculated using GenAlEx 6.5 (Peakall & Smouse, 2012). MICRO-CHECKER 2.2 (Van Oosterhout *et al*., 2004) was used for detecting null alleles, genotyping errors and allelic drop-out. The relationship between individuals was inferred using a principal component analysis (PCA) of the alleles performed using the package Adegenet 2.0.0 (Jombart 2015) in R v. 3.1.2 (R Core Team 2015).

*2.3.6. Population structure and Relatedness*

The genetic relationships between the populations were inferred using a Bayesian clustering analysis in STRUCTURE 2.3.4 (Pritchard *et al*., 2000). Assessments were conducted with the USEPOPINFO = POPFLAG 0 option active. STRUCTURE was run for 5 replicates from K = 1-12, with a run-length of 500,000 repetitions of Markov chain Monte Carlo (MCMC), following the burn-in period of 20,000 iterations. The five values for the estimated ln(Pr(X\K)) were averaged, from which the delta K was calculated (Evanno *et al*., 2005). The K-value with the highest delta K was used as the best K-value for the dataset which was K=4 (Figure 1) (Evanno *et al*., 2005). This was done using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The actual delta K value on the Y-axis however is very low which indicates the K value of four is unlikely in the actual populations (Figure 1).

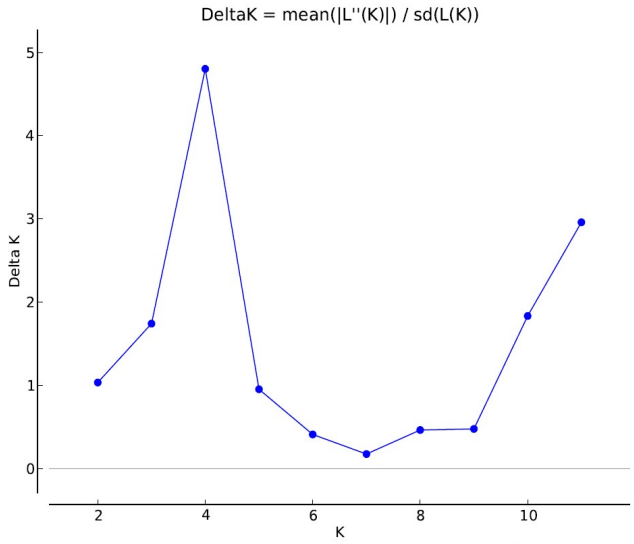


**Figure 1**:Delta K-values for species structures of K=1-12.

The relatedness between individuals was inferred using ML-Relate (Kalinowski *et al*., 2006). ML-Relate uses microsatellite data to calculate maximum likelihood estimates of relatedness and relationship and can be used to differentiate between four common pedigree relationships i.e. parent-offspring (PO), half-siblings (HS), full-siblings (FS) and unrelated individuals (U) (Kalinowski *et al*., 2006). ML-Relate represents relationships between individuals mathematically as probabilities (*k*-coefficients) (Kalinowski *et al*, 2006). If $k_0$ indicates no shared alleles between two individuals, $k_1$ represents one shared allele between two individuals and $k_2$ represents two shared alleles between two individuals, different relationships have different probabilities (Kalinowski *et al*, 2006). For example, if two individuals are parent-offspring $k_0$ equals one and $k_1$ and $k_2$ equal zero (Kalinowski *et al*, 2006). If two individuals are full siblings, $k_0$, $k_1$ and $k_2$, will equal 0.25, 0.5 and 0,25 respectively (Kalinowski *et al*, 2006).

## Results

*Development of microsatellite markers*

A total of 48 novel microsatellite markers were selected based on successful and ease of amplification in the seven samples used for the development of the Pale-winged Starlings genomic library through next-generation sequencing (Appendix 1). Evaluation of these markers found one monomorphic marker and five microsatellite markers (one dinucleotide, two trinucleotides, two tetranucleotides) that were unable to successfully amplify in all seven samples used to create the library. As such, a final 42 microsatellite markers were retained to be used for intraspecific studies of the Pale-winged Starlings. Sequence information and characterisation pertaining to the 48 developed microsatellite markers can be found in Appendix 1.

*Microsatellite Analysis in two Pale-winged Starling populations*

Fifteen microsatellite loci were selected based on their polymorphic ability and genotyped (GenBank accession numbers in Table 2). The primers were used for amplification in the samples from Augrabies Falls Nature Reserve and Ai Ais-Richtersveld Transfrontier Park populations. All fifteen microsatellite markers were found to be polymorphic in both sample populations. The number of alleles per locus (Appendix 2) ranged between 6 and 19 alleles with a mean of 11.73 alleles per locus. The Augrabies Falls Nature Reserve sampled population had the highest number of alleles for On_290548 and On_589878 i.e. 19 alleles and the lowest number of alleles for On_883556 i.e. 8 alleles. The lowest number of alleles in the Ai Ais-Richtersveld Transfrontier Park sampled population was 6 alleles in On_883556 and On_1106367 and the highest number of alleles were 15 alleles in On_1114225. A higher allelic richness was observed in the Augrabies Falls Nature Reserve samples with a mean of 13.2 alleles across fifteen microsatellite markers whereas a mean of 10.27 alleles across the fifteen markers was found in the Ai Ais Richtersveld Transfrontier Park samples. The Augrabies Falls Nature Reserve (n=53) with the larger sample size would be expected to have a greater allelic richness although the allelic richness in the Ai Ais-Richtersveld Transfrontier Park (n=24) samples were relatively high considering this sample size is less than half the size of the Augrabies Falls Nature Reserve sample size.

One microsatellite locus (On_787859) showed evidence of null alleles in MICRO-CHECKER, however, no evidence of scoring errors due to stuttering or evidence for large allele dropout was indicated. The number of alleles, effective alleles (Ne), information index (I), and the number of private alleles present in the two sampled populations of Pale-winged Starlings is shown in Figure 2. Effective alleles are less than the average number of alleles and therefore differ from the allelic richness because they correct for the difference in sample size (Maruyama, 1970) and the information index is used to indicate diversity (Magurran, 1988). More private alleles were found in the Augrabies Falls Nature Reserve sampled population than the Ai Ais-Richtersveld Transfrontier Park sampled

population. Microsatellite On_402628 had no private alleles in the Augrabies Falls Nature Reserve samples. The Ai Ais-Richtersveld Transfrontier Park samples only had private alleles in On_877333, On_968476, On_1114225, On_843840 and On_589878. A comprehensive table of the allele frequencies of private alleles for each marker can be seen in Appendix 3. There were no locally common alleles in the data set. Locally common alleles are alleles which are frequent enough that they can be used in standard marker panels and are not polymorphic (Raychaudhuri, 2011).

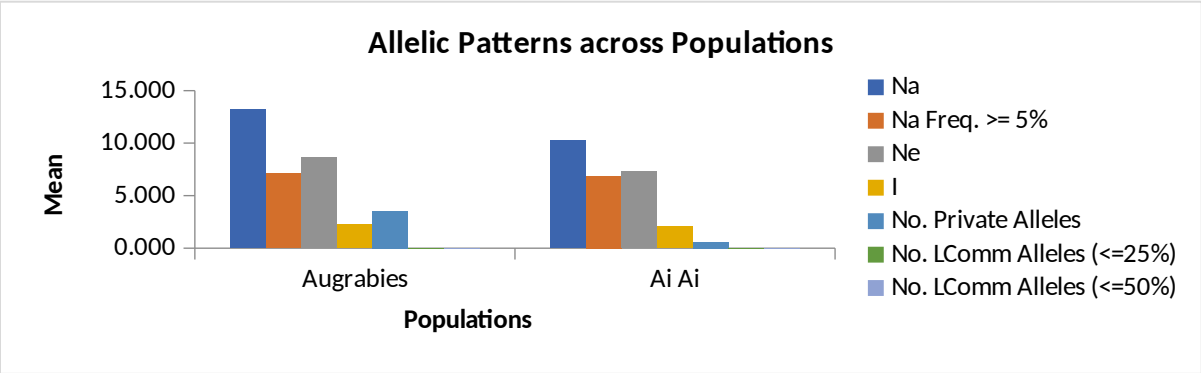


**Figure 2**: Summary of the number of alleles (Na), number of effective alleles (Ne), information index (I) and the number of private alleles.

The Shannon's information index (I) values generally range between 1.5 and 3.5, where the closer the value is to 3.5, the greater the diversity and evenness of the population (Magurran, 1988). The sampled population from the Augrabies Falls Nature Reserve had an information index that lies in the middle of 1.5 and 3.5 and the sampled population from the Ai Ais-Richtersveld Transfrontier Park lies closer to 1.5 indicating genetic diversity in both populations with higher diversity in the sampled population from the Augrabies Falls Nature Reserve (Table 3). Similarly, the mean expected heterozygosity in each sampled population indicated high levels of genetic variation in both sampled populations. The overall Fixation index values were regarded as 0 in each population indicating mixing between the sampled populations (Table 3; Appendix 4).

**Table 2**: Summary of the Information index (I), observed heterozygosity (Ho), expected heterozygosity (He) and F (Fixation index) values per population of Pale-winged Starlings across fifteen loci.

| **Population** | | **Information index (I)** | **Observed heterozygosity (Ho)** | **Expected heterozygosity (He)** | **Fixation index (F)** |
|---|---|---|---|---|---|
| **Augrabies** | **Mean** | 2.264 | 0.902 | 0.872 | -0.034 |
| **Ai Ai** | **Mean** | 2.076 | 0,887 | 0.849 | -0.039 |

Fis values indicate the inbreeding coefficient of an individual relative to the subpopulation per marker (Weir & Cockerham, 1984) (Appendix 5). The mean Fis value (-0.04), which is regarded as 0, indicates a low degree of inbreeding in the subpopulation per individual. The Fst value which is the proportion of the total genetic variance in a subpopulation relative to the total genetic variance was relatively low (0.01) and closer to 0 indicating a low level of genetic divergence/distance within the combined sampled populations. As the Fst value decreases the number of effective migrants increase indicating little difference in the heterozygosity between the subpopulations (Appendix 5). The mean number of effective migrants (N=22.45) between the sampled populations are an indication of the number of migrants entering the population per generation (Appendix 4) (Whitlock & McCauley, 1999).

Hardy-Weinberg statistics (Appendix 6) indicated that the sampled populations from the Augrabies Falls Nature Reserve and the Ai Ais-Richtersveld Transfrontier Park were in Hardy-Weinberg equilibrium with p-values of 0.19 and 0.34, respectively. Using a Bonferroni correction, the level of significance changes from 0.05 to 0.001. No linkage was detected between markers. An AMOVA analysis was conducted to determine the genetic variation between the two sampled populations and within the population samples. The highest variation was seen between individuals within each population rather than between the two populations.

A principal component analysis (PCA) was applied to further assess genotypic variation between individuals and between the two locations. In the PCA, allele frequencies were scaled using the centring option. This analysis showed that the two populations sampled do not have much variability between them with the axis showing PC1 vs PC2 both with only an 18.7% and 11.2% variability in the data set (Figure 3). The two sampled populations did not separate, however, there were five outliers (C5, C8, D3, D6 and I3) from the Augrabies Falls Nature Reserve region (Figure 3).

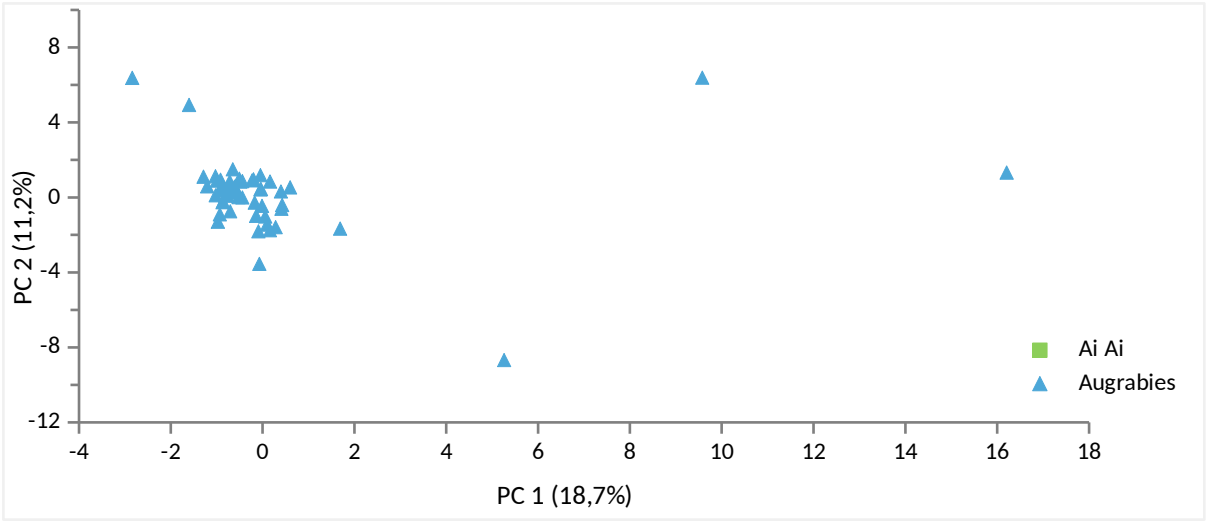


**Figure 3**: A Principal component analysis of fifteen microsatellite markers in two sampled populations of Pale-winged Starlings.

*Population structure and Relatedness*

STRUCTURE analysis was performed using K=4 to get a clear understanding of the genetic structure present in the data set (Figure 4). The data shows that the two sampled populations did not have two distinct genetic clusters, but were made up of a possible four different genetic clusters. It was also observed that the samples from the two populations had similar genetic compositions based on the lengths proportional to estimated membership in each cluster. However the actual delta K values (Figure 1) are low indicating K=4 may not be the actual number of genetic clusters possible.

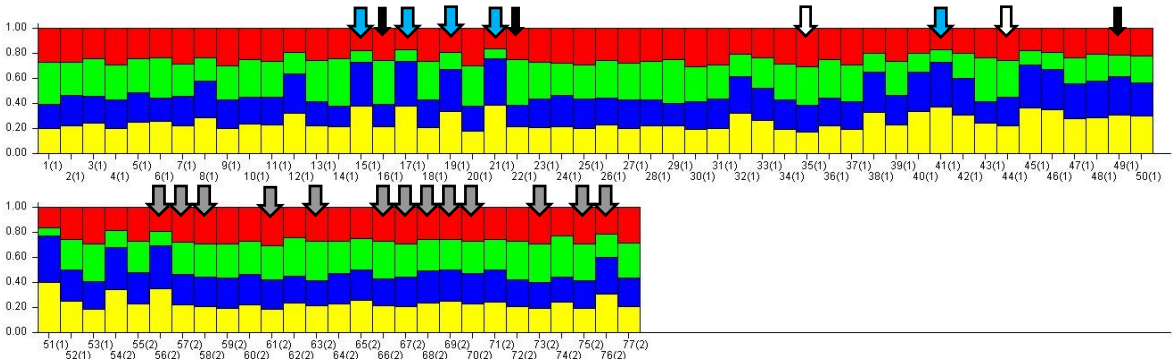


**Figure 4**: STRUCTURE analysis showing the estimated population genetic structure of the Pale-winged Starlings where K=4. Each individual is represented by a single vertical line, with lengths proportional to the estimated membership in each cluster.The y-axis represents probability (q) of each individual assigning to K clusters.

⬇ Samples C6, D7 and J6 from left to right, from Augrabies have no realtives in Ai Ai

⇩ Samples G8 and I7 from left to right, from Augrabies have more relatives in Ai Ai

Thirteen samples from Ai Ai with more relatives in Augrabies

⇩

⇩

Five outliers in the PCA analysis.

*2.4.4. Social structure and Relatedness*

ML-Relate analysis demonstrated that every sampled individual has multiple relatives in the population (Appendix 7). For example, an individual may have multiple half sibling, or full sibling relationships within and between the populations. Table 4 illustrates the cumulative number of relationships each bird had. All the samples except J2, D7, and C6 from the sampled population from Augrabies Falls Nature Reserve region have relatives across the two populations. All three of the exceptions are male. Individuals I7 and G8, both males from the Augrabies Falls Nature Reserve region, had more relatives in the sampled population from the Ai Ais-Richtersveld Transfrontier Park resort region than in the Augrabies Falls Nature Reserve region. A total of thirteen of the twenty-four sampled individuals from the Ai Ais-Richtersveld Transfrontier Park resort have more relatives in the Augrabies Falls Nature Reserve region than in the Ai Ais-Richtersveld Transfrontier Park resort based on the sampled populations. Of the thirteen samples only four are female and nine are male. There were only two confirmed full sibling pairs in the dataset, and they were both from the Augrabies Falls Nature Reserve.

**Table 3**: Collated results of ML-Relate analysis

| | **Both populations** | **Augrabies population** | **Ai Ai population** |
|---|---|---|---|
| **Full sibling** | 0 | 2 | 0 |
| **Half sibling** | 6 | 20 | 3 |
| **Unrelated or Half sibling** | 244 | 277 | 86 |
| **Half sibling or Full sibling** | 9 | 28 | 5 |
| **Unrelated, Half or Full sibling** | 3 | 4 | 1 |

294 **Discussion**

295 All 48 microsatellite markers developed in this study, with the exception of On_772390, were
296 polymorphic in the seven Pale-winged Starling blood samples used to create the library. The single
297 monomorphic microsatellite marker (On_772390) could be a useful tool for population genetic
298 studies. A study in plants by Nazareno and dos Reis (2010) used a dinucleotide monomorphic
299 microsatellite marker to elucidate polymorphic sites in the flanking regions of the monomorphic
300 marker to determine genetic diversity within the population. To our knowledge this technique has not
301 been tested in bird species. The remaining 47 microsatellite markers are comprised of six di-, eighteen
302 tri- and twenty-four tetra-nucleotides. Of these 47 markers, five markers displayed drop outs (no
303 amplification in some samples) (Appendix 1) and as such, 42 markers can be retained for a multitude
304 of analyses ranging from intraspecific population genetics to interspecific comparative genetic
305 studies.

306 From the results of the fifteen microsatellites used for this study we can conclude that the two
307 populations of Pale-winged Starlings, regardless of their geographic distance have a very similar
308 genetic make-up with the largest variation and genetic difference found between individuals rather
309 than between populations. The Brazilian tanager (*Ramphocelus bresilus)* was found to have similar
310 levels of genetic diversity and genetic make-up when comparing different populations across Rio de
311 Janeiro (Nogueira *et al*., 2014). A study on the house sparrow (*Passer domesticus*), also demonstrated
312 low genetic differentiation between distinct populations in Finland, with genetic variation between
313 individuals accounting for most of the variation (Kekkonen *et al*., 2011).

314 The STRUCTURE analysis did not assign the individuals into the two expected population clusters
315 according to their geographic locations. Rather four separate clusters were observed, supporting
316 breeding between the populations even though they are geographically separated by 400km. The low
317 genetic distance between the populations, allows for interbreeding due to the migrants moving
318 between the two populations. However, the program STRUCTURE, detects the uppermost
319 hierarchical level of structure and authors warn that the STRUCTURE results are only an indication
320 of the number of clusters and a guide (Pritchard *et al*., 2000; Pritchard & Wen, 2003). The K-value of
321 four (Figure 1) and the AMOVA analysis both indicate the allelic richness and large amount of
322 variation present in the populations. Five samples appear as outliers in the PCA analysis (Figure 3)
323 from the Augrabies Falls Nature Reserve samples and appear to have a slightly different membership
324 of each genetic cluster when compared to the other samples (Figure 4).

325 The STRUCTURE analysis showing each cluster being made up of individuals from both populations
326 supports the estimated number of migrants, in the population which corresponds with the low Fst
327 values seen in the study and the ML-Relate results. The number of samples from the Ai Ais-
328 Richtersveld Transfrontier Park population (n=24) is much smaller than the number of samples from

Augrabies Falls Nature Reserve population (n=53) however, the variation within the sampled population from the Ai Ais-Richtersveld Transfrontier Park is relatively high. This is supported by the number of effective alleles in each sampled population per locus which takes into account the sample size.

The ML-Relate analysis (Table 3, Appendix 7) revealed that all the individuals had relatives in the populations as well as across the two populations with only three exceptions from the Augrabies Falls Nature Reserve population. Both sample populations are made up of adults and sub-adults with no juveniles or pullus (nestlings unable to fly) present in the data set. There appears to be no bias with regards to sex differences in migration within these two populations. Sex differences in migration are well documented in the literature with all reported cases suggesting females migrate farther than males (Gow & Wiebe, 2014). A study on the migration of the European Robin (*Erithacus rubecula*) found that most males were resident and almost all females were migratory (Adriaensen & Dhondt, 1990). The same is seen in Dark-eyed Juncos (*Junco Hyemalis hyemalis*) and European Blackbirds (*Turdus merula*) (Ketterson & Nolan, 1976; Fudickar *et al*., 2013).

The low genetic variation seen between these two sampled populations are similar to the house sparrows study by Kekkonen *et al*, (2011). House sparrows were found to have low levels of differentiation amongst thirteen different populations from around Finland. This was attributed to a small number of migrants being able to homogenize populations with only a few of these migration events being sufficient to maintain the connectivity between the populations (Franklin, 1980; Frankel & Soule, 1981; Allendorf, 1983). The same could be said about the sampled populations of Pale-winged Starlings from the Augrabies Falls Nature Reserve and Ai Ais-Richtersveld Transfrontier Park region. With an estimated mean number of twenty-two migrants per generation, the homogenization of these populations is very possible, and this finding supports the low Fst values, AMOVA, STRUCTURE, and PCA results.

The socially monogamous Pale-winged Starlings found in the Augrabies Falls Nature Reserve and Ai Ais-Richtersveld Transfrontier Park region are generally sighted in pairs. However social monogamy refers to the long-term living arrangement between an adult male and female which differs from genetic monogamy, where two individuals only reproduce with one another (Reichard & Christophe, 2003). This study supports that the two populations may not exist exclusively in one particular region. Birds from either population migrate and join the other population maintaining gene flow between the two populations. Every bird in the population has a relative. This would indicate that, regardless of the genetic relationships, these birds maintain a stable social environment and harbour strong social bonds between same and opposite sex group members as well as mates. The same conclusions have been drawn in a study conducted on greater ani (*Crotophaga major*) species by Riehl & Strong, (2015). In their study, females lay eggs in the shared nests and adults are unable to identify their own nestlings

or eggs, which results in all community members participating in food delivery and nest defence (Riehl & Jara, 2009; Riehl, 2012; Riehl *et al*., 2015). Although community or population members are genetically unrelated, they maintain stable populations which last over decades at times (Riehl, 2010). Considering the low variation observed between the two populations of Pale-winged Starlings, the social behaviour of these birds could be explained by the inability to recognise differences between the birds based on their phenotypes even though the genetic variation is greatest between individuals. The phenotype matching hypothesis suggests that individuals who resemble their own kin are treated as related (Penn & Frommen, 2010). This hypothesis further supports the stability of these populations regardless of their genetic make-up and origin which maintains the genetic variation.

In conclusion, this study has shown that the two Pale-winged Starling populations are stable and thrive in their environments. These two populations may not exist exclusively in one region, but may move between the two locations, maintaining genetic variation. The social interactions between these birds do not seem to be affected by the presence of migrants. Pale-winged Starlings seem not to regard other Pale-winged Starlings from differing populations as competition and co-exist with them. The behaviour of the Pale-winged Starlings towards their offspring could not be fully elucidated with the lack of juvenile samples. However, we could extrapolate from the data that the populations may work together to secure food and protect their nests, as seen in the greater ani populations, with no discrimination between related and unrelated individuals, as long as their phenotypes match those of the area's population. The microsatellites developed in this study for the Pale-winged Starlings can be used on other Starling species and interspecies studies for comparative analyses with Pale-winged Starlings. Future work will include the addition of samples from the Ai Ais-Richtersveld Transfrontier Park region as well as juvenile birds to get a holistic understanding of these populations.

**Data accessibility statement**

All DNA sequences have been uploaded onto GenBank and accession numbers can be found in Table 1 however, the sequences will only be available online from the 28th February 2021 or upon acceptance of the manuscript.

**Competing interests statement**

We have no competing interests to disclose.

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401

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410 **Appendix 1**

411 Characterisation of 48 novel microsatellite loci isolated from Pale-winged Starlings, (*Onychognathus*

412 *nabouroup*)

413

| Locus | Primer sequences 5′- 3′ | Repeat type | Size bp | No. of alleles | *Genbank accession No. | Comments |
|---|---|---|---|---|---|---|
| On_15877 | F - AGGCATACTTGAGATCCTGGG<br>R – TGAGGTCAATGGAACCCTCG | (TA)16 | 219-249 | 8 | | |
| On_23489 | F - CTTCAAGGATGCACAGGCAG<br>R – TCACTTTCCAGTGAGAAGCC | (ATAG)21 | 178-233 | 8 | MT446477 | |
| On_66272 | F - ACACTTTCTCTCAGTATGTGCAG<br>R – CTTTCAGCATGGGAAGGCAC | (ATCC)12 | 184-231 | 8 | MT446469 | |
| On_96242 | F – GGGTGAGAAAATCTGCAGGC<br>R – GCGTCATGAAAGACCACAGG | (TGGA)12 | 174-259 | 8 | | |
| On_105695 | F – ACTCAGTCGCTGTGGTTTTAC<br>R – TGTCCGGTGAAAATGGCAAG | (TTA)19 | - | - | | |
| On_147152 | F – ACGTATCCTGCTCAGCTGTC<br>R – CAGACCAAAGTGCCTGGTTC | (GACA)7 | 234-240 | 3 | | |
| On_252789 | F – GCGATTTGTCCAACTTCCCC<br>R - TACACGATGCCGAGTAGGAG | (CAG)9 | 127-130 | 2 | | |
| n_290548 | F – GGTGACATCAGTACCTGGGG<br>R – GGGCACAGTGAGGGAATAAC | (TTA)26 | 178-231 | 11 | MT446478 | |
| On_293048 | F - CCCATTCCTACAGATGGGGG<br>R – GCTGTCCCAATGTCAGCAC | (GCG)7 | 222-251 | 7 | | |
| On_324078 | F - ACTGACAAAATTCAAAGCAAAAGTG<br>R - ACTTAGCAGTAAAACAATTGACATC | (ATA)10 | 208-229 | 6 | MT446474 | |
| On_344860 | F - GGATCCTCAGTATGCAAACCC<br>R - AAAAACACCCCAGTGCTTCG | (AGAT)9 | 150-190 | 8 | MT446468 | |
| On_402628 | F – AGAGGCTTTCAGGGGATGTG<br>R - ATCCAGAGCTGGTTCTCCTC | (AGG)8 | 210-233 | 4 | MT446479 | |
| On_507360 | F – GGCAGAACGGGGATGTTTG<br>R - AGATGCTCCCATGTCCACTC | (GGAT)7 | 162-203 | 5 | MT446466 | |
| On_534849 | F – TGCCTTTAGACACATCAGTGG<br>R – GGGGTTTTGATACCTCTGCC | (AT)15 | 118-158 | 5 | MT590769 | |
| On_581501 | F - GGTAACACTGGCAATCTAATCCC<br>R - GCACCAAATTCTCCTAGTCGC | (TTA)16 | 175-208 | 8 | MT590770 | |
| On_589878 | F – GAGGCTCCATATCCCACCAG<br>R - ATCTGCCAGCCAGGATTGTC | (TAA)14 | 224-255 | 7 | MT446467 | |
| On_644315 | F – ATTCTGGAAGGCGAGAGCAG<br>R - CCTCAGGAGCGACCAACATC | (ATAA)8 | 194-206 | 3 | | |
| On_700426 | F - GAGCTGTACTGGACATGTTGC<br>R - TCAGTTTAGAGACAGAAGCAATGAC | (TTA)20 | 192-250 | 9 | | |
| On_772007 | F - GACTGGGGAGAAGCATCAAAG<br>R - CCCCAGGACACTTGGTTTTC | (AAT)27 | 212-274 | 5 | | |
| On_772390 | F - GGGGTGGTGAGCAGTCATAC<br>R - ACCGTATCTGCATCCACCTG | (TC)13 | 180 | 1 | | Monomorphic |
| On_787859 | F - GCTTCCTTGCACAGATAGCAC<br>R - TGGGGATCTGAGTGCATTTTC | (ATCT)15 | 171-187 | 5 | MT446476 | |

| Locus | Primer sequences 5′- 3′ | Repeat type | Size bp | No. of alleles | *Genbank accession No. | Comments |
|---|---|---|---|---|---|---|
| On_806239 | F – TGTAACTGAGAGTTTCCCAAGC<br>R - ACTCCATGTATTTATTCCCAGAGG | (AATA)7 | 165-185 | 5 | | |
| On_821403 | F - GAACCTGCAGAATGTCACCC<br>R - GCCACACTGATTCCTATGAGC | (AGTG)18 | 147-188 | 5 | | |
| On_843840 | F – TGCAGATGCCCCACTTTTTC<br>R - TGGGCAAAACATTGAGTGAATAC | (ATGA)14 | 181-201 | 6 | MT446465 | |
| On_852924 | F - ACTTTTGGAGGTCATTGGCTG<br>R - GCAGAAAGGCTGGTTAGGTC | (GATA)15 | 212-255 | 6 | MT446475 | |
| On_864997 | F - ATTGTTCAGCTGCTTCACGG<br>R - GCTATGAAAGCCAGTGGTGG | (GGA)10 | 141-148 | 3 | MT446471 | |
| On_868467 | F – AGCATGGCAGGTGACTTCTC<br>R - AAACAGTGCTGGCTCAATGG | (CCAT)11 | 221-240 | 6 | MT446470 | |
| On_877333 | F – TGCCTCCTTCGTACCCATTC<br>R - GGGCGTCTGGATGCAAATAG | (TCCA)15 | 159-206 | 8 | MT446472 | |
| On_883556 | F – TATGCAGAAGTGGCTGAGGG<br>R - GTGAACTTGTCAGTGGGCAG | (TATC)18 | 189-206 | 5 | MT446473 | |
| On_894676 | F - CCACAGCGATTGGTGTCTTC<br>R - ACATTCCTGGAGTTGTCCTC | (AATA)7 | 183-187 | 2 | | |
| On_899076 | F - ATTCATGGCACTCCTTTTAGAC<br>R - CCCAGGGTCTTCTTTGATAGC | (TAGA)10 | 212-248 | 5 | | Drop out in samples A4 and A8 |
| On_914873 | F - CGATATCACTGAGGCCAAGC<br>R - AGAAGTCTGAAAGACACAATGGC | (ATT)19(GTT)8 | 155-222 | 5 | | |
| On_943863 | F - ACAACTCAGACGATAAGCTGG<br>R - AGAAATGACTTTGATCCGTGG | (TATC)22 | - | - | | |
| On_950478 | F - ATGGTGACGAGTACCTTGCC<br>R - TCATTCTAGTCCAAACCATGAAAAC | (AT)13 | - | - | | |
| On_968476 | F - ACGTGCAAGAAAAGAGCTGG<br>R - AGAGGTTCCTTTACGTGGGC | (TCA)8 | 224-233 | 3 | MT446480 | |
| On_1016023 | F - TGCTAAACTTTCAATTTTTCCAGTA<br>R - AG TTGAGCATATTGCTGTCTCTTG | (TCTA)18TG (TATC)8 | 102-294 | 15 | | |
| On_1039897 | F – TTTTCAGCCACATGGAGTCG<br>R - TGTCCATGACTGATGCAGAAAC | (ATT)13 | - | - | | |
| On_1061771 | F – TTGCATTACTGGGGGAGGAG<br>R – ACTGCAGGCTTATGAGGGAG | (TATT)20 | 129-180 | 6 | | |
| On_1078206 | F - CTATCTACGTGCAGGTGTGC<br>R – CAGGACCGTCTAGTCCTTGG | (AC)30 | 118-156 | 4 | | Drop out in sample A8 |
| On_1081480 | F – GAGCTATGAACCCACAAGGC<br>R – GGCCAGTAAACCTGAGTCAAAC | (TAGA)17 | 133-190 | 8 | | |
| On_1101365 | F - TCCCAGTAGATTCCCTTTCCAC<br>R – CAGATCCCTCCCAAGTGACC | (ATCC)7 | 147-187 | 7 | | |
| On_1106367 | F - GGGCAGTTATCAGTCCTTGG<br>R – AAGCCATGACTGTCCACCAG | (ACAT)7 | 104-115 | 4 | MT446463 | |
| On_1118845 | F – ATTCCCACCTGTCCCGATCC<br>R - CACTCCCGGAAGGGATTTTTG | (AGG)9 | 125-153 | 8 | | Drop out in sample A8 |
| On_1133203 | F - AAGTCTCCAGTACCTGTGCC<br>R - TGACAACAGGGCGTTTTCAC | (GCT)10 | 191-201 | 3 | | |

| Locus | Primer sequences 5′- 3′ | Repeat type | Size bp | No. of alleles | *Genbank accession No. | Comments |
|---|---|---|---|---|---|---|
| On_1134576 | F - GGATTTGGGCCAGTCCTATG<br>R - GGCATTGCATGCTTCCAGAC | (TTA)21 | 229-265 | 7 | | Drop out in samples A4, A6 and A8 |
| On_1142255 | F - AGCATCACCGTCAGTCCTAC<br>R - AGCCATTTGCTGCACCTATC | (TTA)8 | 163-188 | 7 | MT446464 | |
| On_1157444 | F - TCCTTACCAACCAGACTGCC<br>R - CTTGCTGTCTGTATCACGCC | (GATA)19 | 198-257 | 8 | | Drop out in sample A8 |
| On_1157509 | F - CTAGCGCAGAAGGTATGGTG<br>R - GCTGATGTGTCAACCAGGAG | (TA)11 | - | - | | |

414 * Accession numbers for 20 markers have been uploaded thus far

415

**Appendix 2**

Number of alleles per microsatellite marker in both populations of Pale-winged Starlings.

| Locus | Number of alleles in Augrabies population | Number of alleles in Ai Ai population | Mean | Standard deviation |
|---|---|---|---|---|
| **On_864997** | 11 | 8 | 9.5 | 2.12 |
| **On_877333** | 14 | 10 | 12 | 2.83 |
| **On_883556** | 8 | 6 | 7 | 1.41 |
| **On_324078** | 12 | 9 | 10.5 | 2.12 |
| **On_852925** | 12 | 9 | 10.5 | 2.12 |
| **On_787859** | 13 | 9 | 11 | 2.83 |
| **On_23489** | 15 | 12 | 13.5 | 2.12 |
| **On_290548** | 19 | 13 | 16 | 4.24 |
| **On_402628** | 11 | 11 | 11 | 0 |
| **On_968476** | 11 | 9 | 10 | 1.41 |
| **On_1106367** | 9 | 6 | 7.5 | 2.12 |
| **On_1114225** | 18 | 15 | 16.5 | 2.12 |
| **On_507360** | 14 | 12 | 13 | 1.41 |
| **On_843840** | 12 | 11 | 11.5 | 0.71 |
| **On_589878** | 19 | 14 | 16.5 | 3.54 |
| Mean | 13.2 | 10.27 | 11.73 | 2.07 |
| Standard Deviation | 3.36 | 2.66 | 2.94 | 1.05 |

**Appendix 3**

Summary of Private Alleles by Population

| Augrabies | | | Ai Ai | | |
|---|---|---|---|---|---|
| **Locus** | **Allele** | **Freq** | **Locus** | **Allele** | **Freq** |
| On_864997 | 126 | 0.010 | On_877333 | 174 | 0.021 |
| On_864997 | 150 | 0.039 | On_877333 | 178 | 0.021 |
| On_864997 | 153 | 0.020 | On_968476 | 243 | 0.021 |
| On_877333 | 156 | 0.009 | On_968476 | 252 | 0.021 |
| On_877333 | 184 | 0.075 | On_1114225 | 228 | 0.021 |
| On_877333 | 192 | 0.047 | On_1114225 | 231 | 0.021 |
| On_877333 | 196 | 0.009 | On_843840 | 212 | 0.021 |
| On_877333 | 204 | 0.151 | On_589878 | 210 | 0.021 |
| On_877333 | 208 | 0.019 | On_589878 | 213 | 0.021 |
| On_883556 | 184 | 0.040 | | | |
| On_883556 | 188 | 0.040 | | | |
| On_324078 | 207 | 0.019 | | | |
| On_324078 | 210 | 0.019 | | | |
| On_324078 | 240 | 0.010 | | | |
| On_852925 | 224 | 0.057 | | | |
| On_852925 | 228 | 0.019 | | | |
| On_852925 | 248 | 0.019 | | | |
| On_787859 | 100 | 0.019 | | | |
| On_787859 | 164 | 0.058 | | | |
| On_787859 | 200 | 0.029 | | | |
| On_787859 | 204 | 0.019 | | | |
| On_23489 | 176 | 0.009 | | | |
| On_23489 | 200 | 0.066 | | | |
| On_23489 | 204 | 0.057 | | | |
| On_290548 | 177 | 0.028 | | | |
| On_290548 | 180 | 0.019 | | | |
| On_290548 | 192 | 0.047 | | | |

| | | |
|---|---|---|
| On_290548 | 195 | 0.028 |
| On_290548 | 204 | 0.047 |
| On_290548 | 222 | 0.085 |
| On_968476 | 102 | 0.019 |
| On_968476 | 240 | 0.019 |
| On_968476 | 246 | 0.010 |
| On_968476 | 258 | 0.010 |
| On_110636 7 | 124 | 0.010 |
| On_110636 7 | 172 | 0.010 |
| On_110636 7 | 180 | 0.010 |
| On_111422 5 | 96 | 0.019 |
| On_111422 5 | 132 | 0.010 |
| On_111422 5 | 141 | 0.010 |
| On_111422 5 | 150 | 0.010 |
| On_111422 5 | 198 | 0.019 |
| On_507360 | 208 | 0.075 |
| On_507360 | 216 | 0.009 |
| On_843840 | 172 | 0.009 |
| On_843840 | 220 | 0.009 |
| On_589878 | 198 | 0.009 |
| On_589878 | 216 | 0.028 |
| On_589878 | 231 | 0.047 |
| On_589878 | 252 | 0.028 |
| On_589878 | 261 | 0.009 |
| On_589878 | 264 | 0.009 |
| On_589878 | 321 | 0.019 |

**Appendix 4**

Sample Size (N), No. Alleles (Na), No. Effective Alleles (Ne), Information Index (I), Observed Heterozygosity (Ho), Expected Heterozygosity (He) and Unbiased Expected Heterozygosity (uHe), and Fixation Index (F)

| **Augrabies** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Locus** | **N** | **Na** | **Ne** | **I** | **Ho** | **He** | **uHe** | **F** |
| **On_864997** | 51 | 11.000 | 7.126 | 2.125 | 0.804 | 0.860 | 0.868 | 0.065 |
| **On_877333** | 53 | 14.000 | 9.003 | 2.366 | 0.981 | 0.889 | 0.897 | -0.104 |
| **On_883556** | 50 | 8.000 | 4.907 | 1.744 | 0.840 | 0.796 | 0.804 | -0.055 |
| **On_324078** | 52 | 12.000 | 7.240 | 2.160 | 0.865 | 0.862 | 0.870 | -0.004 |
| **On_852925** | 53 | 12.000 | 7.803 | 2.212 | 0.981 | 0.872 | 0.880 | -0.125 |
| **On_787859** | 52 | 13.000 | 7.895 | 2.274 | 0.750 | 0.873 | 0.882 | 0.141 |
| **On_23489** | 53 | 15.000 | 12.347 | 2.580 | 1.000 | 0.919 | 0.928 | -0.088 |
| **On_290548** | 53 | 19.000 | 14.187 | 2.769 | 0.981 | 0.930 | 0.938 | -0.056 |
| **On_402628** | 52 | 11.000 | 7.005 | 2.159 | 0.904 | 0.857 | 0.866 | -0.054 |
| **On_968476** | 52 | 11.000 | 5.723 | 1.949 | 0.808 | 0.825 | 0.833 | 0.021 |
| **On_1106367** | 51 | 9.000 | 5.363 | 1.814 | 0.922 | 0.814 | 0.822 | -0.133 |
| **On_1114225** | 52 | 18.000 | 11.128 | 2.601 | 0.904 | 0.910 | 0.919 | 0.007 |
| **On_507360** | 53 | 14.000 | 9.620 | 2.392 | 0.981 | 0.896 | 0.905 | -0.095 |
| **On_843840** | 53 | 12.000 | 7.005 | 2.106 | 0.849 | 0.857 | 0.865 | 0.010 |
| **On_589878** | 53 | 19.000 | 13.065 | 2.712 | 0.962 | 0.923 | 0.932 | -0.042 |
| **Ai Ai** | | | | | | | | |
| **On_864997** | 24 | 8.000 | 6.031 | 1.930 | 0.833 | 0.834 | 0.852 | 0.001 |
| **On_877333** | 24 | 10.000 | 4.721 | 1.824 | 1.000 | 0.788 | 0.805 | -0.269 |
| **On_883556** | 23 | 6.000 | 4.560 | 1.605 | 0.522 | 0.781 | 0.798 | 0.332 |
| **On_324078** | 23 | 9.000 | 6.151 | 2.006 | 0.783 | 0.837 | 0.856 | 0.065 |
| **On_852925** | 24 | 9.000 | 7.155 | 2.048 | 0.917 | 0.860 | 0.879 | -0.066 |
| **On_787859** | 24 | 9.000 | 5.760 | 1.901 | 0.792 | 0.826 | 0.844 | 0.042 |
| **On_23489** | 24 | 12.000 | 8.727 | 2.288 | 1.000 | 0.885 | 0.904 | -0.129 |
| **On_290548** | 24 | 13.000 | 9.216 | 2.354 | 0.958 | 0.891 | 0.910 | -0.075 |
| **On_402628** | 24 | 11.000 | 7.945 | 2.210 | 0.958 | 0.874 | 0.893 | -0.096 |
| **On_968476** | 24 | 9.000 | 5.878 | 1.909 | 0.833 | 0.830 | 0.848 | -0.004 |
| **On_1106367** | 24 | 6.000 | 3.866 | 1.488 | 0.833 | 0.741 | 0.757 | -0.124 |
| **On_1114225** | 24 | 15.000 | 9.931 | 2.486 | 0.917 | 0.899 | 0.918 | -0.019 |
| **On_507360** | 24 | 12.000 | 10.017 | 2.375 | 1.000 | 0.900 | 0.919 | -0.111 |
| **On_843840** | 24 | 11.000 | 8.170 | 2.220 | 0.958 | 0.878 | 0.896 | -0.092 |
| **On_589878** | 24 | 14.000 | 11.077 | 2.499 | 1.000 | 0.910 | 0.929 | -0.099 |

426

**Appendix 5**

F-Statistics and estimates of the number of effective migrants (Nm) in both populations (Augrabies Falls Nature Reserve and Ai Ais-Richtersveld Transfrontier Park) for each locus.

| **Locus** | **Fis** | **Fit** | **Fst** | **Nm** |
|---|---|---|---|---|
| **On_864997** | 0.033 | 0.056 | 0.024 | 10.243 |
| **On_877333** | -0.181 | -0.148 | 0.028 | 8.623 |
| **On_883556** | 0.136 | 0.143 | 0.007 | 34.815 |
| **On_324078** | 0.030 | 0.049 | 0.020 | 12.372 |
| **On_852925** | -0.096 | -0.085 | 0.010 | 25.745 |
| **On_787859** | 0.093 | 0.104 | 0.012 | 21.322 |
| **On_23489** | -0.108 | -0.096 | 0.011 | 21.956 |
| **On_290548** | -0.065 | -0.053 | 0.011 | 21.575 |
| **On_402628** | -0.076 | -0.068 | 0.007 | 34.586 |
| **On_968476** | 0.009 | 0.019 | 0.011 | 22.496 |
| **On_1106367** | -0.129 | -0.097 | 0.028 | 8.741 |
| **On_1114225** | -0.006 | 0.000 | 0.006 | 40.585 |
| **On_507360** | -0.103 | -0.090 | 0.011 | 21.675 |
| **On_843840** | -0.042 | -0.032 | 0.009 | 27.062 |
| **On_589878** | -0.070 | -0.060 | 0.010 | 24.970 |
| Mean | **-0.04** | -0.02 | 0.01 | 22.45 |
| Standard deviation | **0.02** | 0.02 | 0.002 | 2.488 |

**Appendix 6**

Hardy-Weinberg statistics per microsatellite marker in two populations of Pale-winged Starlings.

| Population | Locus | DF | P-value |
|---|---|---|---|
| Augrabies | On_864997 | 55 | 0.011 |
| Augrabies | On_877333 | 91 | 0.018 |
| Augrabies | On_883556 | 28 | 0.455 |
| Augrabies | On_324078 | 66 | 0.000 |
| Augrabies | On_852925 | 66 | 0.117 |
| Augrabies | On_787859 | 78 | 0.000 |
| Augrabies | On_23489 | 105 | 0.822 |
| Augrabies | On_290548 | 171 | 0.000 |
| Augrabies | On_402628 | 55 | 0.011 |
| Augrabies | On_968476 | 55 | 0.000 |
| Augrabies | On_1106367 | 36 | 0.000 |
| Augrabies | On_1114225 | 153 | 0.000 |
| Augrabies | On_507360 | 91 | 0.180 |
| Augrabies | On_843840 | 66 | 0.000 |
| Augrabies | On_589878 | 171 | 0.008 |
| Mean | | | 0.108 |
| Ai Ai | On_864997 | 28 | 0.016 |
| Ai Ai | On_877333 | 45 | 0.665 |
| Ai Ai | On_883556 | 15 | 0.107 |
| Ai Ai | On_324078 | 36 | 0.111 |
| Ai Ai | On_852925 | 36 | 0.015 |
| Ai Ai | On_787859 | 36 | 0.000 |
| Ai Ai | On_23489 | 66 | 0.836 |
| Ai Ai | On_290548 | 78 | 0.431 |
| Ai Ai | On_402628 | 55 | 0.857 |
| Ai Ai | On_968476 | 36 | 0.000 |
| Ai Ai | On_1106367 | 15 | 0.627 |
| Ai Ai | On_1114225 | 105 | 0.147 |
| Ai Ai | On_507360 | 66 | 0.555 |
| Ai Ai | On_843840 | 55 | 0.609 |
| Ai Ai | On_589878 | 91 | 0.046 |
| Mean | | | 0.335 |

435 **Appendix 7**

436 ML-Relate results showing each relative pair either Unrelated (U), Half sibling (HS) or Full sibling (FS). Samples from the Augrabies Falls Nature Reserve are highlighted in Yellow and samples from the Ai Ais-Richtersveld Transfrontier Park are highlighted in green.

| Cumulative number of relationships within Augrabies = 331 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Sample | Relationship | Sample | Sample | Relationship | Sample | Sample | Relationship |
| C5 | J6 | FS | A5 | G8 | U, HS | H5 | J2 | U, HS |
| I4 | I9 | FS | A5 | H5 | U, HS | H5 | J3 | U, HS |
| A2 | A6 | HS | A5 | I3 | U, HS | H5 | J6 | U, HS |
| A3 | H8 | HS | A5 | J3 | U, HS | H5 | B57 | U, HS |
| A3 | I7 | HS | A6 | A8 | U, HS | H8 | J0 | U, HS |
| B0 | C1 | HS | A6 | C1 | U, HS | H8 | J3 | U, HS |
| B2 | I9 | HS | A6 | C3 | U, HS | H8 | B55 | U, HS |
| B9 | E6 | HS | A6 | D8 | U, HS | I3 | I4 | U, HS |
| C1 | D7 | HS | A6 | E3 | U, HS | I3 | I8 | U, HS |
| C1 | G1 | HS | A6 | E9 | U, HS | I3 | I9 | U, HS |
| C3 | D4 | HS | A6 | G0 | U, HS | I3 | J0 | U, HS |
| C5 | I3 | HS | A6 | G8 | U, HS | I3 | B55 | U, HS |
| D3 | I3 | HS | A6 | H3 | U, HS | I4 | I5 | U, HS |
| D8 | H8 | HS | A6 | I9 | U, HS | I4 | J0 | U, HS |
| E3 | E6 | HS | A6 | J1 | U, HS | I5 | J3 | U, HS |
| E3 | G0 | HS | A8 | D3 | U, HS | I7 | J3 | U, HS |
| E6 | H3 | HS | A8 | D8 | U, HS | I8 | B55 | U, HS |
| E7 | E9 | HS | A8 | E7 | U, HS | I9 | J0 | U, HS |
| G9 | H3 | HS | A8 | G0 | U, HS | I9 | J1 | U, HS |
| I4 | J1 | HS | A8 | G8 | U, HS | I9 | J3 | U, HS |
| I5 | I8 | HS | A8 | H3 | U, HS | I9 | J6 | U, HS |
| I5 | J1 | HS | A8 | H4 | U, HS | I9 | B57 | U, HS |
| A2 | A3 | U, HS | A8 | H8 | U, HS | J0 | J2 | U, HS |
| A2 | A9 | U, HS | A8 | I4 | U, HS | J0 | J6 | U, HS |
| A2 | C0 | U, HS | A8 | I7 | U, HS | J1 | J3 | U, HS |
| A2 | C6 | U, HS | A9 | B3 | U, HS | B2 | H8 | U, HS |
| A2 | D4 | U, HS | A9 | B9 | U, HS | B2 | I4 | U, HS |
| A2 | E7 | U, HS | A9 | C0 | U, HS | B2 | J1 | U, HS |
| A2 | E9 | U, HS | A9 | C1 | U, HS | B2 | J3 | U, HS |
| A2 | G8 | U, HS | A9 | C8 | U, HS | B2 | J6 | U, HS |
| A2 | H5 | U, HS | A9 | D6 | U, HS | B3 | D3 | U, HS |
| A3 | C0 | U, HS | A9 | E6 | U, HS | B3 | E4 | U, HS |
| A3 | D0 | U, HS | A9 | G1 | U, HS | B3 | E7 | U, HS |
| A3 | D3 | U, HS | A9 | G7 | U, HS | B3 | G8 | U, HS |
| A3 | D4 | U, HS | A9 | H8 | U, HS | B3 | H8 | U, HS |
| A3 | E3 | U, HS | A9 | I5 | U, HS | B3 | J0 | U, HS |
| A3 | E4 | U, HS | A9 | I8 | U, HS | B3 | J2 | U, HS |
| A3 | G7 | U, HS | A9 | J2 | U, HS | B3 | B55 | U, HS |
| A5 | B9 | U, HS | A9 | B57 | U, HS | B3 | B57 | U, HS |
| A5 | C5 | U, HS | B0 | E4 | U, HS | B6 | B9 | U, HS |
| A5 | C8 | U, HS | B0 | G8 | U, HS | B6 | C1 | U, HS |
| A5 | D3 | U, HS | B0 | H4 | U, HS | B6 | C8 | U, HS |
| A5 | D6 | U, HS | B0 | J0 | U, HS | B6 | D3 | U, HS |
| A5 | E3 | U, HS | B0 | B57 | U, HS | B6 | D7 | U, HS |
| A5 | E9 | U, HS | B2 | D6 | U, HS | B6 | E7 | U, HS |
| C0 | I8 | U, HS | D3 | B55 | U, HS | B6 | G7 | U, HS |
| C0 | J3 | U, HS | D4 | D8 | U, HS | B6 | G9 | U, HS |
| C0 | B57 | U, HS | D4 | E4 | U, HS | B6 | H3 | U, HS |
| C1 | C5 | U, HS | D4 | E8 | U, HS | B6 | H5 | U, HS |
| C1 | D8 | U, HS | D4 | G8 | U, HS | B6 | H8 | U, HS |
| C1 | E5 | U, HS | D4 | J2 | U, HS | B6 | J1 | U, HS |
| C1 | E7 | U, HS | D6 | G1 | U, HS | B6 | J3 | U, HS |
| C1 | G0 | U, HS | D6 | H5 | U, HS | B6 | B57 | U, HS |
| C1 | G8 | U, HS | D6 | I4 | U, HS | B9 | C8 | U, HS |
| C1 | J2 | U, HS | D6 | I8 | U, HS | B9 | E3 | U, HS |
| C1 | B55 | U, HS | D6 | I9 | U, HS | B9 | E9 | U, HS |
| C3 | G8 | U, HS | D6 | J0 | U, HS | B9 | G8 | U, HS |
| C3 | H3 | U, HS | D6 | J2 | U, HS | B9 | H8 | U, HS |
| C3 | I4 | U, HS | D6 | J6 | U, HS | B9 | I5 | U, HS |
| C3 | I5 | U, HS | D6 | B55 | U, HS | B9 | J3 | U, HS |
| C5 | D3 | U, HS | D7 | E8 | U, HS | B9 | B57 | U, HS |
| C5 | D4 | U, HS | D7 | G7 | U, HS | C0 | C8 | U, HS |
| C5 | G2 | U, HS | D7 | B19 | U, HS | C0 | G7 | U, HS |
| C5 | G8 | U, HS | D8 | E3 | U, HS | C0 | I4 | U, HS |
| C5 | I7 | U, HS | D8 | I3 | U, HS | C0 | I5 | U, HS |
| C5 | J3 | U, HS | D8 | I8 | U, HS | E6 | I4 | U, HS |
| C5 | B55 | U, HS | D8 | J0 | U, HS | E6 | I8 | U, HS |
| C6 | H5 | U, HS | D8 | J2 | U, HS | E6 | B57 | U, HS |
| C6 | J2 | U, HS | D8 | J3 | U, HS | E7 | G2 | U, HS |
| C6 | B21 | U, HS | D8 | B55 | U, HS | E7 | G7 | U, HS |
| C8 | D3 | U, HS | D8 | B57 | U, HS | E7 | I9 | U, HS |
| C8 | E3 | U, HS | E3 | E4 | U, HS | E7 | J2 | U, HS |

| Cumulative number of relationships within Augrabies = 331 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Sample | Relationship | Sample | Sample | Relationship | Sample | Sample | Relationship |
| C8 | E8 | U, HS | E3 | G2 | U, HS | E7 | J3 | U, HS |
| C8 | G1 | U, HS | E3 | H3 | U, HS | E8 | H8 | U, HS |
| C8 | I5 | U, HS | E4 | E9 | U, HS | E8 | I8 | U, HS |
| C8 | J0 | U, HS | E4 | G0 | U, HS | E9 | G0 | U, HS |
| C8 | J1 | U, HS | E4 | G7 | U, HS | E9 | G2 | U, HS |
| C8 | J2 | U, HS | E4 | H5 | U, HS | E9 | G7 | U, HS |
| D0 | E3 | U, HS | E5 | E6 | U, HS | E9 | H3 | U, HS |
| D0 | E5 | U, HS | E5 | E7 | U, HS | E9 | I7 | U, HS |
| D0 | E7 | U, HS | E5 | E9 | U, HS | E9 | J2 | U, HS |
| D0 | G0 | U, HS | E5 | G7 | U, HS | G0 | G7 | U, HS |
| D0 | G9 | U, HS | E5 | G8 | U, HS | G0 | G8 | U, HS |
| D0 | I5 | U, HS | E5 | G9 | U, HS | G0 | I9 | U, HS |
| D0 | J2 | U, HS | E5 | J1 | U, HS | G0 | J2 | U, HS |
| D0 | J3 | U, HS | E5 | J3 | U, HS | G0 | B55 | U, HS |
| D3 | D8 | U, HS | E5 | J6 | U, HS | G1 | H5 | U, HS |
| D3 | E9 | U, HS | E6 | E8 | U, HS | G1 | I8 | U, HS |
| D3 | G7 | U, HS | E6 | E9 | U, HS | G1 | J1 | U, HS |
| D3 | J2 | U, HS | G2 | G8 | U, HS | C8 | I3 | HS, FS |
| H3 | B55 | U, HS | G2 | H4 | U, HS | C8 | J6 | HS, FS |
| H4 | I9 | U, HS | G2 | I7 | U, HS | D6 | I3 | HS, FS |
| H4 | J1 | U, HS | G2 | J2 | U, HS | D8 | G0 | HS, FS |
| H4 | J6 | U, HS | G7 | I7 | U, HS | D8 | G8 | HS, FS |
| H4 | B57 | U, HS | B2 | H4 | U, HS | E3 | E7 | HS, FS |
| G7 | J2 | U, HS | G9 | I4 | U, HS | E5 | G2 | HS, FS |
| G8 | H3 | U, HS | H3 | I9 | U, HS | G1 | J6 | HS, FS |
| G8 | H5 | U, HS | H3 | J3 | U, HS | H8 | B57 | HS, FS |
| G8 | J2 | U, HS | G2 | G7 | U, HS | A3 | A8 | HS, FS |
| G9 | I5 | U, HS | H3 | I4 | U, HS | A3 | E5 | HS, FS |
| G8 | J3 | U, HS | G2 | J1 | HS, FS | A8 | I9 | HS, FS |
| G8 | B57 | U, HS | G7 | G9 | HS, FS | B0 | B55 | HS, FS |
| G9 | H4 | U, HS | G9 | J1 | HS, FS | D0 | G7 | HS, FS |
| G9 | I3 | U, HS | H4 | I4 | HS, FS | I8 | J0 | HS, FS |
| E6 | H4 | U, HS | H8 | I8 | HS, FS | J0 | B55 | HS, FS |
| B2 | E3 | U, HS | H8 | J6 | HS, FS | J3 | J6 | HS, FS |
| B2 | E4 | U, HS | B2 | D0 | HS, FS | B2 | B55 | U, HS, FS |
| B2 | G1 | U, HS | B6 | J6 | HS, FS | E3 | E9 | U, HS, FS |
| B2 | H3 | U, HS | C0 | I3 | HS, FS | H8 | I9 | U, HS, FS |
| G9 | I7 | U, HS | C5 | D6 | HS, FS | I8 | J6 | U, HS, FS |
| H3 | I7 | U, HS | C5 | G1 | HS, FS | | | |
| | | | | | | | | |

439

440

| Cumulative number of relationships between Augrabies and Ai Ai = 262 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Sample | Relationship | Sample | Sample | Relationship | Sample | Sample | Relationship |
| A2 | B18 | HS | B9 | B10 | U, HS | I5 | YA1 | U, HS |
| C5 | B08 | HS | B9 | B21 | U, HS | I5 | B26 | U, HS |
| E4 | B29 | HS | B9 | B23 | U, HS | I7 | YA1 | U, HS |
| E6 | B41 | HS | B9 | B38 | U, HS | I7 | B13 | U, HS |
| E8 | B38 | HS | B9 | B39 | U, HS | I7 | B22 | U, HS |
| G0 | B38 | HS | B9 | B46 | U, HS | I7 | B34 | U, HS |
| B6 | B34 | U, HS | B9 | B49 | U, HS | I7 | B38 | U, HS |
| B6 | B38 | U, HS | C0 | YA1 | U, HS | I7 | B39 | U, HS |
| B6 | B40 | U, HS | C0 | B10 | U, HS | I7 | B40 | U, HS |
| B6 | B47 | U, HS | C0 | B39 | U, HS | I7 | B45 | U, HS |
| B6 | B49 | U, HS | C1 | B08 | U, HS | I7 | B47 | U, HS |
| A8 | B23 | U, HS | H4 | YA1 | U, HS | A2 | B15 | U, HS |
| A8 | B34 | U, HS | H4 | B15 | U, HS | A2 | B29 | U, HS |
| A8 | B38 | U, HS | H4 | B26 | U, HS | A2 | B41 | U, HS |
| A8 | B49 | U, HS | H4 | B34 | U, HS | A3 | B08 | U, HS |
| A9 | B10 | U, HS | H4 | B40 | U, HS | A3 | B10 | U, HS |
| A9 | B47 | U, HS | H5 | YA1 | U, HS | A3 | B12 | U, HS |
| B0 | B05 | U, HS | H5 | B30 | U, HS | A3 | B18 | U, HS |
| B0 | B08 | U, HS | H5 | B39 | U, HS | A3 | B45 | U, HS |
| B0 | B18 | U, HS | H5 | B40 | U, HS | A5 | B46 | U, HS |
| B0 | B26 | U, HS | H8 | YA1 | U, HS | A5 | B47 | U, HS |
| B0 | B29 | U, HS | H8 | B12 | U, HS | A6 | YA1 | U, HS |
| B0 | B47 | U, HS | H8 | B29 | U, HS | A6 | B10 | U, HS |
| B2 | B05 | U, HS | H8 | B39 | U, HS | A6 | B12 | U, HS |
| B2 | B08 | U, HS | H8 | B46 | U, HS | A6 | B13 | U, HS |
| B2 | B10 | U, HS | I3 | YA1 | U, HS | A6 | B15 | U, HS |
| B2 | B29 | U, HS | I3 | B12 | U, HS | A6 | B18 | U, HS |
| B2 | B38 | U, HS | I3 | B21 | U, HS | A6 | B22 | U, HS |
| B2 | B39 | U, HS | I3 | B22 | U, HS | A6 | B29 | U, HS |
| B3 | B10 | U, HS | I3 | B23 | U, HS | A6 | B30 | U, HS |
| B3 | B13 | U, HS | I3 | B26 | U, HS | A8 | B10 | U, HS |
| B3 | B15 | U, HS | I3 | B30 | U, HS | A8 | B13 | U, HS |
| B3 | B18 | U, HS | I3 | B38 | U, HS | A8 | B15 | U, HS |
| B3 | B34 | U, HS | I3 | B41 | U, HS | E5 | B46 | U, HS |
| B3 | B46 | U, HS | I4 | YA1 | U, HS | E6 | B10 | U, HS |
| B6 | B08 | U, HS | I4 | B08 | U, HS | E6 | B21 | U, HS |
| B6 | B22 | U, HS | I4 | B13 | U, HS | E6 | B46 | U, HS |
| B6 | B23 | U, HS | I4 | B23 | U, HS | E7 | B38 | U, HS |
| B6 | B26 | U, HS | I4 | B26 | U, HS | E9 | B12 | U, HS |
| B6 | B29 | U, HS | I4 | B39 | U, HS | E9 | B18 | U, HS |
| G0 | B15 | U, HS | I4 | B47 | U, HS | E9 | B22 | U, HS |
| G0 | B18 | U, HS | I4 | B49 | U, HS | E9 | B26 | U, HS |
| G0 | B34 | U, HS | C1 | B49 | U, HS | E9 | B30 | U, HS |
| G0 | B40 | U, HS | C3 | B13 | U, HS | E9 | B41 | U, HS |
| G1 | YA1 | U, HS | C3 | B30 | U, HS | E9 | B47 | U, HS |
| G1 | B05 | U, HS | C5 | B10 | U, HS | G0 | B12 | U, HS |
| G1 | B22 | U, HS | C5 | B34 | U, HS | G0 | B13 | U, HS |
| G1 | B30 | U, HS | C8 | YA1 | U, HS | I8 | B41 | U, HS |
| G1 | B34 | U, HS | C8 | B05 | U, HS | I8 | B49 | U, HS |
| G1 | B45 | U, HS | C8 | B29 | U, HS | I9 | YA1 | U, HS |
| G7 | B10 | U, HS | C8 | B30 | U, HS | I9 | B05 | U, HS |
| G7 | B34 | U, HS | C8 | B34 | U, HS | I9 | B08 | U, HS |
| G7 | B38 | U, HS | C8 | B39 | U, HS | I9 | B23 | U, HS |
| G7 | B47 | U, HS | D0 | YA1 | U, HS | I9 | B26 | U, HS |
| G7 | B49 | U, HS | D0 | B12 | U, HS | I9 | B38 | U, HS |
| G8 | B21 | U, HS | D0 | B21 | U, HS | J0 | B08 | U, HS |
| G8 | B26 | U, HS | D0 | B30 | U, HS | J0 | B10 | U, HS |
| G8 | B34 | U, HS | D0 | B40 | U, HS | J0 | B12 | U, HS |
| G8 | B49 | U, HS | D0 | B47 | U, HS | J0 | B29 | U, HS |
| G9 | B22 | U, HS | D3 | B10 | U, HS | J0 | B41 | U, HS |
| G9 | B26 | U, HS | D3 | B22 | U, HS | J1 | B39 | U, HS |
| G9 | B30 | U, HS | D3 | B29 | U, HS | J1 | B45 | U, HS |
| G9 | B38 | U, HS | D3 | B30 | U, HS | J3 | B23 | U, HS |
| G9 | B45 | U, HS | D3 | B34 | U, HS | J3 | B38 | U, HS |
| H3 | B10 | U, HS | D4 | B29 | U, HS | J3 | B47 | U, HS |
| H3 | B12 | U, HS | D4 | B46 | U, HS | J6 | YA1 | U, HS |
| H3 | B18 | U, HS | D6 | YA1 | U, HS | J6 | B22 | U, HS |
| H3 | B22 | U, HS | D6 | B05 | U, HS | J6 | B34 | U, HS |
| H3 | B41 | U, HS | D6 | B18 | U, HS | J6 | B38 | U, HS |
| C1 | B40 | U, HS | D6 | B29 | U, HS | J6 | B47 | U, HS |
| B57 | B05 | U, HS | D6 | B30 | U, HS | B55 | YA1 | U, HS |
| B57 | B10 | U, HS | D6 | B34 | U, HS | B55 | B10 | U, HS |
| B57 | B29 | U, HS | D6 | B45 | U, HS | B55 | B12 | U, HS |
| I5 | B05 | U, HS | D8 | B29 | U, HS | B55 | B29 | U, HS |
| I7 | B05 | U, HS | D8 | B34 | U, HS | B57 | B41 | U, HS |
| I7 | B08 | U, HS | D8 | B39 | U, HS | J0 | B13 | HS, FS |
| E4 | B05 | U, HS | D8 | B41 | U, HS | B55 | B15 | HS, FS |
| G2 | B08 | U, HS | E3 | B13 | U, HS | A6 | B41 | HS, FS |
| G7 | B05 | U, HS | E3 | B34 | U, HS | C3 | B21 | HS, FS |

| Cumulative number of relationships between Augrabies and Ai Ai = 262 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Sample | Relationship | Sample | Sample | Relationship | Sample | Sample | Relationship |
| I8 | B19 | U, HS | E3 | B46 | U, HS | D3 | B47 | HS, FS |
| I8 | B21 | U, HS | E4 | B10 | U, HS | B05 | B12 | HS, FS |
| I8 | B29 | U, HS | E4 | B12 | U, HS | D6 | B08 | HS, FS |
| B55 | B21 | U, HS | E4 | B21 | U, HS | I3 | B08 | HS, FS |
| E4 | B30 | U, HS | B57 | B46 | U, HS | D3 | B08 | HS, FS |
| E5 | B38 | U, HS | B57 | B38 | U, HS | B05 | YA1 | U, HS, FS |
| I8 | B10 | U, HS | B55 | B26 | U, HS | B9 | B34 | U, HS, FS |
| B55 | B30 | U, HS | B55 | B19 | U, HS | H4 | B47 | U, HS, FS |
| | | | | | | J2 | B08 | U, HS, FS |

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| Cumulative number of relationships within Ai Ai = 95 | | | | | |
|---|---|---|---|---|---|
| Sample | Sample | Relationship | Sample | Sample | Relationship |
| B13 | B23 | HS | B30 | B47 | U, HS |
| B15 | B18 | HS | B30 | B49 | U, HS |
| B15 | B23 | HS | B34 | B38 | U, HS |
| YA1 | B10 | U, HS | B34 | B39 | U, HS |
| YA1 | B23 | U, HS | B34 | B45 | U, HS |
| YA1 | B30 | U, HS | B38 | B39 | U, HS |
| YA1 | B34 | U, HS | B38 | B40 | U, HS |
| YA1 | B38 | U, HS | B38 | B49 | U, HS |
| YA1 | B41 | U, HS | B10 | B15 | U, HS |
| YA1 | B47 | U, HS | B10 | B23 | U, HS |
| B13 | B15 | U, HS | B10 | B34 | U, HS |
| B13 | B19 | U, HS | B10 | B46 | U, HS |
| B13 | B22 | U, HS | B10 | B49 | U, HS |
| B13 | B30 | U, HS | B12 | B13 | U, HS |
| B13 | B39 | U, HS | B12 | B23 | U, HS |
| B13 | B40 | U, HS | B12 | B29 | U, HS |
| B13 | B45 | U, HS | B12 | B30 | U, HS |
| B15 | B19 | U, HS | B12 | B40 | U, HS |
| B15 | B34 | U, HS | B12 | B47 | U, HS |
| B15 | B39 | U, HS | B39 | B40 | U, HS |
| B15 | B45 | U, HS | B39 | B47 | U, HS |
| B18 | B22 | U, HS | B40 | B49 | U, HS |
| B18 | B38 | U, HS | B41 | B46 | U, HS |
| B18 | B41 | U, HS | B45 | B47 | U, HS |
| B19 | B21 | U, HS | B29 | B49 | U, HS |
| B19 | B34 | U, HS | B30 | B34 | U, HS |
| B19 | B39 | U, HS | B30 | B38 | U, HS |
| B19 | B41 | U, HS | B30 | B39 | U, HS |
| B21 | B26 | U, HS | B30 | B40 | U, HS |
| B21 | B49 | U, HS | B29 | B30 | U, HS |
| B22 | B29 | U, HS | B29 | B45 | U, HS |
| B22 | B38 | U, HS | B29 | B46 | U, HS |
| B22 | B39 | U, HS | B26 | B29 | U, HS |
| B22 | B41 | U, HS | B05 | B30 | U, HS |
| B22 | B46 | U, HS | B05 | B34 | U, HS |
| B22 | B49 | U, HS | B05 | B38 | U, HS |
| B23 | B34 | U, HS | B05 | B39 | U, HS |
| B23 | B45 | U, HS | B08 | B29 | U, HS |
| B05 | B13 | U, HS | B08 | B30 | U, HS |
| B05 | B18 | U, HS | B08 | B39 | U, HS |
| B05 | B21 | U, HS | B08 | B47 | U, HS |
| B05 | B22 | U, HS | B13 | B18 | HS, FS |
| B05 | B23 | U, HS | B19 | B23 | HS, FS |
| B05 | B40 | U, HS | B21 | B41 | HS, FS |
| B05 | B49 | U, HS | B34 | B49 | HS, FS |
| B08 | B21 | U, HS | B39 | B49 | HS, FS |
| B05 | B26 | U, HS | | | |

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