Homogeneous genetic structuring and microsatellite allele diversities across White-ruffed Manakin (*Corapipo altera*) populations in a highly fragmented Costa Rica landscape

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Abstract

We explored the effects of recent forest fragmentation on fine-scale patterns of population structuring and genetic diversity in populations of White-ruffed Manakins (*Corapipo altera*) inhabiting premontane forest patches of varying size in southwestern Costa Rica. Habitat fragmentation is a major conservation concern for avian populations worldwide, but studies on the genetic effects of fragmentation on Neotropical birds are limited. We sampled 159 manakins from nine forest fragments of varying size and isolation within an 18 kilometer radius, and genotyped these birds at 13 microsatellite loci. Bayesian clustering methods revealed that birds from all fragments comprised a single genetic population, and F-statistics showed only modest levels of differentiation between forest patches. We calculated allelic diversity indices for each fragment but found no correlation between genetic diversity and fragment size. These results suggest two possibilities: first, these manakins may retain substantial connectivity via inter-fragment dispersal despite habitat fragmentation, or if dispersal is currently limited, the short period of a half-century since fragmentation may not have been sufficient to impose genetic structuring or to erode allelic diversity.

Keywords: genetic structure, Bayesian clustering, gene flow, population connectivity, genetic diversity, patch size, habitat fragmentation, Neotropics, birds, microsatellites

Introduction

Habitat loss and fragmentation are primary causes of vertebrate population declines and species extinctions (Sala *et al.* 2003), particularly in the biodiversity-rich Neotropical region (Laurance *et al.* 2002), where birds have received considerable conservation attention. Habitat fragmentation has been shown to adversely affect bird populations via multiple pathways of causation, from within-fragment changes in food availability (Zanette *et al.* 2000), microclimate (Karr & Freemark 1983), nesting success (Sieving 1992, Robinson *et al.* 1995, Robinson *et al.* 2000), and survivorship (Doherty & Grubb 2002), to reductions in dispersal potential between fragments (Laurance *et al.* 2004, Laurance & Gomez 2005) that alter population connectivity (Uezu *et al.* 2005). Fragmentation has led to local extinctions of many species throughout the Neotropics (Kattan 1994, Renjifo 1999, Robinson 1999, Stratford & Stouffer 1999), particularly forest understory insectivores (Stouffer & Bierregaard 1995, Canaday 1996). The ability to disperse through the matrix habitat surrounding forest fragments and maintain connectivity may be the most important determinant of local population persistence in remnant forest patches (Castellon & Sieving 2006, Stouffer *et al.* 2006). Successful mitigation of the adverse effects of fragmentation on Neotropical bird populations thus requires a better understanding of fine-scale and taxon-specific patterns of population connectivity in fragmented landscapes.

Patterns of genetic variation can provide insights into dispersal patterns and population connectivity on scales that direct field measures of dispersal are unable to detect (Koenig *et al.* 1996). Over the course of generations, dispersal limitation may lead to genetic substructuring and a reduction in genetic diversity in isolated fragments (Caizergues *et al.* 2003), effects that likely pose a threat to the long-term viability of populations (Frankham 1996). Despite the recent application of highly sensitive markers such as microsatellites to examine population structure in

many natural populations, we know of no such microsatellite-based study that has investigated fine scale genetic structuring in a Neotropical bird that occupies a fragmented landscape. Most previous work on intra-specific genetic differentiation in Neotropical birds has been based instead on lower resolution markers (usually mitochondrial DNA haplotype variation; e.g., Brown *et al.* 2004) and focused on longer time scales in the context of incipient speciation, exploring effects of landscape features such as rivers (Capparella 1988, 1992), mountains (Brumfield & Capparella 1996), and islands (Ricklefs & Bermingham 2001) on population structure. MtDNA-based studies on the effects of natural forest fragmentation on genetic structure and diversity (Bates 2000, Bates 2002) found limited (if any) effects on genetic structure. Previous work in Southwestern Costa Rica on understory insectivores documents that recent forest fragmentation has led to unstable communities (Borgella *et al.* 2005) and a reduction in genetic diversity (Brown *et al.* 2004) for birds in small isolated fragments, suggesting that fragmentation poses a serious threat to forest-dependent avian populations in this part of the world.

Here we apply a set of microsatellite markers to a population of White-ruffed Manakins (*Corapipo altera*) occupying forest remnants of varying size in the recently fragmented Coto Brus region of Southwestern Costa Rica. Once covered in extensive tropical premontane rainforest (Borgella *et al.* 2001, Borgella & Gavin 2005), the forests of the region have been fragmented by various anthropogenic uses over the past half-century. The White-ruffed Manakin is a common resident of the lower and middle strata of tropical lowland and montane evergreen forests in this region, and in its broader range from eastern Honduras to northwestern Colombia (A.O.U. 1998). This manakin species is mainly frugivorous (Skutch 1967, Rosselli 1994), and males display on moss-covered logs arranged in dispersed leks (Slud 1964, Rosselli 2002).

Individuals generally inhabit intact forest, but may search for food in nearby clearings (Skutch 1967). We expected genetic differentiation among fragments for this species due to increased mortality in matrix habitats during dispersal events (V. Ruiz-Gutierrez, *pers. comm.*), low nesting success (Robinson *et al.* 2000), and low rates of colonization of forest fragments by dispersing individuals due to high lek fidelity (Hoglund and Shorey 2003, Borgella & Gavin 2005).

Our objectives in this study were to explore genetic patterns at two levels: population structuring across the landscape mosaic of forest patches, and within-fragment genetic diversities. To assess the connectivity of the manakins occupying these patches, we use Bayesian clustering methods to estimate the number of genetically distinct populations, and F-statistics to examine patterns of gene flow among forest fragments. We calculate allelic diversity indices to test if there is a reduction of genetic diversity in small forest patches. Considered in concert, the results of these analyses indicate that either these populations of manakins continue to exhibit substantial connectivity, or the time since fragmentation has not been sufficient to impose substantial genetic structuring or to erode allelic diversity.

Methods

Study area and sampling design

Birds were sampled from ten mid-elevation tropical forest sites within the Coto Brus region of Southwestern Costa Rica during June-August 2004 and June-August 2006 (Figure 1). This region was originally forested, but the landscape has become increasingly fragmented over the past half-century, creating a matrix of anthropogenically modified non-forest habitats that may be barriers to dispersal for birds inhabiting the remaining forest fragments. Seven fragments representing a range of patch sizes from 1.35 to 127 hectares (ha) were located within a 7 km radius of the Las Cruces Biological Station (LCBS). Two additional sampling sites were located within the nearby 7,500 ha Guaymi Indigenous Reserve. Anticipating the possibility that differentiation would be low among these nine sites which were all within a 10 km radius, birds were also sampled from one site (Altamira) within the 500,000 ha La Amistad World Biosphere Reserve (Figure 1), which was about 30 km from the remaining sites. Located within large continuous forest tracts, the Guaymi and Altamira sites also provided controls for the effect of patch size on genetic diversity. All sites were between 900 and 1300 meters in elevation. At each site, White-ruffed Manakins were caught in standard 9X12m 35mm mist nets and permanently marked with individually numbered bands as part of a larger long-term demographic study. Blood samples were taken via brachial venipuncture, and the blood samples were preserved in lysis buffer (2% Sodium dodecyl sulfate, 100mM Tris pH 8.0, 100mM Na_2EDTA, 10 mM NaCl). The birds were then released.

DNA extraction and genotyping

Genomic DNA was extracted from each blood sample using Perfect gDNA Blood Mini kits (Eppendorf), following the manufacturer's protocol. DNA was eluted in 200 µl of buffer and stored at -20C until used for genotyping. Microsatellite alleles were amplified via the polymerase chain reaction (PCR) using 15 PCR primer pairs designed specifically for *Corapipo altera* (Barnett *et al.,* in review, See Appendix 1). PCR conditions and cycling profiles followed this publication. Labeled PCR products were analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems), and allele sizes were estimated using GENEMAPPER^{TM} Vers. 3.7 (Applied Biosystems).

Microsatellite validation and variability

We checked each locus for the presence of null alleles using the program MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004), setting the number of iterations to 10000. We further tested for the significance of heterozygote deficiency relative to Hardy-Weinberg equilibrium (HWE), both at each locus in each population and in the whole sample, using the Markov Chain exact test method of GENEPOP version 3.4 (Raymond & Rousset 1995). The default values of the Markov Chain parameters were increased in order to obtain standard errors lower than 0.01 for the P value estimates. All of the birds caught at a given sampling site were considered a "population." For the exact test of each locus in each population, we performed 10,000 dememorizations (default value), 300 batches, and 5,000 iterations per batch. For the global test, we used 10,000 dememorizations, 150 batches, and 4000 iterations per batch.

For each pair of loci, we tested for the presence of linkage disequilibrium with the Markov chain method of GENEPOP (Raymond & Rousset 1995). The default values of the

Markov Chain parameters were again increased to obtain standard errors of less than 0.01. We used 10,000 dememorizations, 500 batches, and 5,000 iterations per batch.

When applicable, we controlled for multiple comparisons by calculating the False Discovery Rate (FDR) –adjusted P-values using the compute.fdr function in R 2.4.1 (Ihaka & Gentleman 1996). The library of the function is available online at http://www.stjuderesearch.org/depts/biostats/documents/fdr-library.R. We used the method of Benjamini & Hochberg (1995).

Population structure

We used two Bayesian clustering approaches, GENELAND version 1.0.8 (Guillot *et al.* 2005a & b) and STUCTURE version 2.1 (Falush *et al.* 2003; Pritchard *et al*. 2000), to explore the population structuring of manakins across this fragmented landscape. Both of these programs use multi-locus genotype information to arrange individuals into groups that most closely fit the expectations of Hardy-Weinberg equilibrium while minimizing linkage disequilibrium. Whereas both GENELAND and STRUCTURE employ similar analytical frameworks, GENELAND has two important differences: first, it allows the number of genetic groups (K) to vary, and second, it allows the spatial coordinates of each sample to be incorporated into the model as a prior. STRUCTURE is currently the most widely adopted method of determining population structure for groups of individuals with unknown population affinities, and thus provides a standard with which to compare the results produced by GENELAND.

In GENELAND, we performed an initial series of runs to determine the number of genetically distinct groups (K). Preliminary runs were performed to adjust the input parameter values so that the MCMC could converge by the end of the runs, as well as to explore the

parameter space comprehensively. We then performed 12 runs of 200,000 MCMC iterations each at the selected parameters: minimum number of genetic groups =1, maximum number of genetic groups =50, maximum number of nuclei in the Poisson-Voronoi tessellation = 500, maximum rate of the Poisson process used to generate the Voronoi cells = 500, allele frequency model = Dirichlet. Multiple runs were performed at these parameters in order to check the consistency of the value inferred for K. The uncertainty associated with the spatial coordinates was set to 400m, based on a 10 ha estimate of home range size in the closely related *C. guttaralis* (Thery 1992) (no estimate of the home range size of *C. altera* is currently available in the literature). In a typical GENELAND analysis, the next step is to assign individuals to the K genetic groups; this step was not necessary here, as the estimated number of genetic groups was 1 for each of the 12 runs (See Results).

In our STRUCTURE analyses we performed runs with values of K set from 1 to 8. Five runs were performed for each value of K, in order to check the consistency of the results between runs with the same K. Each run consisted of 50,000 burn-in iterations and 1,000,000 subsequent MCMC iterations. Because the studied populations may have had some connection to each other before fragmentation occurred – and may still have – we used the correlated allele frequencies model and the admixture model. We used a separate alpha (degree of admixture) for each population. Alpha varied considerably along the runs when we used the default value of alphapropsd (standard deviation of the normal distribution describing alpha), so we decreased it to 0.005 in order to get more accurate estimates of the output parameters (Pritchard *et al.* 2007).

Inferring gene flow patterns among forest fragments

For all of the following analyses, we removed the birds from sites with small sample sizes (Muneco and Brasilia had one individual, and Fragment 5 had seven individuals), leaving for analysis 150 birds from seven sampling sites representing six forest patches.

We employed F-statistics to measure genetic differentiation and index the extent of gene flow among forest fragments. Treating each sampling site as a separate population, we calculated pairwise Weir and Cockerham's (1984) θ's among sample sites, and tested these values for significance using GENETIX version 4.05.2 (Belkhir *et al.*, 1996-2004). This form of Fst quantifies the reduction in heterozygosity within populations relative to the total population, assuming that one single population is the ancestor of all other populations. Although F-statistics, which assume Hardy-Weinberg equilibrium, may be inappropriate for microsatellites because of their high rate of mutation, Weir and Cockerham's (1984) θ is one of the most widely used and conservative approaches to calculating differentiation (Lowe et al. 2004). This statistic thus provides an indication of how genetic variation is distributed among populations that is useful for comparisons with other studies.

We tested for the existence of isolation by distance by testing the correlation between genetic and geographic distances. We performed a Mantel test between Fst/(1-Fst) and the logarithm of the Euclidean geographic distances among fragments (Rousset 1997) using GENEPOP version 3.4 (Raymond & Rousset 1995). Two sets of geographic distance values were calculated: the distance between actual sampling spots, and the closest distance between the edges of each pair of fragments. Since we assumed that birds could move freely within a forest patch, the second set of distance values represented the amount of non-forest habitat that a bird would have to move through in order to reach the other fragment.

Within-population genetic diversity

In order to test whether there was a reduction of genetic diversity in small forest fragments, we calculated within-population genetic diversity metrics for each forest patch (except Muneco, Brasilia, and Fragment 5, because of their small sample sizes) using the program FSTAT version 2.9.3.2 (Goudet 2001) to calculate allelic richness, GENETIX (Belkhir *et al.*, 1996-2004) to calculate observed and expected heterozygosity, and GENALEX (Peakall & Smouse 2006) to calculate number of private alleles. The allelic richness of each locus (corrected for differences in sample size) was summed for each population to get a total allelic richness per forest patch. The Altamira site was eliminated from the following patch-size based analyses because it is uncertain how large the fragment is in terms of suitable manakin habitat – although it is part of the 500,000 ha La Amistad World Biosphere Reserve, much of the mountainous park lies above the elevation range limit for the White-ruffed Manakin. Graphical distributions of the values of allelic richness (R) and observed heterozygosity (Hobs) indicated that neither set of values was normally distributed (we did not use a normality test because the sample sizes were too small). Therefore we used a Spearman test to assess the correlation between both R and fragment size, and between Hobs and fragment size.

Results

Microsatellite validation and variability

A total of 159 birds were genotyped at 15 microsatellite loci (Figure 1), but two of these loci (CoAl21 and CoAl24) were removed from the analysis due to the possible presence of null alleles in five populations for CoAl24 and two populations for CoAl21. All microsatellite loci were polymorphic, with allele diversities ranging from 5 (CoAl68 and CoAl86) to 23 (CoAl02),

and a mean of 10.5 alleles per locus. No significant deficiency of heterozygotes compared to Hardy-Weinberg equilibrium was found for any locus or any population after FDR correction (Table 1). The global test also indicated no significant heterozygote deficiency. No pairs of loci showed significant linkage disequilibrium after correction for multiple comparisons.

Population structure

In all 12 of the GENELAND runs, the modal number of genetic groups estimated along the MCMC was 1. The STRUCTURE analyses likewise indicated that most likely the different samples all belong to the same genetic group. Indeed, the estimated probability of K=1 genetic group was 1, and this trend was confirmed by the plot of the log likelihood values for each value of K: the highest likelihood was obtained with K=1 (Figure 2).

Inferring gene flow patterns among forest fragments

Pairwise θ values were modest for all population pairs, ranging from 0.00079 to 0.03837, although 10 of 21 pairwise comparisons were significant (Table 2). Genetic distance showed a slight positive relationship with both measures of geographic distance, but this correlation was not statistically significant ($p= 0.235$ for distances between sample sites, and $p=0.282$ for distances between fragment edges) (Figure 3).

Within-population genetic diversity

Allelic richness, observed heterozygosity, and number of private alleles did not display any clear relationship with fragment area (Table 1). The scattered pattern of patch size versus allelic richness (Figure 4) was similar for observed heterozygosity and number of private alleles

(not shown). Excluding Altamira, the correlation between allelic richness and fragment size was not significant (Spearman's rho = 0.551, p=0.129), and neither was the correlation between observed heterozygosity and fragment size (Spearman's rho = 0.261, p = 0.309). Guaymi 1, a site within the largest fragment other than Altamira, exhibited the highest values for allelic richness (89.5), observed heterozygosity (0.746), and private alleles (4).

Discussion

The most striking result of this study is that despite the highly fragmented habitat mosaic occupied by this forest-dependent bird, we found little evidence for genetic structuring among White-ruffed Manakins distributed among ten generally isolated forest fragments. Bayesian analyses in both GENELAND and STRUCTURE indicated that all of the sampled birds group into a single genetic population. This conclusion is further supported by analyses of Fsts, which indicated low levels of differentiation and a lack of strong geographic patterning, as expected of single genetic population. Likewise, a global test for heterozygote deficiency found no significant deviation from HWE when all 159 birds were considered as a single group, further indicating that these birds come from a population with little subdivision. Perhaps even more surprisingly, within-population genetic diversity appeared to be unaffected by habitat fragmentation, as allelic diversity and observed heterozygosity showed no relationship with forest patch size. These analyses of both genetic differentiation and genetic variation suggest that habitat fragmentation has not resulted in strong genetic effects on populations of White-ruffed Manakins within the relatively fine spatial and short temporal scales of this study system. This finding of low genetic structure is interesting in ecological and conservation contexts, because forest-dependent tropical birds are typically considered to be highly sensitive to movement limitation due to habitat fragmentation (Laurance *et al.* 2004).

The absence of genetic effects on the study population suggests three possibilities. First, this species may maintain high levels of dispersal potential and population connectivity despite the fragmentation of its forest habitat, as has been shown for the White-starred Robin in Kenya (Galbusera *et al.* 2004). Alternatively, recent fragmentation may have indeed affected the dispersal patterns of this species, but there simply hasn't been enough time for the subsequent

genetic differences to accumulate. Thirdly, the lack of detectable genetic effects may be an issue of small sample sizes and inadequate power – perhaps the methods employed in this study are not robust enough to detect the underlying genetic patterns in this population.

Whereas understory insectivores are the Neotropical bird species most often identified as vulnerable to possible dispersal limitation due to fragmentation, the ecology and movement abilities of the frugivorous White-ruffed Manakin suggests that this species may be able to maintain inter-fragment connectivity in a habitat mosaic. Behavioral observations indicate that although these manakins typically occupy interior forest habitat, individuals do venture into clearings and more open edge habitats in search of fruits, which comprise the majority of their diet (Skutch 1967, Rosselli 1994). In general, frugivorous species tend to forage more widely than insectivores in search of food, and thus may have greater movement capacities. This may help explain why frugivorous birds are less affected than insectivorous birds by the degree of structural connectivity between patches in a fragmented landscape (Uezu *et al.* 2004). The White-ruffed Manakin therefore might be expected to have a higher dispersal capacity than the more understory-dependent, insectivorous species that have been identified as particularly sensitive to habitat fragmentation (Stouffer & Bierregaard 1995, Canaday 1996, Anciaes & Marini 2000, Brown *et al.* 2004). Relative to other frugivorous manakins, White-ruffed Manakins may have stronger movement capacities as suggested by observations that this species tends to frequent the higher strata of the forest when other more understory-dependent manakins are present (Skutch 1967). The White-ruffed Manakin on the Atlantic slope of Costa Rica is further suspected of undertaking a seasonal migration through forest habitat to lower elevations in the non-breeding season (Skutch 1967, Rosselli 1994, Blake & Loiselle 2002), indicating that it might be capable of moving across large distances of potentially unsuitable habitat. This

species may thus possess sufficient movement capabilities to maintain high rates of migration among isolated forest remnants. Movement does not equate with dispersal success, however – just because an individual is capable of reaching a fragment does not mean it will successfully breed there. Even if the White-ruffed Manakin has maintained high rates of movement among fragments, this may not necessarily result in high rates of gene flow. The strong lek fidelity exhibited by this species (Borgella & Gavin 2005) may make it difficult for a migrant new to a small fragment to establish itself as a breeding individual. Furthermore, numerous within-fragment recaptures during the non-breeding season suggest that the study population may not undergo extensive seasonal migrations, instead remaining on territories within fragments (V. Ruiz-Gutierrez, *pers. comm.*).

If limited dispersal among forest remnants is indeed the case for the study population, then the lack of genetic structuring may be due to the relatively short time span since fragmentation of the landscape began. Aerial photographs of the region show that some deforestation had occurred by 1960, and deforestation extents comparable to the present day had been reached by the 1970s. Assuming that the 1960 level of deforestation was enough to impede dispersal, this leaves at most half a century for limited dispersal to result in genetic differentiation among fragments. Even this maximum time frame for the accumulation of genetic differences is relatively short in terms of the probable generation time for these manakins. Although difficult to estimate for this system, the average parental age at which offspring are born is likely at least several years, and probably much longer for males than females (Rosselli 2002).

A final possibility is that the lack of detectable genetic effects stems from markers of insufficient power, limited spatial scale, or small sample sizes. This seems somewhat unlikely in

light of studies that have used as few as six microsatellite markers to reveal fine-scale genetic structure and estimate dispersal in birds with mating clusters such as leks (Hoglund & Shorey 2003) or cooperatively breeding groups (Woxvold *et al.* 2006) at similar spatial scales. Thus, the low differentiation found in the White-ruffed Manakins in this landscape is likely not due to a lack of sensitivity in the molecular markers. On the other hand, a spatial scale of tens of kilometers may be too small to detect differentiation for a species as mobile as the White-ruffed Manakin, as a study of the Long-tailed Manakin found little differentiation between sites separated by 115 km (McDonald 2003), although only four microsatellite markers were used in that study.

The genetic data alone cannot distinguish among the possibility that the sampled birds comprise one currently connected population versus the possibility that they are a collection of now-isolated populations that have simply not been isolated long enough for genetic effects to become detectable. In either case, the White-ruffed Manakins in this study area are unlikely to face immediate genetic threats stemming from the recent fragmentation of their preferred forest habitat. It remains to be seen whether or not fragmentation will affect the genetic viability of these populations in the future. Demographic processes relevant to manakin conservation likely operate at broader regional scales, as local manakin populations have usually not been considered particularly sensitive to habitat fragmentation (Anciaes& Marini 2000, Uezu *et al.* 2005, Anciaes & Peterson 2006). Keeping in mind that different species are affected by habitat fragmentation in disparate ways (e.g. Anciaes & Marini 2000, Galbusera *et al.* 2004, Uezu *et al.* 2005), this study highlights the importance of considering species characteristics along with the appropriate spatial and temporal scales in any assessment of the genetic effects of habitat fragmentation.

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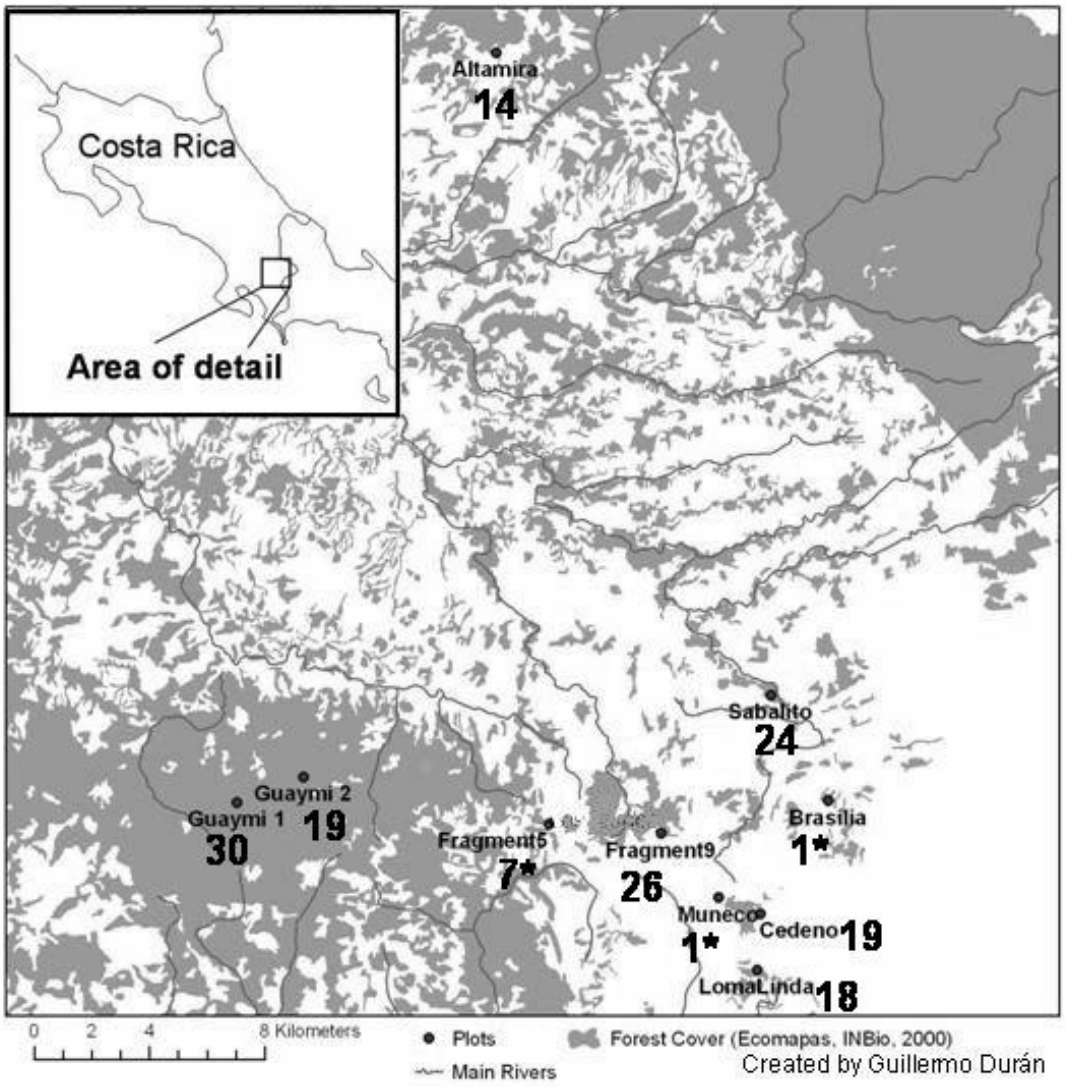

Figure 1. Map of the sampling area, showing the 10 sites where birds were caught, with numbers representing how many White-ruffed Manakins were genotyped per site. A * symbol designates sites not included in the Fst and genetic diversity analyses due to small sample sizes. Gray represents forest cover and white represents non-forest.

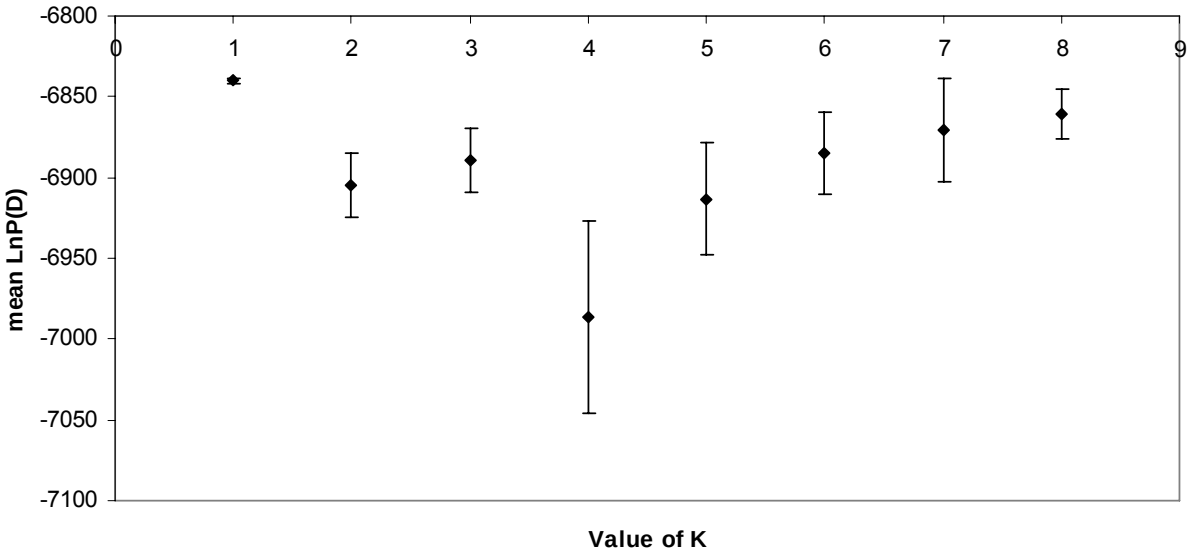

Figure 2. Evolution of the mean log likelihood (mean LnP(D)) as a function of the number of genetic groups (K), calculated over the five STRUCTURE runs made for each value of K. Error bars show one standard deviation above and below the mean of LnP(D).
One appears as the most likely number of genetically distinct manakin populations.

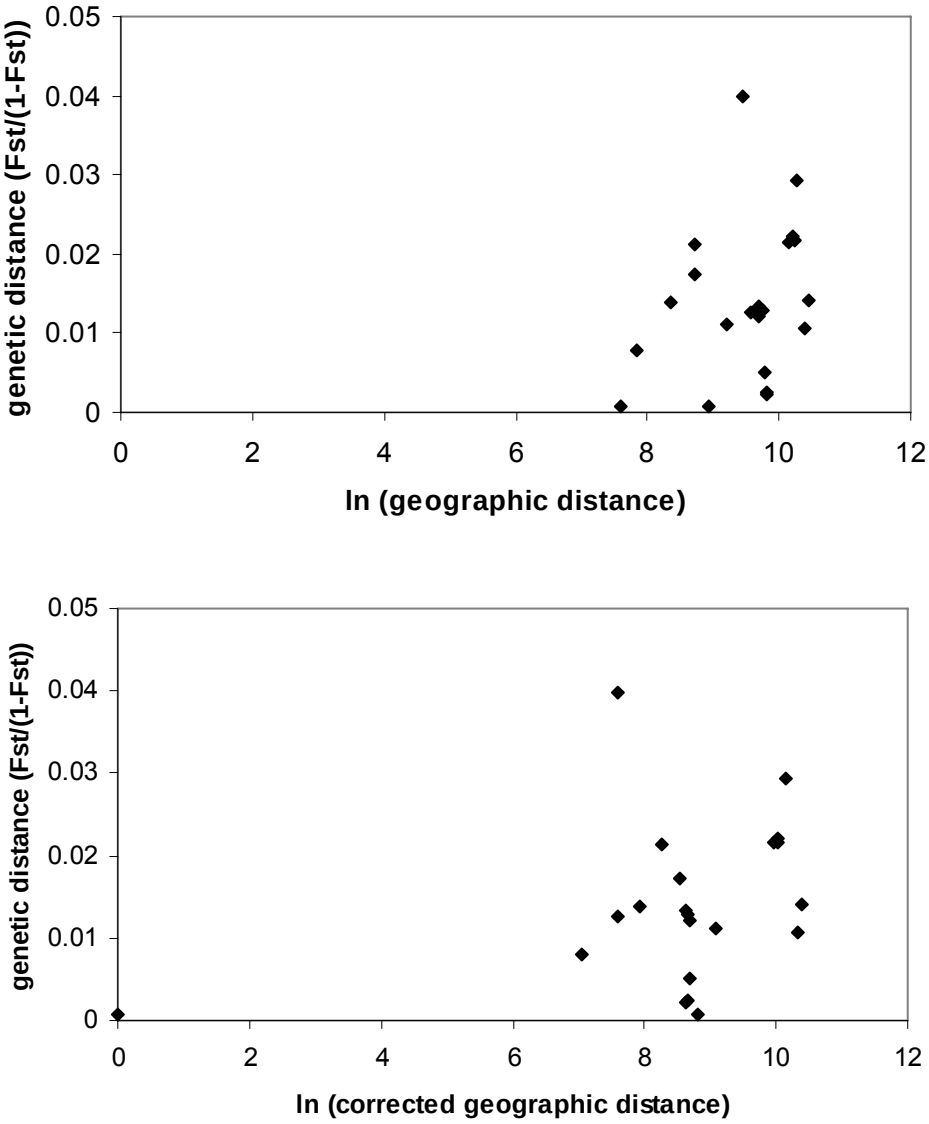

Figure 3. Correlation between pairwise genetic distance and the logarithm of pairwise geographic distance. "Geographic distance" represents the Euclidean distance between spots where birds were sampled, whereas "corrected geographic distance" represents the distance between the edges of forest patches.

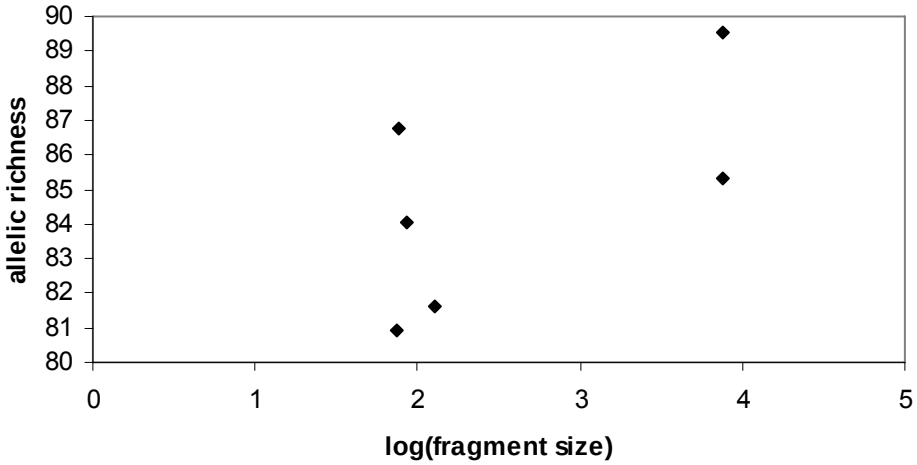

Figure 4. **Relationship between allelic richness and the logarithm of fragment size.**

Table 1. Summary of various genetic diversity estimates by sample site. H_{exp} is the expected heterozygosity, and H_{obs} is the observed heterozygosity. HW p-values are the FDR-corrected p-values of the tests of heterozygote deficiency relative to Hardy-Weinberg equilibrium per site.

Site	fragment size(ha)	sample size	allelic richness	Hexp	H_{obs}	HW p-value	# private alleles.
Altamira	??	14	81.0	0.658	0.659	0.325	3
Cedeno	86.4	19	84.0	0.691	0.717	0.738	2
Fragment 9	73.7	26	80.9	0.682	0.719	0.907	1
Guaymi 1	7500.0	30	89.5	0.719	0.746	0.907	4
Guaymi 2	7500.0	19	85.3	0.706	0.741	0.907	4
Loma Linda	75.9	18	86.7	0.714	0.744	0.907	3
Sabalito	127.2	24	81.6	0.692	0.692	0.738	1

Table 2. Pairwise Weir and Cockerham's θ (upper half-matrix) and the corresponding FDR-corrected p-values of the permutation tests (lower half-matrix). Bolded numerals indicate statistically significant θ at a 0.05 threshold.

θ	Cedeno	Fragment 9	Guaymi 1	Guaymi 2	Loma Linda	Sabalito
Altamira	0.01057	**0.0285**	**0.02113**	**0.02163**	**0.01392**	0.02103
Cedeno		0.01366	0.00504	0.01211	0.00782	0.00079
Fragment 9	0.13125		**0.01243**	**0.03837**	**0.01701**	**0.02094**
Guaymi 1	0.227027	0		0.00084	0.00236	0.00234
Guaymi 2	0.084	0	0.65		**0.01257**	**0.01308**
Loma Linda	0.084	0	0.13125	0		0.01103
Sabalito	0.3675	0	0.227027	0	0.084	

Appendix 1:

Isolation and characterization of microsatellite markers from the White-ruffed Manakin, *Corapipo altera* (Aves, Pipridae)

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Keywords: Corapipo altera, manakins, microsatellites, population structure, Pipridae, Costa Rica

Abstract

We describe 15 polymorphic microsatellite loci from the White-ruffed Manakin (*Corapipo altera*), a common understory bird of Neotropical lowland and montane evergreen forests from eastern Honduras to northwestern Colombia. These markers were developed in order to assess population structure and genetic diversity in a fragmented landscape, and to study gene flow between forest fragments. Primers were tested on a population of 159 individuals from the Coto Brus region of southwestern Costa Rica. We found between 4 and 23 alleles per locus, and observed heterozygosities ranging from 0.23 to 0.93.

The White-ruffed Manakin (Aves: Pipridae: *Corapipo altera*) is a common resident of the lower and middle strata of tropical lowland and montane evergreen forests ranging from eastern Honduras to northwestern Colombia (A.O.U. 1998). The species is mainly frugivorous (Skutch 1967, Rosselli 1994), and males display singly on moss-covered logs arranged in dispersed leks (Aldrich and Bole 1937, Slud 1964, Rosselli 2002). Although microsatellite markers have been developed for species in other genera of manakins (McDonald & Potts 1994; Piertney *et al.* 2002; Francisco *et al.* 2004; Duval & Nutt 2005), no microsatellite loci have previously been isolated from *Corapipo*. The robust set of markers described here will enable studies of the effects of anthropogenic habitat fragmentation on population structure, genetic diversity, and

gene flow, topics that have rarely been addressed in Neotropical birds using high-resolution markers (Bates 2002; Hoglund & Shorey 2003, McDonald 2003).

A DNA library enriched for microsatellites from *C. altera* genomic DNA was created using a universal linker and ligation procedure (Hamilton et al. 1999), with the modifications described in Grant and Bogdanowicz (2006), although for the enrichment step here we used a different set of single-stranded, biotinylated dimeric, trimeric, and tetrameric repeats (dimers: GT_8 and $TC_{9.5}$; trimers TTA_{12}, GAT_7, $GTT_{6.33}$, $GTA_{8.33}$, TTC_7, $GCT_{4.33}$, $GTG_{4.67}$, $GTC_{4.67}$, TCC_5; tetramers: $TTTA_{8.5}$, $GAAT_{5.5}$, $GATA_7$, $GATT_{5.5}$, $GTAT_{6.25}$, $GTTA_{6.25}$, $GTTT_{5.25}$, $TTAC_{6.75}$, $TTTC_6$, $GATG_{4.25}$, $GGTT_4$, $GCTT_{3.75}$, $GTAG_{4.5}$, $GTCA_{4.25}$, $GTCT_{4.25}$, $GTTC_4$, $TCAC_{4.25}$, and $TTCC_{4.25}$).

One hundred positive plasmid clones were sequenced on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) after amplification of the recombinant plasmids with universal M13 primers. Fourty-eight clones contained microsatellite repeats, and the program PrimerSelect version 5.07 (DNASTAR) was used to design 24 polymerase chain reaction (PCR) primer pairs for amplifying microsatellite-containing sites. After optimization and an initial screening for variability on a panel of 16 birds, 15 microsatellite loci appeared suitable for further development and were used to genotype a population of 159 birds from the Coto Brus region of Southwestern Costa Rica.

Blood samples were taken from birds via brachial venipuncture and preserved in lysis buffer (2% Sodium dodecyl sulfate, 100mM Tris pH 8.0, 100mM Na2EDTA, 10mM NaCl). Genomic DNA was extracted from blood using Perfect gDNA Blood Mini kits (Eppendorf), following the manufacturer's protocol and stored at -20C.

Two separate techniques were used to amplify these loci and create fluorescently labeled products via the polymerase chain reaction (PCR). The first was a 'universal tag' method for eight loci (CoAl01, CoAl02, CoAl06, CoAl21, CoAl24, CoAl33, CoAl39, CoAl45) (Schuelke 2000). The reaction uses three primers – an unlabeled locus-specific forward primer with an additional 20-base unpaired sequence tag at the 5' end (5'- CGAGTTTTCCCAGTCACGAC) (Waldbieser et al. 2003), a second 'universal' primer of the same 20 bases but labeled at the 5' end with a fluorescent tag (either PET, 6-FAM, VIC, or NED - Applied Biosystems), and a third, locus-specific reverse primer (described below). All three primers were used in the same reaction. After a small number of PCR cycles, the dye-labeled 'universal' primer can participate in the reaction, labeling the PCR product with the fluorescent tag. The reverse primer used in the reaction was unlabeled, but modified by the addition of a six base-pair 'pigtail' (5'-GTTTCT) to the 5' end to ensure complete adenylation of products and help standardize allele sizes (Brownstein 1996). PCR reactions (10 ul) contained: 10 – 100 ng of genomic DNA, 0.5 units of Jumpstart^{TM} *Taq* Polymerase (Sigma, St. Louis, MO), 10 mM Tris-HCL (pH 8.3), 50 mM KCl, $MgCl_2$ specific to each locus (Table 1), 200 µM of dNTPs (Invitrogen), 0.12 pmol of universal-tag modified locus-specific forward primer, 1.2 pmol of labeled 'universal' forward primer, 1.2 pmol of 'pigtailed' reverse primer, and molecular biology grade H_20 to bring the final volume to 10 µl. PCR reactions were performed using a DYAD® thermal cycler (MJ Research). The cycling profile was one cycle at 94^{o}C for two minutes, 31 cycles of 50 sec at 94º C, 1 minute at the locus-specific annealing temperature (Table 1), and 1 min at 72º C, followed by 11 cycles of 50 sec at 94^{o}C, 1 min at 50^{o}C, and 1 min at 72^{o}C, concluded by a final extension cycle of 30 min at 72º C.

A second method of amplification was used for the other seven loci (CoAl32, CoAl56, CoAl65, CoAl68, CoAl85, CoAl86, and CoAl87). Each locus specific forward primer was modified by the addition of a 5' fluorescent label (PET, 6-FAM, VIC, or NED - Applied Biosystems). Directly labeling the forward primers allowed us to perform PCR reactions of multiple loci in the same tube (multiplexing), thereby reducing the required number of PCR reactions per individual from seven to two (Hailer 2005). Reverse primers were modified by the addition of a six base-pair "pigtail" (5'-GTTTCT) to the 5' end, as described above. The seven primer pairs were multiplexed in two PCR mixes (Mix 1: CoAl56, CoAl86, CoAl87; Mix 2: CoAl32, CoAl65, CoAl68, CoAl85). Each 10 µl PCR reaction contained: 10 – 100 ng of genomic DNA, 0.25 units of Jumpstart™ *Taq* Polymerase (Sigma, St. Louis, MO), 10 mM Tris-HCL (pH 8.3), 50 mM KCl, 3.25 mM $MgCl_2$, 200 µM of dNTPs (Invitrogen), between 1.0 and 2.0 pmol (variable to obtain equal fluorescent signals) of forward and reverse primers for each locus, and H_20 to bring the final volume to 10 µl. PCR reactions were performed using a DYAD® thermal cycler (MJ Research). The cycling profile was one cycle at 95°C for two minutes, 35 cycles of 50 sec at 95° C, 1 minute at the mix-specific annealing temperature (56°C for Mix 1, and 62°C for Mix 2) and 1 min at 72° C, followed by a final extension cycle of 30 min at 72° C.

Labeled PCR products were analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems), and allele sizes were estimated using the GeneScan™ -500 LIZ™ size standard (Applied Biosystems) and GENEMAPPER™ Vers. 3.7 softeware (Applied Biosystems).

Two loci (CoAl21 and CoAl24) showed evidence of null alleles when tested with the program MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004), with the number of iterations set to 10000. Since all analyzed samples amplified at least one allele, we used the

Brookfield 1 equation as a null allele estimator. When all birds were treated as a single population, the estimated frequency of null alleles was 0.0396 for CoAl21 and 0.2243 for CoAl24.

Observed heterozygosity within the 15 loci ranged from 0.2264 to 0.9304 (Table 1). Expected (H_E) and observed (H_O) heterozygosities were calculated with GENETIX version 4.05.2 (Belkhir *et al.*, 1996-2004), using all 15 loci and treating all 159 birds as a single population.

For the 13 loci with no evidence of null alleles, no significant deficiency of heterozygotes was found relative to Hardy-Weinberg equilibrium (HWE) using the Markov Chain exact test method of GENEPOP version 3.4 (Raymond & Rousset 1995). The default values of the Markov Chain parameters were increased in order to obtain standard errors lower than 0.01 for the P value estimates. For the global test, which did not partition individuals into populations, we used 10,000 dememorizations, 150 batches, and 4000 iterations per batch. We controlled for multiple comparisons by calculating the False Discovery Rate (FDR) –adjusted P-values using the compute.fdr function in R 2.4.1 (Ihaka & Gentleman, 1996). The library of the function is available online at http://www.stjuderesearch.org/depts/biostats/documents/fdr-library.R. We used the method of Benjamini & Hochberg (1995).

No pairs of loci showed significant linkage disequilibrium after an FDR correction for multiple testing, as tested by the Markov chain method of GENEPOP (Raymond &Rousset 1995). The default values of the Markov Chain parameters were again increased to obtain standard errors of less than 0.01. We used 10,000 dememorizations, 500 batches, and 5,000 iterations per batch.

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Table 1 Characteristics of microsatellite loci in *Corapipo altera.* T_a, optimized annealing temperature; $MgCl_2$, optimized concentration; n, number of individuals genotyped; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

Locus	Repeat Motif	T_a (ºC)	$MgCl_2$ (mM)	Allele Size Range (bp)	Primer Sequence (5'→ 3')	n	N_A	H_O	H_E	GenBank Accession no.
CoAl01	$(TG)_n$	56	1.5	179-214	F:CTTCACCGTGTCATTCCACATACA R:CAATTGCTGAGCCTCCCTACTTC	159	16	0.8428	0.8884	EF523670
CoAl02	$(GT)_n$	56	1.5	212-269	F:TCTGGAAAAGTAGCGTGAGACTGC R:CACGGAATTGCAACAGCCC	159	23	0.9304	0.9066	EF523671
CoAl06	$(TG)_{10}(TA)(TG)_6$	60	1.5	345-367	F:CTGGCCTGGCACTGGGTGTAAC R:CCTGCTCACAGCCCTGGACAAG	159	10	0.7044	0.6750	EF523672
CoAl21†	$(GT)_{15}$	60	1.0	252-281	F:GGCTGGACCCACCTGACCC R:CTTGTGAATCCAACAGCCCTTATG	159	12	0.7610	0.8307	EF523673
CoAl24†	$CATA(CA)_{16}$	56	1.5	382-388	F:ACCTGCGTTCAAACACCTTCAGAT R:GTGTCCAAGCTGGGAAACAGTCTC	159	4	0.2264	0.5798	EF523674
CoAl32	$(GTAT)_{10}$	62	1*	149-178	F:AAGTTCTACAGTCCAGGCAGCACT R:TGTGATGGGGCAACACCAG	159	6	0.4591	0.4339	EF523675
CoAl33	$(AC)_{18}$	56	1.5	247-263	F:GGTGCACTTTTGGGTGATACTTC R:CTTCCTTCCCTCCATGTCTGA	159	7	0.6541	0.6704	EF523676
CoAl39	$(GA)_{18}$	61	2.0	250-262	F:CAAAGTTCAACCCACCGTCCTG R:GCAGTGAAGCCTTTGCTGTCAGAC	159	7	0.7532	0.7599	EF523677
CoAl45	$(GT)_{14}$	62	2.0	280-299	F:GCCCCCTTCAGGGTCCATT R:TATTAATCAGGCCCAAACAGACTG	159	9	0.6392	0.6039	EF523678
CoAl56	$(GTT)_6…(GAA)_{10}$	56	1*	259-283	F:AGGGGCCCAGAACTGGACACAG R:TTAGGAACTGGCTGGGCATCGTT	159	8	0.7170	0.7071	EF523679
CoAl65	$(CAA)_8C(A)_9$	62	1.5*	294-312	F:TGAGCATCATGTCATGTGAGGACT R:ACGTGTACTGAGTGGGGTGGTTC	159	8	0.7925	0.8012	EF523680
CoAl68	$(CAT)_9$	62	1.5*	201-213	F:CTCACCCAGCCACCCTTCCTG R:TTAGCCACCAGCGTGCTTGTAAGA	159	5	0.6289	0.6060	EF523681
CoAl85	$(GT)_{26}$	62	1.5*	247-299	F:AATACCAAAGTGCACAAGGATACC R:TGTGCTTCAAATGGACATCACC	159	19	0.8616	0.8950	EF523682
CoAl86	$(TG)_{10}$	56	1.5*	226-255	F:GAGACATCAGCAAAGGAGTGACAT R:CTCATGCATCAACTGACTTACAGG	159	5	0.5849	0.6068	EF523683
CoAl87	$(TG)_{20}TA(TG)_2$	56	1.5*	210-239	F:TGCAAGGGGTTAATGCTGTAATC R:ATTTATTCCAGGGGCTCACAAG	159	13	0.7610	0.7906	EF523684

*Indicates individually optimized $MgCl_2$ concentrations for each locus, although concentration was increased to 3.25 mM for each multiplex PCR mix. †Indicates locus may harbour null alleles.