Genetic variation across the historical range of the wild turkey (*Meleagris gallopavo*)

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Abstract

Genetic differences within and among naturally occurring populations of wild turkeys (*Meleagris gallopavo*) were characterized across five subspecies' historical ranges using amplified fragment length polymorphism (AFLP) analysis, microsatellite loci and mitochondrial control region sequencing. Current subspecific designations based on morphological traits were generally supported by these analyses, with the exception of the eastern (*M. g. silvestris*) and Florida (*M. g. osceola*) subspecies, which consistently formed a single unit. The Gould's subspecies was both the most genetically divergent and the least genetically diverse of the subspecies. These genetic patterns were consistent with current and historical patterns of habitat continuity. Merriam's populations showed a positive association between genetic and geographical distance, Rio Grande populations showed a weaker association and the eastern populations showed none, suggesting differing demographic forces at work in these subspecies. We recommend managing turkeys to maintain subspecies integrity, while recognizing the importance of maintaining regional population structure that may reflect important adaptive variation.

Keywords: AFLP, control region, *Meleagris gallopavo*, microsatellite, phylogeography, wild turkey

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Introduction

The wild turkey is indigenous to North America, and had an historical range that included much of the eastern and portions of the southwestern United States, northern Mexico and southeastern Canada. This historical distribution was apparently limited by amount of snowfall to the north and the lack of adequate water and roost trees to the west. By the early 1900s, the original range of the wild turkey had decreased significantly due to habitat alterations and over-harvesting. Since that time, wild turkeys have been re-introduced to some areas and translocated to others, particularly in the midwestern and northeastern USA (Mosby 1949; Mosby 1975; Williams 1981; Kennamer & Kennamer 1996). Programmes to introduce turkeys into new, unoccupied habitat, or to re-introduce them into historical habitat, often have not considered historical species

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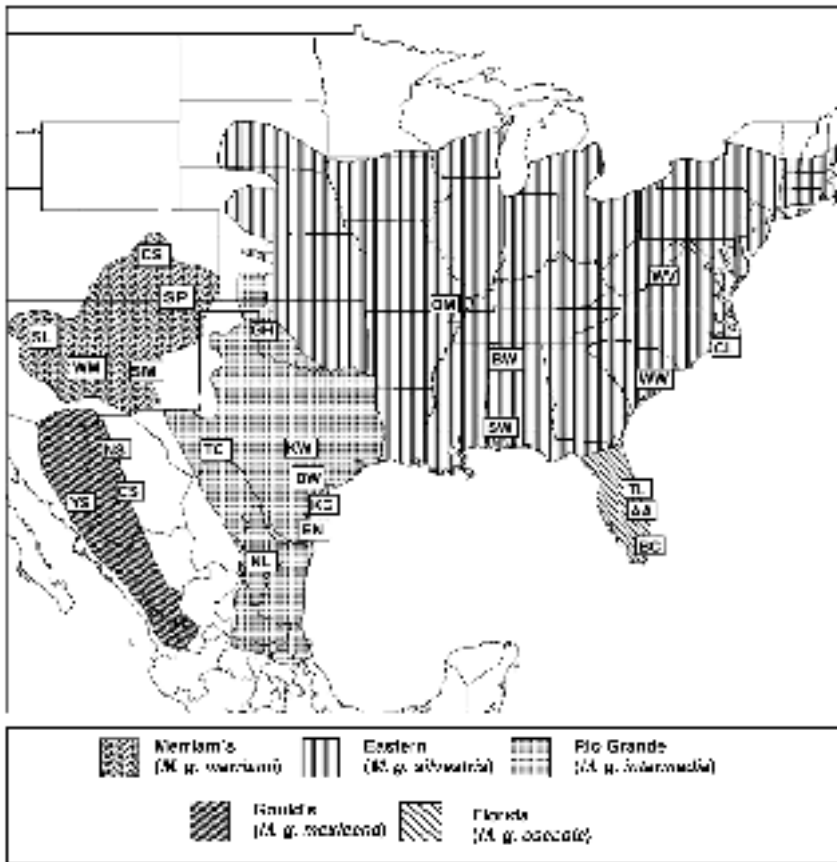
or subspecies ranges. This approach threatens to disrupt the historical patterns of genetic diversity and gene flow and potentially could lead to increased homogenization of subspecies and the loss of locally adapted gene complexes (Dobzhansky 1940; Templeton 1986; Avise 1992; Dickerman & Hubbard 1994). In other areas, habitat degradation and fragmentation may lead to increased isolation of regional populations, resulting in a loss of local genetic diversity (Lacy 1987; Leberg 1991; Leberg *et al*. 1994). Thus, the historical patterns of genetic diversity and gene flow need to be described for wild turkey before they are lost (Leberg 1991).

Six subspecies of the wild turkey have been recognized: the eastern (*Meleagris gallopavo silvestris*), Florida (*M. g. osceola*), Rio Grande (*M. g. intermedia*), Merriam's (*M. g. merriami*), Gould's (*M. g. mexicana*), and south Mexican (*M. g. gallopavo*) (Stangel *et al*. 1992). The eastern subspecies inhabits woodlands ranging from the hardwood forests of the northeastern USA to the oak-hickory forests of the midwestern USA and the pine-oak forests of the southeastern USA. Historically, its range was continuous with that of the Florida subspecies to the south and the Rio Grande subspecies to the west

Fig. 1 Wild turkey sample population locations and historical subspecies boundaries suggested by Schorger (1966). Population location description and sample sizes are provided in Table 1.

(Schorger 1966; Williams 1981; Stangel *et al.* 1992) (Fig. 1). The Merriam's and Gould's subspecies occupy ponderosa pine and pine-oak woodlands of the southwestern USA and northern Mexico. Their ranges are currently isolated from those of the other subspecies and from each other by tracts of unsuitable desert and grassland habitat. However, the ranges of Merriam's, Gould's and Rio Grande subspecies may have been continuous during the expansion of forest habitat in the late Holocene.

Current turkey subspecies designations have been based on body size differences and plumage variation, such as wing-barring patterns, body colour and extent and colour of edging on the tail coverts and rectrices. However, these morphological characteristics have not been quantitatively analysed, and may not be reliable indicators of genetic divergence (Schorger 1966; Stangel *et al.* 1992). Preliminary genetic analyses demonstrated statistically significant differences among all except the eastern and Florida subspecies, with the Gould's subspecies being the most genetically divergent (Mock *et al.* 2001). However, these conclusions were based on a limited data set, used a single marker system, and averaged allele frequencies within subspecies rather than assessing genetic patterns among populations. In this study we expand these earlier genetic analyses (Mock *et al.* 2001) to include additional amplified fragment length polymorphism (AFLP) loci as well microsatellite analysis and mitochondrial control region sequences. Using these data sets, we investigated (i) the putative historical patterns of genetic diversity across the species' range using relict populations, (ii) the genetic validity of current subspecific designations, and (iii) patterns of gene flow across geographical distance within subspecies.

Materials and methods

Sample acquisition and DNA extraction

Because of the long history of human manipulation of wild turkey populations, we focused on those populations that most probably represented 'relicts' of historical patterns of genetic diversity. We defined these populations as those (i) located within the historical geographical range of the species; (ii) having no recorded introductions; and (iii) having no known extirpations or severe declines that might have encouraged introductions from other areas or created a genetic bottleneck. We chose populations that were relatively evenly distributed across the historical range of each subspecies to reduce biases due to geographical proximity. Because of their persistence, most of these populations have been used extensively as translocation sources during

the restoration efforts of the past several decades. Although we feel that these populations are the best available representation of the historical patterns of genetic diversity and divergence in wild turkey, we recognize the possibility that undocumented translocations may have altered these patterns to some extent, particularly in the eastern USA. We sampled a total of 24 populations, representing 379 individuals. These included six eastern populations (total $n = 105$), three Florida populations (total $n = 46$), seven Rio Grande populations (total $n = 77$), five Merriam's populations (total $n = 110$), and three Gould's populations (total $n = 41$) (Table 1, Fig. 1). Additionally, four tissue samples from hunter-killed ocellated turkeys (*Meleagris ocellata*) were obtained from the Yucatan Peninsula, Mexico. All samples were obtained at voluntary hunter check stations from 1995 to 1999, with the exception of the samples from Colorado and Sonora, and a portion of the samples from Missouri and South Carolina, which were collected by winter trapping. Tissue samples collected from hunter-killed birds were pulverized in the field using razor blades and 0.2–2.0 g was immediately suspended in 5 mL lysis buffer (Longmire *et al.* 1988). Blood samples from trapped birds were obtained by jugular venepuncture, and were either collected directly into heparinized tubes and kept on ice or 0.5 mL was collected into a sterile tube containing 5 mL of lysis buffer. Blood samples collected into heparinized tubes were

Table 1 Sample population locations, sample sizes and numbers of samples from each population used for various analyses

Population	Location	Samples (n)	Mito. (n)	Msat (n)	AFLP (n)
Eastern					
ESW	Scotch WMA*, Alabama	20	10	20	20
EWW	Waterhorn WMA, S. Carolina	20	10	19	20
EOM	Ozark Mts, Missouri	18	9	18	18
EWV	various locations, W. Virginia	19	10	19	19
EBW	Black Warrior WMA, Alabama	18	10	—	18
ECL	Camp Lejeune, N. Carolina	10	7	10	10
Totals		105	56	86	105
Florida					
FAA	Avon Air Force Base, Florida	21	10	21	21
FTL	Three Lakes WMA, Florida	9	9	9	9
FBC	Big Cypress WMA, Florida	16	10	16	15
Totals		46	29	46	45
Rio Grande					
RDW	James E. Daughtery WMA, Texas	15	12	—	15
REN	Encino, Texas	9	9	9	9
RTC	Terrell County, Texas	5	5	5	5
RGH	Gene Howe WMA, Texas	10	10	10	10
RKC	Kleberg County, Texas	11	11	11	11
RNL	private ranch, Nuevo Leon, Mexico	7	6	7	7
RKW	Kerr WMA, Texas	20	9	20	20
Totals		77	62	62	77
Merriam's					
MCS	Colorado Springs, Colorado	20	10	20	20
MSP	Spanish Peaks, Colorado	40	20	20	40
MSL	Stoneman Lake, Colorado	16	15	16	16
MWM	White Mountains, Arizona	22	21	23	22
MSM	Ruidoso, New Mexico	12	7	12	12
Totals		110	73	91	110
Gould's					
GYS	Yecora, Sonora, Mexico	21	10	21	21
GNS	northern Sierra Madre, Chihuahua, Mexico	10	6	10	10
GCS	central Sierra Madre, Chihuahua, Mexico	10	9	10	10
Totals		41	25	41	41
O	Ocellated Turkey	4	4	—	—

Mito. = mitochondrial control region sequencing, Msat = microsatellite analysis. Population designations correspond with locations depicted in Fig. 1. The first letter of each population abbreviation refers to the subspecies designation (E = eastern, F = Florida, R = Rio Grande, M = Merriam's, G = Gould's).
*WMA = wildlife management area.

Primer	Sequence	5′-chicken position
LND6-2	5′-aga aaa atc aca aaa taa gtc a	16683 (+ strand)
H125-2	5′-act tgc atg tat atg tct agc a	1351 (– strand)
L16750 (Fumihito *et al.* 1994)	5′-agg act acg gct tga aaa gc	16731 (+ strand)
HD4 (Edwards 1993)	5′-ggt acc atc ttg gca tct tc	1280 (– strand)
NAU313	5′-gcc acc tgt gaa gaa gcc	681 (– strand)
NAU185	5′-acg gct tga aaa gcc att gtt gt	16737 (+ strand)

Table 2 Primers used for control region sequencing in wild turkeys

The positions of the 5′ nucleotides of these primers with reference to the chicken (*Gallus gallus*) mitochondrial genome (Dejardins & Morais 1990; GenBank NC001323) are provided in the last column.

suspended in lysis buffer (0.5 mL sample aliquots in 5 mL lysis buffer) upon arrival in the laboratory. A 1.5-mL aliquot of each of the sample/buffer mixtures was combined with 500 µg proteinase K and 100 µL 0.1 M dithiothreitol and digested overnight in a 55 °C water bath. DNA extraction was performed using a salt–chloroform technique with isopropanol precipitation (Mullenbach *et al.* 1989). The quality and quantity of DNA was assessed by electrophoresis in 0.7% agarose gels stained with ethidium bromide and by comparison to molecular weight and size standards (λ *Hin*dIII digest and 100-bp ladder). Only samples yielding predominantly high quality (> 20 kb) DNA were included in the study.

Mitochondrial control region sequencing and data analysis

We sequenced a 438-bp fragment in domain I of the mitochondrial control region, putatively the most variable portion of the control region in vertebrates (Moritz *et al.* 1987; Randi & Lucchini 1998). A summary of the primers used for this purpose is provided in Table 2. Control region amplicons were obtained for 249 individual birds using the primers LND6-2 and H125-2, and for eight birds (MSL1, GYS2, EWW1, EWW4, EWW12, EOM4, EOM6 and FBC1) using the primers L16750 and HD4. Replicate amplicons were obtained and sequenced as a quality control measure for a total of 32 individual birds, or 13% of the samples, using alternative primer sets. These replicates included seven amplicons obtained using primers LND6-2 and NAU313, eight amplicons obtained using primers NAU185 and HD4, and 18 amplicons obtained using primers L16750 and HD4.

Polymerase chain reaction (PCR) amplification reactions containing 200 µM dNTPs, 2 mM MgCl, 1 × PCR buffer, 0.2 µM primers, 1 U *Taq* polymerase, and 50 ng DNA in a total volume of 50 µL were run for 35 cycles with an annealing temperature of 55 °C. Amplicon quantity and quality were assessed on 0.7% agarose mini-gels with ethidium bromide using size (100 bp ladder) and concentration (λ *Hin*dIII digest) standards. Amplicons producing a single, well-defined band of approximately 1300 bp were purified using Concert™

Rapid PCR Purification tubes and resuspended in TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid).

Sequencing reactions were performed on these amplicons using the ABI BigDye™ kit with the forward primers used to produce the amplicon, and sequencing products were precipitated using the manufacturer's ethanol–sodium acetate procedure. An ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA) was used to generate sequence chromatograms that were manually proofread and aligned using ABI SEQUENCE NAVIGATOR™ software. These sequences were aligned with other galliform sequences and mutations were mapped relative to previously described conserved areas within the control region (Dejardins & Morais 1990; Quinn & Wilson 1993; Fumihito *et al.* 1994; Fumihito *et al.* 1995; Randi & Lucchini 1998). Nuclear copies of mitochondrial genes can confound analyses of mitochondrial sequences (Sorenson & Quinn 1998). However, the confirmation of sequence results with alternate primer pairs, the consistency of results with different tissue sources, the absence of spurious amplicons from blood samples, and the alignment of control region sequences with other galliforms all suggest that the amplicons were mitochondrial in origin.

The haplotype and nucleotide diversities (π) for each subspecies group were calculated using DNA-SP software (Rozas & Rozas 1997). A neighbour-joining tree of mitotypes was created based on the proportion of sequence differences among mitotypes using Phylogenetic Analysis Using Parsimony (PAUP) software (Swofford 1998), assigning the ocellated turkey sequences to the outgroup. Average genetic distances of individuals between each pairwise combination of populations, calculated as the proportion of nucleotide differences, were obtained using MEGA 2.0 (Kumar *et al.* 2001). This matrix was used to construct a neighbour-joining tree of populations using NTSYS software (Rohlf 1993). Mitochondrial genetic structure (F_{ST}) among subspecies was assessed using both the Analysis of Molecular Variation (AMOVA) approach (Weir & Cockerham 1984; Excoffier *et al.* 1992; Weir 1996; Φ_{ST}) and the conventional approach (θ) with ARLEQUIN software (Schneider *et al.* 2000). For the AMOVA analysis, a distance matrix of pairwise

differences (Nei & Li 1979) was constructed using individual mitotypes. For the calculation of θ, mitotypes were pooled according to neighbour-joining results to reduce diversity. The probability of the observed or greater values of both Φ_{ST} and θ was assessed using 1000 permutations of the data.

Microsatellite analysis

Turkeys were genotyped at five microsatellite loci [TUM6 (U79372), TUM12 (U79356), TUM17 (U79312), TUM23 (U79332), and TUM50 (U79306)], originally characterized in domestic turkeys (Huang *et al*. 1999) and later optimized for eastern wild turkeys (Shen 1999). Microsatellite loci were amplified as follows: genomic DNA (30 ng) was used as a template in a 10-μL reaction mixture containing 0.2 mM each dATP, dTTP, dGTP and dCTP; 2 pmol each locus-specific primer, one of which contained an M13 universal primer tag at the 5′ end; 0.1 pmol M13 universal primer with IRD florescent dye tag; 1 × reaction buffer containing 1.5 mM $MgCl_2$, 50 mM KCL, 10 mM Tris–HCL pH 9.0, 0.1% Triton X-100; 1.0 U *Taq* DNA Polymerase (Promega).

PCR reactions were carried out in 96-well microtitre plates and sealed with a microseal A sealing film (MJ Research) under the following conditions: a 5-min denaturation step at 95 °C; four cycles of 95 °C for 45 s, 68 °C (decreasing by 2 °C at each cycle) for 5 min; two cycles of 72 °C for 1 min, 95 °C for 45 s, 58 °C (decreasing by 2 °C per cycle) for two minutes, 72 °C for one minute; 31 cycles of 95 °C for 45 s, 54 °C for 2 min, 72 °C for 1 min; terminal extension step of 72 °C for 10 min. Products were electrophoresed on Long Ranger Single polyacrylamide gels (FMC Bioproducts) using a Licor DNA Analyser 4200. Alleles were assigned using RFLPScan (Scanalytics), with commercial molecular weight markers and duplicate individuals as scoring standards.

Analysis of these microsatellite loci, each consisting of dinucleotide repeats, was performed on 249 samples (Table 1). Microsatellite data were analysed in a hierarchical manner using Tools for Population Genetic Analysis (TFPGA; Miller 1997). Hardy–Weinberg genotypic proportions were tested with TFPGA using a Markov chain approach to estimate the probability of the observed genotypic proportions (Haldane 1954; Guo & Thompson 1992; Miller 1997).

UPGMA cluster analysis of populations based on a matrix of Nei's (1978) unbiased genetic distances was performed using NTSYS (Rohlf 1993) software, and the strength of the major internal nodes was assessed by bootstrapping over loci with 1000 pseudoreplicates using TFPGA software (Miller 1997). In order to compare among- versus within-subspecies genetic distances, we calculated the average (± SE) pairwise Nei's (1978) unbiased distances between all pairs of subspecies and populations within each subspecies. The strength of genetic structuring at both the population and subspecific levels was assessed with Weir & Cockerham's (1984) theta (θ), an estimator of Wright's F_{ST}, using TFPGA

software (Miller 1997). Standard deviations of θ were calculated by jackknifing over loci and 95% confidence intervals for θ were generated by bootstrapping across loci (Miller 1997). Pairwise θ-values between subspecies were calculated using ARLEQUIN software (Schneider *et al*. 2000). The significance of these pairwise values was assessed using 1023 permutations of the data among populations.

Genetic diversity was assessed using microsatellite data by calculating Nei's (1978) unbiased gene diversity (*D*) across all loci within each subspecies and by counting the total number of alleles represented in each subspecies across all five loci. In order to assess the significance of pairwise subspecific differences in *D*, pairwise differences were calculated on a locus-specific basis and a 95% confidence interval for the overall difference was generated using 1000 bootstrap replicates. A 95% confidence interval that did not include zero was taken as evidence of significant difference in *D*. Because sample size can affect the number of alleles observed, the lowest observable frequency was calculated for the Gould's subspecies, which was represented by the smallest number of samples (41). Hence, alleles at frequencies below 0.025 were not counted in the total number of alleles.

In order to investigate the possibility of a recent bottleneck in each subspecies, we used the program BOTTLENECK (Cornuet & Luikart 1996) to calculate the one-tailed probability of excess observed heterozygosity (gene diversity) at these microsatellite loci relative to the expected gene diversity under the two-phased model of mutation, using the Wilcoxon sign-rank test (Luikart *et al*. 1998). If a population has undergone a recent bottleneck, it is expected that the number of alleles in that population will decline more rapidly than the gene diversity, leading to excess gene diversity relative to the expectation based on allele number (Luikart *et al*. 1998). Additionally, we used BOTTLENECK (Cornuet & Luikart 1996) to investigate the shape of the allele frequency distribution, which is expected to deviate from the L-shaped distribution found in stable populations if there has been a recent bottleneck (Luikart & Cornuet 1997).

Microsatellite data were also used to characterize regional patterns of geographical versus genetic distances. Pairwise θ-values were calculated for all pairs of populations within the eastern, Rio Grande and Merriam's subspecies and plotted against the corresponding pairwise geographical distances to provide a visual comparison of patterns among subspecies, following Hutchison & Templeton (1999). Mantel testing was used to assess the strength and significance of the correlation between the pairwise matrices of θ and distance (Mantel 1967) using NTSYS software (Rohlf 1993).

AFLP analysis

AFLP marker profiles were generated for 378 individuals (Table 1) using procedures described by Vos *et al*. (1995)

and modified by Travis *et al*. (1996) and Busch *et al*. (2000). We used a total of eight *Eco*RI/*Mse*I primer combinations in the second selective amplification (selective nucleotides ACG/AGC, ACG/AGG, ACG/ACC, AGG/ACG, ACC/ACG, AGG/ACC, ATC/AGG, and ATC/ACC) to generate markers ranging in size from 90 to 500 bp. Polymorphic markers were selected in the absence of sample labels, and only if they could be scored unambiguously across all samples. Five per cent of the samples were completely replicated to assess the overall error rate associated with the analysis. The error rate was calculated as the number of loci with conflicting scores in replicated samples divided by the total number of loci scored. Because AFLP analysis produces dominant markers, the assumption of Hardy–Weinberg genotypic proportions must be made in order to calculate allele frequencies for statistical analysis (Mueller & Wolfenbarger 1999). Allele frequencies were estimated based on the frequency of the homozygous recessive allele using the Taylor expansion approach of Lynch & Milligan (1994). Cluster analysis, analysis of population and subspecies genetic structure, gene diversity analysis, and analysis of regional patterns of gene flow were performed for AFLP data in the same manner as described above for microsatellite data. In addition, the per cent polymorphic loci (P) was calculated for subspecies as an independent measure of genetic diversity. The 95% confidence intervals were generated for P by scoring each locus as a one (polymorphic according to the 95% criterion) or a zero (monomorphic) for each subspecies and performing 1000 bootstrap replicates.

Results

Mitochondrial sequencing and analysis

Alignment of mitochondrial control region sequences from 245 individual birds across all five subspecies (Tables 1 and 3, excluding the ocellated turkey) yielded 42 mitotypes, with 31 polymorphic nucleotide sites, 23 of which were phylogenetically informative (Table 3). All informative nucleotide variation was in the form of transitions, and most mitotypes differed by only one to two mutations. Mutations among wild turkeys occurred primarily in clusters between known conserved areas in domain I of the galliform control region (Randi & Lucchini 1998). The neighbour-joining tree describing phylogenetic relationships among mitotypes suggested a lack of diagnostic structure among the eastern, Florida and Rio Grande mitotypes, but strong clustering of the Merriam's mitotypes (Fig. 2). The Gould's populations were fixed for a single mitotype, with the exception of a closely related mitotype found in a single individual. One of the mutations found uniquely in Gould's mitotypes and in one Florida mitotype (represented by a single individual) was located in the central conserved region (domain

Table 3 Mitotypes found in populations of wild turkey (**Gen-Bank alignment accession numbers AF486875s–AF487121)

Mitotype	Populations
A	MCS(8), MRM(7), MSL(7), MSM(3), MSP(7), MWM(11)
B	MSM(1), MSP(1)
C	MSM(2)
D	MCS(1), MSL(2), MWM(1)
E	MWM(3)
F	MWM(3)
G	MRM(1), MSP(1)
H	MSL(5), MSM(1)
I	MWM(2)
J	RGH(2)
K	RDW(1), RKW(1), RTC(1), REN(1), RKC(3), RKW(4), MSL(1)
L	RDW(3)
M	GNS(5), GCS(9), GYS(10)
N	EWW(5), FAA(2), FBC(5), FTL(4), RDW(2), RGH(3)
O	FBC(4)
P	FAA(2), FBC(1), ESW(5), EOM(6), EBW(3), MCS(1), MRM(1), REN(1), RGH(3), RKW(1), RTC(3), RKC(1)
Q	EWV(2)
R	FTL(1), FAA(3)
S	ECL(4)
T	REN(1), RGH(1), RKC(2), RNL(3)
U	EBW(3)
V	EOM(1), ESW(2), EWV(1), RDW(2)
W	EBW(4)
X	EOM(2)
Y	FTL(2), EWV(2), FAA(2)
Z	ECL(1), EWV(4), RKC(2), EWW(1)
AA	MRM(1), MSP(1), MWM(1)
AB	EWW(2)
AC	RDW(2)
AD	RDW(1), REN(5), RGH(2), RKC(3), RKW(2), RNL(3)
AE	ECL(2), EWW(2)
gnc15	GNC(1)
rdw11	RDW(1)
rkw3	RKW(1)
esw3	ESW(1)
esw1	ESW(1)
eom5	EOM(1)
ewv6	EWV(1)
ftl1	FTL(1)
ftl9	FTL(1)
faa23	FAA(1)
ren3	REN(1)

Numbers in parentheses following the population codes refer to the number of individuals within each population.

II) of the control region. The neighbour-joining tree based on differences among individuals (Fig. 3) followed the same general pattern seen in the haplotype tree (Fig. 2), suggesting that the Gould's subspecies was the most divergent, followed by the Merriam's and Rio Grande subspecies.
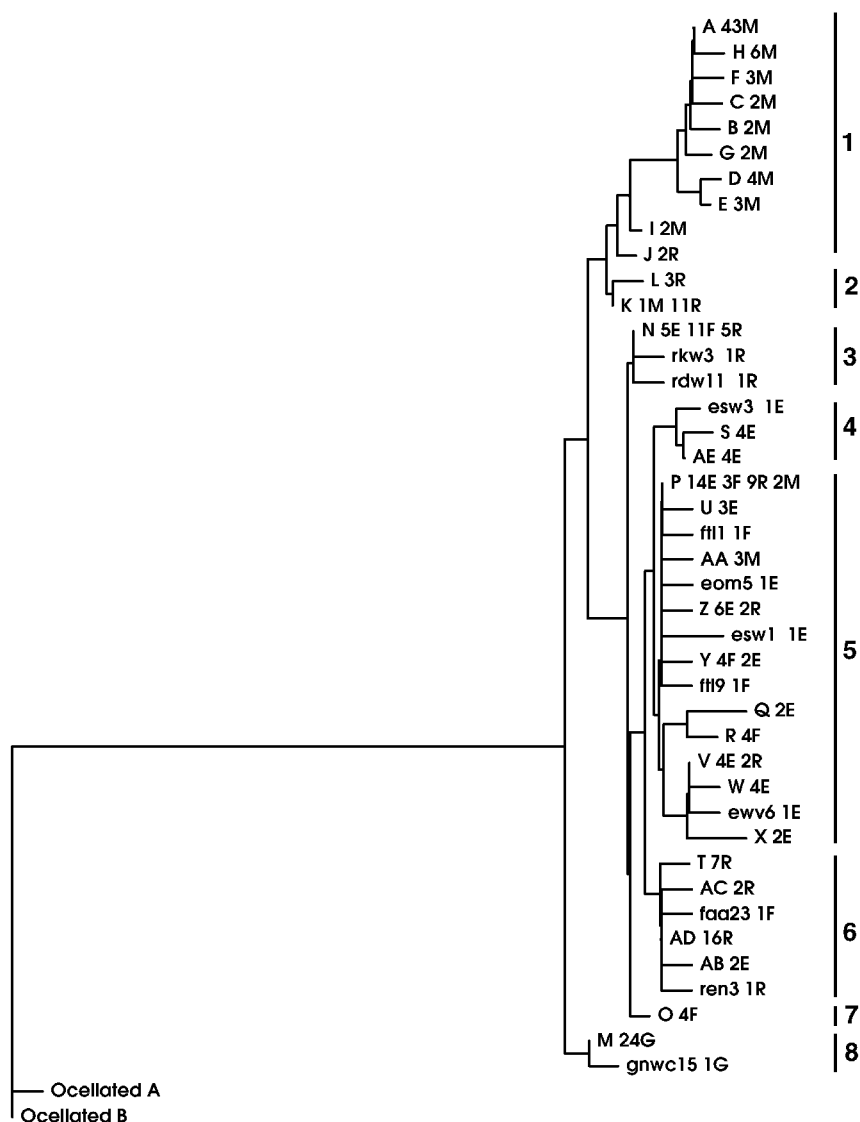
Fig. 2 Neighbour-joining dendrogram of wild turkey mitotypes based on control region sequence data. Mitotypes are designated by letter, number of individuals represented, and subspecies: eastern subspecies (E), Florida subspecies (F), Rio Grande subspecies (R), Merriam's subspecies (M), Gould's subspecies (G). Numbers along the right margin designate mitotype clusters used for calculation of F-statistics.

Eight mitotype clusters were assigned, based on the neighbour-joining mitotype tree (Fig. 2), excluding the ocellated turkey samples, in order to estimate F_{ST} using θ. A very high degree of mitotype structuring among subspecies was detected ($\Phi_{ST} = 0.613$, $\theta = 0.546$ (P (both) < 0.0001). Pairwise θ-values among subspecies (Table 4) suggested that the eastern and Florida subspecies were the least distinct, and the θ calculated for this pair of subspecies was the only nonsignificant comparison ($P = 0.009$) using the Bonferroni-adjusted significance level of 0.005.

Mitotype diversity (h) and nucleotide diversity (π) were similar among subspecies with the exception of the Gould's subspecies, which was less diverse by an order of magnitude according to both measures (Table 5). The remaining subspecies were characterized by high h and low π. The eastern and Rio Grande subspecies showed the greatest diversity according to both measures.

Microsatellite analysis

Microsatellite markers provide multiallelic, co-dominant genetic data to contrast AFLP analysis and control region sequencing. Amplification of microsatellite loci was successful on all but 28 of the 1655 total (individual by locus) reactions. The number of alleles detected per locus was highly variable: TUM6 (two alleles, 145–147 bp), TUM12 (12 alleles, 191–223 bp), TUM17 (14 alleles, 161–189 bp), TUM23 (12 alleles, 144–166 bp), and TUM50 (38 alleles, 113–160 bp). Genotypic frequencies were significantly different ($P < 0.05$) from Hardy–Weinberg expectations in eight of the 105, or 7.6%, of the population by locus data sets for which there was more than one segregating allele. The deviations were found in the following populations (and loci): FBC (TUM12), RDW (TUM50), RKC (TUM12), RKW (TUM50), EOM (TUM 12), MWM (TUM17), EWV

Table 4 Pairwise θ-values among subspecies of wild turkey using mitochondrial sequences (above diagonal), AFLP data (above diagonal, italics), and microsatellite data (below diagonal)

	Eastern	Florida	Gould's	Merriam's	Rio Grande
Eastern	—	0.120* *0.035*	0.687 *0.320*	0.679 *0.176*	0.252 *0.106*
Florida	0.003[a]	—	0.654 *0.336*	0.658 *0.181*	0.178 *0.126*
Gould's	0.010	0.010	—	0.888 *0.287*	0.554 *0.255*
Merriam's	0.010	0.010	0.014	—	0.559 *0.151*
Rio Grande	0.003	0.003	0.009	0.008	—

*not significant ($P \geq 0.005$, with Bonferroni correction).
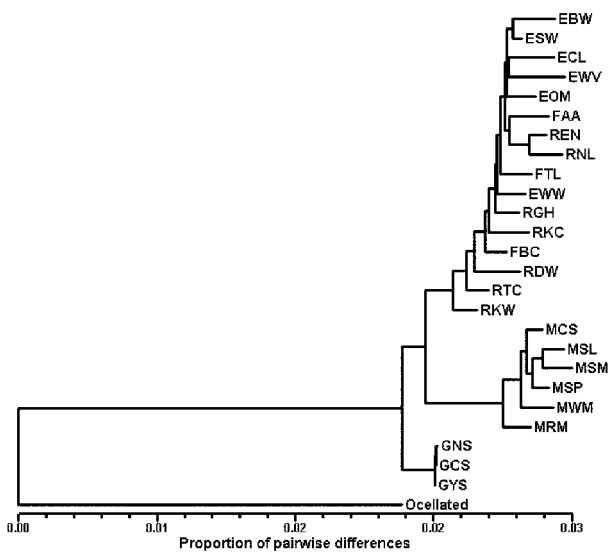
Fig. 3. Neighbour-joining dendrogram of wild turkey populations based on a matrix of the average proportion of control region nucleotide differences. Population codes are described in Table 1.

Table 5 Haplotype (h) and nucleotide (π) diversities (Nei 1987) for wild turkey subspecies based on mitochondrial control region sequencing

Subspecies	h (SD)	π (SD)
Eastern	0.895 (0.03)	0.00451 (0.0004)
Florida	0.813 (0.05)	0.00402 (0.0005)
Rio Grande	0.868 (0.02)	0.00547 (0.0003)
Merriam's	0.643 (0.06)	0.00378 (0.0008)
Gould's	0.080 (0.07)	0.00018 (0.0002)

(TUM 12), GYS (TUM 17). Thus, there was no apparent concentration of these deviations within a particular population, subspecies, or locus.

UPGMA cluster analysis of populations (Fig. 4a) was consistent with current subspecific designations with the exception of the eastern and Florida subspecies, which formed a single cluster. Bootstrap support for the major cluster topology, however, was weak. Average genetic distances between pairs of populations within subspecies were consistently and significantly less than distances among subspecies except the eastern and Florida subspecies (Table 6). The average genetic distance between eastern and Florida populations was within a standard error of the average genetic distance between eastern populations. Genetic structuring was stronger at the population

level ($\theta = 0.173$, SD = 0.05) than at the subspecies level ($\theta = 0.113$, SD = 0.03), and none of the 95% confidence intervals for these values included zero (Fig. 5).

Values of θ for pairwise subspecies (Table 4) suggested that the least structuring was between eastern and Florida, eastern and Rio Grande, and Rio Grande and Florida subspecies, while the greatest degree of structuring was between the Merriam's and Gould's subspecies. All pairwise θ-values were significant ($P < 0.005$, with a Bonferroni correction for pairwise comparisons) except for the eastern and Florida subspecies ($P = 0.0185$). Values of θ for populations within subspecies suggested that the Merriam's populations were the most highly structured, followed by Rio Grande populations (Fig. 5). There was a negative correlation ($r = 0.81$) between the number of alleles at a locus and the θ-value calculated for that locus. This correlation was nonsignificant ($P = 0.10$) with only five loci.

According to the analysis of paired, microsatellite allele-specific differences in D, the eastern subspecies was significantly more diverse than any of the other species, and the Rio Grande subspecies was more diverse than either the Gould's or Merriam's subspecies. Other differences in D among pairs of subspecies were not significant. The total number of microsatellite alleles found across all five loci were as follows: Merriam's (22), Gould's (24),
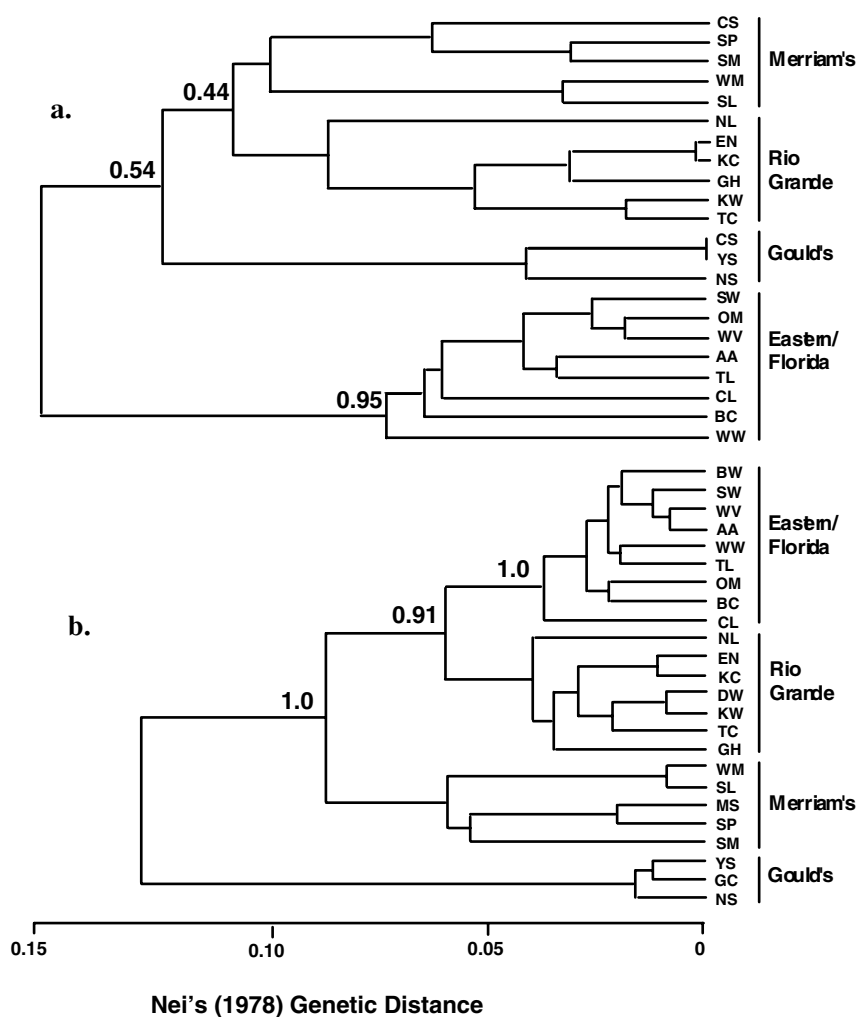
Fig. 4 UPGMA cluster analysis dendrograms based on Nei's (1978) distance calculated from (a) five microsatellite loci and (b) 83 AFLP loci in wild turkey populations representing five subspecies. Numbers at interior nodes represent the proportion of 1000 bootstrap replicates that clustered the same taxa.

Nei's (1978) Genetic Distance

Table 6 Matrix of average pairwise Nei's (1978) unbiased genetic distances (± SE) within and among subspecies using AFLP (A, above the diagonal) and microsatellite data (M, below the diagonal). Intrasubspecific contrasts are italicized

	Eastern	Florida	Rio Grande	Merriam's	Gould's
Eastern	*0.0312 (A) (0.003)* *0.1269 (M) (0.027)*	0.0311 (0.002)	0.0644 (0.003)	0.0943 (0.002)	0.1451 (0.004)
Florida	0.1710 (0.019)	*0.0263 (A) (0.004)* *0.1049 (M) (0.025)*	0.0638 (0.004)	0.0885 (0.003)	0.1425 (0.004)
Rio Grande	0.3490 (0.038)	0.4929 (0.044)	*0.0356 (A) (0.003)* *0.1362 (M) (0.022)*	0.0900 (0.003)	0.1209 (0.005)
Merriam's	0.6255 (0.058)	0.8119 (0.048)	0.2780 (0.020)	*0.0537 (A) (0.006)* *0.1590 (M) (0.026)*	0.1318 (0.005)
Gould's	0.4409 (0.065)	0.4409 (0.065)	0.3265 (0.022)	0.3367 (0.027)	*0.0197 (A) (0.002)* *0.0344 (M) (0.017)*

Florida (31), Rio Grande (32), and eastern (34). The Florida subspecies did show evidence of a recent bottleneck according to the one-tailed Wilcoxon test for gene diversity excess ($P = 0.03125$). The results of this test were not significant for the Merriam's ($P = 0.969$), Rio Grande ($P = 0.500$), eastern ($P = 0.109$), or Gould's ($P = 0.406$) subspecies. None of the subspecies showed a deviation from the L-shaped distribution of allele frequencies expected for stable populations (Luikart *et al.* 1998). Of the three subspecies tested for a pattern of isolation by distance using microsatellite
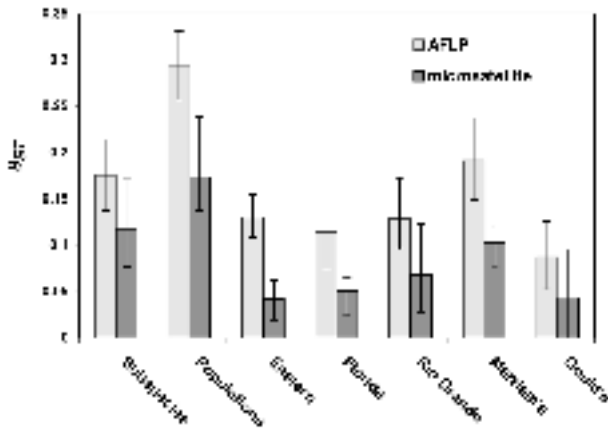
Fig. 5 Values of θ and 95% confidence intervals for AFLP and microsatellite data among subspecies, all populations, and populations within subspecies.

AFLP analysis

AFLP analysis yielded 83 polymorphic loci that could be scored for all samples. The technique error rate was estimated at 2.5%, which is comparable to the error rates found in other AFLP studies on natural populations (Busch *et al.* 2000). Genetic clustering (Fig. 4b) followed existing subspecific boundaries, with the exception of the eastern and Florida subspecies, supporting the microsatellite results (Fig. 4a). Bootstrap support for the major cluster topology was greater than 90%. Consistent with the microsatellite results, average genetic distances between pairs of populations within subspecies were significantly less than distances among subspecies with the exception of the eastern and Florida subspecies (Table 6).

As with microsatellite data, genetic structuring with AFLP data was found to be greater at the population level (θ = 0.294, SD = 0.02) than at the subspecies level (θ = 0.175, SD = 0.02), and at neither level did the 95% confidence intervals of θ include zero. Within subspecies, the Merriam's populations showed the highest levels of genetic structuring (θ) (Fig. 5). AFLP data yielded higher θ-values
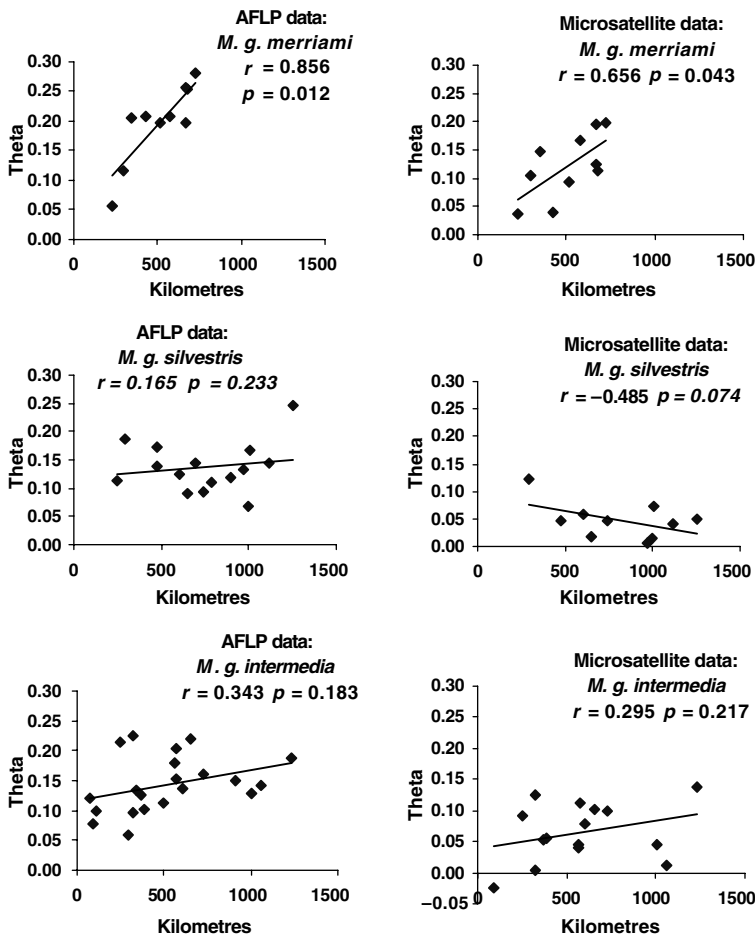
data, only the Merriam's subspecies showed a significant correlation (*r* = 0.656) between the pairwise matrices of θ and geographical distance with Mantel testing (*P* = 0.043) (Fig. 6).

Fig. 6 Pairwise population comparisons of theta (θ) versus geographical distance in three subspecies of wild turkey using AFLP and microsatellite data. Values of *r* and *P* provided in each graph were calculated from a Mantel test comparing matrices of θ and geographical distance.

Table 7 Allele-specific differences in D between all pairs of subspecies expressed as 95% confidence intervals for AFLP (above diagonal) and microsatellite (below diagonal) data

	Eastern	Florida	Rio Grande	Merriam's	Gould's
Eastern	P (AFLP) = 0.50−0.70	0.008−0.034	−0.049−0.011	−0.051−0.028	−0.039−0.054
Florida	0.006−0.133	P (AFLP) = 0.39−0.61	−0.062−0.001	−0.021−0.065	−0.045−0.050
Rio Grande	0.020−0.060	−0.108−0.052	P (AFLP) = 0.37−0.58	−0.042−0.031	−0.071−0.018
Merriam's	0.076−0.287	−0.255−0.003	−0.254 to −0.023	P (AFLP) = 0.46−0.67	−0.066−0.024
Gould's	0.045−0.344	−0.312−0.073	−0.308 to −0.001	−0.284−0.248	P (AFLP) = 0.54−0.73

Confidence intervals in bold did not include zero. Per cent polymorphic loci (P) for each subspecies, also expressed as 95% confidence intervals, are provided along the diagonal.

than microsatellite data at both the species and subspecies levels (Fig. 5). The eastern subspecies had a significantly higher value of D than the Florida subspecies, but all other pairwise differences in D among subspecies were nonsignificant (Table 7). The 95% confidence intervals for P were overlapping for all subspecies.

As with the microsatellite data, only the Merriam's subspecies showed a significant correlation ($r = 0.856$) between pairwise matrices of θ and geographical distance with Mantel testing ($P = 0.012$) (Fig. 6).

Discussion

Genetic differentiation and subspecific designation

Our data generally support current subspecies designations for wild turkey, with the exception of the eastern and Florida subspecies, which together form a genetically distinct group. The geographical differentiation of turkey populations along subspecies lines is consistent with the geographical potential for gene flow and isolation that has historically existed between current subspecies ranges. Continuous habitat has linked populations of eastern and Florida wild turkeys both currently and historically (Schorger 1966; Aldrich 1967), and gene flow between the two has apparently been great enough to prevent major genetic differentiation detectable with neutral markers. Subspecific plumage characteristics are intermediate in turkeys from the areas where the ranges of these two putative subspecies meet in the southeastern United States (Aldrich 1967), a finding consistent with the hypothesis of significant gene flow between these two areas. In northern Florida and southern Georgia, Remington (1968) described a 'suture zone' of putative secondary contact in a variety of terrestrial taxa between endemic forms in Florida and continental forms. We found no evidence of endemic forms of wild turkey in Florida, although such a pattern could have been eliminated by extensive historical and present-day gene flow. Undocumented translocations between eastern and Florida subspecies

may also have obscured historical differences among these two groups, but our sample populations were chosen specifically to minimize this possibility. The Rio Grande subspecies historically occupied habitat continuous with that of the eastern/Florida subspecies as well (Schorger 1966; Aldrich 1967). It is possible that these subspecies may now appear to be genetically distinct only because intermediate populations occupying habitat near the historical range boundaries were extirpated in the early 1900s. However, there was no indication of an east–west cline in genetic differences across eastern populations, as might be expected in such a situation.

Genetic differentiation of the Merriam's and Gould's subspecies is most likely the result of their relatively long geographical isolation from other subspecies by unsuitable desert habitats. Lack of palaeontological evidence of turkeys in the current range of the Merriam's subspecies has led to the hypothesis that this subspecies was introduced into its current range less than 1500 years ago by Pueblo Indian cultures (Hargrave 1970; Rea 1980; McKusick 1986; Breitburg 1988). According to these hypotheses, Merriam's turkey was derived from either eastern or Gould's populations. Our data do not support a strong link between Merriam's and either of these two groups. An alternative hypothesis (Shaw & Mollohan 1992) argues that Merriam's turkey was isolated from Rio Grande and/or Gould's turkeys when suitable woodland habitat contracted and was replaced by desert habitats at the end of the Pleistocene and early Holocene. The mitochondrial data presented here suggests that the Merriam's subspecies has been more recently associated with the Rio Grande subspecies than with the Gould's subspecies.

Although both nuclear techniques supported the same subspecific groupings, they differed with respect to relationships among the subspecies. The pattern of clustering in the mitochondrial data (Figs 2 and 3) resembled that found with AFLP data, suggesting that the Gould's subspecies was the most divergent, followed by the Merriam's subspecies. Given the relatively small number of microsatellite loci and the weak bootstrap support for the microsatellite

groupings, the relationships suggested by AFLP analysis appear the more robust of the two. The relationships suggested by the microsatellite analysis, with the eastern and Florida subspecies being the most divergent, would also be difficult to explain based on our understanding of historical habitat connectivity. The development of additional microsatellite loci for use in wild turkeys would allow a more direct comparison of these techniques. One limitation of AFLP data is the need to assume Hardy–Weinberg equilibrium in order to estimate allele frequencies. Because we also used microsatellites to characterize the same sample set, we were able to assess the validity of this assumption, at least with respect to the microsatellite loci analysed. Our results suggest that the number of population by locus data sets that were significantly ($P < 0.05$) different from Hardy–Weinberg expectations (7.6%) was only slightly greater than what would be expected by chance alone (5%). Therefore, the assumption of Hardy–Weinberg equilibrium in our sample populations seems reasonable.

Genetic diversity within subspecies

While population-level genetic diversity measures suggested that no one subspecies was consistently more diverse than the others (Tables 4 and 6), the eastern subspecies tended to be among the most diverse and the Gould's subspecies tended to be the least diverse, particularly with respect to mitochondrial diversity. The lack of mitochondrial diversity in Gould's turkeys could be due to a population bottleneck (Grant & Bowen 1998), but the approach of Luikart *et al.* (1998) for detecting recent bottlenecks based on relative rates of heterozygosity and allele number losses failed to provide evidence of such a process in the Gould's subspecies. This lack of diversity could also be due to a persistently low population size in this subspecies. Unfortunately, information on population trends in this subspecies is not available.

The remaining subspecies showed a general pattern of high mitotype diversity and low nucleotide diversity among mitotypes. Grant & Bowen (1998) suggest that such a pattern could be attributed to a recent expansion from a period of low population sizes, where the low sequence diversity among mitotypes is due to a recent coalescence, and the high mitotype diversity is due to the retention of mutations in an expanding population. In wild turkeys, this may be a reflection of rapid expansion following the retreat of glacial maxima at the end of the Pleistocene.

Hierarchical genetic structuring

Genetic structuring (θ) across the species complex was evident at both the population and subspecies levels (Fig. 5). The magnitude of θ at the subspecies level, in combination with the cluster analysis results, suggests that the currently designated subspecies do reflect groups of populations that share a long evolutionary history, with the exception of the eastern and Florida subspecies. Values of θ estimated using AFLPs were greater than those estimated using microsatellite data both within and among subspecies (Fig. 5). This may be an artefact of the high level of polymorphism in microsatellite loci (Hedrick 1999; Balloux *et al.* 2000), a conclusion supported by a negative correlation between the number of microsatellite alleles at a locus and the locus-specific value of θ. The comparative magnitude of θ within subspecies suggests that there is greater genetic differentiation among the Merriam's and Rio Grande populations than among populations within the other subspecies. This may reflect the less continuous nature of the habitat historically occupied by these subspecies.

Geographic versus genetic distance among subspecies

In the absence of human intervention, dispersal in wild turkeys is most likely by juvenile (predominantly female) birds moving out of the home range of their natal flock into adjacent areas over relatively small geographical distances (Ellis & Lewis 1967; Eaton *et al.* 1976; Exum *et al.* 1985). As a result, wild turkey populations may correspond relatively well to predictions based on two-dimensional stepping-stone models of isolation by distance (Kimura 1953; Malècot 1955; Kimura & Weiss 1964). These models predict a positive relationship between geographical and genetic distance in populations approximating demographic equilibrium (Malecot 1955; Slatkin 1993; Rousset 1997; Hutchison & Templeton 1999). The absence of a strong isolation by distance pattern may have a variety of interpretations (Rousset 1997; Hutchison & Templeton 1999).

The eastern wild turkey and Rio Grande populations we analysed did not show significant isolation by distance effects, and both showed less structuring among populations than the Merriam's populations (Figs 5 and 6). The Rio Grande pattern suggests some effect of distance on proximal populations, although the relationship is not significant. It is possible that a pattern of isolation by distance would fail to be detected in these subspecies if distances between sampled populations were too large (Rousset 1997). The eastern and Rio Grande subspecies were sampled over somewhat greater geographical distances than was the Merriam's subspecies. However, even at distances of under 800 km, the pattern of isolation by distance was more apparent among Merriam's populations than among eastern or Rio Grande populations, and the values of θ among pairwise populations in both the Rio Grande and eastern subspecies tended to be lower than those among Merriam's populations. The apparent absence of a significant isolation

by distance pattern among the Rio Grande and eastern populations could also be interpreted as the result of post-Pleistocene colonization into a relatively continuous habitat in these subspecies. High levels of gene flow among populations could result in a weaker relationship between genetic and geographical distance relative to the Merriam's subspecies. This interpretation is consistent with our understanding of subspecies-specific differences in historical habitat continuity: the eastern and Rio Grande subspecies occupy habitat that is far more continuous than that of the Merriam's subspecies. It is also possible that this pattern could reflect the influence of widespread reintroduction programmes during the last century, producing a pattern consistent with recent range expansion from a common source population (Hutchison & Templeton 1999). These reintroduction programmes were particularly prevalent in the eastern subspecies but also occurred in the Rio Grande subspecies. However, Leberg *et al.* (1994) have demonstrated that the genetic effects of re-introductions on neighbouring populations may be relatively slow to develop. Additionally, our study focused on those populations with the best record of having remained historically strong and which tended to serve as source populations for reintroduction programmes.

In contrast to eastern and Rio Grande populations, pairwise θ-values among pairs of Merriam's populations showed a significantly positive relationship with geographical distance with both nuclear marker systems (Fig. 6). This pattern is consistent with expectations for populations that have experienced restricted geographical dispersal for long enough to have approached regional demographic equilibrium. Because suitable habitat for Merriam's turkey often forms an archipelago of high-elevation habitat patches separated by unsuitable desert environments, Merriam's populations are likely to experience lower levels of interpopulational gene flow than eastern and Rio Grande populations, resulting in a more strongly positive relationship between geographical distance and genetic distance (Rousset 1997; Hutchison & Templeton 1999). However, further reduction in the limited habitat connectivity that exists among Merriam's populations or an increase in translocations could alter the relative effects of genetic drift or gene flow, resulting in a gradual loss of this isolation by distance pattern in the future (Leberg 1991; Waples 1998; Hutchison & Templeton 1999).

Given the many assumptions underlying isolation by distance models and the multitude of factors that can affect population structure, hypotheses like those outlined above must remain tentative (Slatkin 1993). Nevertheless, these analyses indicate that the demographic factors that have resulted in the current population structure have been different for the Merriam's versus the Rio Grande and eastern populations.

Management implications

Based on extant populations that best represent the historical pattern of genetic differentiation, our analyses support current subspecies designations, except for the eastern and Florida subspecies. These two subspecies appear to be a single unit genetically. The remaining subspecies appear genetically distinct and may exhibit different demographic structures. Therefore, managers should avoid translocations that would threaten currently recognized subspecies population integrity.

Here we have used analyses of putatively neutral markers to delineate groups of populations that share a common evolutionary history. However, it is possible that important adaptive traits will not follow patterns in neutral variation (Endler 1986; Knapp & Rice 1998; Crandall *et al.* 2000). Adaptive variation may develop despite high levels of gene flow, or selective pressures may prevent adaptive divergence despite neutral divergence. In general, we are lacking data on heritable, adaptive variation in wild turkeys. In order to address adaptive variation directly, managers should maintain records of translocation outcomes and attempt to correlate these with regional ecological data. Until data on the ecological equivalency of subspecies is available, conservative management should focus at the level of regional populations while respecting subspecies boundaries.

The Gould's subspecies appears to be the most divergent of the five subspecies, but is the least studied in terms of habitat requirements and availability, demographic history and current status. Addressing these questions should become a high priority for managers concerned with the conservation of species-wide genetic diversity in wild turkeys.

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