Genetic differentiation in *Oxalis* (Oxalidaceae): A tale of rarity and abundance in the Cape Floristic Region

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Abstract

*Oxalis* L. is the largest and most diverse genus in the family Oxalidaceae. Within southern Africa, *Oxalis* is represented by ca. 270 taxa, the majority occurring in the Cape Region. Although many of the species are widespread, ca. 25% are considered rare. The aim of this paper is to assess the degree of genetic differentiation between two rare and highly localized species (*Oxalis hygrophila* Dreyer and *Oxalis oligophylla* Salter) and the more widespread *Oxalis tomentosa* L.f. For comparative purposes, we also include *Oxalis purpurea* L., one of the most widely distributed species in South Africa. Chloroplast sequences of the *trnH*/*psbA* spacer revealed low genetic diversity for *O. oligophylla* and *O. tomentosa* compared to the widespread *O. purpurea*. High genetic diversity in *O. purpurea* might, in combination with other ecological and reproductive factors, account for the success of this species. In contrast, low variation might contribute to rarity in *O. oligophylla* and ultimately ground *O. tomentosa* to become rare. The latter two species were not monophyletic with a shared haplotype. Coalescent modelling revealed low levels of gene flow (<1 migrant per generation) between them and we argue that the genetic pattern is the result of the retention of ancestral polymorphism following a recent divergence.

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1. Introduction

The southern tip of Africa is characterized by a unique and exceptionally rich flora. It includes both the Cape Floristic Region (CFR) and the Succulent Karoo Region (Van Wyk and Smith, 2001), each characterized by a specialized flora rich in species and endemics. *Oxalis* represents the seventh largest genus within the CFR and shows remarkable species richness in both the CFR and the Succulent Karoo Region (Linder, 2003). All southern African members of *Oxalis* still exhibit tristyly, a breeding system characterized by a genetic polymorphism in which three floral morphs (long, mid and short, according to stigma height) are present within populations (Salter, 1944). Tristyly is an out-crossing mechanism that limits seed production to pollination between different morph types which, in turn, reduces the negative effects of close in-breeding within populations (Barrett, 1992; Weller, 1992).

Ecological gradients (e.g. soils, aridity, altitude,) are thought to have been pivotal in speciation and species maintenance within the CFR (Linder, 2003; Verboom et al., 2004; Linder and Hardy, 2005). Many species have narrow natural tolerances and restricted distribution ranges. Although a small proportion of *Oxalis* species have very wide distributions and ecological tolerances, most conform to the pattern in other CFR taxa by being highly localized, often also with specific habitat preferences. Primack (1993) and Pullin (2002) consider a species to be rare if it displays one or more of the following characteristics: (i) if it occupies a narrow geographical distribution range, (ii) if it has highly specific habitat requirements, or (iii) if it is found only in small populations. Many *Oxalis* species can thus be regarded as rare, because they occupy narrow geographic ranges and/or have very specific habitat requirements and/or are restricted to very small populations. This has resulted in an estimated 25% of southern African *Oxalis* species being classified as rare (Hilton-Taylor, 1996).

As a world biodiversity hotspot, conservation prioritization is of particular importance in the Cape Region. Maintenance of
biodiversity may, in part, be achieved by directing conservation resources to species and ecosystems most at risk, and/or by controlling or reversing the processes that place them at risk. However, in order to do this, we need to understand what those processes are. A review of recent literature revealed a paucity of information pertaining to the reproductive systems of geophytes in the Cape Region. There is a generally consistent view held amongst terrestrial ecologists (Prochez et al., 2006) that geophytes are the floral element least affected by typical fragmentation processes of the Succulent Karoo, Renosterveld and Fynbos biomes due to vegetative propagation capabilities of most geophytes. Geophytic forms are resilient to the disturbance regimes of both the Succulent and Nama Karoo biomes (drought and fire), and it is believed that this growth form thrived even during periods of climatic changes and associated successive replacements of the two biomes (Midgley et al., 2001). Although this may apply to southern African members of Oxalis, fitness and abundance of Oxalis taxa may be influenced both by their geophytic habit and the potentially restrictive tristylist breeding. Different levels of expression of these attributes may, in part, explain why some species are rare, while others are common and/or weedy (Burne et al., 2003).

To understand the causes of rarity in Oxalis, given the biological complexity of the genus in southern Africa, it is important to identify the spatial distribution of genetic variation across species and populations. Observed phylogeographic patterns are the result of historic (and to a lesser extent current) space–time processes. These include current levels of gene flow amongst isolated populations, historic migrations and past demographic changes (see Avise, 2000 and references therein). These kinds of information are readily inferred from the relationships among intra- and interspecific DNA sequences. Additionally, genetic analyses can address whether populations represent units with independent evolutionary trajectories and provide insights into the evolutionary processes that determine the degree to which they are genetically distinct.

This paper aims to assess the degree of genetic differentiation within a well-supported monophyletic lineage including two rare and highly localized species: Oxalis hygrophiila Dreyer and Oxalis oligophylla Salter, as sister taxa to the widespread Oxalis tomentosa L.f. (Oberlander et al., 2004). Our specific objective was to determine whether geographic occurrence (highly localized vs. widespread vs. weedy) is reflected in the species’ respective genetic profiles with weedy species expected to have the highest genetic diversity with geographically localized species being characterized by lower levels of variation. We employed sequence data from the chloroplast intergenic spacer trnH/pshA to assess population parameters including genetic variability and the level of gene flow among populations. For comparative purposes, we also include Oxalis purpurea L., one of the most widely distributed and ecologically tolerant species in South Africa. The distribution and amounts of genetic diversity within and among populations of rare plants are likely to depend on whether a species has always been rare (naturally rare endemics) or whether it has recently become so as a result of human influences. Plant species that have a history of being rare occur naturally in sparsely distributed and small populations, and they may even have adaptations that compensate for the disadvantage of rarity. In contrast, formerly widely distributed species that have experienced severe reductions in population sizes may be more susceptible to genetic stresses imposed by small population size (Falk and Holsinger, 1991; Gustafsson and Sjögren-Gulve, 2002).

2. Methods

2.1. Study species

O. tomentosa, flowering from April to June, is common and well-represented all over the Western Cape Province (South Africa), but is mostly restricted to shale-derived clay soils supporting Renosterveld vegetation (J. Zietsman, personal observation). Although widely distributed, populations of this species are subjected to habitat fragmentation and disturbance within its natural distribution range, as large portions of Renosterveld have been lost to agriculture (Kemper et al., 1998). O. oligophylla, flowering from May to June, is a Gifberg endemic (Salter, 1944) that grows in sandstone Fynbos. It is restricted rock crevices in the escarpment of the Gifberg Mountain, where it is present at extremely low densities. The Gifberg Mountain comprises mainly of refugium Arid Fynbos surrounded by succulent Karoo shrublands, causing this area to be isolated from adjacent Fynbos (J. Zietsman, personal observation). O. hygrophiila, which flowers only during November, has been recorded from a single locality in the Pakhuis Pass (Salter, 1944; Kumwenda et al., 2004). This species occurs in a very specific habitat patch in these sandstone mountains, comprising a seasonally waterlogged, natural water seepage shale band composed of Renosterveld vegetation. O. purpurea has an extended flowering period from May to September. This weedy species is very abundant in the Cape Region and mostly grows in large, dense populations (Salter, 1944; Goldblatt and Manning, 2000). It has no specific habitat preferences and often colonizes very disturbed areas.

2.2. Samples

Three populations of the widely distributed O. tomentosa (located at Darling, Saron and Elandsberg, Fig. 1) were included in these studies representing populations from across the entire distribution range of this species. The Darling population is very disturbed and fragmented. An assessment of several reproductive traits, including the expression of tristyly, natural seed production and clonality, suggests that clonal growth is the only mode for reproduction in this population (J. Zietsman, personal observation). In contrast, O. tomentosa populations at the Saron and Elandsberg localities are large and relatively undisturbed. Seed production was observed within these populations, but they also display extensive levels of clonality, observed by the clumped distribution pattern of plants that form clusters of the same morph type (J. Zietsman, personal observation). A single population of the rare and highly localized O. oligophylla, located in the Gifberg, and O. hygrophiila in the Pakhuis Pass (Fig. 1) were included. Both of these are natural populations, but O. oligophylla has an extremely low density. Due to the more solitary distribution of individuals, clonal growth is presumably low. The exact location of the type locality (the only known population) of O. hygrophiila was visited on various
occasions during its flowering period in 2005, but not a single plant was found (J. Zietsman, personal observation). As a result, only one individual sampled during a previous visit was available for inclusion in this study. *O. purpurea* is represented by a large, weedy population sampled in Stellenbosch (Fig. 1). Within this population, extensive levels of clonal growth accompany extensive seed production.

With the exception of *O. hygrophila*, for which only a single specimen was available, we included between 15 and 20 individual plants per population (Table 1). Since *Oxalis* is capable of vegetative reproduction via bulbil formation, collections were made at 1–2 meter intervals to prevent the sampling of clones. To further avoid the inclusion of vegetative samples, only a single individual was sampled per clump. Sampled material was dried and stored in silica gel matrix (Merck Laboratory Supplies (Pty) Ltd, Darmstadt, Germany).

### 2.3. Laboratory methodology

DNA was extracted from 0.01–0.02 g of dried leaf tissue using the 2× CTAB extraction protocol adapted from Doyle and Doyle (1987). In short, leaf tissue was ground in liquid nitrogen with a mortar and pestle and incubated in 2× CTAB isolation buffer at 60 °C. Chloroform–isoamylalcohol (24:1 v/v) was added and centrifuged to separate a DNA containing aqueous phase. Cold isopropanol (−20 °C) was used to precipitate nucleic acids and subsequently washed with a buffer containing 1 part of 40 mM ammonium acetate to 3 parts of ethanol by volume. The dried precipitants were resuspended in TE buffer.

To investigate the spatial distribution of genetic variation among populations, we employed the *trn*H/chloroplast spacer marker. This region was amplified using the primers *trn*H (GUG) and *psb*A described by Hamilton (1999). PCR reactions were carried out in 30 µl reaction volumes and included 2 µl of genomic DNA, 3 µl of a 10× reaction buffer, 3 µl of 2 mM MgCl₂, 3 µl of 1 mM dNTP solution, 3 pmol of each primer and 1 unit of Taq polymerase (Super-Therm). The final volume was adjusted with deionised distilled water. The cycling parameters included an initial denaturation step at 96 °C for 5 min followed by 30 cycles of 96 °C for 30 s, 50 °C for 30 s and 72 °C for 40 s. A final extension step at 72 °C for 5 min completed the reactions. PCR products were purified with the

| Species      | Sampling locality | N  | Haplotypes                  | $h$            | $\pi$          |
|--------------|-------------------|----|-----------------------------|----------------|---------------|
| *O. oligophylla* | Gilberg          | 20 | A ($n=15$), B ($n=5$)       | 0.395±0.101    | 0.022±0.013   |
| *O. hygrophila* | Pakhuis Pass     | 1  | H ($n=1$)                   | –              | –             |
| *O. tomentosa*  | Saron            | 20 | B ($n=16$), D ($n=3$), I ($n=1$) | 0.352±0.123    | 0.011         |
|               | Elandsberg       | 15 | B ($n=15$)                  | 0              | 0             |
|               | Darling          | 20 | C ($n=20$)                  | 0              | 0             |
| *O. purpurea*  | Stellenbosch     | 15 | E ($n=6$), F ($n=3$), G ($n=4$), J ($n=1$), K ($n=1$) | 0.771±0.072    | 0.011±0.007   |

Fig. 1. The geographical distribution of *O. hygrophila* (A), *O. oligophylla* (B), *O. tomentosa* (C) and *O. purpurea* (D) in the Western Cape Province, South Africa. Specific study sites of each species are indicated in black.
Wizard SV Gel and PCR clean-up system (Promega, U.S.A.) according to the manufacturer’s recommendations.

Amplicons were cycle sequenced using BigDye chemistry (Applied Biosystems, U.S.A.). Unincorporated dye label was removed by sephadex columns before the samples were run on an ABI 3100 automated sequencer (Applied Biosystems, U.S.A.). Electropherograms of the raw data were manually checked and edited with Sequence Editor™ software (Applied Biosystems, U.S.A.). All Oxalis sequences generated in this study were deposited in GenBank (EF040587–EF040597).

2.4. Sequence analyses

Sequences were aligned with Clustal X (Thompson et al., 1997) using the multiple alignment mode and checked manually. Several insertions/deletions (indels) of various sizes were present in our data. We assumed that these indels represent single events rather than multiple events. In following Jansen Van Vuuren and Robinson (1997), we coded all indels as single characters (see Results for detail discussion). PAUP* (Swofford, 2001) was used to assess the nucleotide composition and the number of parsimony-informative sites. Haplotype and nucleotide diversities were calculated in Arlequin version 3.1 (Excoffier et al., 2005). Haplotype frequencies were identified using MacClade 4 (Maddison and Maddison, 2000) and verified in Arlequin.

To depict the evolutionary relationship among species and populations, we followed a hierarchical approach. At the species level, phylogenetic trees were constructed for all taxa using both a parsimony and maximum likelihood approach. Parsimony analyses were based on heuristic searches with 100 random additions of taxa and TBR branch swapping. Gaps were considered as a 5th character state (for detailed discussion see Result below). The evolutionary model that best fitted our data (HKY; Hasegawa et al., 1985) was determined with Modeltest version 3.7 (Posada and Crandall, 1998). To assess the robustness of resultant topologies, we obtained 1000 bootstrap replications for parsimony and maximum likelihood analyses. At the population level we constructed a minimum spanning network to depict relationships among haplotypes using TCS (Clement et al., 2000).

To distinguish historical high levels of gene flow from recent population divergence, we followed a coalescence approach. MDIV (Nielsen and Wakeley, 2001) was used to calculate theta (an indication of population size; $\theta=N_e\mu$) and directional migration or gene flow ($M=N_e m$). The 95% credibility intervals were calculated whenever possible. To ensure convergence of the ergotic values, pairwise simulations were run for 2, 5, and 50 million generations with Mmax set at 5 or 10, while Tmax was set at 10 or 100. The analyses were conducted under the infinite sites model. This model was selected since it would allow the inclusion of gaps (recorded as nucleotides; see below). Pairwise comparisons were confined to populations and species with shared haplotypes. For the calculations we assumed a generation time of one year.

3. Results

3.1. Genetic diversity, indel treatment and haplotype frequencies

Our final data set comprised 374 bp of the chloroplast trnH/psbA intergenic spacer for 91 specimens sampled from four species (Table 1 and Fig. 1). In total, six populations were sampled which included the rare and localized $O. oligophylla$ ($n=20$) and $O. hygrophila$ ($n=1$) as well as three geographically separated populations of $O. tomentosa$ (Saron, $n=20$; Elandsberg, $n=15$; Darling, $n=20$). For comparative purposes we included the weedy $O. purpurea$ (Stellenbosch, $n=15$). Our data showed a nucleotide bias towards A (0.462) and T (0.324) with a noticeably lower C (0.115) and G (0.099) content. Homogeneity of base frequencies across taxa was not rejected ($p=1$). Of the 374 bp, 346 characters were constant. The 28 variable characters were all parsimony informative. The transition:transversion ratio was estimated at 1:1.

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Our data set (including the distantly related $O. purpurea$) contained a large number of indels varying in length between 1 bp and 112 bp. Although most of these were introduced to align $O. purpurea$ with the other three species, indels also had...
to be introduced in the alignment of ingroup taxa including between the O. tomentosa populations. We argue that it is highly unlikely that an indel of, for instance 10 characters would represent 10 independent events, but rather reflect a single insertion/deletion event. In order to avoid any bias in our results, all gaps were recoded as single characters (0 if the gap was absent and 1 if the gap was present), reducing our data set to 230 bp. The coalescence methodology, implemented in MDIV, does not recognize indels as characters. Since we believe that these indel characters add valuable information to our data (indeed, the majority of the information in the data are indel events), we recoded all indel characters (0 and 1) as nucleotides (A or G) in our coalescent analyses.

Eleven haplotypes were found for the 91 Oxalis specimens. The distribution of haplotypes across the different populations is indicated in Table 1. A shared haplotype (haplotype B) was found for the Saron and Elandsberg populations of O. tomentosa. Interestingly, and perhaps somewhat unexpectedly, this haplotype B is also shared between O. oligophylla and O. tomentosa. Five different haplotypes characterized the O. purpurea population from Stellenbosch. The high genetic diversity found in O. purpurea compared to the other species is reflected in their haplotype diversity values (see Table 1).

3.2. Phylogenetic relationships based on haplotypes

Phylogenetic relationships were reconstructed among the six populations included in this study. Irrespective of the method of analyses (parsimony or maximum likelihood), we obtained near identical results (see below). For parsimony analyses, a single most parsimonious tree of 55 steps was found (CI=0.865) (Fig. 2). It comprises three well-supported clades that correspond largely to O. purpurea, O. tomentosa and O. oligophylla/O. hygrophila. Sequence divergence values between clades I (haplotypes A, H, D) and II (B, I, C) were, on average, 1.8% compared to divergence values within clades which were always <1%. Divergence values within O. purpurea were similarly low (<1%); however, this weedy species was separated from the other clades by an average divergence of 10.8%. The maximum likelihood topology differed only in the placement of haplotype D within clade II rather than clade I. This difference in tree topologies is most likely the result of the way in which these different analysis methods treat gap characters.

At the population level, we constructed a minimum spanning network among haplotypes. The 95% connection limit was set at 6 steps. We could therefore not include all haplotypes into a single network. Rather, three networks resulted (Fig. 2) which corresponded to the three clades detected in our parsimony analyses.

3.3. Coalescent modelling

All analyses were run for 2, 5 and 50 million generations and in all cases estimates of theta and M were very similar. Migration rates among populations were invariably low (Table 2) with less than 1 migrant detected between O. oligophylla, O. tomentosa (Saron) and O. tomentosa (Elandsberg). θ, an indicator of population size, was relatively low and varied between 0.245 (O. oligophylla vs. O. tomentosa Elandsberg) and 1.072 (O. oligophylla vs. O. tomentosa Saron). When we tried to model the distribution of T, we encountered several problems in that the posterior distribution did not form a distinct peak with an asymptote that converged to zero. This would happen when the Monte Carlo variance for T is large combined with a relatively flat likelihood surface making reliable estimates impossible. Also and perhaps more importantly, the integrated likelihood value may have several peaks which can be explained when there were only a limited number of migration events during the history of the species/populations (see Fig. 3c in Nielsen and Wakeley, 2001). It was therefore impossible for us to obtain any reasonable estimates of T and TMRCA.

4. Discussion

4.1. Genetic patterns

Our investigation of the spatial distribution of genetic variation in four Oxalis species revealed three well-supported clades (Fig. 2). These clades are, however, not congruent with current taxonomy. This results from the clustering of haplotype D (identified in O. tomentosa sampled at Saron) with haplotypes A (O. oligophylla) and H (O. hygrophila), rather than with haplotypes B, I, and C (which characterize O. tomentosa populations). More importantly, our analyses further revealed the presence of shared haplotypes between O. oligophylla and O. tomentosa, where haplotype B characterizes specimens from the gifberg (O. oligophylla), Saron (O. tomentosa) as well as Elandsberg (O. tomentosa). Although the inclusion of an erroneously assumed outgroup could affect ingroup relationships (i.e. that the suggested paraphyly between these species is the result of an erroneous root), we argue that this is not likely to be case here. Haplotype are shared between species; a result that would not change with the inclusion of different outgroups.

One of the most problematic questions in conservation biology when faced with shared haplotypes between presumed distinct lineages or species is to separate short divergence times with low gene flow from long divergence times with moderate gene flow. In the former instance, one could argue different evolutionary
trajectories for taxa whereas this would be inappropriate when current gene flow is moderate to high. Species that are not reciprocally monophyletic, in combination with a pattern of shared haplotypes, could indicate (high levels of) current gene flow between alleged species. We strongly argue that this is not the case for Oxalis, and that the phylogeographic patterns evident in this genus are the result of shared ancestral polymorphism between species. Otherwise put, the observed pattern could be remnants of recent evolutionary divergence events with insufficient time for populations/species to reach a state of reciprocal monophyly. Although we could not determine the time of population divergence (see Results for explanation), coalescent modelling unequivocally indicates very low levels of gene flow between species with the number of migrants exchanged between populations being invariably less than 1 migrant per generation (Table 2). Our result of limited gene flow is further supported by the geographical (spatial) separation (approximately 300 km separate populations) of these species together with short dispersal distance of seeds. Oxalis seeds are explosively dehisced from the capsule through rupture of the outer testa layer (Salter, 1944). This results in highly localized seed dispersal, with a dispersal distance of no more than 2 m. Gene flow is further restricted by the complex tristylos breeding system, which limits successful pollination events even within a population (Ornduff, 1964) and could thus further complicate pollination events between two species. This is further corroborated by the absence of recorded occurrence of natural interspecific hybrids (Salter, 1944).

Low levels of genetic variation characterized both the O. oligophylla population as well as the three O. tomentosa populations. For example, a single haplotype characterized all specimens included from the Darling and Elandsberg populations of O. tomentosa (Table 1). Genetic variation within a particular population may be negatively influenced by restrictions to seed production, as equal availability of morph types as well as an effective pollen vector is required for the tristylos breeding system to function effectively (Ornduff, 1964). Failure of these requirements mostly leads to no natural seed production. This may indeed be the case for the O. tomentosa populations, and perhaps more specifically for the Darling population, where vegetative reproduction was observed to be the only mode of reproduction. These low levels of genetic variation stand in sharp contrast to the much higher levels of genetic variation observed in the weedy O. purpurea, where five haplotypes characterized 15 specimens. Importantly, the success of weedy species is often linked to higher levels of genetic variation (Karron, 1987).

4.2. Conservation implications

From a conservation perspective, small populations are particular vulnerable to extinction due to low genetic variation, inbreeding depression and stochastic events, amongst others (Primack, 1993). Patterns or levels of genetic diversity may have a significant influence on the long-term persistence of local populations, and revealing such information is important in protecting rare species (Gustafsson and Sjögren-Gulve, 2002). Effective population sizes are influenced by extensive vegetative reproduction via clonal growth, given that flowering is strongly influenced by temperature and rainfall (Dreyer et al., 2006). Furthermore, sexual reproduction in Oxalis is also influenced by the complex tristylos breeding system, as seed production is limited to highly specific pollination events between specific morph types (Ornduff, 1964; Barrett, 1992; Weller, 1992). Theoretically, our estimates of the theta (and therefore by implication effective population sizes) are small and given the above mentioned implications of Oxalis reproductive biology, the number of reproducing individuals is not equal to reproductive success. In fact, reproductive success would be much less than the number of reproducing individuals.

Although the rare species (O. oligophylla and/or O. hygrophila) are not monophyletic in our study, our results suggest that they are recent endemics (derived from O. tomentosa) that are naturally rare and should be conserved as recently diverged species that have never occupied an extensive range. These species appear to occur naturally in sparsely distributed/small populations, which could be the results of limited available habitat. It is therefore of extreme importance that the habitat of such species is being conserved.

A combination of various biological attributes (lack of specific habitat requirements, efficient ability to reproduce both vegetatively and through high levels of seed production, an extended flowering period, geographical flower colour variation etc.) may explain the wide distribution and abundance of O. purpurea. The high level of genetic variability found in this species relative to the genetic variability of the more restricted species was therefore not unexpected. This high level of genetic variability, as detected from the 15 individuals studied, may reflect the potential of this species to colonize such a variety of habitats. In contrast, low levels of genetic variability could compel Oxalis species to a narrow geographical distribution range and specific habitat patches as it is unable to adapt to different environmental conditions. These low levels of genetic variability detected in the studied species possibly result from a combination of small population sizes, restrictions to sexual reproduction and also a limited availability of their natural species specific habitats. Low levels of genetic variation in neoendemics may also result from low variability in the ancestral population, and the proportion of that variation represented in founder individuals (Karron, 1987).

The low genetic variability of both O. oligophylla and O. tomentosa contrasts with the assumption that widespread species can be predicted to exhibit higher levels of genetic variability than rare congeners (Hamrick and Godt, 1989). Moreover, an investigation by Gitzendanner and Solis (2000) on 34 pairs of restricted and widespread congeners revealed that rare species often exhibit levels of diversity equal to, or sometimes higher than, widespread congeners. Although O. tomentosa is common in the Western Cape, it could possibly have displayed a much larger, continuous population in the past as large parts of its natural habitat has been transformed by agriculture. Fragmentation of these populations into smaller patches could further erode the genetic variation, especially if seed production is disrupted (e.g. the Darling population) and could cause some populations to become endangered. This agrees with the suggestions by Van Rossum et al. (2004) who argue that commonness is not
always a guarantee for long-term survival, especially in fragmented habitats.

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