Bird Conservation International

CAMBRIDGE UNIVERSITY PRESS

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Journal:	*Bird Conservation International*
Manuscript ID:	BCI-MP-2013-0115
Manuscript Type:	Main Paper
Date Submitted by the Author:	09-Sep-2013
Complete List of Authors:	Di Giacomo, Adrian; Universidad de Buenos Aires, Ecología, Genética y Evolución Di Giacomo, Alejandro; Aves Argentinas, Departamento de Conservación Kliger, Rafi; Universidad de Buenos Aires, Ecología, Genética y Evolución Reboreda, Juan; Universidad de Buenos Aires, Ecología, Genética y Evolución Tiedemann, Ralph; University of Potsdam, Unit of Evolutionary Biology/Systematic Zoology Mahler, Bettina; Universidad de Buenos Aires, Ecología, Genética y Evolución
Keywords:	Alectrurus risora, genetic variability, habitat fragmentation, neotropical birds, Strange-tailed Tyrant

No evidence of genetic variation in microsatellite and mitochondrial DNA markers among remaining populations of the Strange-tailed Tyrant *Alectrurus risora*, an endangered grassland species

ADRIAN S. DI GIACOMO, ALEJANDRO G. DI GIACOMO, RAFI KLIGER, JUAN C. REBOREDA, RALPH TIEDEMANN AND BETTINA MAHLER

Running head: Conservation genetics of the Strange-tailed Tyrant

Author head: A. S. Di Giacomo et al.

Keywords: *Alectrurus risora*, genetic structure, habitat fragmentation, neotropical birds, Strange-tailed Tyrant

SUMMARY

The Strange-tailed Tyrant *Alectrurus risora* (Aves: Tyrannidae) is an endemic species of southern South American grasslands that suffered a 90% reduction of its original distribution due to habitat transformation. This has led the species to be classified as globally vulnerable. By the beginning of last century, populations were partially migratory and moved south during the breeding season. Currently, the main breeding population inhabits Iberá wetlands in the province of Corrientes, NE Argentina, where it is resident all year round. There are two remaining small populations in the province of Formosa, NE Argentina, and in Southern Paraguay, which are separated from the main population by the Parana-Paraguay River and its continuous riverine forest habitat. The populations of Corrientes and Formosa are separated by 300 km and the grasslands between populations are non-continuous due to habitat transformation. We used mtDNA sequences and microsatellite loci to test if there were evidences of genetic isolation between Argentinean populations. We found no evidence of genetic structure between populations ($\Phi_{ST} = 0.004$, $P = 0.32$; $F\text{st} = 0.01$, $P = 0.06$), which can be explained by either retained ancestral polymorphism or by dispersal between populations. We found no evidence for a recent demographic bottleneck in nuclear loci. Our results indicate that these populations could be managed as a single conservation unit on a regional scale. Conservation actions should be focused on preserving the remaining network of areas with natural grasslands to guarantee reproduction, dispersal and prevent further decline of populations.

RESUMEN

El Yetapa de Collar *Alectrurus risora* (Aves: Tyrannidae) es una especie endémica del cono sur de Sudamérica que ha sufrido una reducción del 90% de su distribución geográfica histórica debido a la transformación del hábitat. Esto condujo a que la especie sea clasificada como vulnerable a nivel global. A principios del siglo pasado, las poblaciones eran parcialmente migratorias migrando al sur durante la temporada reproductiva. Actualmente la principal población reproductiva habita en los Esteros del Iberá en la provincia de Corrientes, en el noreste de Argentina, donde es residente durante todo el año. Existen otras dos pequeñas poblaciones restantes en la provincia de Formosa, en el noreste de Argentina, y en el sur del Paraguay, que están separadas de la población principal por el río Paraná-Paraguay y los hábitats de selva ribereña continua. Las poblaciones de Corrientes y Formosa están separadas por 300 km y los pastizales entre ambas poblaciones no son continuos debido a la fragmentación por los cambios recientes en el uso de la tierra. El objetivo de este trabajo fue estudiar la estructura genética entre ambas poblaciones utilizando secuencias de ADN mitocondrial y microsatélites, con el fin de inferir patrones de aislamiento entre las poblaciones. No se encontraron evidencias de estructura genética entre las poblaciones de Argentina ($\Phi_{ST} = 0.004$, $P = 0.32$; $F\text{st} = 0.01$, $P = 0.06$), lo que puede explicarse ya sea por la retención de un polimorfismo ancestral o por la dispersión actual de los individuos entre ambas poblaciones. Tampoco se encontraron evidencias de un cuello de botella demográfico reciente. Estas poblaciones podrían ser manejadas como una única unidad de manejo a escala regional y las acciones de conservación deberían enfocarse en la conservación de una red de áreas con pastizales naturales que garanticen la reproducción, la dispersión y eviten así la disminución de las poblaciones actuales de la especie.

INTRODUCTION

Loss of suitable habitat and the subsequent fragmentation of populations are recognized as the major threat affecting birds (Owens and Bennett 2000, Fischer and Lindenmayer 2007, Hoffman *et al.* 2010, Rands *et al.* 2010). Among the variety of biological processes negatively affected by habitat fragmentation, one of the most important is the geographic structure of genetic diversity and the reduction in gene flow among populations (Höglund 2009, Frankham *et al.* 2010). Also, the small population sizes in remaining fragments may cause increased levels of inbreeding and a stronger effect of random genetic drift (Wade 2001). Both processes result in a loss of genetic variability within populations, which generally causes a reduction in individual fitness and population adaptability and ultimately may lead to local and global extinction (Gilpin and Soulé 1986, Hedrick and Kalinowski 2000, Höglund 2009).

Loss of gene flow because of reduced dispersal, and reduction in genetic diversity because of genetic drift, can re-distribute genetic variability among spatially structured populations over the course of a few generations only (Harrison and Hastings 1996, Caizergues *et al.* 2003). Patterns of genetic variation can provide insights into dispersal patterns and population connectivity on scales that direct field measures of dispersal are unable to address (Koenig *et al*. 1996). Thus, genetic information, including data on population genetic structure, has become relevant in species conservation (Frankham 2010, Haig *et al*. 2011). Ryder (1986) introduced the concept of evolutionarily significant units (ESU) to account for genetic diversity below recognized taxonomical levels. Although ESU definitions might differ (Moritz 1994, Crandall *et al.* 2000, Kizirian and Donnelly 2004, Degner *et al.* 2007), genetic data always play a critical role for identifying evolutionary units for conservation purposes (for a review see Palsbøll *et al.* 2006) and the protection of genetically distinct populations helps to maintain high levels of biodiversity minimizing extinction risks (Waples 1991).

The Strange-tailed Tyrant *Alectrurus risora* belongs to the group of Tyrant flycatchers (Aves: Tyrannidae), which are endemic of the New World. Thirteen percent of the 429 species of this group (Fitzpatrick 2004) are threatened (10 Endangered and 19 Vulnerable species) or near threatened (27 species) at global level (Birdlife International 2013). Most of the tyrant flycatchers are typical forest inhabitants (Fitzpatrick 1980, 2004), although some of them have diversified towards open habitats occupying grasslands, steppes and wetlands along the Andes, Patagonia and Pampas in western and southern South America (Ohlson *et al.* 2008). Populations of endemic Neotropical grassland birds, including tyrant flycatchers, have declined dramatically over the recent decades in these habitats where they now exist mostly in isolated fragments scattered in an agricultural matrix (BirdLife International 2008, Azpiroz *et al.* 2012).

The Strange-tailed Tyrant has suffered a large contraction reaching a 90% loss of its original range. This was associated with the conversion of natural grasslands to agriculture during the last century (Di Giacomo and Di Giacomo 2004). At present, it is classified as a globally vulnerable species and further decline in range and number is expected because suitable habitats are subject to extensive agricultural modification, intensification of livestock farming and afforestation (Collar *et al.* 1992, BirdLife International 2013). Range contraction was accompanied by a change in migration behavior. While one century ago the species was partially migratory and reproduction also occurred in Central Argentina, at present all populations are resident and breed in grasslands of Northern Argentina and Paraguay (Di Giacomo and Di Giacomo 2004, Di Giacomo *et al.* 2011a). The current main breeding population inhabits Iberá wetlands in the province of Corrientes, NE Argentina, while other small populations occur in the province of Formosa, NE Argentina, and in southern Paraguay (Birdlife International 2013). The Parana-Paraguay River and its continuous riverine forest habitat historically separate these small populations from the main one. As regard the Argentinean populations, they are separated by 300 km and the grasslands between them are non-continuous due to fragmentation by changes in land use. However, a comprehensive study that has been carried out on the Formosa population for the last ten years showed that banded fledglings are rarely seen in the population in the following years, suggesting that young individuals might disperse from their natal area (Di Giacomo *et al*. 2011b).

The aim of this study was to analyze the genetic variability and the genetic structure of the main population and one isolated Argentinean population of the Strange-tailed Tyrant, using both mtDNA and microsatellite molecular markers. Current conservation and management efforts are focused on preserving a high degree of biodiversity (Allendorf and Luikart 2007), which can be evaluated through ecological and/or genetic means (Schwartz *et al*. 2006, Pertoldi *et al.* 2007). Therefore, to determine how intraspecific genetic diversity is geographically partitioned in natural populations may help to identify which populations are genetically depleted or fragmented (Stoffer 1999, Allendorf *et al.* 2008) and to identify the presence of evolutionarily significant units.

MATERIALS AND METHODS

Study site and sample collection

We mist-netted individuals between 2004 and 2011 during the reproductive (September-January) and non-reproductive (February-August) seasons at 'Reserva Ecológica El Bagual', Formosa Province, (26° 10’ S; 58° 56’ W), and 'Reserva Natural del Iberá', Corrientes Province (28° 36’ S; 57° 49’ W), Argentina. These populations are approximately 300 km apart. Before release, we took a small amount (15–30 μL) of blood through brachial vein puncture with a 31G needle.

Blood was collected with an 80 µL heparinized capillary tube, immediately added to 0.5 mL of lysis buffer (100mM Tris pH 8, 10mM NaCl, 100mM EDTA, 2% SDS), and stored at room temperature until analysis. In total, we captured 68 adult individuals: 46 in Formosa and 22 in Corrientes.

Genetic analysis

We extracted DNA from blood samples with QIAGEN (Hilden, Germany) extraction kit and amplified a 700 bp fragment of mtDNA cytochrome *b* gene using the following primers: H15767 (Edwards *et al.* 1991) and Aris (ATGAAGGGATGTTCTACTGGTTG). We performed PCR amplifications in 10 µl reaction volumes using 50-100 ng DNA, 0.5 µM forward and reverse primers, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, and 0.25 units Taq-Polymerase (Invitrogen), with annealing temperatures set at 56^{o}C for 30 cycles. We amplified products on an Applied Biosystems Model 3130xl Genetic Analyzer using ABI Big Dye^{TM} Terminator Chemistry.

For microsatellite loci, we performed PCR amplifications in 25 µL reaction volumes using 10–80 ng of DNA template, 136 nM forward and reverse primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 units Taq-Polymerase (Q-BIOgene Taq Core Kit 10). We used 8 published primer pairs (Beheler *et al*. 2007, Tarof *et al*. 2001, Watson *et al*. 2002) that have been successfully tested in our study species (Mahler *et al*. 2013). We used multiplex PCR reactions with fluorescent-labelled forward primers (fluorescent dyes were 6-FAM, VIC, NED, and PET) and non-labelled reverse primers. Reactions were cycled: 5 min at 94 °C, then 30 s at 94 °C, 60 s at the annealing temperature + 3 °C, 45 s at 72 °C, 3 times, then 30 s at 94 °C, 60 s at the annealing temperature, 45 s at 72 °C, 37 times, and finally 10 min at 72 °C. Annealing temperatures for each pair of primers are detailed in Mahler *et al*. (2013).

Statistical analysis

We edited Cytochrome *b* sequences with the software BioEdit v7.0 (Hall 1999), performed the alignment with CLUSTAL W (Thompson *et al.* 1994), and determined the number of haplotypes and their frequencies. We constructed a haplotype network using the Median-joining algorithm as implemented in Network v4.6.1.0 (Bandelt *et al*. 1999). Networks are better suited to depict intraspecific phylogenies than are tree algorithms because they allow for the coexistence of ancestral and descendant alleles in a sample, whereas trees treat all sequences as terminal taxa (Posada and Crandall 2001). We used Arlequin v3.11 (Excoffier *et al.* 2005) to calculate genetic diversity indices.

We analyzed polymorphic loci with GeneMapper v3.7 (Applied Biosystems). We used MicroChecker v2.2.3 (Van Oosterhout *et al.* 2004) to assess the quality of polymorphic loci scoring, calculated observed and expected heterozygosities and allelic richness with GenAlEx v6 (Peakall and Smouse 2006), and tested for Hardy–Weinberg disequilibrium and for departures from linkage equilibrium using a Markov chain method with Genepop v4.0 (Raymond and Rousset 1995a, Rousset 2008). We tested for differences in genetic variability parameters between populations with a Mann-Whitney test. We used the software Bottleneck v1.2.02 (Cornuet and Luikart 1997) to detect recent effective population size reductions as a consequence of habitat contraction. For this analysis we used 1000 iterations and the infinite alleles model.

We analyzed genetic structure between populations using Arlequin v3.11 and used AMOVA (Excoffier *et al.* 1992) and the Exact Test of Population Differentiation (Raymond and Rousset 1995b) to calculate Φ_{ST} analogous of Wright's (1965) F statistics for mtDNA and F_{ST}

for microsatellites. Significance levels were determined using permutation procedures as implemented in Arlequin. For microsatellites we also evaluated population structure using Bayesian cluster analysis with STRUCTURE 2.1 (Pritchard *et al.* 2000). We carried out Markov chain Monte Carlo simulations without prior information on the admixture model and using correlated allele frequencies. We assumed that the maximum number of populations (*K*) varied between one and four. We set the number of steps for burn in and simulations to 50,000 and 100,000, respectively, and performed three replications for each *K*.

RESULTS

Mitochondrial DNA

We amplified the cytochrome *b* fragment for 58 adult individuals, 42 belonging to the Formosa population and 16 to the Corrientes population. The amplified fragment contained 12 variable sites that yielded 11 different haplotypes (Table 1). One haplotype (H1) was very frequent, while the others were found at low frequencies and in most cases in only one individual (Table 1). The haplotype network showed a star-like fashion, with H1 occupying the central position and the remaining haplotypes diverging in only one site, except for H9 and H10 (Figure 1). Three haplotypes (H1, H5 and H6) were found in both populations, while the remaining were found in only one population (Figure 1). Haplotype number was similar for both populations ($N = 7$), but haplotype diversity was higher for the Corrientes population ($H = 0.69$ vs. $H = 0.38$).

AMOVA results showed that less than 1% of the variation is explained among populations and that there is no haplotype frequency difference between them ($\Phi_{ST} = 0.004$, $P = 0.32$). The exact test showed similar results ($P = 0.06$).

Microsatellites

We genotyped 68 individuals, 46 belonging to Formosa and 22 to Corrientes populations. Seven polymorphic loci were in Hardy–Weinberg equilibrium (Table 2), while one locus (SAP94) was significantly heterozygote deficient and not in Hardy–Weinberg equilibrium ($P < 0.05$). MicroChecker results and preliminary data on family groups indicate that locus SAP94 might have a high proportion of null alleles and was consequently eliminated from posterior analyses. Expected ($Z = 0.18$, $P = 0.85$) and observed ($Z = 0.23$, $P = 0.81$) heterozygozities, as well as allelic richness ($Z = 0.57$, $P = 0.56$), did not differ between populations. Pairwise tests for linkage between the seven polymorphic loci revealed significant genotypic linkage disequilibrium between one set of paired loci (SAP39-SAP156).

AMOVA results showed that the percentage of variation explained among populations was 1.45 % and that population differentiation was not significant ($F_{ST} = 0.01$, $P = 0.06$). We found similar results with the exact test ($P = 0.19$). We did not find evidence for a recent demographic bottleneck in nuclear loci (Sign test: Formosa population, $P = 0.47$; Iberá population, $P = 0.56$; Global population, $P = 0.47$).

Bayesian clustering analysis showed the lowest likelihood for a population structure of $K = 2$ (Table 3) and assigned the individuals equally to any of the populations (Figure 2a). The assignation of individuals to populations with $K = 3$ and $K = 4$ was not associated with sampling locations. Figure 2b shows the individual assignation to $K = 3$ populations, which showed the highest likelihood value (Table 3).

DISCUSSION

Strange-tailed Tyrants, together with other species such as the Saffron-cowled Blackbird *Xanthopsar flavus*, Black-and-White Monjita *Xolmis dominicanus* and Pampas Meadowlark *Sturnella defilippii*, are good examples of threatened species inhabiting South American grasslands that have suffered a sharp decline in their populations during the last century due to extensive changes in land use (see Azpiroz *et al*. 2012). Historically, these species were widespread throughout southern South American grasslands but they have experienced local extinctions and fragmentation of their populations, which at present are small and isolated.

The effect of grassland fragmentation on genetic diversity in southern South America has been previously documented only for flightless Greater Rheas *Rhea americana* (Bouzat 2001). Our paper represents the first study on the genetic diversity of a globally threatened grassland passerine with documented extinctions at broad scale that could affect the maintenance of its adaptive evolutionary potential (Frankham *et al*. 2010).

The loss of natural grasslands has been very pronounced during the last century in southern South America (Azpiroz *et al.* 2012) and approximately 90% of the Strange-tailed Tyrant's habitat has disappeared (Di Giacomo and Di Giacomo 2004). The Strange-tailed Tyrant is currently confined to several areas or patches of natural grasslands containing a few resident populations in Argentina and Paraguay and total population size is estimated in 6,000 - 15,000 mature individuals (Birdlife International 2013). The genetic analysis of the main populations of the species in Argentina showed an absence of genetic differentiation between them and no evidence of a recent demographic bottleneck. The star-like pattern of the haplotype network might indicate a recent expansion of the species on evolutionary time (Bandelt *et al.* 1995) as has been recently reported for other grassland birds of southern South America (Campagna *et al.* 2012).

The lack of genetic structure between our study populations could be explained by the short time elapsed since these populations became isolated or by movements of individuals among populations. Genetic similarity might reflect retained ancestral polymorphism in both populations, indicating that a bottlenecked population is absent or genetic processes acting on it are less pronounced than expected. The time necessary to detect the genetic consequences of recent processes of fragmentation and loss of habitat in endangered birds can be of only a few generations. For example, a study of Cerrado dry forests fragmentation and its association with the genetic structure of Pfrimer's Parakeet *Pyrrhura pfrimeri* populations suggested a 35-year lag between deforestation and its effect on genetic structure (Miller *et al.* 2013). On the other hand, the species' ability to disperse might account for the genetic pattern. For example, populations of the Florida Grasshopper Sparrow *Ammodramus savannarum floridanus*, a threatened grassland passerine of North America that has suffered significant population decline due to habitat destruction and fragmentation during the last century, showed limited genetic structure, likely as a result of dispersal among populations (Mylecraine *et al.* 2008). On the contrary, the Sierra Madre Sparrow *Xenospiza baileyi*, another threatened grassland bird with current fragmented populations due to habitat loss in Mexico, showed significant genetic differentiation suggesting the species' inability to cross areas of unsuitable habitat (Oliveras de Ita *et al.* 2012).

One unexpected result of our study was that Bayesian clustering showed the highest probability of individuals belonging to three populations, although these panmictic units were not associated to current geographical distribution. One possible explanation for this pattern is that it reflects ancestral reproductive behavior. If individuals were phylopatric for reproductive areas, each of these three groups might be reflecting different areas to which individuals migrated during the reproductive season. Transformation of grasslands in the Southern and

Northern part of the species distribution has led this species to change from migratory to resident approximately 50 years ago (Di Giacomo and Di Giacomo 2004). Thus, current populations are not migratory and remain only in the areas where in the past they were resident all year round or were also occupied by wintering individuals from southward breeding populations. Consequently, individuals of the studied populations might be assigned with higher probability to ancestral reproductive areas and more generations have to go past to cause genetic homogenization. Further studies conducted on the Paraguayan population of Strange-tailed Tyrants may help to better understand how recent changes in migratory behavior in this species are imprinted on the genetic pattern observed within and between populations.

CONSERVATION IMPLICATIONS

Populations that are genetically distinct may require separate management (*sensu* Moritz 1994), whereas source populations for reintroduction or translocation projects should be as genetically similar as possible to the historical population in an area (IUCN/SSC 2013). In absence of genetic differentiation, the populations of Strange-tailed Tyrants could be managed as a single management unit at a regional scale rather than as two separated management units. Fortunately, both populations are within protected natural reserves, where the number of individuals has not declined during the last 20 years (Di Giacomo 2005, Di Giacomo *et al*. 2011). However, because of the transformation of natural grasslands to agriculture, conservation actions for this species should be first directed to the protection of breeding sites to provide a network of natural grassland areas that ensure the recruitment and further dispersal of individuals. Finally, considering that a population increase and the dispersal of young individuals might allow the establishment of new reproductive populations that may even be founded through artificial translocation of individuals from other populations, the conservation of further areas with natural grasslands should be encouraged.

ACKNOWLEDGEMENTS

We are grateful to Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2008-1599), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 114-201101-00329) and Universidad de Buenos Aires (UBACyT 0708) for financial support, and to Deutscher Akademischer Austauschdienst (DAAD) and CONICET for supporting BM’s stay at the University of Potsdam. BM, ASDG, and JCR are Research fellows of CONICET. We also thank Alparamis SA, Aves Argentinas/Asociación Ornitológica del Plata, Dirección de Recursos Naturales de Corrientes, Dirección de Parques y Reservas de Corrientes and The Conservation Land Trust for allowing us to conduct this study at Reserva El Bagual and Reserva Natural del Iberá, and K. Havenstein for laboratory support.

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ADRIAN S. DI GIACOMO, RAFI KLIGER, JUAN C. REBOREDA, BETTINA MAHLER*

Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II Ciudad Universitaria, C1428EGA Buenos Aires, Argentina

ALEJANDRO G. DI GIACOMO,

Departamento de Conservación, Aves Argentinas/Asociación Ornitológica del Plata, Matheu 1246, C1249AAB, Buenos Aires, Argentina

RALPH TIEDEMANN

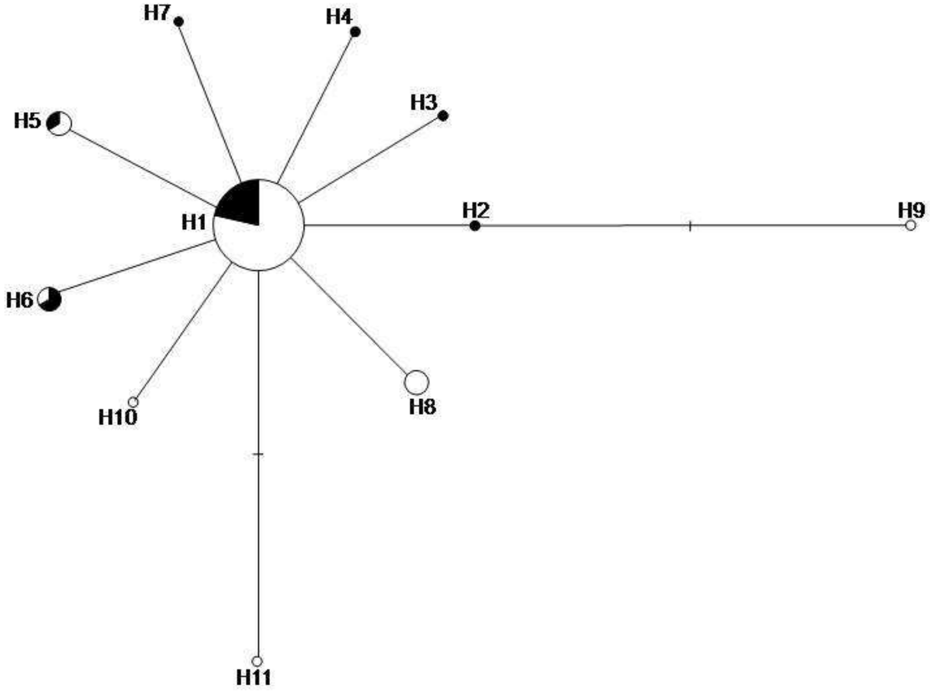
Unit of Evolutionary Biology/Systematic Zoology, Institute of Biochemistry and Biology, University of Potsdam, Potsdam-Golm, Germany

*Author for correspondence; e-mail: bemahler@ege.fcen.uba.ar

FIGURE CAPTIONS

FIGURE 1. Network for eleven haplotypes belonging to a 700-bp fragment of the cytochrome *b* gene sequenced for 58 Strange-tailed Tyrants. The size of the circle represents haplotype frequency; color distribution within the circle corresponds to the proportion of individuals found in each population (white: Formosa; black: Corrientes). Each segment represents one variable site.

FIGURE 2. Bar plot of population assignment probabilities of individuals of Strange-tailed Tyrants for $K = 2$ and $K = 3$ estimated by STRUCTURE. Each individual is represented by a single vertical line divided into the two (light gray and dark gray) or three (light gray, dark gray and black) inferred clusters.


H7
H4
H3
H5
H1
H2
H9
H6
H10
H8
H11

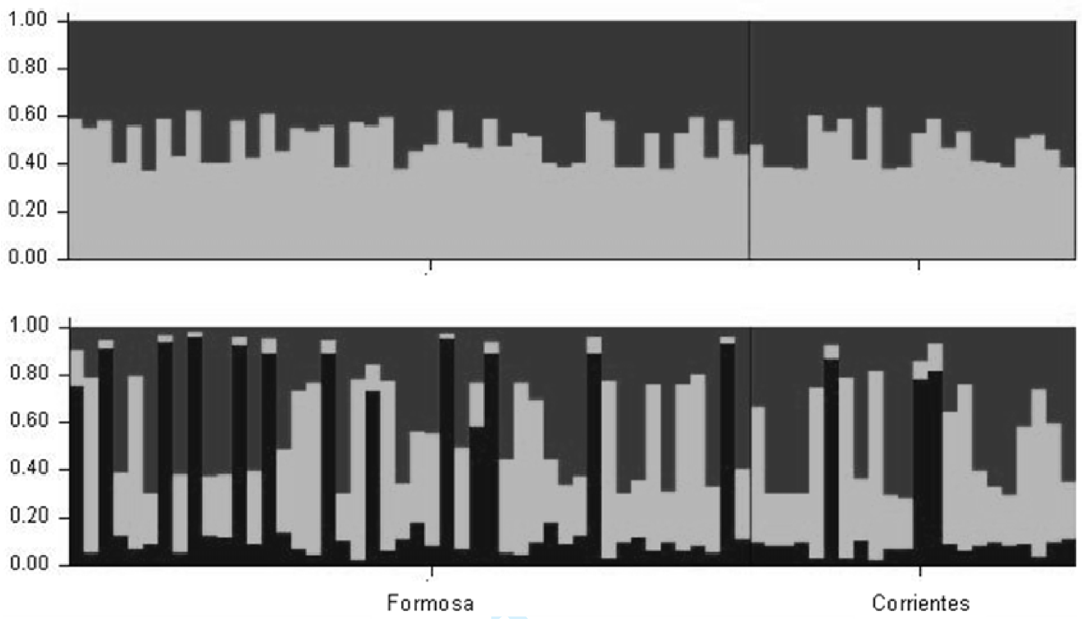

1.00
0.80
0.60
0.40
0.20
0.00
1.00
0.80
0.60
0.40
0.20
0.00
Formosa
Corrientes

TABLE 1. Variable sites and frequencies (with number of individuals) for eleven haplotypes (H1 – H11) determined for the 700 bp cytochrome *b* fragment sequenced for 58 Strange-tailed Tyrants.

	52	62	154	158	286	466	490	526	613	628	667	710	Frequency % (*N*)
H1	G	G	T	C	C	T	C	A	G	A	T	C	72,41 (42)
H2	-	-	-	-	-	-	-	-	-	-	C	-	1,72 (1)
H3	-	-	-	T	-	-	-	-	-	-	-	-	1,72 (1)
H4	-	-	-	-	-	-	-	-	-	C	-	-	1,72 (1)
H5	A	-	-	-	-	-	-	-	-	-	-	-	5,17 (3)
H6	-	-	-	-	-	-	-	G	-	-	-	-	5,17 (3)
H7	-	-	-	-	-	-	T	-	-	-	-	-	1,72 (1)
H8	-	-	-	-	T	-	-	-	-	-	-	-	5,17 (3)
H9	-	-	-	-	-	C	-	-	A	-	C	-	1,72 (1)
H10	-	A	-	-	-	-	-	-	-	-	-	-	1,72 (1)
H11	-	-	C	-	-	-	-	-	-	-	-	G	1,72 (1)

TABLE 2. Genetic diversity measured as number of alleles (*A*), expected heterocigocity (H_E), observed heterocigocity (H_O) and deviation from Hardy-Weinberg proportion (P_{HW}) for eight loci studied for two Strange-tailed Tyrant populations: Formosa (N = 46) and Corrientes (N = 22).

	Formosa				Corrientes			
	A	H_E	H_O	P_{HW}	*A*	H_E	H_O	P_{HW}
EMIZ1	3	0,362	0,435	0,422	3	0,241	0,273	0,908
SAP94	3	0,397	0,109	0,000	3	0,397	0,109	0,000
EMIZ27	6	0,553	0,457	0,043	6	0,645	0,682	0,780
SAP66	2	0,502	0,565	0,559	2	0,449	0,318	0,171
EMID46	3	0,200	0,217	1,000	2	0,210	0,238	0,536
SAP39	6	0,462	0,457	0,622	5	0,353	0,318	0,883
SAP108	2	0,436	0,413	0,742	2	0,496	0,636	0,184
SAP156	6	0,431	0,435	0,898	5	0,353	0,318	0,883

TABLE 3. Mean maximum likelihood values (± standard deviation) calculated with the software Structure for different *K*.

K	Mean likelihood ± s.d.
1	-742,8 ± 0,26
2	-758,7 ± 19,2
3	-727,2 ± 10,0
4	-748,4 ± 16,7