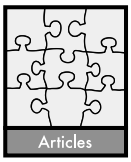

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Intraspecific genetic analysis of the summer tanager *Piranga rubra*: implications for species limits and conservation

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The summer tanager *Piranga rubra* is a Neotropical migrant that has experienced noted declines in the southwestern United States caused by extensive habitat loss of native riparian woodlands. This species is composed of two morphologically and behaviorally distinct taxa that traditionally have been recognized as subspecies, each occupying unique habitats in the southern part of North America. Genetic analyses of intraspecific variation are important in studies of threatened or endangered species because they can indicate whether smaller management units exist below the species level and they also provide estimates of within population variability. Using a mitochondrial DNA marker, the intraspecific genetic variation of this species is explored to determine whether the morphologically and behaviorally distinct subspecies are also genetically unique. By using traditional phylogenetic methods and building haplotype networks, results from this study indicate that the subspecies represent two phylogenetic species and should be managed as separate units. In addition, the level of gene flow among geographically isolated populations of the western subspecies is explored using Nested Clade Phylogeographic Analysis and population genetic tests. These analyses show that populations are genetically diverse and that haplotypes are shared across populations. Newly colonized populations are as diverse as older populations. This suggests that as habitat degrades in traditional breeding areas of the summer tanager, if suitable habitat elsewhere becomes available for new populations, these new colonies should be genetically diverse.

Although the summer tanager *Piranga rubra* is fairly common throughout most of its breeding range, the status of this species in the southwestern United States is of increasing concern due to degradation of the native riparian habitat it resides in. Significant declines in historic populations along the lower Colorado River have been attributed to various anthropogenic disturbances, including hydrology alteration, exotic plant invasion, and conversion of habitat to agricultural land (Rosenberg et al. 1991). Genetic analyses have become an important tool in many studies of threatened or endangered species (Moritz 1994, Haig and Avise 1996), and by using genetic markers, the evolutionary history of a group can be investigated to determine whether smaller management units may exist below the species level (Moritz 1994, Knapen et al. 2003). Using a mitochondrial marker, this study examines intraspecific variation within the summer tanager and the population history of this species in the southwestern United States.

Piranga rubra is a Neotropical migrant songbird that is easily distinguished from similar species by its large, omnivorous bill and the conspicuous red plumage of adult males. Each spring, this species migrates north into the United States to breed. Its breeding range encompasses much of the southern United States, as well as portions of northern Mexico (Robinson 1996). On its breeding grounds, the summer tanager occupies a variety of habitats, ranging from deciduous forests in the East to riparian woodlands in the West (Robinson 1996). In late summer and early fall, the summer tanager departs for its wintering grounds (Oberholser 1974). The winter range of this species extends from central Mexico south

to northern portions of South America (Robinson 1996).

The summer tanager includes two recognized subspecies, the Cooper's summer tanager and the eastern summer tanager (American Ornithologists' Union 1957). The Cooper's summer tanager *P. r. cooperi* breeds from southern California into western Texas including portions of north central Mexico. The eastern summer tanager *P. r. rubra* breeds throughout the eastern United States into central Texas and northeastern Mexico, making its way as far north as 40°N (Blake 1953, Robinson 1996). Wintering distributions of the subspecies are also distinct. *P. r. cooperi* winters from southern Baja California and along portions of southwestern Mexico, including south Sinaloa to western Oaxaca (Robinson 1996), while *P. r. rubra* winters in southeast Mexico, from Veracruz to the Yucatan Peninsula and south to western Brazil (Robinson 1996). The extent to which the two subspecies occur sympatrically in western Texas or northeastern Mexico during the breeding season is largely unknown. A generalized geographic distribution illustrated in Oberholser (1974) indicates the smallest distance separating breeding individuals of the two subspecies in west Texas is approximately 200 km, with the major dividing feature being the Pecos River.

The two subspecies have distinct morphologies and behaviors. Ridgway (1869) was first to describe morphological differences between these subspecies. In his description, he pointed out that the Cooper's summer tanager is easily distinguished from the eastern subspecies by its larger body size, longer and more swollen bill, and wings that are more pointed (Ridgway 1869). Overall, male *P. r. cooperi* display paler red plumage and females are more grayish yellow when compared to the smaller and brighter eastern race (Ridgway 1869, 1902, Rea 1970). The two subspecies also exhibit unique habitat preferences and display geographic variation in song (Shy 1983, 1984, 1985, Robinson 1996). The summer tanager is the only species of North American tanager to reside in distinct habitat types in different regions of its geographic range (Shy 1983). In the East, *P. r. rubra* tends to occupy open deciduous forests dominated by mixed oak woodlands and oak-pine woodlands (Robinson 1996, Isler and Isler 1999). *P. r. cooperi,* on the other hand, prefers the cottonwood (*Populus* spp.) and willow (*Salix* spp.) association of western riparian areas (Brown 1994, Robinson 1996, Isler and Isler 1999). Rosenberg et al. (1982, 1991) reported that *P. r. cooperi* will also venture into areas dominated by mesquite (*Prosopis* spp.) at higher elevations. Shy (1983, 1984, 1985) found that the two subspecies also display differences in song. Compared to *P. r. cooperi*, the song of *P. r. rubra* has a lower maximum frequency, a lower minimum frequency, and a narrower frequency range (Shy 1983). Shy (1983) also reported that the song of *P. r. rubra* has a faster tempo marked by shorter pauses between subfigures. Shy (1983, 1985) hypothesized that the geographical variation in song was due to differences in tree density between the habitats of the two subspecies, with the eastern habitat containing a significantly larger amount of small trees. The use of a narrower frequency range may be an adaptation of eastern populations living among dense vegetation because it allows the birds to broadcast greater power per Hz bandwidth (Shy 1983). The sound propagation characteristics of a species' habitat, which may effect the development of song, could consequently contribute to the accumulation of intraspecific variation. Thus, if a species occupies different habitats at different areas of its range, like *P. rubra*, this occurrence may encourage the development of divergent phenotypes.

This study explores the evolutionary history of the summer tanager, while looking specifically at the population history of the western subspecies, *P. r. cooperi*. First, mtDNA data are used to investigate whether the two morphologically and behaviorally distinct subspecies are also genetically unique. For this part of the study, sequences of the mitochondrial cytochrome *b* (cyt *b*) gene are used to construct phylogenetic trees. Second, the study includes a population-level analysis of key colonies along an east-west transect within the western subspecies. This aspect of the study assesses the gene flow and distinctiveness of geographically isolated populations within the southwestern United States based on cyt *b* sequences, population genetic analyses, and haplotype network reconstruction. Results of this study will aid in management decisions of southwestern populations, which have experienced noted declines along the lower Colorado River in the past 100 years (Rosenberg et al. 1991). Contrasting with recent declines along the Colorado River, a documented range expansion within *P. r. cooperi* has also occurred into southern California over the past five decades (Johnson 1994). A molecular investigation of this recent range expansion is completed to determine if this documented range expansion can be detected with the genetic data.

Materials and methods

Sample collection

During the 2002 and 2003 breeding seasons, 43 summer tanagers, representing *P. r. cooperi*, were sampled from four populations in southern California and Arizona using a mist net, playback tape and decoy (Fig. 1, Table 1). Populations sampled in California (population A: South Fork Kern River, Kern County, and population B: San Felipe Creek, San Diego

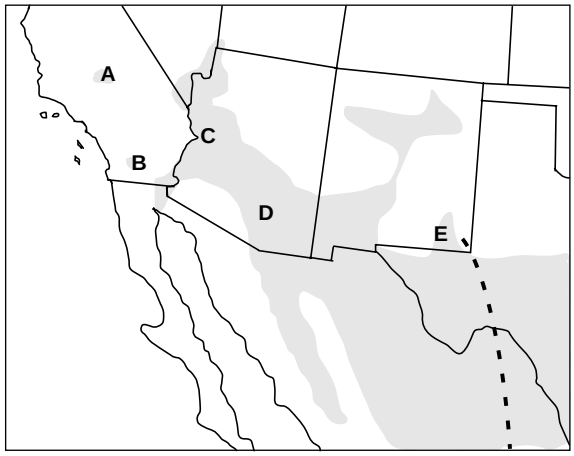

Fig. 1. Breeding distribution of summer tanager in the southwestern U.S., modified from Robinson (1996). Shaded areas indicate the breeding distribution of this species. Dashed line shows approximate subspecies boundary, with *P. rubra cooperi* occurring west of the boundary. Sampling localities are shown by letters A – E (population A: South Fork Kern River, California, population B: San Felipe Creek, California, population C: Bill Williams River, Arizona, population D: San Pedro River, Arizona, population E: New Mexico, further locality details are provided in Table 1).

County) represent two of the largest breeding colonies in the state (Small 1994). A third colony is located on the Mohave River in San Bernardino County (Small 1994), but it was not sampled. The populations sampled in Arizona (population C: Bill Williams River, La Paz County and population D: San Pedro River, Pinal County) are the largest breeding colonies in the state (Phillips et al. 1964, Rosenberg et al. 1991). In addition to these colonies, *P. r. cooperi* also nests in scattered pairs where suitable riparian habitat is available (Small 1994). The populations sampled are located along an approximate east-west transect in the southwestern United States (Fig. 1). Following each capture, birds received a U.S. Fish and Wildlife Service numbered, stainless steel leg band, morphological measurements were taken, and blood and feather samples were collected. Voucher specimens were not collected because the species has special conservation status in California. The small blood sample needed for DNA analysis was acquired using a toenail clip procedure. Blood was stored in Alsever's solution (Alsever and Ainslie 1941), which acts as an anticoagulant and preservative, until returning to the lab, where it was separated and frozen at −80°C. Feathers were preserved in 100% EtOH. In addition to the 43 birds specifically sampled for this study, one additional tissue sample of *P. r. cooperi* (population E from New Mexico) was obtained from the Museum of Southwestern Biology (Table 1). To represent the eastern subspecies, seven individuals were used. Four were sequenced for this study from tissues obtained from the Field Museum (Table 1), and three were obtained from GenBank (Accession numbers AF011779, AF011780, AY955196). Individuals from the other eight species in the genus *Piranga* were included as outgroup taxa based on their close relationship to the summer tanager (GenBank Accession numbers AF006247, AF006248, AF011759-AF0011781, Burns 1998). To root the tree of *Piranga* relationships, we also included representatives of the genera *Habia* and *Chlorothraupis* (GenBank Accession numbers AF006233, AF006219) which are closely related to *Piranga* (Burns 1997).

DNA sequencing

Template DNA was extracted from feather pulp using Chelex (BioRad, Hercules, California). If no feather sample was available, DNA was extracted from blood or tissue using a Qiagen DNeasy tissue kit (Qiagen, Valencia, California). Avian specific primers were used with standard polymerase chain reaction (PCR) procedures to amplify 1213 bases of mitochondrial DNA (mtDNA; Hillis et al. 1996). For this study, the region sequenced included a small portion of the ND5 gene (37 bp), two intergenic spacers, a small segment of $tRNA^{Thr}$ (46 bp), and the entire cyt *b* gene (1120 bp). This segment was sequenced in three overlapping sections using primer pairs L14851/H15297, L15206/H15710 and L15656/H16058 (Groth 1998). Initial PCR of 10 µl amplifications was accomplished in capillary tubes via a hot-air thermocycler using the following conditions: 40 cycles of denaturation at 94°C for 3 s, annealing at 43°C for 0 s and extension at 71°C for 30 s. PCR product was verified by running 5 µl of sample on a 2% agarose mini-gel stained with ethidium bromide. Samples were then plugged from the gel, melted and reamplified in 50 µl reactions using the following conditions: 41 cycles of denaturation at 94°C for 12 s, annealing at 52°C for 4 s and extension at 71°C for 26 s. Next, samples were purified using a GeneClean kit (Qbiogene, Carlsbad, California). Cycle sequencing of both light and heavy strands was accomplished using BigDye version 3.0 (Applied Biosystems, Foster City, California) under the following conditions: initial denaturation at 96°C for 1 m, followed by 28 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 15 s and extension at 60°C for 4 m. Cycle sequencing products were then centrifuged through a Sephadex column (Sigma-Aldrich, St. Louis, Missouri) and sequenced using an ABI 377 automated sequencer (Applied Biosystems). Remaining blood and feather samples were archived in the San Diego State University vertebrate collections (Table 1).

Table 1. Locality, voucher, and band information. SDSU = Vertebrate Collections, San Diego State University, FMNH = Field Museum of Natural History, MSB = Museum of Southwestern Biology, University of New Mexico, LSUMZ = Louisiana State University Museum of Natural Sciences.

Site	Ind.	Subspecies	Voucher #	Band #	Locality
A	1	*P. r. cooperi*	SDSU 2733	1621–12001	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	2	*P. r. cooperi*	SDSU 2734	1621–12002	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	3	*P. r. cooperi*	SDSU 2735	1621–12003	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	4	*P. r. cooperi*	SDSU 2736	1621–12004	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	5	*P. r. cooperi*	SDSU 2737	1621–12005	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	6	*P. r. cooperi*	SDSU 2738	1621–12006	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	7	*P. r. cooperi*	SDSU 2739	1621–12007	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	8	*P. r. cooperi*	SDSU 2740	1621–12008	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	9	*P. r. cooperi*	SDSU 2741	1621–12009	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
	10	*P. r. cooperi*	SDSU 2742	1621–12010	USA: California, Kern County, South Fork Kern River, 35°40′22.08″N, 118°18′25.65″W
B	1	*P. r. cooperi*	SDSU 2729	1711–97952	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	2	*P. r. cooperi*	SDSU 2730	1711–97953	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	3	*P. r. cooperi*	SDSU 2731	1711–97954	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	4	*P. r. cooperi*	SDSU 2732	1711–97961	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	5	*P. r. cooperi*	SDSU 2743	1621–12011	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	6	*P. r. cooperi*	SDSU 2744	1621–12012	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	7	*P. r. cooperi*	SDSU 2652	1621–12035	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	8	*P. r. cooperi*	SDSU 2653	1621–12036	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	9	*P. r. cooperi*	SDSU 2654	1621–12037	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
	10	*P. r. cooperi*	SDSU 2655	1621–12038	USA: California, San Diego County, San Felipe Creek, 33°6′19.74″N, 116°29′58.56″W
C	1	*P. r. cooperi*	SDSU 2622	1621–12013	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°16′29.16″N, 114°2′52.8″W
	2	*P. r. cooperi*	SDSU 2623	1621–12015	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°16′29.16″N, 114°2′52.8″W
	3	*P. r. cooperi*	SDSU 2624	1621–12016	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°16′29.16″N, 114°2′52.8″W
	4	*P. r. cooperi*	SDSU 2625	1621–12017	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°16′29.16″N, 114°2′52.8″W
	5	*P. r. cooperi*	SDSU 2626	1621–12018	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°16′29.16″N, 114°2′52.8″W
	6	*P. r. cooperi*	SDSU 2627	1621–12019	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°° 16′29.16″N, 114°2′52.8″W
	7	*P. r. cooperi*	SDSU 2656	1621–12039	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°16′29.16″N, 114°2′52.8″W
	8	*P. r. cooperi*	SDSU 2657	1621–12040	USA: Arizona, La Paz County, Bill Williams National Wildlife Refuge, 34°16′29.16″N, 114°2′52.8″W
D	1	*P. r. cooperi*	SDSU 2628	1621–12020	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	2	*P. r. cooperi*	SDSU 2629	1621–12021	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	3	*P. r. cooperi*	SDSU 2630	1621–12022	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	4	*P. r. cooperi*	SDSU 2631	1621–12023	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	5	*P. r. cooperi*	SDSU 2632	1621–12024	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	6	*P. r. cooperi*	SDSU 2633	1621–12025	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	7	*P. r. cooperi*	SDSU 2634	1621–12026	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	8	*P. r. cooperi*	SDSU 2635	1621–12027	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	9	*P. r. cooperi*	SDSU 2636	1621–12028	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	10	*P. r. cooperi*	SDSU 2637	1621–12029	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
D	11	*P. r. cooperi*	SDSU 2638	1621–12030	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	12	*P. r. cooperi*	SDSU 2639	1621–12031	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	13	*P. r. cooperi*	SDSU 2640	1621–12032	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	14	*P. r. cooperi*	SDSU 2641	1621–12033	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W
	15	*P. r. cooperi*	SDSU 2642	1621–12034	USA: Arizona, Pinal County, San Pedro River Preserve, 32°55′38.88″N, 110°44′32.16″W

Table 1 (*Continued*)

Site	Ind.	Subspecies	Voucher #	Band #	Locality
E	1	*P. r. cooperi*	MSB 20268	N/A	USA: New Mexico, Eddy County, White City, 9.65 km W, 8.05 km S = Black River
F	1	*P. r. rubra*	FMNH 363314	N/A	USA: Minnesota, Crow Wing County, Borden Lake, north side
G	1	*P. r. rubra*	FMNH 383080	N/A	USA: Illinois, Cook County, Chicago, Foster Avenue Beach
	2	*P. r. rubra*	FMNH 383081	N/A	USA: Illinois, Lake County, Waukegan, Illinois Beach State Park
H	1	*P. r. rubra*	LSUMZ B3319	N/A	USA: Louisiana, Cameron Parish, Garner Ridge, ca. 5 km W Johnson's Bayou School
I	1	*P. r. rubra*	LSUMZ B19749	N/A	USA: Louisiana, Iberville Parish, 6.4 km N St. Gabriel, 435 Pecan Drive
J	1	*P. r. rubra*	FMNH 394093	N/A	Mexico: Oaxaca, Rio Salado, 10 km N San Gabriel Mixtepec, Sierra de Miahuatlan at 1400m
K	1	*P. r. rubra*	LSUMZ B16276	N/A	Costa Rica: Prov. Heredia, 5 km by road S. Puerto Viejo

Data analysis

Sequences were aligned and edited using SEQUENCHER v. 3.1 (Gene Code Corporation, Ann Arbor, Michigan). All sequences were deposited in GenBank (Accession numbers AY649495-AY649542). Cyt *b* was chosen because it has been shown to be an effective phylogenetic marker for intraspecific avian research (Klicka et al. 1999, Sgariglia and Burns 2003, Burns and Barhoum 2006); cyt *b* is a particularly valuable genetic marker for resolving recent splitting events (Moore and DeFilippis 1997). A phylogenetic tree developed using a mitochondrial marker should have a better chance of accurately recovering recent splitting events because its effective population size is one-fourth that of a nuclear gene (Moore 1995). Also, mtDNA is useful as a genetic marker because it has a high average rate of mutation (Wenink et al. 1993).

To determine whether population structure exists within this species, an analysis of molecular variation (AMOVA) was used to calculate Φ-statistics (Excoffier et al. 1992) and to quantify the percent variation among and within populations. An additional AMOVA was computed to test for population structure among the more rigorously sampled *P. r. cooperi*. All analyses of molecular variation were computed using the number of pairwise distances with distance matrices calculated during each analysis, implemented in the program ARLEQUIN version 2.0 (Schneider et al. 2000).

Nucleotide diversity (π), which is the average number of base differences per site between two sequences (Nei 1987), was calculated for each population represented by more than one individual using ARLEQUIN. Nucleotide diversity estimates have been used to attempt to infer the direction of range expansion in many species of birds (Zink et al. 2000, 2001, Sgariglia and Burns 2003) based on the assumption that smaller nucleotide diversity values will be observed in recently colonized populations. Given that historical data suggest that a northwestern range expansion has occurred in *P. r. cooperi* over the past 45 years (Johnson 1994), we were interested in seeing if newly colonized populations were less diverse.

Another method of inferring population growth is to plot a mismatch distribution and compare it against the expected distribution under a sudden expansion model (Rogers and Harpending 1992, Rogers 1995). A unimodal distribution of pairwise differences often indicates a population has undergone a recent expansion event (Rogers and Harpending 1992). A mismatch distribution and Harpending's raggedness index (Harpending 1994) were calculated in DnaSP version 4.0 (Rozas et al. 2003) and ARLEQUIN, respectively, for the western subspecies. Tajima's D (Tajima 1989a,b) and Fu's F_s (Fu 1997) were calculated using ARLE-

QUIN. Assuming the marker being used is neutral, these statistics can be used to infer population growth because samples containing an excess of new mutations tend to produce a significantly negative value. To further examine the documented range expansion of *P. r. cooperi*, an additional test statistic, R_2, was calculated using DnaSP. Ramos-Onsins and Rozas (2002) showed that R_2 is the most powerful test statistic for detecting population growth with small sample sizes, when compared to more conservative tests based on a mismatch distribution, such as the raggedness index.

Phylogenetic analyses were conducted using Bayesian, maximum likelihood, and parsimony approaches. Bayesian estimation of phylogeny was implemented in the program MrBayes version 3.0 (Huelsenbeck and Ronquist 2001). Bayesian inference of phylogeny constructs evolutionary relationships by using a maximum likelihood framework, and posterior probability values are approximated using a Metropolis-coupled Markov chain Monte Carlo algorithm (Huelsenbeck et al. 2001, Huelsenbeck and Imennov 2002). The appropriate nucleotide substitution model was determined using MrModeltest version 1.1 (Nylander 2002) with exact model parameters estimated during analyses. A total of five separate Bayesian searches were conducted to decrease the probability of entrapment on a suboptimal peak (Huelsenbeck and Bollback 2001). Each analysis was initiated with a random starting tree and run for 1.0×10^6 generations. Four separate Markov chains were employed in each run to adequately explore the parameter space, and parameters were saved every 100 generations. To verify stationarity, log-likelihood scores were plotted against generation and burn-in trees were discarded. A majority-rule consensus tree based on the 45,000 saved trees was constructed in the program PAUP* 4.0b10 (Swofford 2002). To explore the robustness of our data to different phylogenetic methods, we also analyzed the data using maximum likelihood and parsimony as implemented in PAUP* 4.0b10. For maximum likelihood analysis, we again used the results from MrModeltest to identify the best fit model and parameters. For parsimony analyses, we used the heuristic search option with characters weighted equally and 1000 random addition replicates.

To diagram relationships among individuals within the western subspecies, a haplotype network was also constructed using the program TCS version 1.13 (Clement et al. 2000). This program uses statistical parsimony to estimate a cladogram based on the methods of Templeton et al. (1992). The *P. r. cooperi* network was taken through Nested Clade Phylogeographic Analysis (NCPA, Templeton 1998, 2004), with nesting following rules outlined in Templeton et al. (1987) and symmetries and ambiguities resolved following the convention of Templeton and Sing (1993). With the input of geographic locality information, the program GEODIS 2.0 (Posada et al. 2000) was used to calculate clade distances (D_c: a measure of the geographical range of a given clade), nested clade distances (D_n: a measure of the geographic distribution of a particular clade relative to its evolutionary sister clades) and interior-tip contrasts (I-T: a measure of the difference between interior and tip clades in D_c and D_n values; Templeton 1998). Within GEODIS, the null hypothesis of a random geographic association was tested for each clade based on 10 000 random permutation contingency tests. Significant values ($P < 0.05$) were interpreted using the 2004 inference key (Posada and Templeton 2004, Templeton 2004) in order to separate current population structure (e.g. ongoing gene flow) from historical processes (e.g. range expansion and fragmentation events).

Results

Sequence variation

Among the *P. rubra* individuals sequenced, 37 of the 1213 sites were polymorphic, of which 18 were parsimony informative. Within the entire *P. rubra* complex, 25 unique haplotypes were identified. Of the 44 individuals of the subspecies *P. r. cooperi* examined, 20 unique haplotypes were revealed. Five unique haplotypes were contained within the seven *P. r. rubra* analyzed. Variation within the sequences included 31 transitions and six transversions. The transition-transversion ratios by codon position were: first position, 9:1, second position, 4:0, and third position, 18:5. The average uncorrected pairwise sequence distance within *P. rubra* was 0.36%, with a range of 0–1.30%. Within *P. r. cooperi*, the average pairwise distance was 0.25% (range, 0–0.77%), while *P. r. rubra* had an average of 0.36% (range, 0–0.67%). The average pairwise distance between *P. r. rubra* and *P. r. cooperi* was 0.70% (range, 0.34–1.30%). All *P. r. rubra* and *P. r. cooperi* samples were distinguishable from each other by at least three fixed nucleotide differences (positions 387, 975 and 1179), thus these groups are clearly distinct genetically.

Descriptive statistics

When data were subjected to an AMOVA in which populations were not categorized into subgroups, geographic structure was indicated with 31% of the molecular variation partitioned among populations (Table 2). To determine whether geographic structure exists between the two subspecies, an additional AMOVA was performed with eastern and western

Table 2. Results of AMOVA for *Piranga rubra* showing the percent variation and associated Φ_{ST} value. Results are first displayed for an analysis in which populations were not divided into groups and next for an analysis where populations were divided into eastern (A–E) and western (G–I) subspecies. P-values are the probability of getting a more extreme Φ_{ST} value by chance.

Groups	Source of variation	Percent variation	Φ-statistic	P
None specified	Among populations	31.1	0.31	<0.001
	Within populations	68.9		
East vs. west	Among groups	62.7	0.63	<0.001
	Among populations within groups	1.6	0.64	<0.001
	Within populations	35.7		

populations grouped separately. This analysis showed 63% of the variation is partitioned between subspecies (Table 2) indicating a genetic split exists between these groups. As a result, little of the variation is explained by genetic differences among populations within subspecies. When western populations were analyzed independently using AMOVA, no population substructure was revealed; rather, all variation was distributed within populations ($\Phi_{ST} = -0.01$, $P = 0.632$, Table not shown).

Overall, nucleotide diversity (π) values for populations within this species were comparable with published values on other bird populations (Milot et al. 2000, Zink et al. 2001, Sgariglia and Burns 2003). Values of the western subspecies pooled together (0.002) were lower than those of the eastern subspecies (0.004); however, when a two-sample t-test was performed, the values were not found to be significantly different ($P = 0.32$). For the western subspecies (Table 3), nucleotide diversity values for 'new' populations (A, B) were unexpectedly higher than those of historic populations (C, D), but again, the values were not significantly different ($P = 0.18$).

The mismatch distribution for the western subspecies (Fig. 2) was unimodal and skewed left, a similar shape to that expected of an expanding population. However, the distribution differed significantly from that of a growing population (raggedness index = 0.077, $P = 0.015$). Although negative, Tajima's D did not differ significantly from zero ($D = -1.3721$, $P = 0.07$). However, Fu's F_s test indicated a departure from population equilibrium, suggesting a recent demographic expansion may have occurred in the western subspecies ($F_s = -7.9571$, $P = 0.001$). In addition, the R_2 statistic was significant for the *P. r. cooperi* data ($R_2 = 0.162$, $P < 0.001$), which is consistent with a recent population expansion.

Table 3. Estimates of genetic diversity for summer tanager populations in which more than one individual was available. See Table 1 for locality information.

Site	N	Number of haplotypes	Nucleotide diversity (π)
A	10	9	0.0029
B	10	9	0.0028
C	8	5	0.0017
D	15	10	0.0023
G	2	2	0.0026

Phylogenetic analysis

For phylogenetic analysis of *P. rubra* and related species, the most appropriate evolutionary model to explain the data was specified as GTR + Γ by MrModeltest. The majority-rule consensus tree (Fig. 3) from the Bayesian analyses identified the sister taxon to *P. rubra* as a clade containing *P. ludoviciana*, *P. bidentata*, and *P. flava*. The monophyly of all *P. rubra* individuals is highly supported with a posterior probability value of 1.0 (Fig. 3). The tree also illustrates that haplotypes of the western subspecies form a separate monophyletic group, which is also significantly supported (posterior probability = 0.95). The eastern subspecies forms a paraphyletic group with respect to *P. r. cooperi*, but the paraphyly is not highly supported (posterior probabilities < 0.95). Within the western subspecies, there is no clear geographic structuring, as haplotypes from the same population are distributed throughout the western clade, and identical haplotypes are found in distant populations. The tree produced from maximum likelihood analysis (-Ln likelihood = 5040.45, not shown) was largely congruent with the Bayesian analyses. Both trees showed the

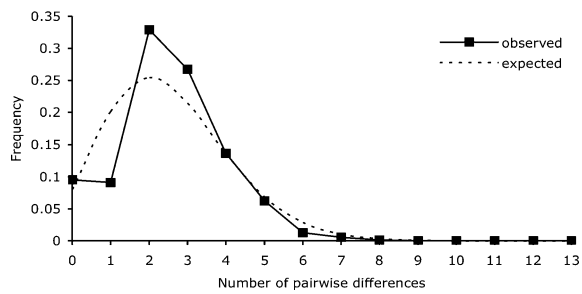

Fig. 2. Mismatch distribution of haplotypes of *P. r. cooperi.* Solid line indicates the observed distribution of pairwise differences and dashed line shows the expected distribution under a model of sudden expansion (Rogers and Harpending 1992).

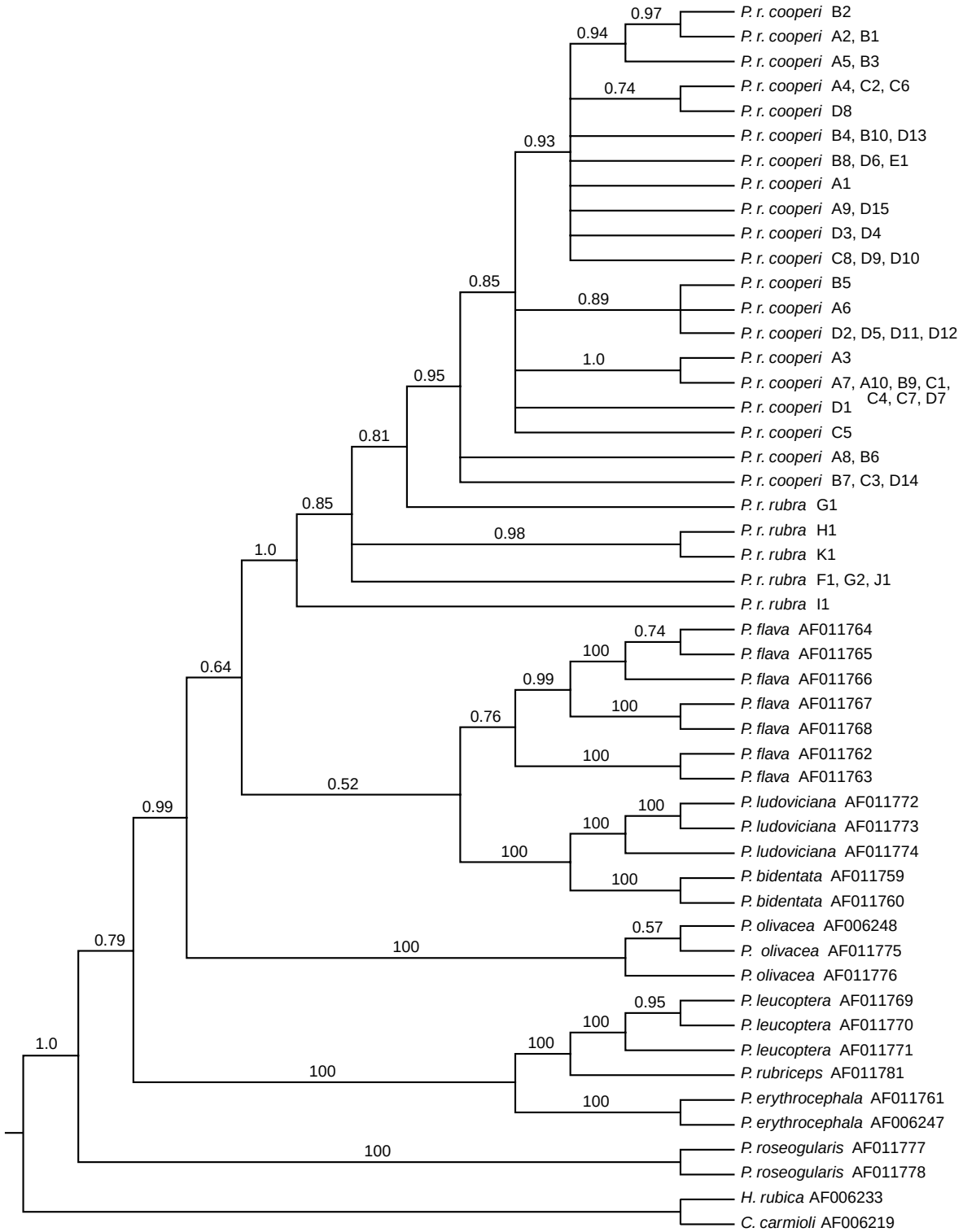

Fig. 3. Majority-rule consensus tree using GTR+Γ model and Bayesian analyses. Terminal haplotypes are coded by individual according to Table 1. Numbers on branches indicate posterior probability values. Voucher numbers for all individuals are given in Table 1.

same species-level relationships. Differences between the maximum likelihood tree and the Bayesian tree were restricted to the relationships of haplotypes within *P. r. cooperi*. Parsimony analyses resulted in 465 equally parsimonious trees of 698 steps. The strict consensus of these trees (not shown) displayed much less resolution than the Bayesian and maximum likelihood trees. However, the resolved portions of the strict consensus parsimony tree agreed with that of the other analyses.

Species-level relationships of *Piranga* identified in this study are largely congruent with those presented in Burns (1998), which was also based on cyt *b* but with fewer taxa sampled. Burns (1998) presented three trees: a maximum likelihood tree, an equally weighted parsimony consensus tree, and a transversion-weighted parsimony tree. Species-level relationships found in the present study (Fig. 3) are in complete agreement with the maximum likelihood tree of Burns (1998). In addition, the present study (Fig. 3) agrees at most nodes with the parsimony trees of Burns (1998). However, the equally-weighted parsimony tree of Burns (1998) differs in the placement of *P. olivacea,* and the transversion-weighted parsimony tree of Burns (1998) differs in the placement of *P. roseogularis*. Placement of these taxa in the trees of Burns (1998) had low support (less than 50% bootstrap values). The current study involves increased sampling, more rigorous analyses, and higher support values. Thus, we consider the trees presented here as superior to the parsimony trees presented in Burns (1998).

Nested clade phylogeographic analysis

The haplotype network of *P. r. cooperi* was constructed such that all connections between haplotypes have a 95% plausible association (Fig. 4). Ambiguities and loops were resolved using neutral coalescent theory and parsimony, taking into account the idea that rare haplotypes are more likely to be derived from more common haplotypes (Crandall et al. 1994) and associations of haplotypes from the same locality are favored (Crandall and Templeton 1993). The network reveals that populations are diverse genetically, and haplotypes found in one population are usually also found in geographically distant populations. The cladogram was nested at first-step, second-step and third-step levels, resulting in a total cladogram nested within four levels. A clade may be taken though the inference key if it contains both geographic and genetic variation and shows a significant D_c or D_n value ($P < 0.05$) (Templeton 1998). The nested geographic analysis of *P. r. cooperi* resulted in one rejection of the null hypothesis of no geographic association, revealed within clade 2–3. For this clade, NCPA infers restricted gene flow with isolation-by-distance.

Discussion

Taxonomic status of *P. r. rubra* and *P. r. cooperi*

The high level of genetic diversity revealed within mtDNA sequences of *P. rubra* allowed for the evolutionary history of this species to be explored. Twenty-five unique haplotypes were uncovered within the 51 individuals analyzed, which is comparable to results of similar avian studies using cyt *b* as a genetic marker (Sgariglia and Burns 2003, Burns and Barhoum 2006). The molecular analysis of *P. rubra* reveals that the morphologically and behaviorally distinct subspecies also represent two genetically distinct lineages. Existence of three fixed nucleotide differences between these groups allows them to be unequivocally sorted to type using a diagnostic genetic marker; moreover, the presence of these fixed differences indicates that *P. r. rubra* and *P. r. cooperi* are on separate evolutionary trajectories (for a similar example see Klicka et al. 1999). The range of sequence divergence uncovered between *P. r. rubra* and *P. r. cooperi* (range, 0.34–1.30%, mean = 0.70%,) is similar to that observed among many sister species of North American birds (Johnson and Cicero 2004).

Assuming a molecular clock rate of 1.6% corrected cyt *b* sequence divergence per million years (Fleischer et al. 1998), the western populations of the summer tanager began diverging from eastern populations no earlier than approximately 800 000 years ago (maximum Kimura 2-parameter corrected divergence between *P. r. rubra* and *P. r. cooperi* populations is 1.3%). The divergence of a gene tree occurs prior to population divergence (Knowles and Maddison 2002), and therefore, the split of *P. r. cooperi* from *P. r. rubra* occurred less than 800 000 years ago. A more exact approximation of their population divergence cannot be estimated with the available data. Like the divergence of these subspecies, similar divergences between eastern and western taxa have been reported in numerous North American birds (Klicka and Zink 1999, Milot et al. 2000). Timing of the isolation of taxa within *P. rubra* indicates divergence was most likely caused by climatic changes during the Pleistocene. Recently, there has been much debate over origination timing in avian species and whether most species experienced a Pleistocene origin or an earlier Pliocene origin (for discussion see Klicka and Zink 1997, 1999, Avise and Walker 1998, and Johnson and Cicero 2004). Results of this study support the idea that climatic events in the Pleistocene played a major role in the isolation of *P. r. cooperi* populations from those of *P. r. rubra*.

Phylogenetic analysis of this species shows that *P. r. rubra* is paraphyletic with respect to *P. r. cooperi* (Fig. 3). Such a paraphyletic pattern is predicted for taxa that diverged from each other recently in the late

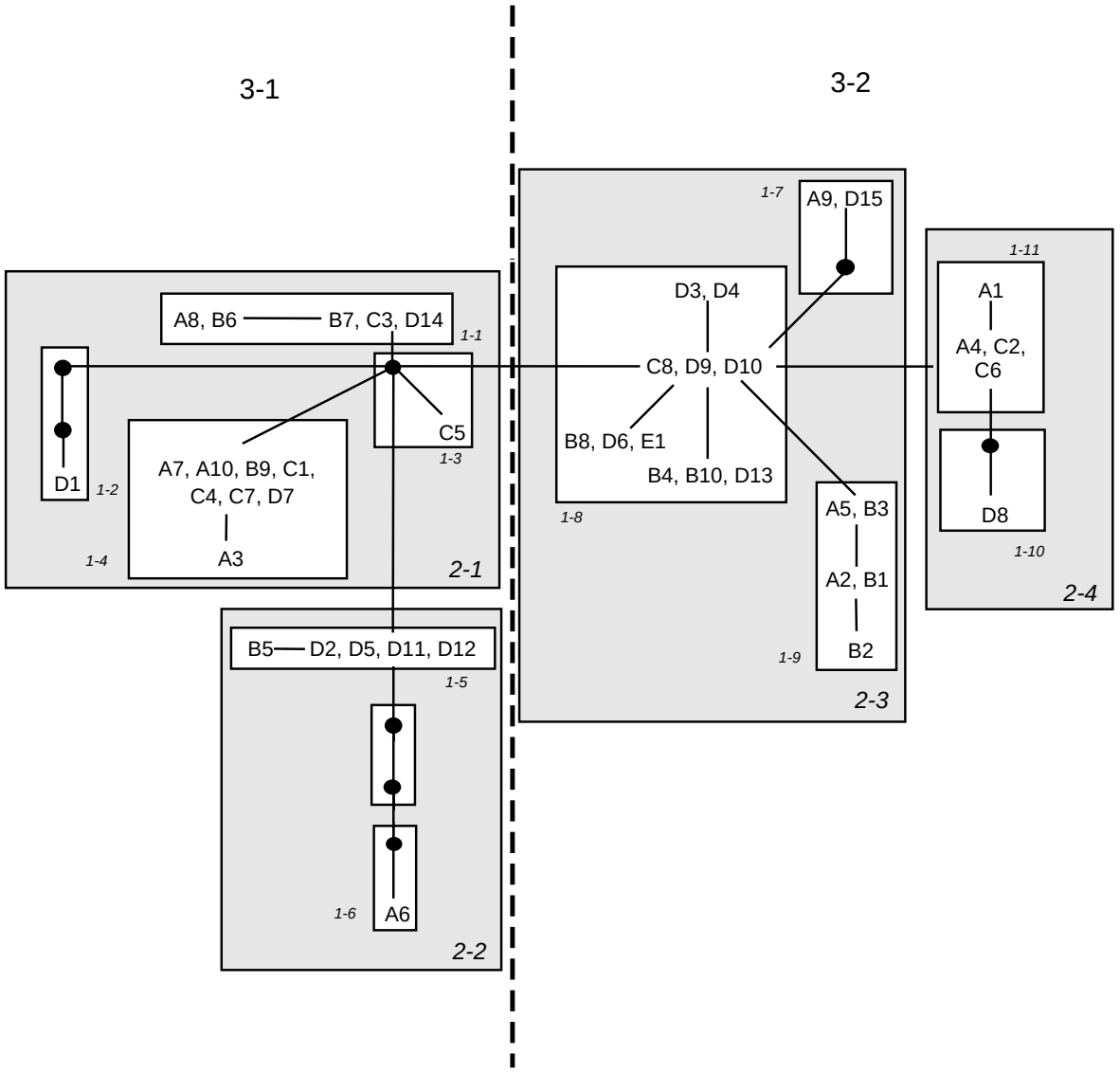

Fig. 4. Nested haplotype network for *P. r. cooperi*. A solid branch indicates a single base pair mutation. Labeled nodes represent sampled haplotypes. Unsampled haplotypes are indicated with solid black nodes. Every letter/number combination represents a single individual from its corresponding locality (Fig. 1, Table 1). In some cases, haplotypes are shared by more than one individual. One-level clades are contained in white boxes, two-level clades are contained in grey boxes, and dashed lines outline three-level clades.

Pleistocene (Klicka and Zink 1997). However, there are many possible reasons, besides incomplete lineage sorting, for observing paraphyly between taxa in a gene tree including saturation due to homoplasy and introgressive hybridization (Funk and Omland 2003). Paraphyly in this group may be caused by incomplete lineage sorting because *P. r. rubra* and *P. r. cooperi* have only recently split, and the paraphyletic pattern indicates the western group is derived from the eastern form. If *P. r. cooperi* is in fact derived from *P. r. rubra*, this supports the theory that many western birds in North America have evolved from an eastern ancestor (see Mengel's Model; Mengel 1970). The result, that *P. r. cooperi* and *P. r. rubra* are not reciprocally monophyletic, does not mean that ongoing gene flow continues. Incomplete lineage sorting could simply be the result of insufficient passage of time since separation (on average, it will take $4N_e$ generations to achieve reciprocal monophyly, see Avise 1994), and if the effective population size of a group is large, it will take more generations for reciprocal monophyly to be achieved.

Large Φ_{ST} values between subspecies and the high posterior probability value for *P. r. cooperi* monophyly in the Bayesian tree both suggest the subspecies of summer tanager are two species, and the presence of three fixed characters that diagnose taxon limits mean they fit the qualifications of two phylogenetic species (Nixon and Wheeler 1990). Furthermore, the many morphological differences, which include body size, bill size, wing shape, and plumage coloration, plus the unique song and habitat types all support the naming of *P. r. cooperi* and *P. r. rubra* as unique species. However, stating whether *P. r. cooperi* and *P. r. rubra* are two biological species (Mayr 1963) would benefit from further research. Additional research to resolve this question should include sampling of individuals where the ranges of these groups potentially meet in western Texas and northeastern Mexico and determining whether hybridization occurs among them.

Low level of population structure in *P. r. cooperi*

Because no previous research exists that documents the level of site fidelity within populations of the summer tanager, this study is the first to explore the level of genetic movement among breeding populations within this species. Population-level analysis of the western subspecies reveals there are probably high levels of migration between breeding populations. The Bayesian phylogenetic analysis (Fig. 3) did not uncover any clear geographic groupings of haplotypes, and a low Φ_{ST} value for among population comparisons of *P. r. cooperi* indicates that little variation is structured among populations.

NCPA takes the distribution of particular clades and their geographic locality into consideration, and thus can provide higher resolution of phylogenetic data. This can then allow specific evolutionary processes to be identified, such as range expansion or fragmentation and can distinguish from current population-level processes such as ongoing gene flow and isolation-by-distance. Most clades within the network of *P. r. cooperi* haplotypes (including the overall cladogram) showed no significant geographic association, suggesting widespread gene flow among populations. However, within clade 2–3, limited gene flow was inferred with isolation-by-distance. Within this clade, the interior clade (1–8) includes representatives from all populations except Kern River, which is at the northern edge of the breeding range. The tip clade (1–7) is geographically widespread (including individuals from Kern River and from San Pedro River), while the other tip clade (1–9) is geographically limited (all individuals hail from Kern River or San Felipe Creek). Because clade 2–3 contains a restricted tip clade (1–9) that overlies geographically with a widespread interior clade (1–8), limited gene flow is inferred with isolation-by-distance for clade 2–3.

Although the NCPA identified isolation-by-distance for this part of the network, the lack of geographic association for most haplotypes in the NCPA agrees with the low level of population structure identified by the AMOVA of *P. r. cooperi* populations. An implication from this molecular analysis is that low levels of individual site fidelity occur in this species, but field data would help corroborate this finding. Evidence of geographic structuring within migratory birds has been reported in a number of species (Milot et al. 2000, Wenink et al. 1993), but within *P. r. cooperi* high gene flow and/or multiple colonizations have overcome any effect of genetic drift. Other avian studies have uncovered low geographic structure within some bird species, including the red-winged blackbird (Ball et al. 1988), swamp sparrow (Greenberg et al. 1998) and California gnatcatcher (Zink et al. 2000).

Recent range expansion and colonization

Over the past several decades, northward range expansions have been documented among many southwestern U.S. bird species; the expansion of nesting distributions have been attributed primarily to climatic change, including enhanced summer moisture and rising temperatures (Johnson 1994). Among the numerous species documented with a northward range expansion is the summer tanager, specifically the western subspecies, which has increased its breeding range to include northern New Mexico, southeast Utah, southern Nevada and southern California (Johnson 1994). Fu's F_s and the R_2 test both indicate a demographic expansion which is consistent with the historic data indicating that a range expansion occurred in *P. r. cooperi*. The historic occurrence of *P. r. cooperi* in Arizona along the San Pedro River and Colorado River (Phillips et al. 1964) and in southeastern California at the Colorado River (Grinnell and Miller 1944) has long been established, but new breeding colonies have only recently appeared in California along the South Fork Kern River (population A) and the San Felipe Creek in the Anza Borrego Desert (population B) (T. Gallion and P. Jorgensen, pers. comm.). Consequently, it was expected that California populations would show lower nucleotide diversity values compared to Arizona populations; however, nucleotide diversity values do not indicate a direction to the population expansion (Table 3). The mismatch distribution (Fig. 2) was unimodal and markedly skewed left, a similar shape to that expected of an expanding population (Rogers and Harpending 1992). However, the distribution was significantly different from that of a growing population (raggedness index = 0.077, P =

0.015) and Tajima's D ($D = -1.372$, $P = 0.07$) was consistent with neutral equilibrium. This discrepancy may be caused by various factors and merely signifies that the data do not correspond with the specific expectations under these two models of evolution. Ramos-Onsins and Rozas (2002) also show that the raggedness index and Tajima's D tend to be more conservative tests, and therefore have lower power in rejecting the null hypothesis (constant population size) when the alternative hypothesis (population growth) is true.

Past range expansion was not inferred using NCPA. When all clades were tested for no association of the haplotype tree with geography, the null hypothesis was rejected for only one clade (2–3). Often, in species such as birds with high dispersal capabilities, there is a failure to reject the hypothesis of no geographical association (Templeton 2004). Therefore, the range expansion within *P. r. cooperi* may not have been uncovered because of high dispersal. Also, inadequate sampling may have contributed to a failure to reject the null hypothesis of no geographic association and thus, led to a failure to uncover the documented expansion (Templeton 2004).

Conservation implications

The western form of the summer tanager has experienced noted declines throughout its historic range along the lower Colorado River valley. Recent studies indicate this bird is on the verge of disappearing from this region (Rosenberg et al. 1991). The drop in numbers has largely been attributed to the loss of native riparian habitat, which is essential for this species' survival in the West. This habitat has been destroyed and fragmented by conversion to agricultural land (Robinson 1996). Additional threats to *P. r. cooperi* may include cowbird parasitism, fire and unnatural water regimes that increase non-native plant species such as *Tamarix* (Rosenberg et al. 1991, Robinson 1996). In California, the summer tanager is recognized as a Species of Special Concern, but has been highly recommended for listing as a threatened or endangered species (Hunter 1984). The decline of *P. r. cooperi* in Arizona and southeastern California has been slightly offset by a rise in numbers at South Fork Kern River valley and San Felipe Creek.

Results of the *P. r. cooperi* population-level study indicate that the populations we studied do not form distinct genetic groupings on a very small geographic scale. This contrasts with patterns of geographic structuring found within populations of the yellow warbler, also a Neotropical migrant (Milot et al. 2000). Instead, within the western subspecies of summer tanager, most of the results indicate very high levels of movement among populations. The level of genetic diversity present within populations of a species is an important factor when considering its future viability (Soule 1980). Results of this study indicate that *P. r. cooperi* possesses high levels of genetic diversity within the region of mtDNA analyzed. Also, recently colonized populations in southern California (A, B) exhibit levels of nucleotide diversity of a similar magnitude as historic Arizona populations (C, D; Table 3). These results are encouraging for the future viability of the western subspecies of summer tanager. They suggest that as habitat degrades along the lower Colorado River, if suitable habitat elsewhere becomes available for new colonies to be established, these new colonies should be diverse genetically. This finding indicates populations of summer tanager in the southwestern U.S. probably do not risk a reduction in evolutionary potential due to low genetic diversity.

Of greater consequence, the genetic distinctness revealed between *P. r. cooperi* and *P. r. rubra* confirms the summer tanager consists of two independently evolving lineages. Exhibiting three fixed character differences, these two subspecies easily fit the definition of species under the phylogenetic species concept (Nixon and Wheeler 1990). Also, given these subspecies display unique habitat preferences and have distinct songs (Shy 1985), an important component in avian mate recognition, they are probably reproductively isolated. Further field research, though, is needed to confirm that they are two biological species. However, results from this study clearly indicate *P. r. cooperi* and *P. r. rubra* need to be managed as two distinct units. For the purpose of immediate management decisions, the eastern and western forms should be considered two evolutionarily significant units (Crandall et al. 2000). Under Crandall's et al.'s (2000) concept, two groups should be managed independently if they display both adaptive distinctiveness and historical and reproductive isolation (Crandall et al. 2000). *P. r. cooperi* and *P. r. rubra* show adaptive distinctiveness due to great differences in morphology, song and habitat preference. Also, historical isolation is indicated by fixed genetic differences. Therefore, *P. r. cooperi* and *P. r. rubra* qualify as evolutionarily significant units because they meet the criteria for Case 2 (rejection of both recent/historic genetic exchangeability and recent ecological exchangeability) under Crandall et al.'s (2000) guidelines. Furthermore, Crandall et al. (2000) state that if units being compared meet the criteria for Case 2, they should be managed as separate species.

Management of *P. r. cooperi* and *P. r. rubra* as two distinct species requires that high priority be given to preserving these separate units and the ability of their current populations to grow. Anthropogenic disturbances, including cattle grazing, alterations in hydrology and introduction of exotic plants, are widespread among native riparian stands of the southwestern

United States (Brown 1994). Effective management that includes the protection of riparian habitat in the west will be needed to promote the ongoing survival of *P. r. cooperi*, as well as other riparian-obligate species (Skagen et al. 1998).

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