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A genetic and stable isotope interpretation of behaviour and social structure in Pale-winged Starling *Onychognathus nabouroup*

by

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Thesis

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Declaration

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Structure of the Thesis

This thesis comprises of four chapters. Chapter one presents an introduction to the study, together with a review of the literature that served to inform the study. Chapter two and three are presented in the format of research papers so that they can be read independently. Each consists of a brief introduction, methods and materials, results and discussion. Chapter two discusses and explains the isolation and development of the species-specific microsatellites and interprets the outcome of the microsatellite genotyping in determining the social and genetic structure of the two populations of Pale-winged Starlings analysed in this study. Chapter two has been submitted to *Ecology and Evolution,* (Manuscript ID: ECE-2021-04-00641). The third chapter discusses the results of the isotope analysis for the two populations. The final fourth chapter discusses the main findings of the study, the link between the genetic and isotope analyses, and highlights the conclusions of the study. A single reference list has been compiled at the end of the thesis.

Research Outputs

Oral Presentations

Munshi N., Symes C., De Maayer P., L. Craig A., Henry L., Lange L, Hausberger M. and Mollett J. 2019. A genetic and stable isotope interpretation of behaviour and social structure in Pale-winged Starlings, *Onychognathus nabouroup*. 10th Cross Faculty Postgraduate Symposium. 3-4 September 2019. New Commerce Building, Braamfontein, Wits West Campus. Student - Oral presentation.

Poster Presentations

Munshi N., Jackson D., Symes C., De Maayer P., Craig A., Henry L., Lange L, Hausberger M. and Mollett J. 2019. Molecular Markers for Starlings. Advancing Conservation Consciousness. 10th Oppenheimer Research Conference. 1-3 October 2019. Randjiesfontein, Midrand.

Munshi N., Symes C., De Maayer P., Craig A., Henry L., Lange L, Hausberger M. and Mollett J. 2019. A genetic and stable isotope interpretation of behaviour and social structure in Pale-winged Starling *Onychognathus nabouroup*. University of the Witwatersrand Molecular Biosciences Research Thrust (MBRT) Research Day. Medical School, 28 November 2019. Poster presentation won second place Best Poster Award

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Abstract

Starlings have received much attention in the field of vocalisation and the link between song and social structure. The aim of the present study was to use the microsatellite markers developed in this study alongside carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) to understand the population structure and social structure in Pale-winged Starlings (*Onychognathus nabouroup*), songbirds found in southern Africa. Using next-generation sequencing, 48 microsatellite markers 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 in Namibia (n=24), respectively and genotyped using fifteen polymorphic microsatellites. STRUCTURE analysis revealed a lack of genetic structure and low genetic variation between the two populations. Each population has a high degree of genetic diversity present between individuals. Movement of individuals between the two populations appear frequently and maintains gene flow between them. Stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) in feathers revealed a clear distinction between the two populations of Pale-winged Starlings sampled from the Augrabies Falls Nature Reserve and the Ai Ais-Richtersveld Transfrontier Park. Male Pale-winged Starlings are larger than females and were found to occupy higher trophic levels than females in samples from both the populations. Based on differences in male and female physiology we can propose that males have access to food of higher trophic levels because of their size. Individual foraging specialisation within each population is also apparent. 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.

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List of Abbreviations

AMOVA	Analysis of molecular variance
CO_2	Carbon dioxide
DFA	Discriminant Function Analysis
DNA	Deoxyribose nucleic acid
F	Fixation index
FS	Full sibling
He	Expected heterozygosity
HS	Half sibling
Ho	Observed heterozygosity
I	Information Index
Ne	Number of effective alleles
Nm	Number of migrants
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIC	Polymorphic Information Content
U	Unrelated

Chapter 1

Introduction

1.1. Genetic markers in population genetic studies

The introduction of molecular markers into population ecology studies has revolutionised the field (Avise, 1994; Freeland *et al*., 2011). The way we view mating systems, estimations of genetic variation and gene flow among populations has been enhanced with the use of molecular markers (Avise *et al*., 2002; Griffith *et al*., 2002; Uller & Olser, 2008; Roberts & Byme, 2011). The amount of information attainable from studies on population structures and parentage continues to soar as the technologies for the development of these markers become more efficient and accessible (Morin *et al*., 2004; Anderson & Garza, 2006; Dawson *et al*., 2013). Determining the genetic diversity of populations serves to track the ability of populations to adapt to changing environments (Templeton, 2001). The general impact of human activities and climate change on genetic diversity disrupts or diminishes the capacity for adaptation (Templeton, 2001). This impact can ultimately diminish biodiversity at all levels (Templeton, 2001). Therefore, determining the genetic variation present in species will enable us to inform the conservation of biodiversity in those species. Genetic variation can be easily assessed with the use of molecular markers (Ellegren, 2000).

The use of nucleotide sequence differences in a single gene to investigate evolutionary relationships were first utilised in 1977 by Carl Woese (Balch *et al.*, 1977). Sequence differences in a conserved gene such as ribosomal RNA (rRNA) were initially used to infer phylogenetic relationships (Woese, 2000). It was later discovered that closely related organisms could not be differentiated using rRNA due to its slow evolving rate (Woese, 2000). Genes that evolve faster were then explored to determine if these rapidly evolving genes, such as mtDNA, were able to differentiate between closely related species (Brown *et al.*, 1979).

The high mutation rate of mtDNA makes it a popular marker for biodiversity studies. MtDNA has a rapid mutation rate of approximately 6.2×10^{-8} per site allowing an increased chance of accumulating variations (Avise, 1994; Haag-Liautard *et al*., 2008; Sturge *et al*., 2016). It has been extensively used to investigate intraspecific and interspecific evolutionary relationships and for disentangling rapid, recent speciation events in vertebrate phylogenetic studies (Saetre *et al*., 2001). It has since become a widely employed marker in phylogenetic and population genetic studies as it has a higher mutation rate than nuclear DNA, allowing for the accumulation of differences between closely related species (Brown *et al*., 1979). MtDNA is maternally inherited and is regarded as clonal (Galtier *et al*., 2009). The whole mitochondrial genome behaves as a single, non-recombining locus. Its enhanced mutation rate compared to that of the nuclear genome enhances its popularity along with the high copy number present in each cell; allowing easy isolation (Leese & Held, 2011). However, in most studies mtDNA markers are coupled with microsatellite markers to encompass a large pool of genetic variation to inform interand intraspecific species interactions and genetic diversity (Lu *et al*., 2001; Crochet *et al*., 2003; Toews *et al*., 2011; Coetzer *et al*., 2015; Weinman *et al*., 2015; Gariboldi *et al*., 2016; Germain-Aubrey *et al*., 2016; Samani *et al*., 2016).

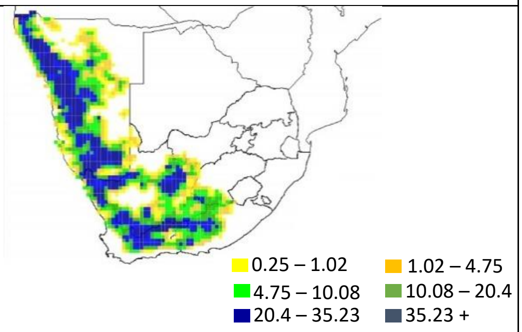
Microsatellites are short tandem repeats of DNA, characteristically less than five base pairs in length with high variability based on the repeat numbers. Microsatellite mutation rates differ among alleles, loci and species (Ellegren, 2000), and depend on a variety of features such as length in base pairs, number of repeated units, and repeated motif (Webster *et al*., 2002). The mechanism of slippage is responsible for the higher number of repeats and the high mutation rates found in microsatellite markers. Slippage occurs during replication when the nascent strand and template strand do not align and are out of register. This alters the repeat number due to the replication synthesis continuing as normal (Webster *et al*., 2002). Microsatellites have become a useful tool to ecologists due to their abundance throughout the genome, highly polymorphic nature and Mendelian inheritance (Morgante & Olivieri, 1993; Shamjana *et al*., 2015; Germain-Aubrey *et al*., 2016). The microsatellite flanking regions are generally conserved across individuals of the same species and occasionally of different species allowing microsatellite loci to be identified by the sequence of the flanking regions (Selkoe & Toonen, 2006). Differences in alleles are not only determined by the variation in repeat number, the substitutions, insertions and deletions in the flanking regions are also relevant (Viard *et al*., 1998). Microsatellite markers have been used in multiple studies to link heterozygosity and genetic diversity to behavioural patterns and social structure in different species. A study by Hill *et al*. (2010) used microsatellites to infer the genetic diversity of Song Sparrows (*Melospiza melodia*). Microsatellite profiling was used to determine extra pair paternity present in the population and determine the role genetic diversity played in mate choice.

Transferability of microsatellite markers between species of the same genus is often successful based on the flanking region consisting of homologous DNA sequences however, the successful amplification rate declines as the genetic divergence increases (Primmer & Merilä, 2002). Studies on birds by Ellegren *et al*. (1995) have shown that the degree of microsatellite polymorphism is not transferable between species. High levels of polymorphism detected in one species may not be found at the same locus of another species using the same primers (Ellegren *et al*., 1995). The challenge of microsatellite transferability in birds was illustrated by Lillandt *et al.* (2003) as well. Lillandt *et al*. (2003) isolated four microsatellite markers from the Siberian Jay (*Perisoreus infaustus*) which were all polymorphic and they tested 64 heterologous microsatellite markers which were isolated from other passerine birds. From these microsatellite markers only eight were able to amplify in the Siberian Jay and were polymorphic. The nine microsatellite markers which were polymorphic including the four isolated from the Siberian Jay were tested for cross amplification. Only eight of the nine markers were able to cross amplify in the Common Jay (*Garrulus glandarius*) and five markers were monomorphic in the Great Reed Warbler (*Acrocephalus arundinaceus*). This outcome displayed the difficulty of microsatellite transferability by testing species specific markers compared to common heterologous markers of the same family. Based on the results of the research described above, markers generated specifically for Pale-winged Starlings would therefore ensure the best results for the highest degree of polymorphism to be detected in the species.

1.2. Pale-winged Starlings in southern Africa

In the last few years research into starling species has increased and revealed interesting findings which contribute to understanding the impact of social density on vocal diversity, the tolerance of starlings for birds which have variants of their species' song and a link between song temporal structuring and social organisation (Henry *et al*., 2015a; LIA VOCOM now called IRP (International Research Project) VOCOM, communication). The Pale-winged Starling (*Onychognathus nabouroup*), a songbird commonly found in southern Africa, is the focus species for this study. Table 1.1 illustrates the distribution and defining characteristics of Pale-winged Starlings. Not much has been published to date about this species in a genetic context. The timing of moult and the sexing of these birds have been researched (Craig *et al*., 2015; Henry *et al*., 2015). Pale-winged Starlings are sexually monomorphic and a Discriminant Function Analysis (DFA) using morphometrics to aid in sexing Pale-winged Starlings in the field has been developed (Henry *et al*., 2015).

Table 1. 1: The distribution of Pale-winged Starling (Onychognathus nabouroup) in southern Africa as reported by SABAP2. Map from Brookes (2018).

Species	**Characteristics**	**Distribution and incidence of sighting (%)**
 Photo by: Daniel Danckwerts Pale-winged Starling	•Colonial species. •Monogamous species. •Breeding season: Oct – Apr. •Glossy, black plumage, a narrow pale rufous panel visible on the folded wing.	

Henry *et al*. (2015) reported that the socially monogamous Pale-winged Starlings from the Graaff-Reinet area display both categories of song (whistles and warbling) as well as alternating and overlapping song interactions. Females choose their mates based on traits such as song repertoire size and plumage ornamentation (Foerster *et al*., 2003; Marshall *et al*., 2003). These sexual traits are linked with high genetic heterozygosity (Foerster *et al*., 2003). There is growing evidence that supports female birds choosing mating partners based on genetic heterozygosity because they choose partners with highly heterozygous features (Brown, 1997; Foerster *et al*., 2003). A female's strategy of choosing a male with exaggerated sexual traits may involve finding the alleles that best suit her own to pass on to some of her offspring (Brown, 1997). She would identify these alleles by identifying heterozygosity by means of the males' features. The advantage of heterozygous offspring is assumed to result in more vigour and fitness which then improves survival and mating success (Aparicio *et al.*, 2001). Genetic information on Pale-winged Starlings will allow us to identify the role genetic variation plays in social structures and the success of these birds.

Microsatellite markers have been isolated and developed for the Spotless Starling (*Sturnus unicolor*) to assess interspecific variation in starlings (Celis *et al.*, 2007). These markers are able to cross amplify in the European Starling and can be used to make comparisons between different starling species. However, these markers would not easily detect intraspecific and population genetic variation in any other species besides the Spotless Starling. For intraspecific and population diversity to be detected easily and efficiently, with high polymorphic information content (PIC) values the microsatellite markers would need to be species specific. Species specific markers will be sensitive enough to detect fine scale variation within the populations which can then be used to understand the behavioural and social structure of Pale-winged Starlings.

Genetic markers, such as microsatellites, have become valuable tools in understanding population genetics and connectivity. They have also been applied in tracking populations alongside isotopic markers (Wenink & Baker, 1996; Haig *et al.*, 1997; Wennerberg, 2001). Information on population connectivity and relatedness has also been obtained in many studies via isotopic analysis of animal tissues, such as feathers alongside microsatellite markers (Marra *et al.*, 1998; Hobson, 1999; Chamberlain *et al.*, 2000; Hobson *et al.*, 2001; Meehan *et al.*, 2001; Rubenstein *et al.*, 2002).

1.3. Isotopes and population connectivity

The use of stable isotope ratios of several light elements has become an asset in ecological studies (Inger & Bearhop, 2008). Avian ecologists have developed multiple applications of the stable isotope procedures; with a number of studies focusing on the diets and migration patterns of birds (Hobson & Wassenaar, 1997; Marra *et al.*, 1998; Schäfer, 2003; Pain *et al.*, 2004; Rubenstein & Hobson, 2004; Bearhop *et al.*, 2005; Procházka *et al.*, 2008; Symes & Woodborne, 2010; Oppel *et al.*, 2011). In nature, each element occurs in one or more forms, where the number of neutrons they contain differ. These different forms are called isotopes (Fox & Bearhop, 2008; Hoefs, 2009). Stable isotopes, unlike radiogenic isotopes, do not decay over time (Inger & Bearhop, 2008). The more neutrons, the heavier the atom; the heavier the atomic mass (Inger & Bearhop, 2008). The relative abundance differences of these isotopes can be measured using a mass spectrometer and is expressed as the ratio of heavy to light form (Bigeleisen, 1965; Ehleringer & Osmond, 1989; McKechnie, 2004; Inger & Bearhop, 2008; Hoefs, 2009).

The use of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes allows the reconstruction and quantification of the proportions of isotopically distinct diets in animals (Inger & Bearhop, 2008; Symes, 2012). $\delta^{13}C$ is generally used to understand the importance of particular carbon pools to a consumer. Terrestrial plants for example, have different $\delta^{13}C$ values based on their differing photosynthetic pathways (Marshall *et al*., 2007), and marine algae have a higher $\delta^{13}C$ value than all terrestrial plants (Kelly, 2000; Marshall *et al*., 2007). These differences reflect in the tissues of primary consumers and can be detected at all trophic levels within the food web (Kelly, 2000). $\delta^{15}N$ is effective in identifying the trophic level (Inger & Bearhop, 2008). This is because of the loss of light ^{14}N to nitrogenous waste products (Michener & Kaufman, 2007), leaving consumer tissues enriched with ^{15}N. This trophic enrichment is typically between 2-4‰ at each trophic level (Post, 2002).

The use of feathers or claws reflect the highly specific isotopic environment in which they grow; and this signature is stored until they are shed because these tissues are metabolically inactive (Chamberlain *et al*., 1997; Hobson, 1999; Hobson, 2005; Fox & Bearhop, 2008). Feathers can reflect information on the period of growth whereas claws can infer dietary and habitat information over a period varying from weeks to months (Bearhop *et al*., 2003). Metabolically active tissue will reflect information according to the tissues turnover rate (Hobson & Clark, 1992a; Hobson, 1999; Hobson, 2005; Podlesak *et al*., 2005; Inger & Bearhop, 2008; Larson & Hobson, 2009; Hobson, 2011). Studies using isotope ratios have been conducted on a variety of avian species including Amur Falcon (*Falco amurensis*), Willow Warblers (*Phylloscopus trochilus*), Aquatic Warblers (*Acrocephalus paludicola*), Reed and Great Reed Warblers (*Acrocephalus scirpaceus*) (Hedenström & Pettersson, 1987; Schäfer, 2003; Pain *et al*., 2004; Procházka *et al*., 2008). A study by Clegg *et al*. (2003) used stable isotopes and genetic markers to reveal population connectivity and migration patterns in Wilson's Warblers (*Wilsonia pusilla*). The genetic markers provided information about the genetic diversity present in the populations and the orientation of the population structure (Clegg *et al*., 2003). Unfortunately, the study did not include carbon and nitrogen isotope ratios, although this would have enhanced the understanding of these birds' social, geographical and behavioural interactions.

1.4. The present study

The present study used a combined analysis of genetic markers and isotopic ratios to interpret the role genetic variation and diversity play in the behavioural patterns, social, and population structure of Pale-winged Starlings. This study attempts to uncover the genetic variation present in two Pale-winged Starling populations and determine the effect the changing environment and human impact has on these birds based on their genetic variation and their foraging habits. This study will add to the research currently being conducted by the International Associated Laboratory (LIA) project called "Evolution of vocal communication: testing the impact of social systems, phylogeny and conditions of life" (VOCOM). LIA is now referred to as International Research Project (IRP). VOCOM is led by the University of Rennes1, which focuses mainly on the evolution of vocal communication and the factors involved. VOCOM has successfully ringed 70% of the local Pale-winged Starling population in Augrabies Falls Nature Reserve and are currently involved in following the vocal and social dynamics of this species whose song has never been studied. The present study will analyse samples from the same study site as Henry *et al*. (2015) from the Augrabies Falls Nature Reserve in the Northern Cape Province of South Africa. The study by Henry *et al*. (2015) investigated the sexing of Pale-winged Starlings using morphometric measurements as accurate sexing is important when analysing these birds' singing patterns and population or social structures. The second population of Pale-winged Starlings that will be studied are from a study site in the Ai Ais-Richtersveld Transfrontier Park located in Namibia. The isotopic analysis of the feathers will allow inferences of diet and trophic levels in individual Pale-winged Starlings found in southern Africa to be compared and investigated. The stable isotope analysis in conjunction with the genetic information will allow us to understand how these birds relate to one another and form social structures and populations. The research conducted on the Pale-winged Starlings will add to the understanding of the behavioural patterns seen in these populations. Two populations will be analysed and can be compared to determine variation between and within them based on habitat and geographic distances. This will provide knowledge and insight into the biodiversity present in these Pale-winged Starling populations and will contribute to the understanding of genetic variation, social structure, and behaviour of these birds.

Chapter 2[*]

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

2.1. 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 present study aimed 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 in Namibia (n=24), respectively. Fifteen polymorphic microsatellite markers were genotyped. A Bayesian clustering software revealed four different genetic clusters within the two populations. There is low genetic variation (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 move freely 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 contributed information for the success of this species. Social structure, relatedness and behaviour were inferred and Pale-winged Starlings maintain stable social environments regardless of genetic relationships.

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

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[*] This Chapter (manuscript) has been submitted to *Ecology and Evolution*. Manuscript ID: ECE-2021-04-00641

2.2. Introduction

The study of sociogenomics encompasses the understanding of social life in molecular terms (Robinson *et al*., 2005). 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 range 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.).

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 genetics is needed.

Microsatellites have become common practice in studies investigating parentage or species relatedness (Crochet *et al*. 2003; Germain-Aubrey *et al*. 2016). Relatedness refers to the proportion of shared genes between individuals (Koenig & Dickinson, 2016). It is an influential factor in interpreting how altruism works in nature and influences the behaviour of populations, communities, or species (Hamilton, 1982). Studies have found a link between offspring survival, increased longevity and territorial establishment of social birds with strong and stable social bonds (van Overveld, 2020). The relatedness of individuals in a population affects the social structures and behaviour of the populations. 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 (Toth *et al*., 2009). 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). This bird species had increased individual fitness due to the establishment of stable relationships between unrelated females (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 determine genetic diversity and to demonstrate the positive influence of genetic diversity on mating success and ornamentation (van Dongen & Mulder, 2009).

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 from 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. This can be determined by calculating the polymorphism information content (PIC) of the markers (Serrote *et al*., 2020). The PIC measures the ability of markers to detect polymorphisms (Serrote *et al*., 2020). PIC values range between 0 and 1 where a PIC value of 0,44 is considered a moderately informative marker and a PIC value greater than 0,7 is a highly informative marker (Miller *et al*., 1992).

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.

2.3. Methods and materials

2.3.1. 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. 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. The VOCOM team also provided the mass, wing length, tarsus length and sex which were used in this study. All the samples collected were of adult and sub-adult birds. Of the 77 samples 36 were male and 41 were female. The samples had an average mass, wing length and tarsus length of 96,9gr, 141,5mm and 31,5mm respectively. The VOCOM team included qualified ringers recognised by SAFRING (Permit 296) who captured and ringed the birds. Blood and feather samples were collected under the license number R-2012MH01, and ethics approval code RU-LAD-15-09-0001 and agreement number HAUM1381.

2.3.2. Genomic DNA extraction

Total genomic DNA extractions from the blood samples 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).

2.3.3. Development of microsatellite markers

A library was prepared using the combined 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.

2.3.4. Microsatellite Analysis

Fifteen polymorphic loci out of the total 48 microsatellite loci were selected by Ecogenics based on the successful amplification during the isolation and development of the markers from the genomic library of *O. nabouroup*. These 15 markers were then used for the analysis of the 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 2.1). Microsatellite PCR product sizes were determined using an ABI3100 Genetic Analyzer (Central Analytical Facility, Stellenbosch University), with a Genescan^{TM} 500 Liz^{TM} (Applied Biosystem Inc., Central Analytical Facility, Stellenbosch University) internal size standard. Peak Scanner Software^{TM} v1.0 was used to visualise and determine allele sizes.

Table 2.1: 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 R- AGCCATTTGCTGCACCTATC	(TTA)8	FAM	163-188	7	1	MT446464
On_507360	F- GGCAGAACGGGGATGTTTG R- AGATGCTCCCATGTCCACTC	(GGAT)7	ATTO565	162-203	5	1	MT446466
On_1106367	F- GGGCAGTTATCAGTCCTTGG R- AAGCCATGACTGTCCACCAG	(ACAT)7	ATTO532	104-115	4	1	MT446463
On_843840	F- TGCAGATGCCCCACTTTTTC R- TGGGCAAAACATTGAGTGAATAC	(ATGA)14	ATTO550	181-201	6	1	MT446465
On_589878	F- GAGGCTCCATATCCCACCAG R- ATCTGCCAGCCAGGATTGTC	(TAA)14	ATTO532	224-255	7	1	MT446467
On_324078	F- ACTGACAAAATTCAAAGCAAAAGTG R- ACTTAGCAGTAAAACAATTGACATC	(ATA)10	ATTO532	208-229	6	2	MT446474
On_852924	F- ACTTTTGGAGGTCATTGGCTG R- GCAGAAAGGCTGGTTAGGTC	(GATA)15	FAM	212-255	6	2	MT446475
On_864997	F- ATTGTTCAGCTGCTTCACGG R- GCTATGAAAGCCAGTGGTGG	(GGA)10	FAM	141-148	3	2	MT446471

Locus	Primer sequences 5′- 3′	Repeat type	Fluourescent dye	Size bp	No. Of Alleles	Multiplex panel	Genbank accession No.
On_877333	F- TGCCTCCTTCGTACCCATTC R- GGGCGTCTGGATGCAAATAG	(TCCA)15	ATTO565	159-206	8	2	MT446472
On_883556	F- TATGCAGAAGTGGCTGAGGG R- GTGAACTTGTCAGTGGGCAG	(TATC)18	ATTO550	189-206	5	2	MT446473
On_23489	F- CTTCAAGGATGCACAGGCAG R- TCACTTTCCAGTGAGAAGCC	(ATAG)21	ATTO565	178-233	8	3	MT446477
On_290548	F- GGTGACATCAGTACCTGGGG R- GGGCACAGTGAGGGAATAAC	(TTA)26	ATTO590	178-231	11	3	MT446478
On_402628	F- AGAGGCTTTCAGGGGATGTG R- ATCCAGAGCTGGTTCTCCTC	(AGG)8	ATTO532	210-233	4	3	MT446479
On_787859	F- GCTTCCTTGCACAGATAGCAC R- TGGGGATCTGAGTGCATTTTC	(ATCT)15	FAM	171-187	5	3	MT446476
On_968476	F- ACGTGCAAGAAAAGAGCTGG R- AGAGGTTCCTTTACGTGGGC	(TCA)8	FAM	224-233	3	3	MT446480

The mean number of alleles (as determined on the basis of fragment lengths from the multiplex PCR) per locus, deviations from Hardy-Weinberg proportions, observed heterozygosities, expected heterozygosities, 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). The PCA analysis was used to determine the distribution of the genetic variation.

2.3.5. 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). Delta K is the most common way of determining the number of clusters or sub populations present in the total population. The K-value with the highest delta K was used as the best K-value for the dataset which was K=4 (Figure 2.1) (Evanno *et al.*, 2005). This was done using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The delta K-values on the Y-axis however are very low which indicates the K-value of four is unlikely in the actual populations (Figure 2.1).

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).

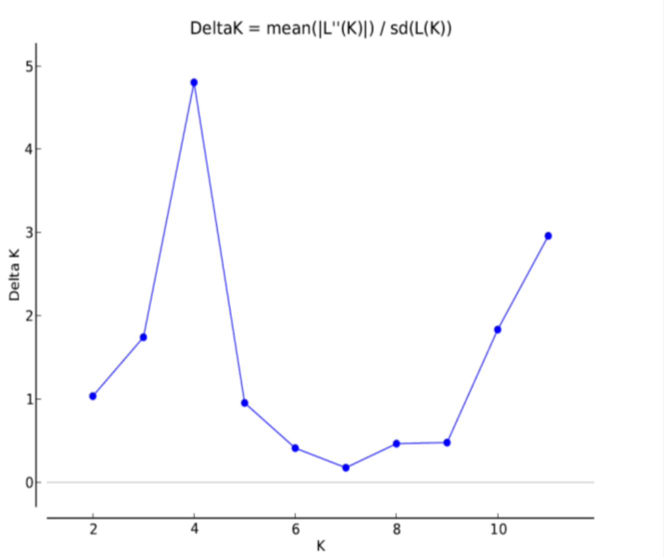

Figure 2.1: Delta K values for species structures of K=1-12.

Table 2.2: Ln(P) of K values used in STRUCTURE analysis

K	LnP(D)	K	LnP(D)
1	-5347,6	7	-7339,4
2	-5416,4	8	-6897,3
3	-6127,3	9	-8435,5
4	-6014,6	10	-8312,7
5	-7030,1	11	-7978,1
6	-6667,6	12	-8247,7

2.4. Results

2.4.1. 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 which can 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.

2.4.2. Microsatellite Analysis in two Pale-winged Starling populations

Fifteen microsatellite loci were selected based on their polymorphic strength and genotyped (GenBank accession numbers in Table 2.1). Every marker had a PIC value between 0,78 and 0,94 which was calculated using the formula PIC= $1-\sum (Pi)^2$, where Pi depicts the proportion of samples carrying the ith allele. 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 effective population size was determined using the formula $N_e = (4 \times N_m \times N_f) / N_m + N_f$, where N_m and N_f represent the number of breeding males and females in the population. The effective population size in the Augrabies Falls Nature Reserve is 49,28 and 26,96 in the Ai Ais-Richtersveld Transfrontier Park. 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 2.2. 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 those which are frequent enough that they can be used in standard marker panels and are not polymorphic (Raychaudhuri, 2011).

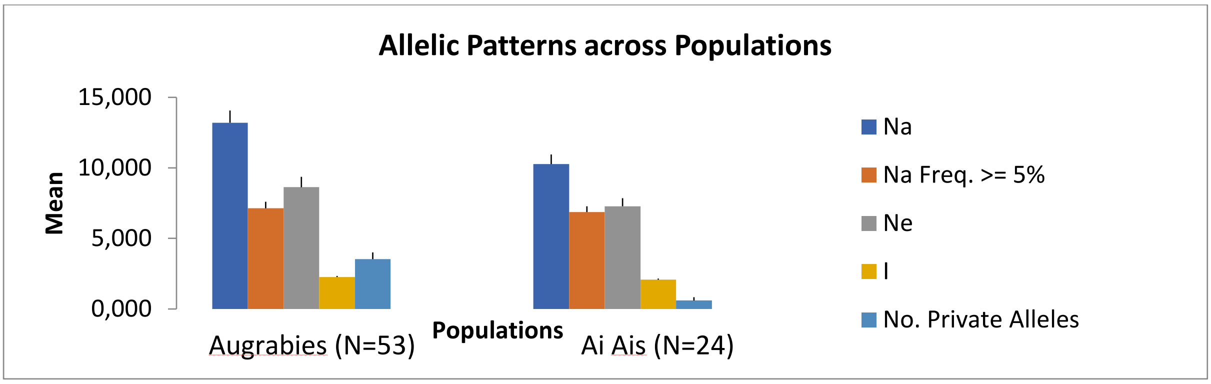

Figure 2.2: Summary of the number of alleles (Na), number of effective alleles (Ne), information index (I) and the number of private alleles.

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 2.3). 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 2.3; Appendix 4).

Table 2.3: 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 Ais	**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 variation/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.

Table 2.4: Analysis of molecular variance (AMOVA) for Pale-winged Starlings across 15 microsatellite markers.

Source of variation	Sum of squares	Variance components	Percentage variation	Fst (p value)
Among populations	10,31	0,04	0,56	0,368
Among individuals within populations	567,05	0,51	7,05	0,076
Within individuals	506,5	6,65	92,39	0,804

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 2.3). 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 2.3).

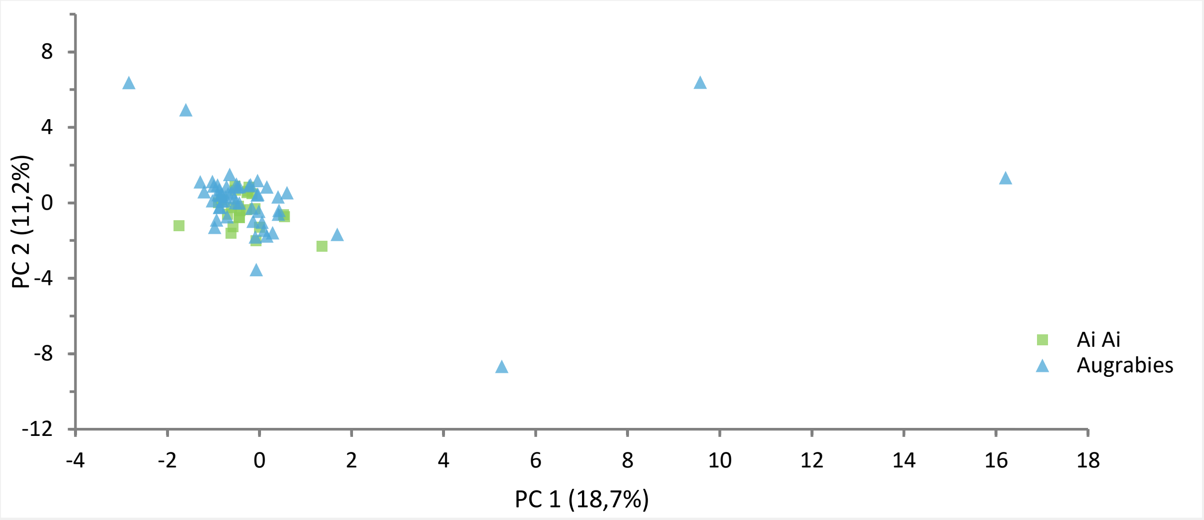

Figure 2.3: A Principal component analysis plot indicating the distribution of genetic variation in two populations of Pale-winged Starlings population based on fifteen microsatellite markers.

2.4.3. Population structure and Relatedness

STRUCTURE analysis was performed using K=4 to get a clear understanding of the genetic structure and clusters present in the data set (Figure 2.4). The data showed 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 2.1) are low indicating K=4 may not be the actual number of genetic clusters possible. The data appears to represent less than four genetic clusters. Figure 2.5 and Figure 2.6 illustrate both K=1 and K=2 respectively.

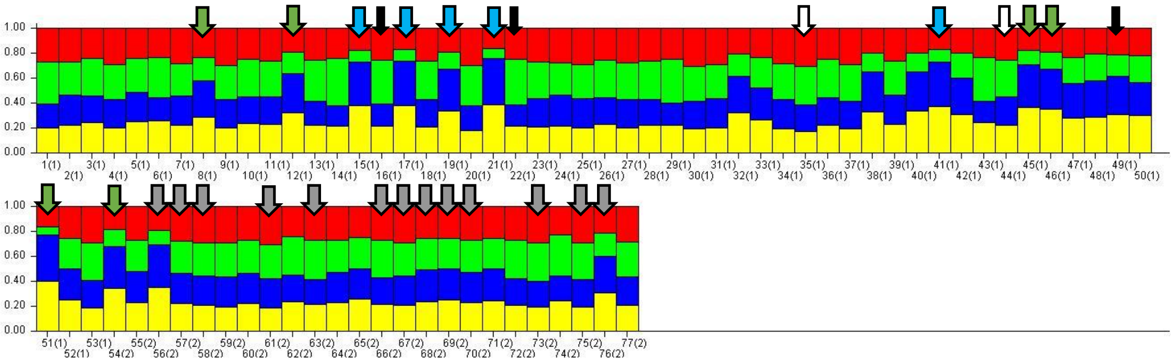

Figure 2.4: A STRUCTURE membership assignment plot of 77 Pale-winged Starling individuals for K=4. Each individual is represented by a single vertical line, with lengths proportional to the estimated membership in each cluster. The y-axis represents probability (q) of each individual assigning to K clusters.

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

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

⬇ Thirteen samples from Ai Ais with more relatives in Augrabies

⬇ Five outliers in the PCA analysis.

⬇ Similar genetic membership as the outliers

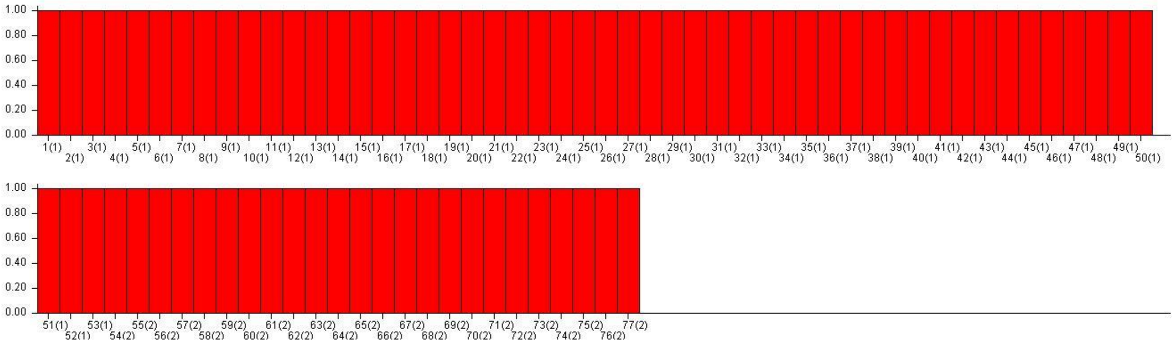

Figure 2.5: A STRUCTURE membership assignment plot of 77 Pale-winged Starling individuals for K=1. Each individual is represented by a single vertical line, with lengths proportional to the estimated membership in each cluster. The y-axis represents probability (q) of each individual assigning to K clusters.

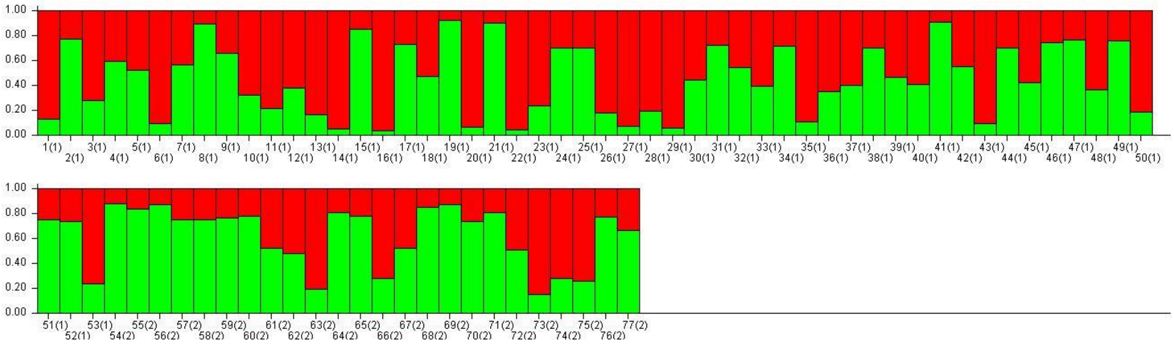

Figure 2.6: STRUCTURE membership assignment plot of 77 Pale-winged Starling individuals for K=2. Each individual is represented by a single vertical line, with lengths proportional to the estimated membership in each cluster. The y-axis represents probability (q) of each individual assigning to K clusters.

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 2.5 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 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 have more relatives in the Augrabies Falls Nature Reserve region than in the Ai Ais-Richtersveld Transfrontier Park 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 2.5: Collated results of ML-Relate analysis

	Both populations	**Augrabies population**	**Ai Ais 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

2.5. Discussion

All 48 microsatellite markers developed in this study, with the exception of On_772390, were polymorphic in the seven Pale-winged Starling blood samples used to create the library. This single monomorphic microsatellite marker could, however, still be a useful tool for population genetic studies. A study in plants by Nazareno and dos Reis (2010) used a dinucleotide monomorphic microsatellite marker to elucidate polymorphic sites in the flanking regions of the monomorphic marker to determine genetic diversity within the population. To our knowledge using a monomorphic marker has not been tested in bird species. The remaining 47 microsatellite markers are comprised of six di-, eighteen tri- and twenty-four tetra-nucleotides. Of these 47 markers, five markers displayed drop-outs (no amplification in some samples) (Appendix 1) and as such, 42 markers can be retained for a multitude of analyses ranging from intraspecific population genetics to interspecific comparative genetic studies. With 90% of bird species being socially monogamous (Hoi and Griggio, 2010), results obtained from the application of these markers regarding genetic diversity and social structures, and further differentiation between socially and sexually monogamous species, can be used to infer similar trends in other bird species.

A total of fifteen markers were chosen for further analysis. These markers were chosen based on the ease of amplification during the development of the markers. The PIC vales of all fifteen markers were above 0,7 which indicates a highly informative marker (Miller *et al*., 1992). From the results of the fifteen microsatellites used to evaluate Pale-winged Starlings, we can conclude that the two populations of Pale-winged Starlings, regardless of their geographic distance are very similar based on the chosen molecular markers with the largest variation and genetic difference found between individuals rather than between populations. The Brazilian tanager (*Ramphocelus bresilus)* across different populations had similar genetic diversity levels and genetic make-up when comparing different populations across Rio de Janeiro (Nogueira *et al*., 2014). A study on the house sparrow (*Passer domesticus*), also demonstrated low genetic differentiation between distinct populations in Finland, with genetic variation between individuals accounting for most of the variation (Kekkonen *et al*., 2011).

The STRUCTURE analysis did not assign the individuals into the two expected population clusters according to their geographic locations. Rather four separate clusters were observed, supporting breeding between the populations even though they are geographically separated by 400km. The low genetic distance between the populations, allows for interbreeding due to the migrants moving between the two populations. However, the program STRUCTURE, detects the uppermost hierarchical level of population structure and authors have warned that STRUCTURE results are only an indication of the number of clusters and a guide (Pritchard *et al*., 2000; Pritchard & Wen, 2003). The K-value of four (Figure 1) and the AMOVA analysis (Table 2.4) both indicate the allelic richness and large amount of variation present in the populations. However, the STRUCTURE analysis appears to represent K=1 or K=2. This would indicate a better representation of the populations and supports breeding between the two populations regardless of their geographical location. The K=1 shows panmixia of the two populations which is the random mating within a breeding population.

The STRUCTURE analysis showed that each genetic cluster is comprised of individuals from both populations, which supports the estimated number of migrants in the population. This corresponds with the low Fst values seen in the study and the ML-Relate results. The number of samples from the Ai Ais-Richtersveld Transfrontier Park population (n=24) is much smaller than the number of samples from the 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 2.3, 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 showed 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 indicates that the two populations may not exist exclusively in one particular region. Birds from either population migrate and join other populations 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 differentiation 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 indicates 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. 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 based on the transferability of markers within species. 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 as well as samples from different areas to determine the genetic variation and possible differentiation.

2.6. Data accessibility statement

All DNA sequences have been uploaded onto GenBank and accession numbers can be found in Table 2.1.

2.7. Appendix 1

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

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

Locus	Primer sequences 5′- 3′	Repeat type	Size bp	No. of alleles	*Genbank accession No.	Comments
On_772390	F - GGGGTGGTGAGCAGTCATAC R - ACCGTATCTGCATCCACCTG	(TC)13	180	1		Monomorphic
On_787859	F - GCTTCCTTGCACAGATAGCAC R - TGGGGATCTGAGTGCATTTTC	(ATCT)15	171-187	5	MT446476	
On_806239	F – TGTAACTGAGAGTTTCCCAAGC R - ACTCCATGTATTTATTCCCAGAGG	(AATA)7	165-185	5		
On_821403	F - GAACCTGCAGAATGTCACCC R - GCCACACTGATTCCTATGAGC	(AGTG)18	147-188	5		
On_843840	F – TGCAGATGCCCCACTTTTTC R - TGGGCAAAACATTGAGTGAATAC	(ATGA)14	181-201	6	MT446465	
On_852924	F - ACTTTTGGAGGTCATTGGCTG R - GCAGAAAGGCTGGTTAGGTC	(GATA)15	212-255	6	MT446475	
On_864997	F - ATTGTTCAGCTGCTTCACGG R - GCTATGAAAGCCAGTGGTGG	(GGA)10	141-148	3	MT446471	
On_868467	F – AGCATGGCAGGTGACTTCTC R - AAACAGTGCTGGCTCAATGG	(CCAT)11	221-240	6	MT446470	
On_877333	F – TGCCTCCTTCGTACCCATTC R - GGGCGTCTGGATGCAAATAG	(TCCA)15	159-206	8	MT446472	
On_883556	F – TATGCAGAAGTGGCTGAGGG R - GTGAACTTGTCAGTGGGCAG	(TATC)18	189-206	5	MT446473	
On_894676	F - CCACAGCGATTGGTGTCTTC R - ACATTCCTGGAGTTGTCCTC	(AATA)7	183-187	2		
On_899076	F - ATTCATGGCACTCCTTTTAGAC R - CCCAGGGTCTTCTTTGATAGC	(TAGA)10	212-248	5		Drop out in samples A4 and A8
On_914873	F - CGATATCACTGAGGCCAAGC R - AGAAGTCTGAAAGACACAATGGC	(ATT)19(GTT)8	155-222	5		
On_943863	F - ACAACTCAGACGATAAGCTGG R - AGAAATGACTTTGATCCGTGG	(TATC)22	-	-		
On_950478	F - ATGGTGACGAGTACCTTGCC R - TCATTCTAGTCCAAACCATGAAAAC	(AT)13	-	-		
On_968476	F - ACGTGCAAGAAAAGAGCTGG R - AGAGGTTCCTTTACGTGGGC	(TCA)8	224-233	3	MT446480	
On_1016023	F - TGCTAAACTTTCAATTTTTCCAGTA R - AG TTGAGCATATTGCTGTCTCTTG	(TCTA)18TG (TATC)8	102-294	15		
On_1039897	F – TTTTCAGCCACATGGAGTCG R - TGTCCATGACTGATGCAGAAAC	(ATT)13	-	-		
On_1061771	F – TTGCATTACTGGGGGAGGAG R – ACTGCAGGCTTATGAGGGAG	(TATT)20	129-180	6		
On_1078206	F - CTATCTACGTGCAGGTGTGC R – CAGGACCGTCTAGTCCTTGG	(AC)30	118-156	4		Drop out in sample A8
On_1081480	F – GAGCTATGAACCCACAAGGC R – GGCCAGTAAACCTGAGTCAAAC	(TAGA)17	133-190	8		
On_1101365	F - TCCCAGTAGATTCCCTTTCCAC R – CAGATCCCTCCCAAGTGACC	(ATCC)7	147-187	7		

Locus	Primer sequences 5′- 3′	Repeat type	Size bp	No. of alleles	*Genbank accession No.	Comments
On_1106367	F - GGGCAGTTATCAGTCCTTGG R – AAGCCATGACTGTCCACCAG	(ACAT)7	104-115	4	MT446463	
On_1118845	F – ATTCCCACCTGTCCCGATCC R - CACTCCCGGAAGGGATTTTTG	(AGG)9	125-153	8		Drop out in sample A8
On_1133203	F - AAGTCTCCAGTACCTGTGCC R - TGACAACAGGGCGTTTTCAC	(GCT)10	191-201	3		
On_1134576	F - GGATTTGGGCCAGTCCTATG R - GGCATTGCATGCTTCCAGAC	(TTA)21	229-265	7		Drop out in samples A4, A6 andA8
On_1142255	F - AGCATCACCGTCAGTCCTAC R - AGCCATTTGCTGCACCTATC	(TTA)8	163-188	7	MT446464	
On_1157444	F - TCCTTACCAACCAGACTGCC R - CTTGCTGTCTGTATCACGCC	(GATA)19	198-257	8		Drop out in sample A8
On_1157509	F - CTAGCGCAGAAGGTATGGTG R - GCTGATGTGTCAACCAGGAG	(TA)11	-	-		

* Accession numbers for 20 markers have been uploaded thus far

2.8. Appendix 2

Raw allele data of fifteen microsatellite markers of 77 Pale-winged Starlings

Sample	Population	Locus6		Locus7		Locus8		Locus9		Locus10		Locus11		Locus12	
1	Augrabies	141	144	184	184	0	0	225	228	220	224	176	176	184	192
2	Augrabies	132	135	160	184	196	208	210	225	236	240	172	184	192	216
4	Augrabies	132	156	164	200	204	208	222	228	236	252	164	164	200	232
5	Augrabies	144	141	160	184	200	200	216	216	216	220	184	184	228	232
7	Augrabies	138	138	188	208	196	200	216	231	220	236	172	180	176	216
8	Augrabies	144	156	168	204	200	208	0	0	216	232	188	188	204	224
9	Augrabies	156	156	164	204	208	208	222	225	228	240	180	180	184	220
11	Augrabies	141	159	176	200	196	212	216	222	216	240	176	180	188	212
12	Augrabies	156	156	172	204	200	208	222	228	220	236	160	196	212	232
15	Augrabies	0	0	188	200	204	208	222	228	236	244	168	180	192	220
18	Augrabies	0	0	188	200	204	204	204	213	220	252	168	188	220	232
19	Augrabies	138	138	184	200	196	204	225	225	232	240	172	172	212	224
20	Augrabies	150	156	168	184	0	0	222	225	224	228	180	180	204	228
22	Augrabies	144	141	180	188	184	200	216	240	212	244	164	184	208	228
24	Augrabies	144	156	168	192	204	208	222	225	216	252	180	180	212	232
25	Augrabies	141	147	180	196	204	204	207	207	220	240	100	100	184	200
27	Augrabies	138	135	188	200	196	208	228	228	216	236	172	188	200	224
29	Augrabies	132	156	164	176	196	204	216	222	216	240	184	192	200	208
32	Augrabies	156	156	192	204	208	208	222	228	216	236	172	188	192	220
33	Augrabies	147	147	184	192	0	0	213	222	224	224	0	0	188	192
35	Augrabies	135	135	188	200	208	208	222	231	216	236	176	184	204	232
36	Augrabies	150	150	200	204	184	200	219	225	216	224	204	204	196	220
37	Augrabies	135	147	192	204	192	200	219	231	220	236	188	188	208	228
42	Augrabies	132	156	172	204	200	212	222	228	220	236	168	184	200	212
43	Augrabies	147	156	164	176	196	204	222	222	232	240	176	184	192	212
44	Augrabies	132	147	160	180	188	208	210	225	212	256	180	192	192	216
45	Augrabies	138	150	180	204	188	200	204	213	232	256	168	168	208	224
46	Augrabies	144	156	180	204	200	208	222	228	220	240	160	200	204	216
47	Augrabies	138	153	160	184	188	208	213	222	220	232	200	200	188	208
48	Augrabies	141	156	180	204	200	208	222	228	220	232	168	176	188	216
49	Augrabies	132	159	172	204	200	204	222	231	220	236	176	180	200	228
50	Augrabies	135	159	168	188	204	208	216	228	216	236	180	188	204	212
51	Augrabies	141	156	180	208	208	212	225	234	232	248	180	196	196	216
56	Augrabies	132	156	172	204	200	200	222	225	216	232	172	184	196	192
57	Augrabies	138	147	180	204	200	204	204	225	212	224	160	188	216	232
58	Augrabies	135	135	160	200	200	208	225	234	232	244	168	184	196	208
61	Augrabies	138	144	180	200	200	200	204	216	232	256	176	180	200	220
62	Augrabies	138	135	188	200	200	208	225	234	232	240	176	180	184	208
63	Augrabies	141	147	164	200	196	204	222	228	220	236	176	180	188	212
67	Augrabies	138	135	160	200	188	208	219	231	220	240	180	188	192	212
70	Augrabies	138	135	192	200	196	204	225	231	232	240	172	184	192	232
71	Augrabies	138	135	160	200	196	208	216	231	216	240	164	180	180	224
72	Augrabies	135	126	160	180	184	204	216	216	216	240	168	188	208	224
74	Augrabies	132	159	156	180	200	208	228	234	232	252	160	180	196	212
75	Augrabies	138	135	160	200	204	208	216	231	220	240	172	188	208	224
76	Augrabies	138	135	160	200	204	208	216	231	220	240	180	196	192	216
77	Augrabies	135	156	172	200	196	208	216	216	240	256	172	188	192	228
78	Augrabies	135	135	160	200	184	200	216	234	236	248	164	180	208	224
79	Augrabies	147	156	164	204	196	204	222	225	232	240	160	188	216	228
80	Augrabies	138	135	164	180	192	204	219	222	220	240	180	164	204	224
83	Augrabies	138	135	188	200	204	208	222	228	220	236	172	180	192	212
88	Augrabies	135	156	200	204	196	204	216	231	216	240	172	180	188	220
90	Augrabies	138	153	172	204	196	204	213	225	220	244	180	188	184	212
102	Ai Ais	135	135	188	200	204	204	228	228	216	240	172	180	184	232
103	Ai Ais	135	159	172	200	0	0	222	228	240	252	184	184	212	232
105	Ai Ais	132	156	168	200	208	208	225	234	232	240	168	184	192	212
107	Ai Ais	144	144	172	174	200	204	213	216	216	216	172	180	188	192
109	Ai Ais	132	135	168	200	196	196	216	231	220	240	176	184	220	228
110	Ai Ais	132	135	172	188	196	212	216	216	236	252	172	184	180	232
112	Ai Ais	141	159	188	200	200	200	216	231	216	236	172	184	184	232
115	Ai Ais	141	144	164	172	200	208	213	219	220	236	176	184	184	232
116	Ai Ais	135	159	188	200	212	212	231	231	240	252	172	172	196	208
118	Ai Ais	141	144	180	200	204	204	204	219	240	252	172	184	192	208
119	Ai Ais	138	159	188	200	208	208	228	228	216	232	176	184	192	228
120	Ai Ais	135	159	164	188	204	212	216	231	244	252	172	172	192	220
123	Ai Ais	138	135	160	178	196	204	222	225	216	232	160	196	208	220
126	Ai Ais	147	159	160	200	196	208	219	228	216	244	176	188	192	220
127	Ai Ais	132	156	188	200	196	208	228	234	216	232	184	188	184	208
131	Ai Ais	138	159	172	188	200	200	228	228	236	252	180	188	208	232
135	Ai Ais	159	159	172	200	196	208	213	228	220	232	180	180	216	232
136	Ai Ais	135	159	188	200	196	196	216	228	232	252	180	188	184	212
137	Ai Ais	132	159	176	200	196	208	222	228	236	252	168	184	184	228
138	Ai Ais	141	144	180	200	192	204	204	213	220	256	172	184	196	232
142	Ai Ais	135	159	160	188	208	208	0	0	236	244	192	192	196	224
143	Ai Ais	132	147	172	200	200	208	213	219	212	212	180	188	192	212
144	Ai Ais	156	156	188	200	208	208	222	228	232	240	180	188	196	220
146	Ai Ais	138	159	172	188	196	204	228	234	232	240	180	188	180	232

Sample	Population	Locus13		Locus14		Locus15		Locus16		Locus17		Locus18		Locus19		Locus20	
1	Augrabies	192	207	225	231	231	231	172	180	0	0	168	180	188	200	225	234
2	Augrabies	186	216	213	213	222	231	108	116	159	180	160	180	180	192	219	246
4	Augrabies	201	216	216	231	225	234	112	116	168	168	164	200	172	220	231	234
5	Augrabies	207	216	219	222	228	231	108	112	171	177	180	192	196	196	225	237
7	Augrabies	186	216	213	225	225	231	108	116	159	177	160	192	184	200	237	243
8	Augrabies	201	219	216	225	228	234	116	116	141	150	168	188	200	200	198	219
9	Augrabies	198	222	210	228	225	231	100	112	174	186	188	200	176	176	225	243
11	Augrabies	189	219	213	222	225	225	108	112	180	201	188	20	180	204	237	258
12	Augrabies	198	222	216	231	225	231	112	116	165	180	172	188	192	192	243	246
15	Augrabies	180	228	222	237	219	225	104	116	162	180	192	204	184	200	228	246
18	Augrabies	201	219	216	222	228	234	108	116	180	198	180	200	180	200	228	234
19	Augrabies	207	219	213	225	228	234	0	0	96	96	180	204	188	192	321	321
20	Augrabies	189	222	207	228	228	231	108	116	171	189	192	208	180	184	228	237
22	Augrabies	177	231	228	234	225	258	104	116	159	180	188	216	188	196	216	249
24	Augrabies	201	219	213	222	222	222	100	112	162	186	184	208	184	192	234	240
25	Augrabies	192	225	0	0	102	102	100	112	162	198	188	204	188	208	222	231
27	Augrabies	207	219	222	222	228	237	100	112	162	186	172	200	188	200	234	252
29	Augrabies	192	204	213	222	228	240	108	116	165	180	176	204	180	200	225	237
32	Augrabies	189	216	213	231	222	237	108	116	177	186	192	192	188	204	246	255
33	Augrabies	177	177	213	213	0	0	112	116	159	174	172	200	192	208	216	216
35	Augrabies	189	201	213	222	231	234	100	112	162	186	160	188	188	204	240	258
36	Augrabies	198	231	222	237	219	231	108	124	168	189	168	196	180	200	228	243
37	Augrabies	201	222	219	231	225	231	108	112	159	171	192	200	180	188	219	249
42	Augrabies	183	207	213	213	225	225	108	112	171	180	180	200	180	200	234	240
43	Augrabies	207	225	213	228	222	231	108	120	174	180	188	200	192	196	246	252
44	Augrabies	186	222	213	222	228	234	116	116	180	201	180	208	180	200	240	249
45	Augrabies	204	225	216	228	225	231	104	112	171	180	180	200	200	200	234	258
46	Augrabies	180	207	213	225	225	240	116	116	171	201	184	200	200	200	237	240
47	Augrabies	219	231	222	237	219	246	108	120	171	186	172	196	200	216	219	252
48	Augrabies	207	216	210	219	228	228	104	116	168	180	180	200	184	196	234	255
49	Augrabies	186	222	219	234	228	231	108	120	180	201	180	200	188	196	237	249
50	Augrabies	207	225	216	222	222	231	100	112	177	189	184	208	180	180	231	264
51	Augrabies	189	225	213	222	228	237	104	112	183	183	180	208	200	200	240	246
56	Augrabies	192	213	213	213	219	228	116	116	180	201	184	204	188	200	237	246
57	Augrabies	201	222	219	231	225	231	0	0	159	183	200	208	184	196	225	231
58	Augrabies	183	204	213	222	219	228	108	116	183	201	184	204	184	204	249	261
61	Augrabies	183	216	213	228	225	231	104	112	183	183	184	204	184	204	237	246
62	Augrabies	189	210	210	219	225	228	108	120	159	180	168	180	200	204	240	258
63	Augrabies	189	210	222	231	231	234	100	120	192	192	168	204	184	196	231	243
67	Augrabies	195	201	216	237	225	225	100	112	159	180	160	188	180	196	219	246
70	Augrabies	186	216	213	231	228	234	100	112	162	186	192	200	188	204	234	249
71	Augrabies	204	231	219	234	225	234	104	112	159	180	176	204	200	204	237	258
72	Augrabies	198	219	216	225	228	228	104	120	132	192	172	204	180	196	222	234
74	Augrabies	216	219	213	234	222	228	108	120	159	183	160	184	184	192	219	246
75	Augrabies	204	231	207	216	225	231	112	120	162	186	188	208	188	200	234	255
76	Augrabies	189	219	213	234	225	231	112	116	177	189	184	200	196	200	237	258
77	Augrabies	192	222	207	234	225	231	100	112	177	186	172	188	188	192	234	240
78	Augrabies	189	225	213	222	228	237	104	112	177	201	180	204	180	200	228	240
79	Augrabies	189	219	213	222	228	234	104	112	162	186	188	200	188	192	237	258
80	Augrabies	195	219	222	231	225	234	108	116	183	201	184	204	184	200	234	249
83	Augrabies	195	210	213	222	225	225	100	112	162	180	184	208	180	200	234	249
88	Augrabies	198	222	216	228	222	231	100	112	171	201	188	200	192	204	225	249
90	Augrabies	189	201	216	237	225	225	112	116	174	180	168	180	188	196	219	243
102	Ai Ais	189	219	213	222	228	234	112	120	159	171	176	188	180	196	237	246
103	Ai Ais	201	219	213	222	219	228	104	112	174	189	172	184	176	196	225	237
105	Ai Ais	189	210	222	231	222	231	104	112	159	186	192	200	188	204	240	258
107	Ai Ais	216	219	213	216	228	231	108	112	180	186	180	188	192	200	243	246
109	Ai Ais	207	216	213	234	219	228	104	112	168	174	160	164	180	196	225	246
110	Ai Ais	186	216	219	234	225	231	104	116	171	183	172	196	188	192	222	237
112	Ai Ais	198	210	210	225	222	231	104	112	177	201	168	196	192	208	246	249
115	Ai Ais	186	207	210	210	231	231	104	116	171	183	160	172	176	188	228	246
116	Ai Ais	231	231	213	237	222	222	104	104	165	171	168	184	180	196	210	213
118	Ai Ais	201	231	222	228	225	231	104	116	162	180	164	200	188	212	225	255
119	Ai Ais	207	216	213	219	219	225	104	104	165	183	184	192	192	208	228	255
120	Ai Ais	186	219	210	219	228	234	104	104	168	177	184	196	200	200	246	258
123	Ai Ais	186	228	219	228	225	228	104	116	159	174	172	200	176	204	228	255
126	Ai Ais	207	216	207	228	225	231	112	116	174	174	188	192	188	196	222	258
127	Ai Ais	216	183	219	222	222	222	100	116	180	192	172	188	188	196	225	249
131	Ai Ais	201	219	213	219	222	231	112	116	180	186	184	200	180	200	222	246
135	Ai Ais	186	219	213	222	219	225	116	120	180	201	184	200	188	216	228	249
136	Ai Ais	201	231	213	231	222	237	104	116	180	180	200	204	188	208	219	243
137	Ai Ais	186	213	207	234	243	252	116	120	180	189	168	196	184	204	225	249
138	Ai Ais	207	216	207	228	225	234	104	112	165	171	168	192	188	196	219	234
142	Ai Ais	186	225	213	231	222	231	108	112	228	231	164	196	188	192	222	258
143	Ai Ais	207	225	216	228	222	228	112	112	165	168	180	188	204	208	234	255
144	Ai Ais	210	216	210	231	228	234	108	116	180	186	164	176	180	200	219	249
146	Ai Ais	213	231	207	213	231	231	104	116	168	180	192	200	184	200	225	243

2.9. Appendix 3

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

Locus	**Number of alleles in Augrabies population**	**Number of alleles in Ai Ais 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

2.10. 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
Average	52,2	13,2	8,63	2,26	0,90	0,87	0,88	-0,03
Ai Ais								
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
Average	23,87	10,27	7,28	2,08	0,87	0,85	0,87	-0,04

2.11. 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 calculated using Genalex.

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

2.12. 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 Ais	On_864997	28	0.016
Ai Ais	On_877333	45	0.665
Ai Ais	On_883556	15	0.107
Ai Ais	On_324078	36	0.111
Ai Ais	On_852925	36	0.015
Ai Ais	On_787859	36	0.000
Ai Ais	On_23489	66	0.836
Ai Ais	On_290548	78	0.431
Ai Ais	On_402628	55	0.857
Ai Ais	On_968476	36	0.000
Ai Ais	On_1106367	15	0.627
Ai Ais	On_1114225	105	0.147
Ai Ais	On_507360	66	0.555
Ai Ais	On_843840	55	0.609
Ai Ais	On_589878	91	0.046
Mean			0.335

2.13. Appendix 7

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

Cumulative number of relationships within Augrabies = 331								
Sample	Sample	Relationship	Sample	Sample	Relationship	Sample	Sample	Relationship
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
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			

Cumulative number of relationships between Augrabies and Ai Ais = 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 Ais = 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

Cumulative number of relationships within Ai Ais = 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			

Chapter 3

Sex partitioning revealed in Pale-winged Starlings from two regions in southern Africa using stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes

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3.1 Abstract

Avian ecologists successfully use stable isotopes in tissues to study bird diets. The use of food resources and the extent to which different individuals or genders within a population use them are an important aspect of foraging ecology. Stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) in feathers of Pale-winged Starlings from Augrabies Falls Nature Reserve (n= 53) and Ai Ais-Richtersveld Transfrontier Park (n= 24) were analysed. This allowed investigation of foraging specialisation and differences in foraging. Pale-winged Starlings sampled from the Augrabies Falls Nature Reserve and the Ai Ais-Richtersveld Transfrontier Park have a clear distinction between the two populations based on their isotope signatures. $\delta^{13}C$ and $\delta^{15}N$ values were different between males and females within each population. Males were found to occupy higher trophic levels than females in both the populations. From this we can infer that trophic variations in Pale-winged Starlings are driven by differences in physiology, enabling males to feed on higher trophic levels because they are larger. There is also individual foraging specialisation within each population that is apparent.

Keywords: Stable isotopes, Pale-winged Starlings, sex partitioning.

3.2 Introduction

In recent decades (Kelly & Finch, 1998; Hobson, 1999; Inger & Bearhop, 2008; Navarro *et al*., 2021) the use of stable isotopes analyses in studies of avian nutritional ecology has increased tremendously (Kelly & Finch, 1998; Hobson, 1999). There is a clear differentiation between radiogenic isotopes and stable isotopes. Radioactive isotopes undergo radioactive decay (Klein, 2014). It is also mainly used to determine the absolute age of a samples and to trace the origin of a sample and are more suited for measuring time in old geological systems (Klein, 2014). Stable isotopes do not decay over time (Inger & Bearhop, 2008). The isotopes differ in mass where the lightest stable isotope is much more common (Fry, 2006). The differences in the abundance of these isotopes can be expressed as the ratio of the heavier isotope to the lighter form (Inger & Bearhop, 2008). The ratios are standardised against international reference samples and reported in the delta (δ) notation as parts per thousand (or per mill, ‰) (Inger & Bearhop, 2008).

Stable isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) can be used to delineate dietary patterns in animal populations (Peterson & Fry, 1987; Ehleringer & Osmond, 1989; Smith *et al*., 2021). Carbon isotope ratios are used to indicate relative contributions of differing carbon sources (Fry *et al*., 1983). Higher plants are commonly divided into C_3 or C_4 plants based on their carbon fixation pathways during photosynthesis (Vogel, 1993). Based on the different approaches for isotopic fractionation in the initial fixation of CO_2 in the two pathways, the $^{13}C/^{12}C$ ratios also differ. C_3 plants have depleted amounts of ^{13}C relative to C_4 plants (Vogel, 1993; Hobbie & Werner, 2003). C_3 plants will have more negative $\delta^{13}C$ values compared to C_4 plants (Hobbie & Werner, 2003). Stable carbon isotope ratios display a limited increase (0-1‰) between trophic levels (Fox & Bearhop, 2008).

Most natural materials have $\delta^{15}N$ values between -20 and +45‰ (DeNiro & Hastorf, 1985; Mizutani & Wada, 1988). The use of nitrogen isotopes relies on the distribution of nitrogen in foods and how they are incorporated into the tissues of consumers (Peterson & Fry, 1988). Stable nitrogen isotope ratios have been shown to exhibit stepwise enrichment through food chains. This allows nitrogen isotopes to provide trophic level information (Minagawa & Wada, 1984; Ambrose & DeNiro, 1986; Hobson & Montevecchi, 1991). A 3-4‰ enrichment in $\delta^{15}N$ values should be expected with each trophic step (Peterson & Fry, 1987; Boutton, 1991; Ambrose, 1993; Koch *et al*., 1994). A more positive $\delta^{15}N$ reflects a higher trophic level or more of a carnivore (Hobbie & Werner, 2003).

When stable isotope ratios of carbon and nitrogen are combined, they can be used to discriminate between species based on dietary input (Hobson, 1991). Most studies require internal tissues which requires the killing of individuals (Rau *et al*., 1992; Winker, 2000; Anchin-Murguzur *et al*., 2021; Tarroux *et al*., 2021). However, Hobson and Clark (1993) used avian blood while Schaffner and Swart (1991) used eggshells to assess the diet of seabirds. In studies of birds, feathers are a non-destructive alternative which can provide information relating to the diet and trophic position of an individual

(Thompson & Furness, 1995). Feathers are chemically inert and show isotope values characteristic of keratin (Michalik *el al*., 2010; Deme *et al*., 2021). Studies using isotope ratios have been conducted on a variety of avian species such as Amur Falcon (*Falco amurensis*) for migratory connectivity and conservation using δD, $\delta^{13}C$ and $\delta^{15}N$; Willow Warblers (*Phylloscopus trochilus*), using $\delta^{13}C$ and $\delta^{15}N$ isotopes for migratory connectivity and diving seabirds using $\delta^{13}C$ and $\delta^{15}N$ isotopes to investigate sex-specific and individual foraging specialisation (Bearhop *et al*., 2006; Bensch *et al*., 2009; Symes & Woodborne, 2010).

Pale-winged Starlings from the Augrabies Falls Nature Reserve in the Northern Cape Province of South Africa and from the Ai Ais-Richtersveld Transfrontier Park in Namibia were the focus of this study. These birds are generalist omnivores, feeding on a range of fruit and insects. Carbon and nitrogen isotope ratios were used to investigate the trophic levels present in the two populations. The trophic levels were compared within each population, between individuals and between males and females to determine whether there are differences in foraging habits based on gender.

3.3. Methods and materials

Isotope analysis

3.3.1. Feather collection

Wing and contour feather samples of two populations of Pale-winged Starlings (N=143) were collected: Augrabies Falls Nature Reserve, Northern Cape Province, South Africa (n=93) and Ai Ais-Richtersveld Transfrontier Park, Namibia (n=50). A total of 53 samples from Augrabies Falls Nature Reserve and 24 samples from Ai Ais-Richtersveld Transfrontier Park, were used for the isotope analysis. The sample sites are located 400 km from each other. Feather 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. The VOCOM team also provided the mass, wing length, tarsus length and sex which were used in this study. A single feather was used per individual with replicates from different parts of the feather.

3.3.2. Feather sample preparation

Before feather samples were subjected to mass spectrometry, feather samples were cleaned with a washing mixture of chloroform-methanol 2:1 to remove surface oils (Botempo *et* al., 2014). Once the feathers were cleaned, they were air-dried in a fume hood for 24 hours to remove the remaining solvent. The sample size required for $\delta^{13}C$ and $\delta^{15}N$ isotope assays are between 0.40 – 0.50 mg. Sectional cutting with surgical scissors were used to fraction the feathers. Pre-tared 6mm x 4mm tin cups were used for $\delta^{13}C$ and $\delta^{15}N$ analyses. Two proteinaceous Merck gels (Merck, Germany) and two urea samples were placed every twenty-four samples as standards throughout the tray at varying weights of 0.2, 0.4, and 0.6 mg respectively.

3.3.3. Feather sample analysis

The samples were analysed at iThemba LABS (University of the Witwatersrand) using a Flash HT Plus elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer by a ConFloIV interface (all equipment supplied by ThermoFisher, Bremen, Germany). Carbon and nitrogen isotope values were corrected against an in-house standard (Merck Gel) and a Urea Working Standard (IVA Analysentechnik E.K., Meerbusch, Germany). The isotope ratio was calculated using the formula $\delta X = [(R_{sample} – R_{standard}) – 1] \times 1000$, where δX is the standard isotopic notation of the heavier isotope, and R is the ratio of heavy to light isotopes of the sample and standard. The values are expressed in parts per thousand (‰, per mil) (Peterson and Fry, 1987). A higher delta value will indicate an increase in the presence of the heavier isotope relative to the standard and vice versa (Peterson & Fry, 1987). The $\delta^{13}C$ will allow the determination of the primary energy production source and higher $\delta^{15}N$ levels will indicate organisms which are higher-up in the trophic pyramid as they would have accumulated increased levels of ^{15}N relative to their food source (Schoeninger & DeNiro, 1983; O'Leary, 1988). This will enable us to compare the trophic levels individually within the population and between the two populations.

3.3.4. Isotope data analysis

A Kruskal-Wallis rank sum test was performed to determine whether there are statistically significant differences between the two populations. These tests were performed on both the $\delta^{13}C$ and $\delta^{15}N$ isotopic data. A multivariate analysis was performed using $\delta^{13}C$ and $\delta^{15}N$ values, mass, wing length, tarsus length and sex using the Analyze add on in Excel using Joint PCA and correlation biplot parameters.

3.4.1. Trophic differences between and within two populations

The Kruskal-Wallis test showed that at a 0.05 significance level there is no significant difference between males and females in the Augrabies Falls Nature Reserve sampled population for both isotopic nitrogen and carbon signatures. There is, however, a significant difference between males and females in the Ai Ais-Richtersveld Transfrontier Park sampled population for the nitrogen signatures. No significant difference was observed for the carbon signatures for the latter sample cohort. Overall, the two sampled populations are significantly different at a 0.05 significance level.

Table 3.1: Kruskal-Wallis rank sum test of different comparison groups.

Kruskal-Wallis rank sum test						
Comparison	**Nitrogen p-value**	**Standard deviation**	**Significant difference at 0.05 significance level**	**Carbon p-value**	**Standard deviation**	**Significant difference at 0.05 significance level**
Males vs females in Augrabies	0.67	0.70	No	0.94	0.93	No
Males vs females in Ai Ais	0.02	0.93	Yes	0.28	0.81	No
Males in Augrabies vs Ai Ais	7 x 10^{-4}	0.86	Yes	2.25 x 10^{-5}	1.09	Yes
Females in Augrabies vs Ai Ais	1.55 x 10^{-5}	1.19	Yes	2.3 x 10^{-3}	1.07	Yes
Augrabies vs Ai Ais	1.65E - 08	1.05	Yes	2.52E – 07	1.08	Yes

Samples from the Augrabies Falls Nature Reserve region have a higher nitrogen signature when compared to samples from the Ai Ais-Richtersveld Transfrontier Park sampled population (Figure 3.1). The $\delta^{15}N$ values in the Augrabies Falls Nature Reserve population ranged between 9 to 13‰ and those of the Ai Ais-Richtersveld Transfrontier Park population between 8 to 12‰. The $\delta^{13}C$ values in the Augrabies Falls Nature Reserve population ranged between -18 to -23‰, while those for the Ai Ais-Richtersveld Transfrontier Park population ranged between -20 to -24‰. On the basis of the $\delta^{15}N$ and $\delta^{13}C$ values it can be concluded that multiple trophic levels exist in the two populations.

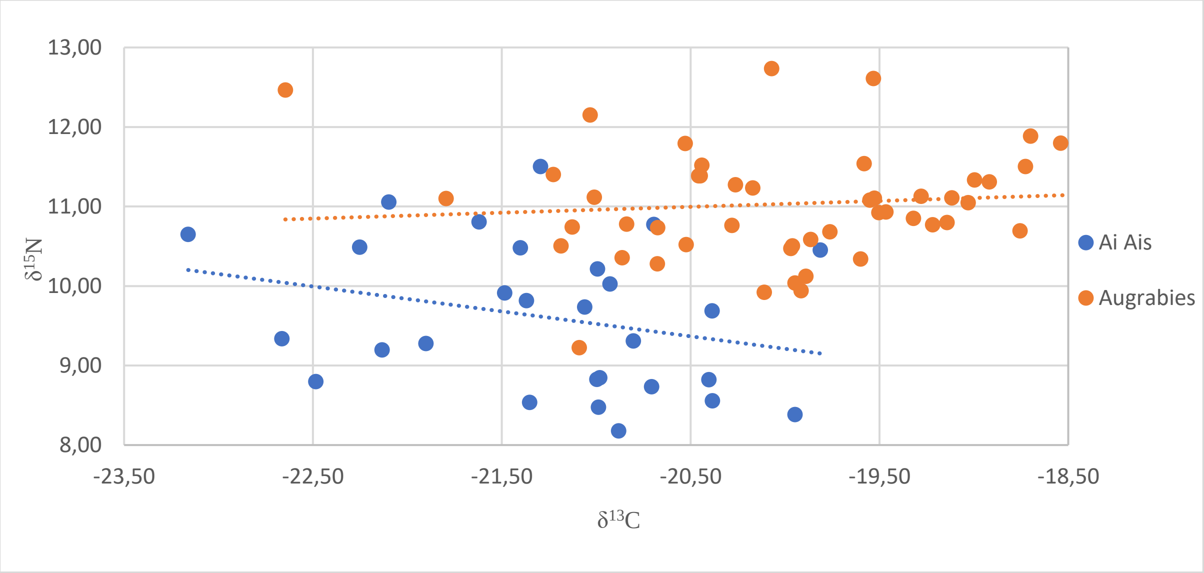

Figure 3. 1: Isotope ratios of males and females sampled from both the Augrabies Falls Nature Reserve and Ai Ais-Richtersveld Transfrontier Park, with the Augrabies Falls Nature Reserve trendline higher than the Ai Ais-Richtersveld Transfrontier Park.

Male and female carbon and nitrogen signatures in the Augrabies Falls Nature Reserve population is very clustered with few outliers (Figure 3.2). The outliers are predominantly female. Most males in the Augrabies Falls Nature Reserve sampled population are at the same trophic level according to the nitrogen signatures. Males sampled from the Augrabies Falls Nature Reserve are at a higher trophic level than females with N values beginning at 10‰ up to 13‰ (Figure 3.2).

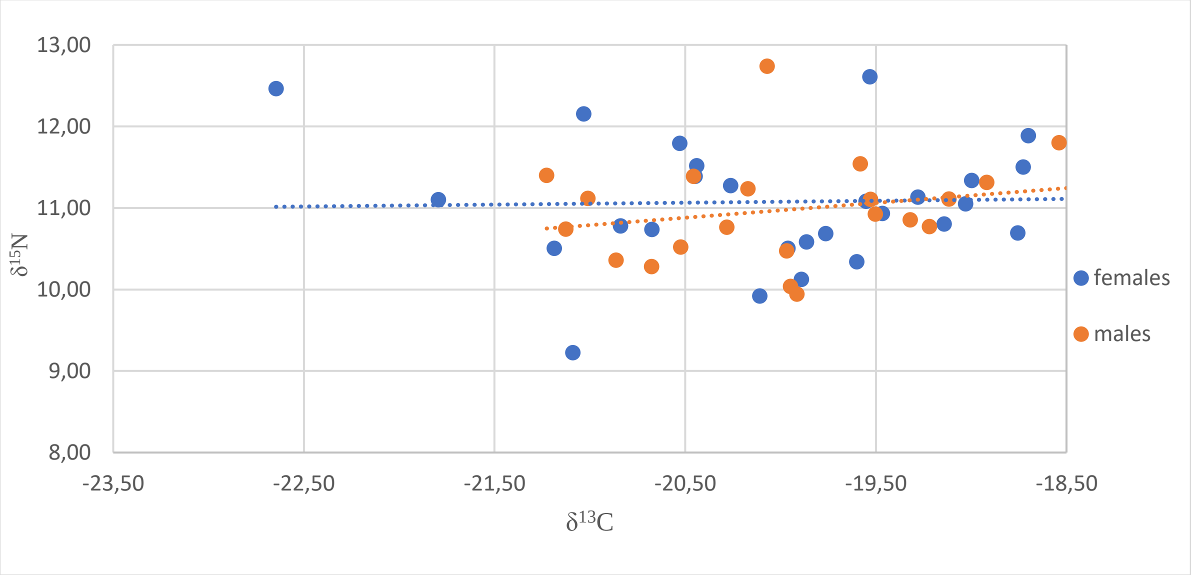

Figure 3.2: Isotope ratios of males and females sampled from the Augrabies Falls Nature Reserve with the trendline for males slightly higher than the trendline for females.

Male and female carbon and nitrogen signatures are more dispersed in the Ai Ais-Richtersveld Transfrontier Park sampled population with outliers that are both male and female (Figure 3.3). Males are generally at a higher trophic level than females. The carbon ratios of males sampled from the Ai Ais-Richtersveld Transfrontier Park population are more negative and begin two trophic levels lower (-20‰) than the males sampled from the Augrabies Falls Nature Reserve population (-18‰).

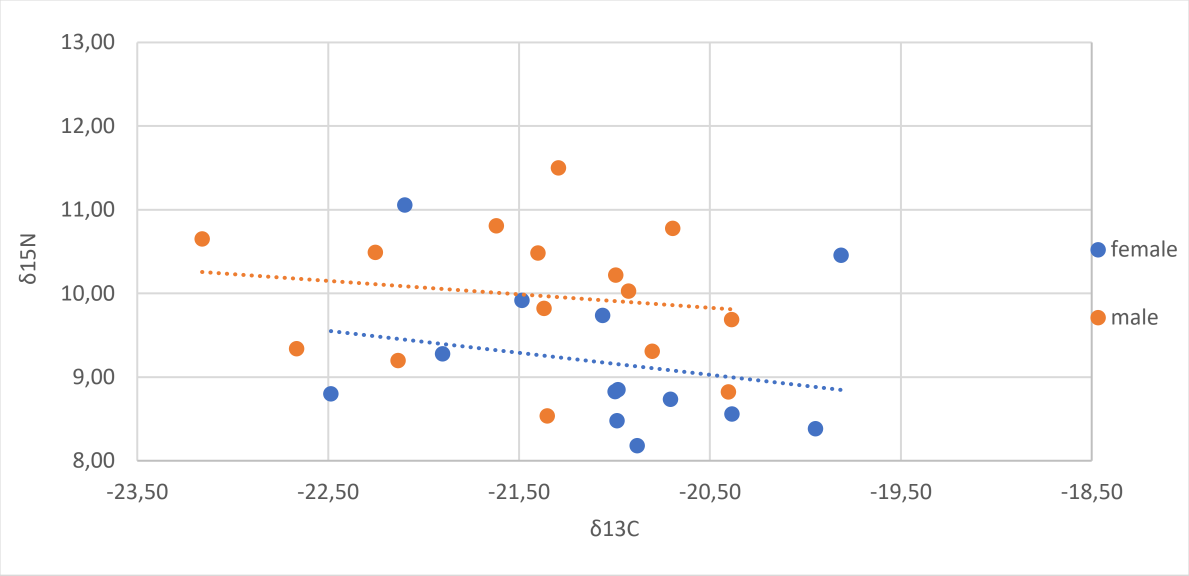

Figure 3.3: Isotope ratios of males and females sampled from the Ai Ais-Richtersveld Transfrontier Park with the trendline higher for males than for females.

3.4.2. Sexual segregation within each population

Multivariate analyses of the Augrabies Falls Nature Reserve and Ai Ais-Richtersveld Transfrontier Park sampled populations showed that males occupy higher trophic levels than females. In the Augrabies Falls Nature Reserve samples there is a positive correlation between male mass, tarsus size, wing length and $\delta^{13}C$ and $\delta^{15}N$ values (Figure 3.4). Female $\delta^{13}C$ and $\delta^{15}N$ values in Augrabies Falls Nature Reserve samples, however, have an almost negative correlation with female mass, tarsus size and wing length. The $\delta^{15}N$ values in the samples from the Augrabies Falls Nature Reserve for males and females are perfectly correlated and are positively correlated with both male and female $\delta^{13}C$ values. Male and female $\delta^{13}C$ values, however, are almost negatively correlated in the Augrabies Falls Nature Reserve sampled population (Figure 3.5). Male $\delta^{15}N$ has more variation than female $\delta^{15}N$, whereas females have more variation in $\delta^{13}C$ than males.

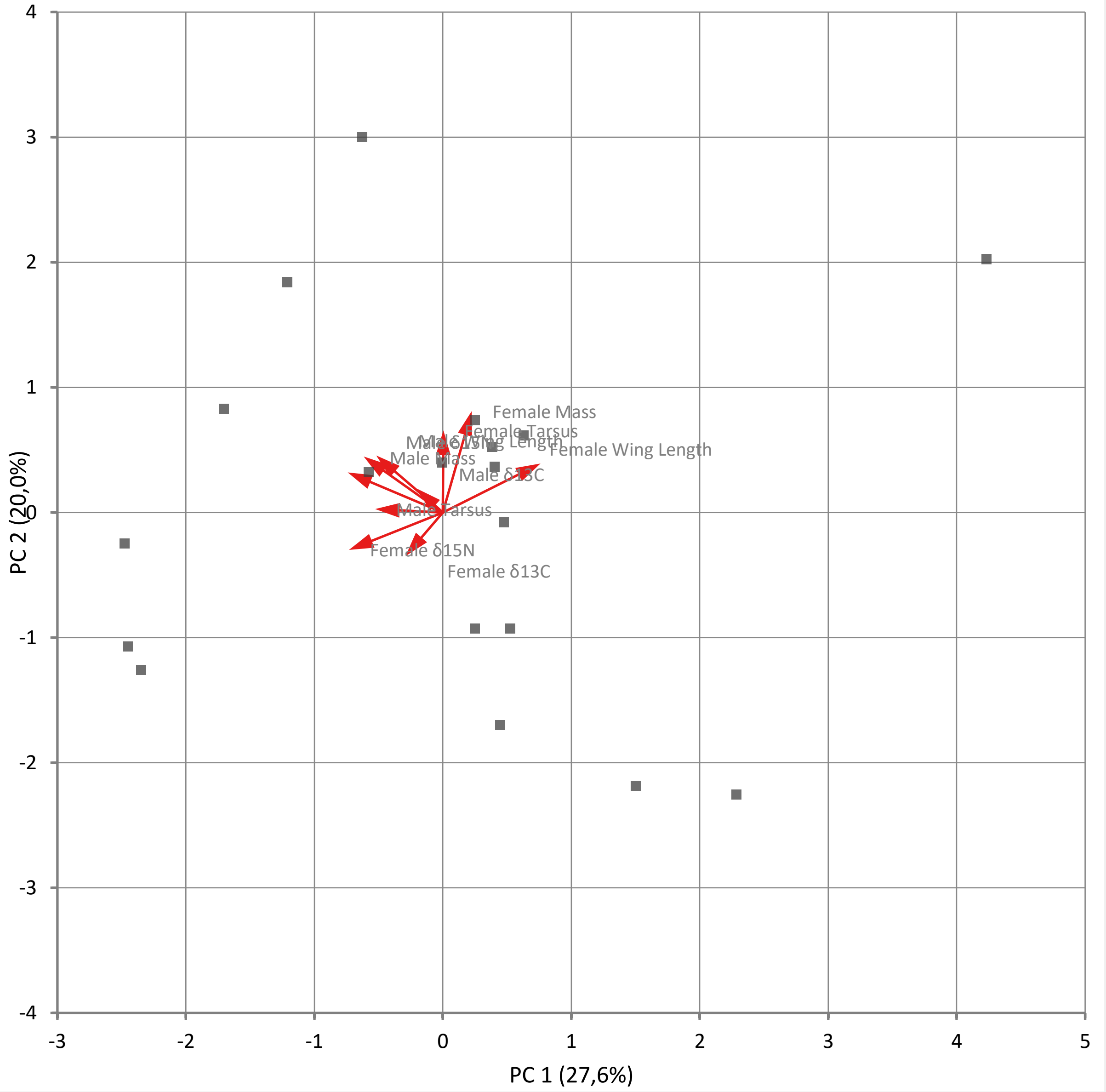

Figure 3.4: Multivariate analysis of mass, tarsus size, wing length, δ13C and δ15N values of 53 Pale-winged Starlings sampled from the Augrabies Falls Nature Reserve. This display represents 47.6% of the total data variance. The longer arrows represent more discrimination while the angles between the arrows show the correlations between the measures. Positively correlated variables are grouped together, and negatively correlated variables are positioned on opposite sides of the plot.

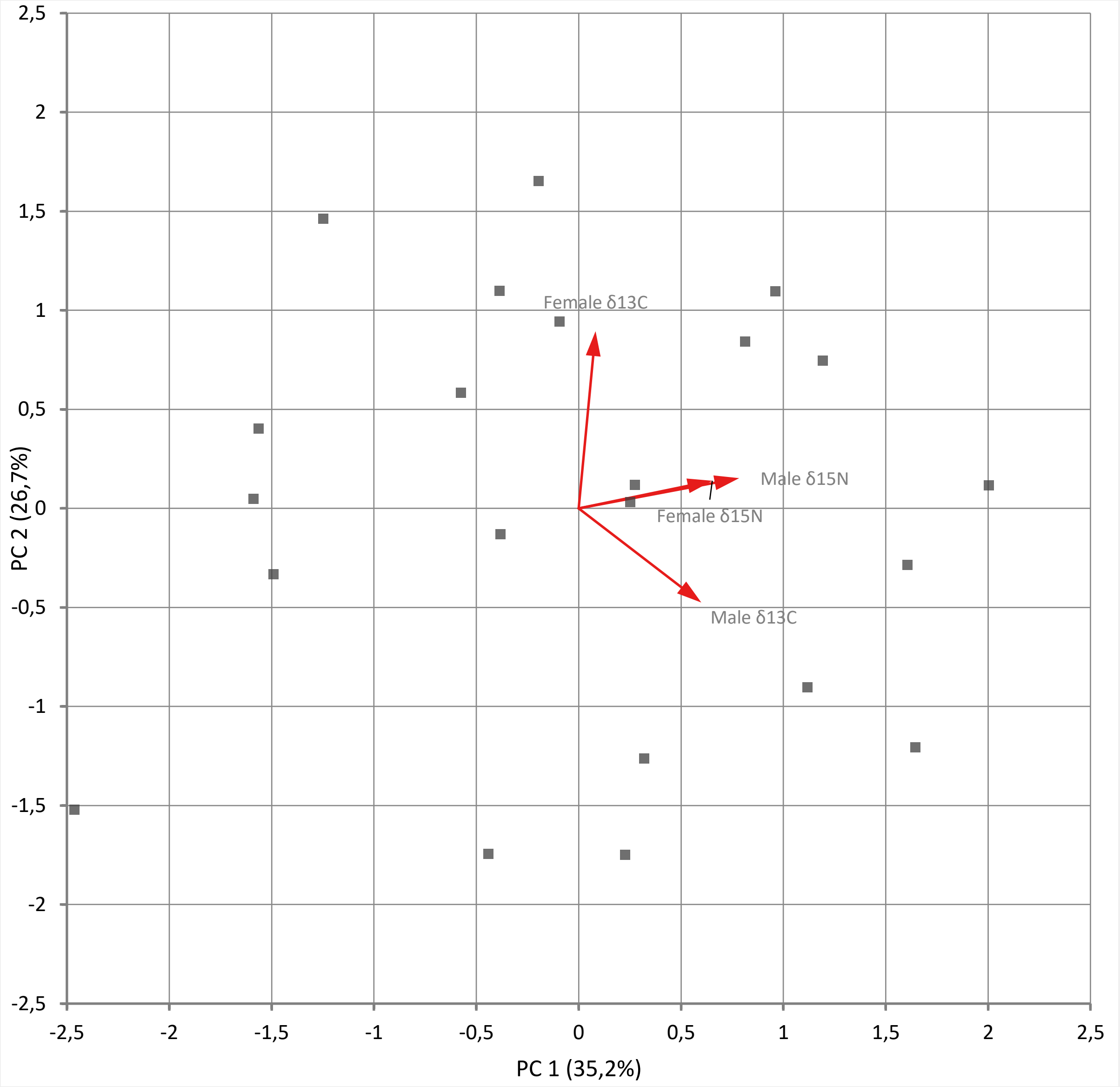

Figure 3.5: Analysis of δ13C and δ15N values of 53 Pale-winged Starlings sampled from the Augrabies Falls Nature Reserve. This display represents 61.9% of the total data variance. The longer arrows represent more discrimination while the angles between the arrows show the correlations between the measures. Positively correlated variables are grouped together, and negatively correlated variables are positioned on opposite sides of the plot.

In the Ai Ais-Richtersveld Transfrontier Park sampled population male mass, wing length, tarsus size and δ^{13}C values are positively correlated. Female mass, wing length, tarsus size and δ^{15}N values are also positively correlated (Figure 3.6). Female and male δ^{15}N are positively correlated and male and female δ^{13}C values are also positively correlated. Male δ^{13}C and δ^{15}N as well as female δ^{13}C and δ^{15}N values are negatively correlated (Figure 3.7).

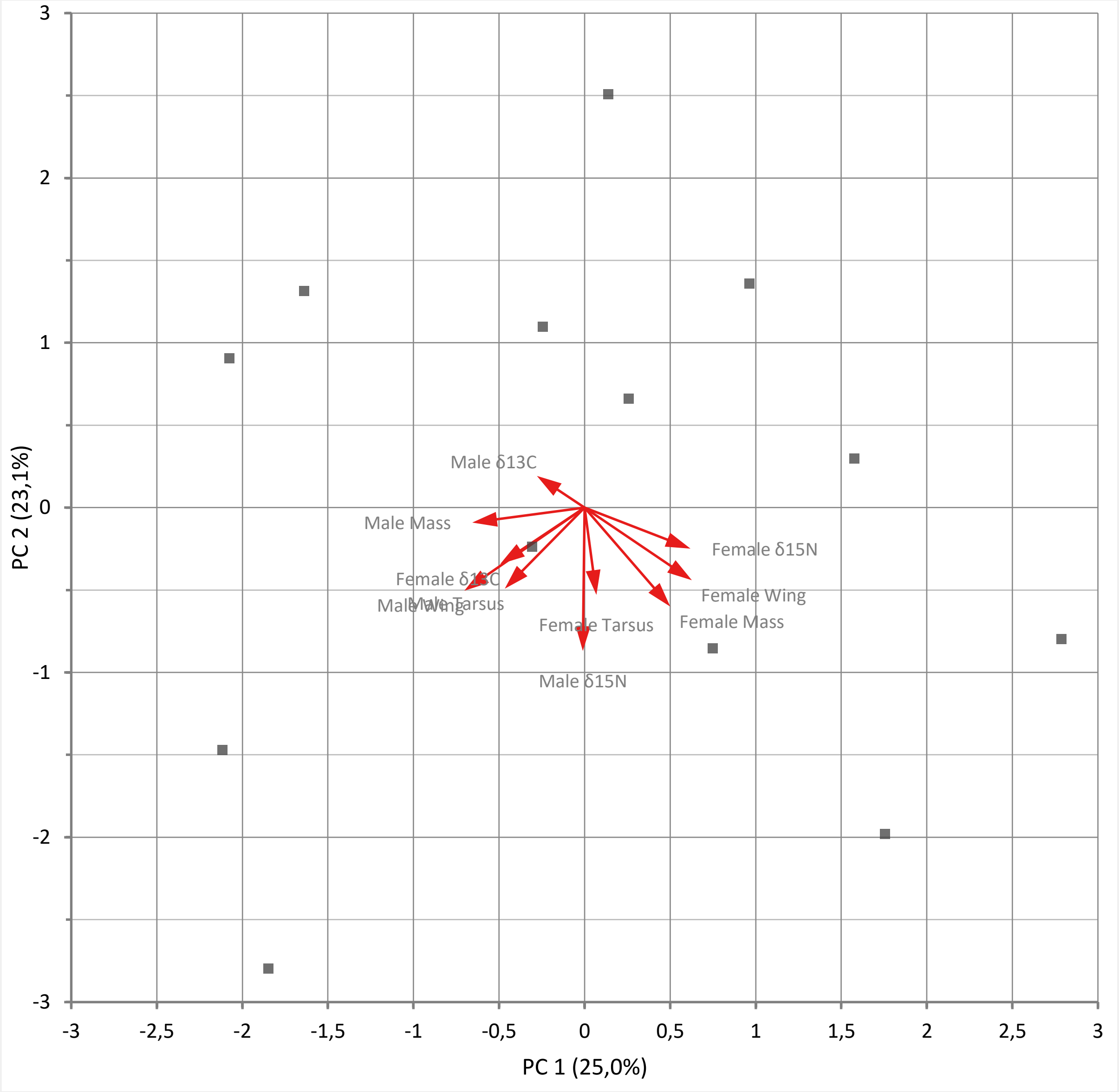

Figure 3.6: Multivariate analysis of mass, tarsus size, wing length, δ13C and δ15N values of 24 Pale-winged Starlings sampled from the Ai Ais-Richtersveld Transfrontier Park. This display represents 48.1% of the total data variance. The longer arrows represent more discrimination while the angles between the arrows show the correlations between the measures. Positively correlated variables are grouped together, and negatively correlated variables are positioned on opposite sides of the plot.

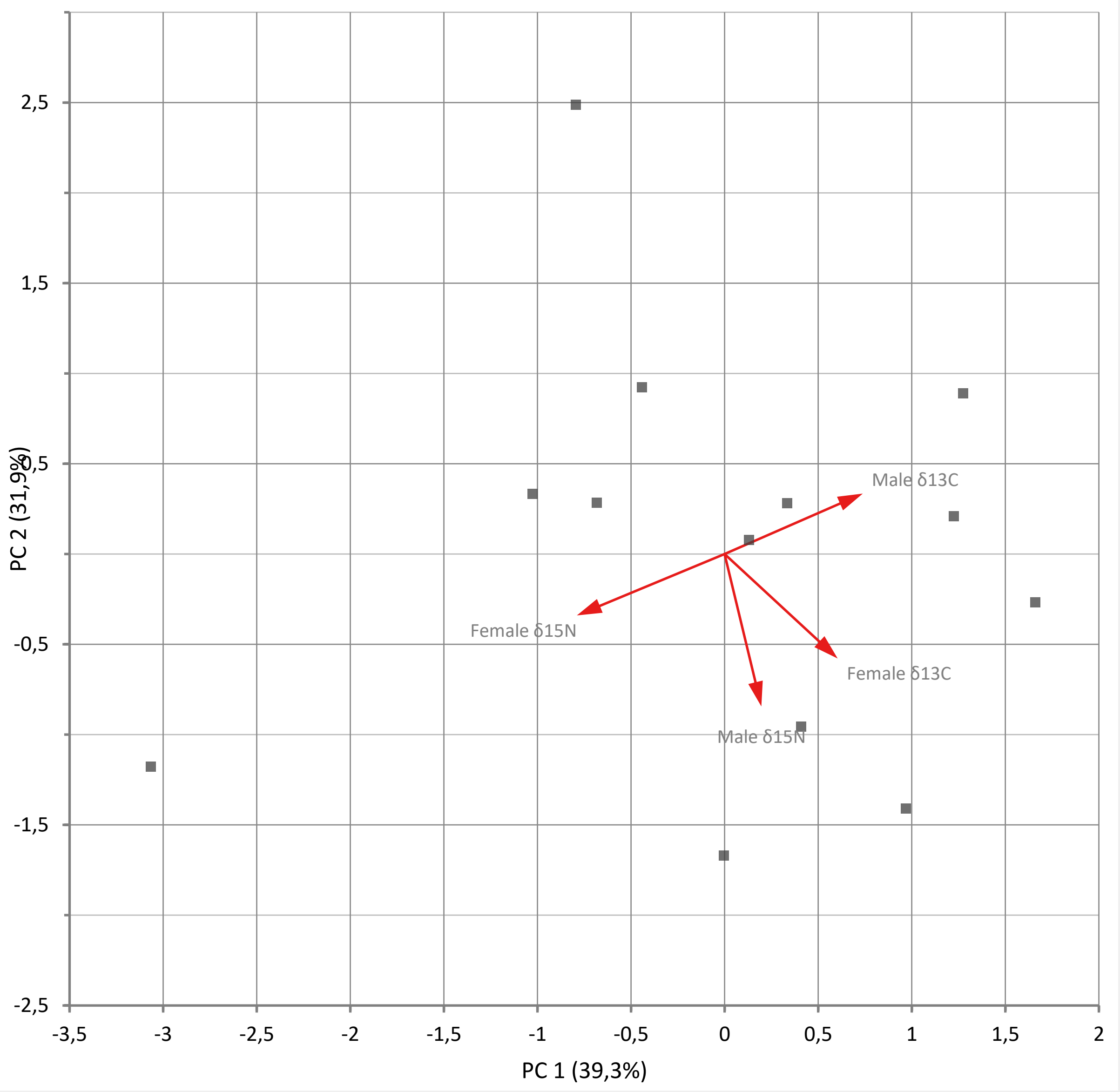

Figure 3.7: Analysis of δ13C and δ15N values of 24 Pale-winged Starlings from the Ai Ais-Richtersveld Transfrontier Park. This display represents 71.2% of the total data variance. The longer arrows represent more discrimination while the angles between the arrows show the correlations between the measures. Positively correlated variables are grouped together, and negatively correlated variables are positioned on opposite sides of the plot.

3.5 Discussion

The isotopic data collected in this study on the Pale-winged Starlings sampled from both the Augrabies Falls Nature Reserve and the Ai Ais-Richtersveld Transfrontier Park show that there are significant differences between and within the two populations. Males and females sampled show differences in trophic levels within both populations. When the two sampled populations are closely analysed there are differences between the male and female trophic levels according to the carbon and nitrogen signatures.

A 3-4‰ enrichment in $\delta^{15}N$ values should be expected with each trophic step (Peterson & Fry, 1987; Boutton, 1991; Ambrose, 1993; Koch *et al.*, 1994), while $\delta^{13}C$ values display a limited increase (0-1‰) between trophic levels (Fox & Bearhop, 2008). The Augrabies Falls Nature Reserve sampled population has a broader $\delta^{13}C$ (-18‰ to -23‰) signature than the sampled population from the Ai Ais-Richtersveld Transfrontier Park (-20‰ to -23‰). The $\delta^{15}N$ signature of Augrabies Falls Nature Reserve (9‰ to 13‰) has the same range size as the Ai Ais-Richtersveld Transfrontier Park population (8‰ to12‰). The $\delta^{15}N$ signatures have greater variation in the sampled male population from the Augrabies Falls Nature Reserve than females. The $\delta^{13}C$ and $\delta^{15}N$ signatures of the samples from the Ai Ais-Richtersveld Transfrontier Park have similar variation in both males and females. Sampled males from the Augrabies Falls Nature Reserve region and the Ai Ais-Richtersveld Transfrontier Park region occupy higher trophic levels than the females sampled from these two regions.

From the data, the Pale-winged Starlings sampled from the Augrabies Falls Nature Reserve region appear higher-up in the trophic pyramid than those in the Ai Ais-Richtersveld Transfrontier Park population. This observation is supported by the ecology of these regions. The Augrabies Falls Nature Reserve region has several grape farms adjacent to the Orange River which supports a frugivorous diet (Bezuidenhout, 1996). The Augrabies Falls Nature Reserve area is made up of five main vegetation units namely *Aloe dichotoma*, *Schotia afra*, *Acacia mellifera*, *Stipagrostis* species and *Ziziphus mucronate* (Bezuidenhout, 1996). These are representative of CAM, C_3 and C_4 plants allowing a varied diet, less competition and possibly the ability to develop specialised feeding habits (Werger & Coetzee 1977; Bezuidenhout, 1996). C_3 plants are predominantly trees and have higher nitrogen signatures than C_4 plants which are grasses (Swap *et al.*, 2004). C_3 plants also have a more negative carbon signature (Swap *et al.*, 2004).

The Ai Ais-Richtersveld Transfrontier Park region's vegetation is largely made up of ephemeral grasses and dwarf shrubs, *Aloidendron dichotomum, A. pillansii* and *Pachypodium namaquanum* and occasional trees, (Burke *et al.,* 2002). With only occasional trees in the Ai Ais-Richtersveld Transfrontier Park region, it can be expected that the carbon signature is less negative, which was observed in the data. Carbon signatures are generally between -33‰ to -24‰ for C_3 plants, between -16‰ to -10‰ for C_4 plants and between -20‰ to -10‰ for CAM plants (O'Leary, 1988). Carbon signatures in Augrabies Falls Nature Reserve are between -18‰ to -23‰ and -20‰ to -23‰ in the Ai Ais-Richtersveld Transfrontier Park region. These signatures range across all three types of carbon sources with more negative values in the Ai Ais-Richtersveld Transfrontier Park region. The Augrabies Falls Nature Reserve region has many settlements and lodges close by. This could influence the diet of the Pale-winged Starlings in this region. Plants treated with mineral nitrogen fertilizers often have less positive $\delta^{15}N$ values compared to untreated plants (Huelsemann *et al.*,2013). The average $\delta^{15}N$ of vegetables treated with fertilizers are between 3.1‰ to 6.8‰ and approximately 9.8‰ in vegetables fertilized with organic or animal derived manure (Huelsemann *et al*.,2013). $\delta^{15}N$ signatures (9‰ to 13‰) are much higher in our study than reported by Martinelli *et al*, (2020), allowing us to infer the use of organic fertilizers or animal-based manure in both Augrabies Falls Nature Reserve and Ai Ais-Richtersveld Transfrontier Park.

The study by Martinelli *et al*. (2020) provided $\delta^{13}C$ signatures of plant and animal based processed foods. The $\delta^{13}C$ signatures ranged between -12‰ to 29.5‰ for plant based processed foods and -12‰ to 21‰ for animal based processed foods. Diets could also include and be made up of processed foods which will directly affect their trophic levels. In our study Pale-winged Starlings had $\delta^{13}C$ signatures (-18‰ to -23‰ within the range reported by Martinelli *et al*, (2020). The consumption of processed foods by these birds is supported by the settlements and holiday resorts in the Augrabies Falls Nature Reserve vicinity and the campsites in Ai Ais-Richtersveld Transfrontier Park. Augrabies Falls Nature Reserve has accommodation within the park as well as multiple lodges and camping sites. Ai Ais-Richtersveld Transfrontier Park has campsites, however according to SanParks the park has limited visitor facilities, with only three wilderness camps, five serviced and an overnight camp. The differing level of urbanization of the two regions provides further support for the higher trophic levels seen in Pale-winged Starlings from the Augrabies Falls Nature Reserve region. A study by Aranibar *et al*, (2007) found that as land use intensity increases so does $\delta^{15}N$ signatures thus supporting the higher $\delta^{15}N$ signatures seen in the Augrabies Falls Nature Reserve population.

Further analysis of the data showed that both populations are made up of generalised feeders and specialised individuals, with males sampled from both populations occupying higher trophic levels than females. Pale-winged Starling males are known to be statistically larger than females (Henry *et* al., 2015). In a number of invertebrates bigger individuals often occupy higher trophic levels, and thus have higher $\delta^{15}N$ signatures (Lindsay *et al*., 1998, Schmidt *et al*., 2003), which was also observed in our study. Sexual segregation in foraging areas has been observed in passerine species (Marra & Holmes, 2001). Males and females may share similar foraging areas but specialise on different types of prey (Newton, 1998). The niche variation hypothesis expects that populations with broader niches are more adjustable than populations with narrow niches (van Valen, 1965). Morphological differences, usually size, have been explored in attempts to understand whether individuals within a species or population partition resources (Schoener, 1967; Werner & Gilliam, 1984). Bolnick *et al*, (2007) further discussed that a morphology driven factor is likely to underestimate the extent of resource and ecological partitioning among individuals because such variation arises due to behavioural decisions. Studies also found that more generalised populations exhibit higher variation among individuals (Bolnick *et al*., 2007).

From the trophic niches observed in the data we see the behaviour of males and females differ in feeding habits. Variability in physiology, specialisation of roles or even the acquiring of specific skills can be reflected by variances in foraging selection among individuals or between sexes (Selander, 1966; Durell, 2000). From their feeding behaviour and trends, we can deduce that the Pale-winged Starlings sampled from the Ai Ais-Richtersveld Transfrontier Park and the Augrabies Falls Nature Reserve feed in multiple trophic niches from multiple sources and avoid direct competition with each other within the population. Competition for resources may not be a huge concern within the Augrabies Falls Nature Reserve region due to the diverse ecology and vegetation and landscape developments in this region also enhances feeding options. Anthropogenic food available in urban areas have led many bird species to have more diverse diets which allows more individual variation in diet (Murray *et al*., 2015). This was seen in wading birds and coyotes amongst others (Fedriani *et al*., 2001) which supports individual foraging specialisation seen in both populations.

Augrabies Falls Nature Reserve being more developed than Ai Ais-Richtersveld Transfrontier Park shows higher trophic niches occupied by Pale-winged Starlings in the former location. The feeding trends and social behaviour of males and females are similar in both sampled populations. Males are larger than females and occupy higher trophic levels than females. From this we hypothesize that trophic differences in Pale-winged Starlings are driven by differences in physiology, with males able to access food resources of higher trophic levels because they are larger. There is also individual specialisation within each population.

Chapter 4

Discussion / Conclusion

The development of species-specific microsatellite markers in Pale-winged Starlings revealed the genetic structure, social structure and variation present in two sampled populations of Pale-winged Starlings i.e., Augrabies Falls Nature Reserve and the Ai Ais-Richtersveld Transfontier Park. The microsatellite analysis alongside the isotope analysis sheds light on the population connectivity, social structure, and behaviour of the sampled populations of Pale-winged Starlings.

The microsatellite variation detected a lack of genetic structure which can be attributed to current/continuous gene flow (Clegg *et al*., 2003). According to the literature, geographical distances, genetic distances, and overall low variance in genetic differentiation should be completely unrelated (Hutchison & Templeton, 1999). This pattern of unrelatedness between genetic distance and geographical distance is expected to persist if gene flow remains relatively high between populations (Hutchison & Templeton, 1999). As seen in the Wilson’s warblers (Clegg *et al*., 2003), Pale-winged Starlings sampled in this study, show a lack of relationship between geographical and genetic distance with low levels of variance in genetic differentiation between populations. We can thus hypothesize that long distance gene flow plays a role in limiting genetic differentiation between the Augrabies Falls Nature Reserve and the Ai Ais-Richtersveld Park Pale-winged Starling populations.

All individuals had multiple relatives within and between the two sampled populations except for three males from the Augrabies Falls Nature Reserve. Three males sampled from the Augrabies Falls Nature Reserve had more relatives in the Ai Ais-Richtersveld Transfontier Park population and thirteen samples (nine of which are male) from the Ai Ais-Richtersveld Transfontier Park had more relatives in the sampled population from the Augrabies Falls Nature Reserve. This observation ties in with the results of the isotope analysis regarding the behaviour of males in the sampled populations.

The isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ in the feather samples of the two populations of Pale-winged Starlings showed that the pale-winged Starling population from Augrabies Falls Nature Reserve are at a higher trophic level than those from the Ai Ais-Richtersveld Transfontier Park. The isotope analysis also revealed males in both populations feed at a higher trophic level than females. Sexual segregation within the same habitat is a common phenomenon where males and females feed within the same area but specialise on different types of food resources (Newton, 1998). Individual feeding specialisation has also been reported in several species such as waders, gulls and skuas (Bearhop *et al*., 2000).

Male Pale-winged Starlings are statistically larger than females. As seen in diving seabirds (Bearhop *et al*., 2006), this may allow them access to prey of higher trophic levels. Pale-winged Starlings are semi-colonial birds which are highly social (Craig & Feare, 2020). Their foraging area may be prescribed and thus drives individual specialisation within populations to avoid competition (Wanless *et al*., 1992). Males which are often regarded as the dominant individuals could exclude subordinates, which are usually female, from high quality foraging areas leading to the higher trophic levels seen in males (Gauthreauxm 1978). The male interactions and multiple relationships across and within the populations, revealed by the microsatellite analyses are supported by males being the dominant individuals within the populations. Males appear to migrate and join the other population more frequently than females. There are individual females especially within both sampled populations that feed at similar trophic levels as the males. Since Pale-winged Starlings are socially monogamous and are almost always sighted in pairs these females may be foraging alongside their mate while others feed independently.

The microsatellite analysis provided information with regards to the genetic variation present in the two populations, however, the lack of samples of different age groups and from different areas limited the scope of the markers. The addition of known offspring or juveniles in the data set would have enhanced the isotope analysis. Inferences of foraging habits passed on from the parents could have been determined. The sampling of different food sources from the two regions would have provided more information on the changes in food selections based on the developments and human impact in the areas.

The present study does however lay the foundation for further analysis of Pale-winged Starlings and other socially monogamous birds as the finding of this study and the application of these markers regarding genetic diversity and social structures, and further differentiation between socially and sexually monogamous species, can be used to infer similar trends in other bird species. The isotope analysis provides insight into the effects of human impact on the trophic levels of this species. These findings and methods can be used in other species and applied in biodiversity conservation studies. These techniques could be applied in threatened species and in areas which are being urbanized and developed. The impact of changing environments on species diet and survival can be easily traced and monitored.

In conclusion our study on Pale-winged Starlings sampled from two regions in southern Africa, approximately 400km apart, showed gene flow between the two sampled populations with minimal genetic differentiation between them. The highest genetic diversity is seen between individuals within each population. Pale-winged Starlings appear to join and migrate between the two populations and the origin of the bird does not appear to affect their social interactions. Males feed at a higher trophic level than females within each population and have more relatives across the two populations sampled which may be attributed to them being the dominant individuals. There is individual foraging specialisation within each population by both males and females. The 48 microsatellites developed in this study could potentially be used in other Starling species for comparative analyses and interspecific studies. Future work on Pale-winged Starlings should include the addition of juvenile samples into the dataset as well as additional samples from the Ai Ais-Richtersveld Transfontier Park. Isotope analysis of blood samples alongside the feather samples would also add to the research being done on the connectivity and social structure of Pale-winged Starlings. Samples from different areas should also be added to the study and the transferability of the microsatellite markers should be tested to enhance the current study.

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