Report

Current Biology

Speciation Associated with Shifts in Migratory Behavior in an Avian Radiation

Highlights

- Loss of migration promoted speciation in fork-tailed flycatchers (*Tyrannus savana*)
- Divergence is associated with the presence or absence of migratory behavior
- Loss of migration resulting in speciation has likely happened across flycatchers

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In Brief

Gómez-Bahamón et al. show that speciation is associated with changes in migratory behavior in fork-tailed flycatchers (*Tyrannus savana*). Divergence occurred through loss of migratory behavior of a single lineage. This mode of speciation likely occurred across New World flycatchers (Tyrannidae).

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Current Biology
Report

Speciation Associated with Shifts in Migratory Behavior in an Avian Radiation

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SUMMARY

Migratory animals move up to thousands of kilometers every year [1]. Losses of migration (i.e., migratory drop-offs) occur when individuals of a migratory species stop migrating and establish founder sedentary populations, a phenomenon documented in birds [2–5] and butterflies [6]. In theory, losses—and also gains—of migration might promote speciation if sedentary and migratory populations become reproductively isolated [7–9]. Because migratory and sedentary strategies involve alternative physiological, behavioral, and morphological traits [10–13], divergence along multiple axes of organismal function is expected to accompany switches in migratory behavior, potentially accelerating speciation. We present evidence of speciation driven by a migratory drop-off in the fork-tailed flycatcher (*Tyrannus savana*) resulting in reproductive isolation likely driven by changes in breeding schedules (allochronic speciation [13–15]) and geographic isolation of breeding grounds (allopatric speciation [16]). Phylogenetic analyses across New World flycatchers (Tyrannidae) showed that an association between speciation and drop-offs is also observable at a macroevolutionary scale. Loss of migration was significantly more frequent than its gain, and speciation rates of migratory and partially migratory lineages (i.e., species having both migratory and sedentary populations) exceeded those of sedentary lineages. Models of trait evolution indicated that partial migration is an intermediate step between migratory and sedentary states in this family. Given that partial migration is widespread across migratory animals (e.g., of all migratory birds, ca. 51% are partially migratory [5]), speciation via switches in migratory behavior might be an important yet overlooked mechanism of animal diversification.

RESULTS AND DISCUSSION

Speciation in Fork-Tailed Flycatchers

We tested the hypothesis that an evolutionary switch in migratory behavior promoted speciation in the fork-tailed flycatcher (*Tyrannus savana*). This common New World bird in the tyrant-flycatcher family (Tyrannidae) comprises four subspecies; three of them (*monachus*, *sanctaemartae*, and *circumdatus*) are sedentary in Central America and northern South America, and one (*savana*) is a long-distance migrant that breeds from central Brazil to Argentina and spends the non-breeding season in northern South America, where it overlaps with the resident subspecies [17–20] (Figure 1). Subspecies were described on the basis of geographic variation in wing feathers of adult males, which differ in the extent of attenuation of a notch at the tips of the primary feathers (Figure 1) [21], a trait used during social displays to produce non-vocal acoustic signals [22].

Genome-wide differentiation assessed with thousands of markers generated through genotyping by sequencing (GBS) revealed patterns consistent with the hypothesis that migratory and sedentary fork-tailed flycatchers are on separate evolutionary trajectories. In both neighbor-joining (Figure 2A) and maximum-likelihood (Figure S1A) phylogenetic analyses of SNP data, sedentary fork-tailed flycatcher subspecies formed a monophyletic group nested within a clade in which deep branches corresponded to a paraphyletic, migratory *T. s. savana*. This topology suggests that a sedentary lineage evolved through loss of migration from migratory ancestral populations. Both admixture proportions and principal-component analyses (PCAs) revealed substantial genetic differentiation between migratory and sedentary birds (Figures 2B and 2C). Moreover, population structure as measured by F_{ST} values was greater

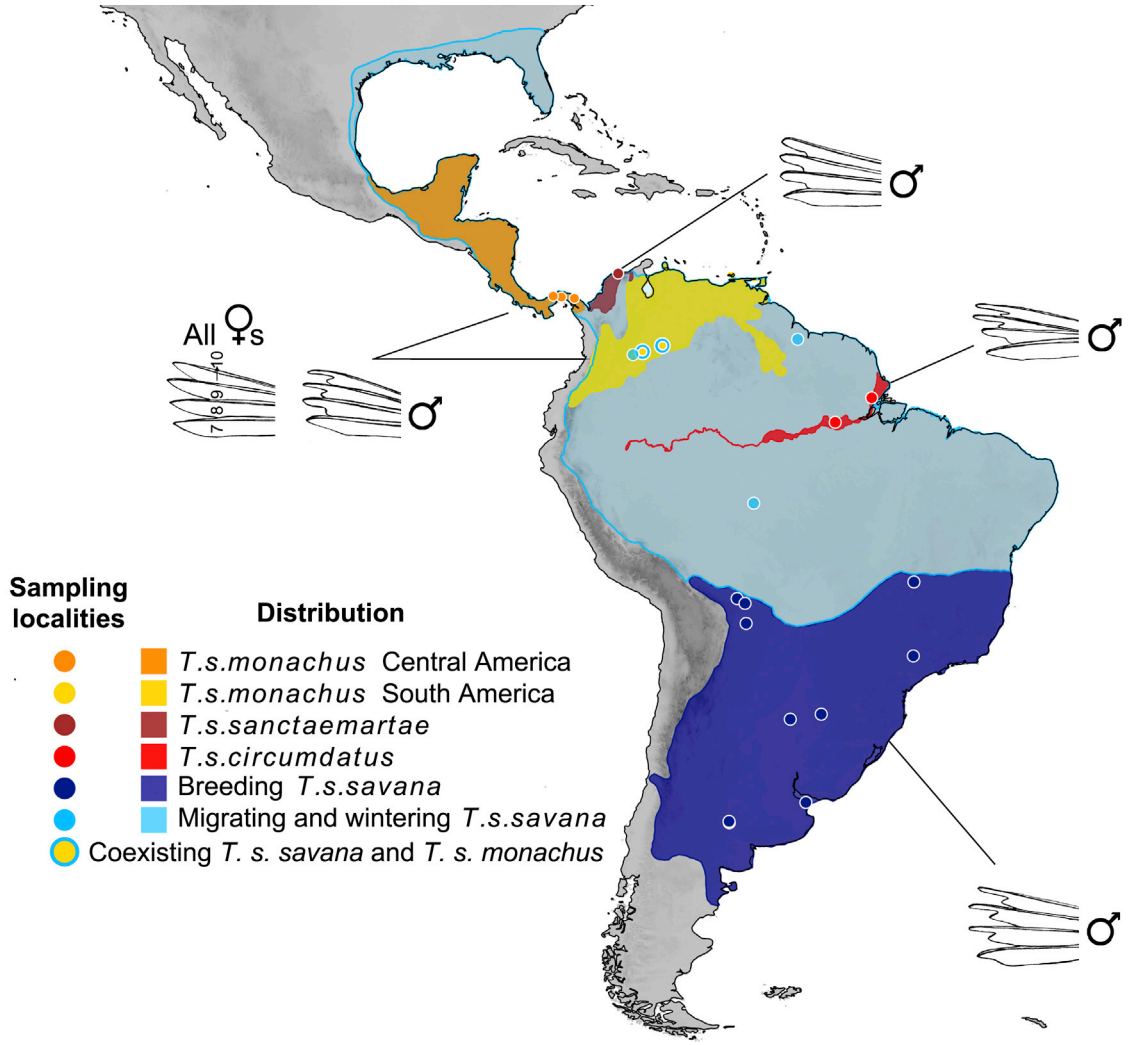

Figure 1. Geographic Distribution of Fork-Tailed Flycatchers and Sampling Localities

Polygons indicate ranges: sedentary subspecies *Tyrannus savana circumdatus* (red), *T. s. sanctaemartae* (brown), and *T. s. monachus* (yellow) in northern South America and the long-distance migrant *T. s. savana*, which breeds from central Brazil to Argentina (dark blue) and winters in northern South America and Central America (light blue). Circles represent sampling localities. Illustrations indicate primary feather morphology of males in each subspecies. Females of all subspecies lack notches in all primaries.

between migratory and sedentary populations than within behavioral classes (Figure S1C). A weak effect of isolation by distance (Mantel r = 0.238, p = 0.014) (Figure S1D) suggests that genetic differentiation is not solely the result of restricted dispersal and geographic isolation. In fact, migratory and resident individuals sampled at the same site belonged to distinct genetic clusters.

Positive values for Tajima's D estimated for the sedentary lineage are consistent with a reduction in effective population size (Figure 2F). Likewise, observed heterozygosity in sedentary lineages is a small fraction of that observed in migratory populations (Figure 2E). Together, these results are consistent with a reduction in population size associated with the origin of the sedentary lineage, likely reflecting founder event speciation [23, 24]. Moreover, consistent with the hypothesis that migratory populations are larger and older, there were more private mitochondrial DNA haplotypes in the migratory taxon *T. s. savana* than in the resident clade of fork-tailed flycatchers. However, an analysis of genetic differentiation among populations on the basis of this marker showed no discernible pattern of population structure with respect to geography or migratory status (Figure S1B) (overall ϕ_{ST} = 0.116, AMOVA [analysis of molecular variance], $\sigma^2 = 1.901 \times 10^{-7}$, p = 0.115).

Sedentary and migratory lineages diverged from each other in the Pleistocene (~1.08 million years ago [mya]) (Figure 2G) according to demographic parameter optimization on the basis of the site-frequency spectrum [25]. Estimates of effective population size of the sedentary lineage (N_e 66,448) were less than half of that of the ancestral migratory lineage (N_e 160,020), also supporting a scenario in which sedentary birds stemmed from a migratory ancestor via a founder event [23]. Given that fork-tailed flycatchers primarily use savanna and woodland habitats [20],

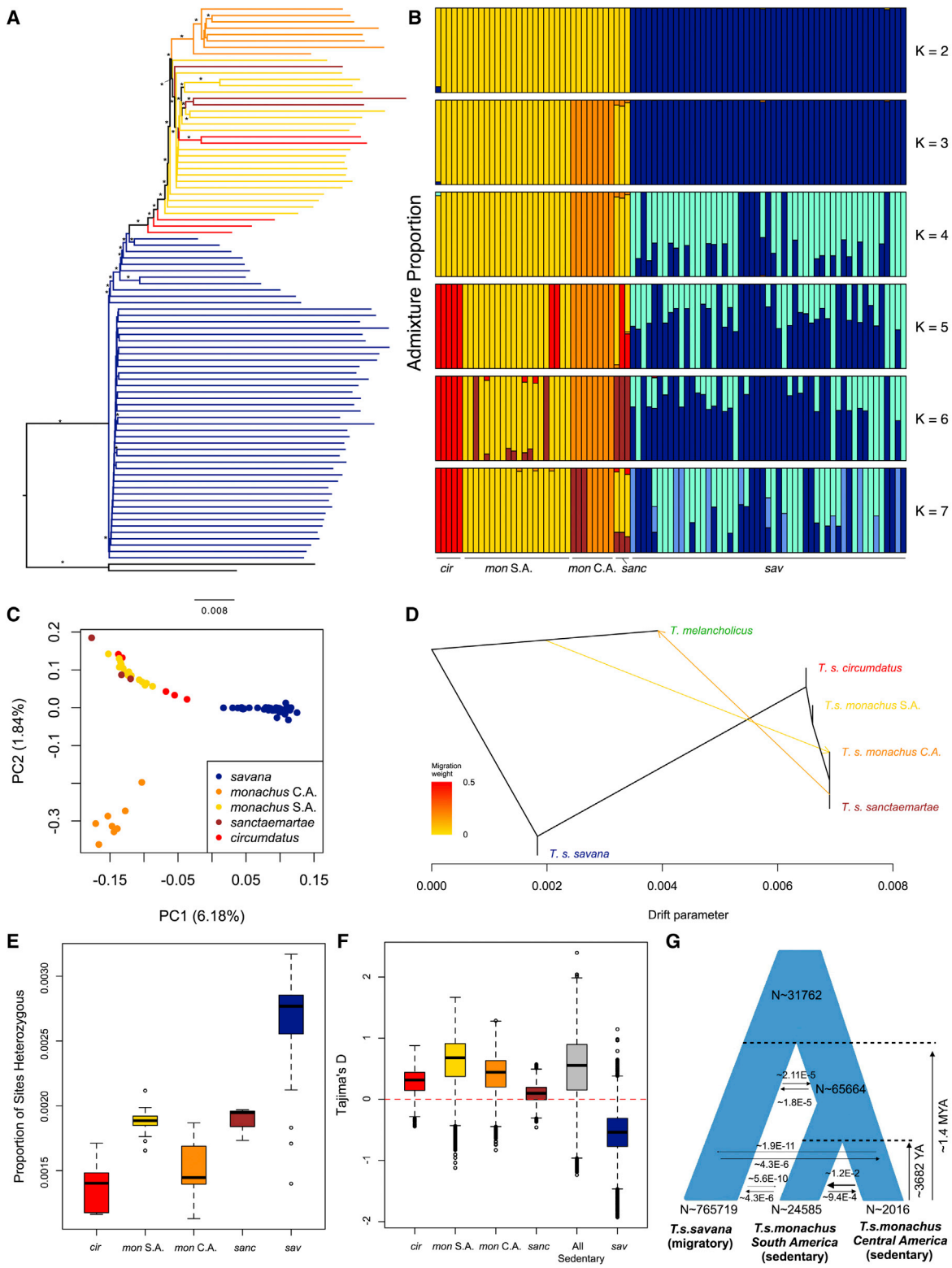

Figure 2. Population Genetic Analyses

(A) Neighbor-joining tree based on genetic distances obtained from genotype likelihoods. All sedentary subspecies (warm colors) formed a clade within a migratory subspecies (blue), suggesting that sedentary populations derived from migratory ancestors.

(legend continued on next page)

warm and dry climatic conditions during periods of the Pleistocene might have promoted the establishment of a founder sedentary population in areas of northern South America that might have resulted from the spread of savannas in tropical South America at that time. Alternatively, dispersal of temperate-breeding populations across the Amazon basin linked to Pleistocene climate change resulting in a savanna corridor might have allowed the establishment of a sedentary breeding population in open areas of northern South America [26, 27]. In either case, population divergence likely arose as a result of the establishment of a sedentary lineage breeding in savanna areas in northern South America.

Estimates of gene flow rates between migratory and sedentary lineages after their divergence were low (the probability that a gene from the migratory population is passed to the sedentary population per generation and vice versa was $2.1e^{-5}$ and $2.6e^{-6}$, respectively; Figure 2G). Similar results were observed estimating gene flow with a TreeMix model [28], which provided no evidence of significant gene flow between sedentary and migratory populations (Figure 2D). In fact, the only two putative gene flow events estimated involved a species used as an outgroup (tropical kingbird, *Tyrannus melancholicus*) and the sedentary subspecies *T. s. monachus* and *T. s. sanctaemartae* (Figure 2D). This pattern might reflect incomplete lineage sorting rather than gene exchange. Lack of recurrent gene flow suggests that a single loss of migration, as opposed to continued establishment of individuals, gave rise to a nascent sedentary clade. Altogether, population genetic evidence indicates that the migratory clade is likely older and has larger effective size than the sedentary clade, which appears to have been subject to a decline associated with its origin. Our inference of reduced or no gene flow also indicates lack of interbreeding between sedentary and migratory birds.

Consistent with our genetic analyses, our field research in eastern Colombia demonstrated that migratory and sedentary fork-tailed flycatchers are reproductively isolated (Figure 3A). At times when *T. s. monachus* and *T. s. savana* were sympatric, we observed sedentary individuals in pairs tending nests and defending territories in isolated "Chaparro" trees (*Curatella americana*). In turn, migratory birds formed large flocks (50–100 individuals) foraging for fruits as is typical for other migratory birds, which are more frugivorous than insectivorous in their tropical wintering grounds [29]. Roosting behavior also differed: migrant flocks of *T. s. savana* roosted in large and dense trees, whereas sedentary *T. s. monachus* roosted in pairs on Chaparro trees within their territories, except for immature sedentary birds which joined migrant flocks. We found 33 active nests, all of which were attended by pairs of sedentary *T. s. monachus*. Of 19 females and 27 males of *T. s. monachus* we captured, 85% were in breeding condition (i.e., females had an incubation patch and males a cloacal protuberance). In contrast, we captured 43 females and 83 males of the migratory *T. s. savana*, none of which were in breeding condition (Figure 3B). Because migration is energetically costly, it rarely overlaps with reproduction or molting in birds [30, 31]. The breeding season of sedentary fork-tailed flycatchers occurs after the rainy season in tropical savannas of South America. It is likely that adaptation to breeding in ecologically suitable conditions might have shifted the breeding schedule of sedentary birds resulting in allochronic reproductive isolation [13], a pattern rarely seen in other birds coexisting in sympatry [32].

We examined whether differences in selection pressures for efficient flight presumably faced by sedentary and migratory birds might have promoted evolutionary divergence along separate morphological trajectories. We found morphometric divergence between migratory and sedentary birds in traits associated with flight performance (measurements of wings and tails; Figures 3C–3F). These patterns are consistent with adaptive differences between migratory and sedentary populations in other birds [33–35]: longer, more pointed wings are more efficient for powered flight [36, 37] whereas shorter and more rounded wings enhance maneuverability [37], which is likely adaptive for hunting insects [38]. Tails were shorter in migratory birds (both males and females; Figures 3C–3F), possibly because of tradeoffs resulting from opposing pressures of sexual and natural selection [39].

Fork-tailed flycatchers use their tails during courtship displays, suggesting that differences between males and females reflect sexual selection pressures. Moreover, larger differences between migratory and sedentary males in comparison to migratory and sedentary females might reflect that the strength of natural selection for aerodynamic efficiency in males is stronger because they need to reach breeding grounds early enough to secure mating territories [40–42]. Indeed, migratory males of the fork-tailed flycatcher arriving earlier at breeding grounds have higher reproductive success [43]. Our observations of morphological divergence between sedentary and migratory populations contrast with findings from migratory divides (i.e., divergence driven by populations migrating in different directions) in which phenotypic traits under natural or sexual selection need not diverge in the process of speciation [44].

In summary, migratory and tropical sedentary fork-tailed flycatchers are reproductively isolated due to spatial and temporal separation in breeding activities as a result of changes in

(B) Admixture proportions with various numbers of clusters (K = 2–7). Divergence between migratory (*T. s. savana*) and sedentary birds (other subspecies) is observed; *cir*, *T. s. circumdatus*; *monS.A.*, *T. s. monachus* from South America; *monC.A.*, *T. s. monachus* from Central America; *sanc*, *T. s. sanctaemartae*; *sav*, *T. s. savana*.

(C) PCA using genotype likelihoods sampled across the genome. Genetic differentiation is observed between migratory (blue) and sedentary individuals (*T. s. monachus* from Central America [C.A.] and South America [S.A.], *T. s. sanctaemartae*, and *T. s. circumdatus*).

(D) TreeMix population graph chosen on the basis of likelihood (with linkage groups k = 1, migration edges m = 2).

(E) Genome-wide estimates of heterozygosity of fork-tailed flycatcher subspecies. Note that sedentary subspecies have lower genetic variability than the migratory subspecies *T. s. savana* (blue).

(F) Values of Tajima's D per taxon. Each and all sedentary subspecies have positive values, consistent with a contraction in population size, presumably reflecting a bottleneck associated with the loss of migration, whereas the migratory subspecies has negative values, reflecting population expansion.

(G) Demographic model with parameter optimization values of demographic history for populations of fork-tailed flycatchers with more than 8 samples.

See also Figure S1 and Table S1.

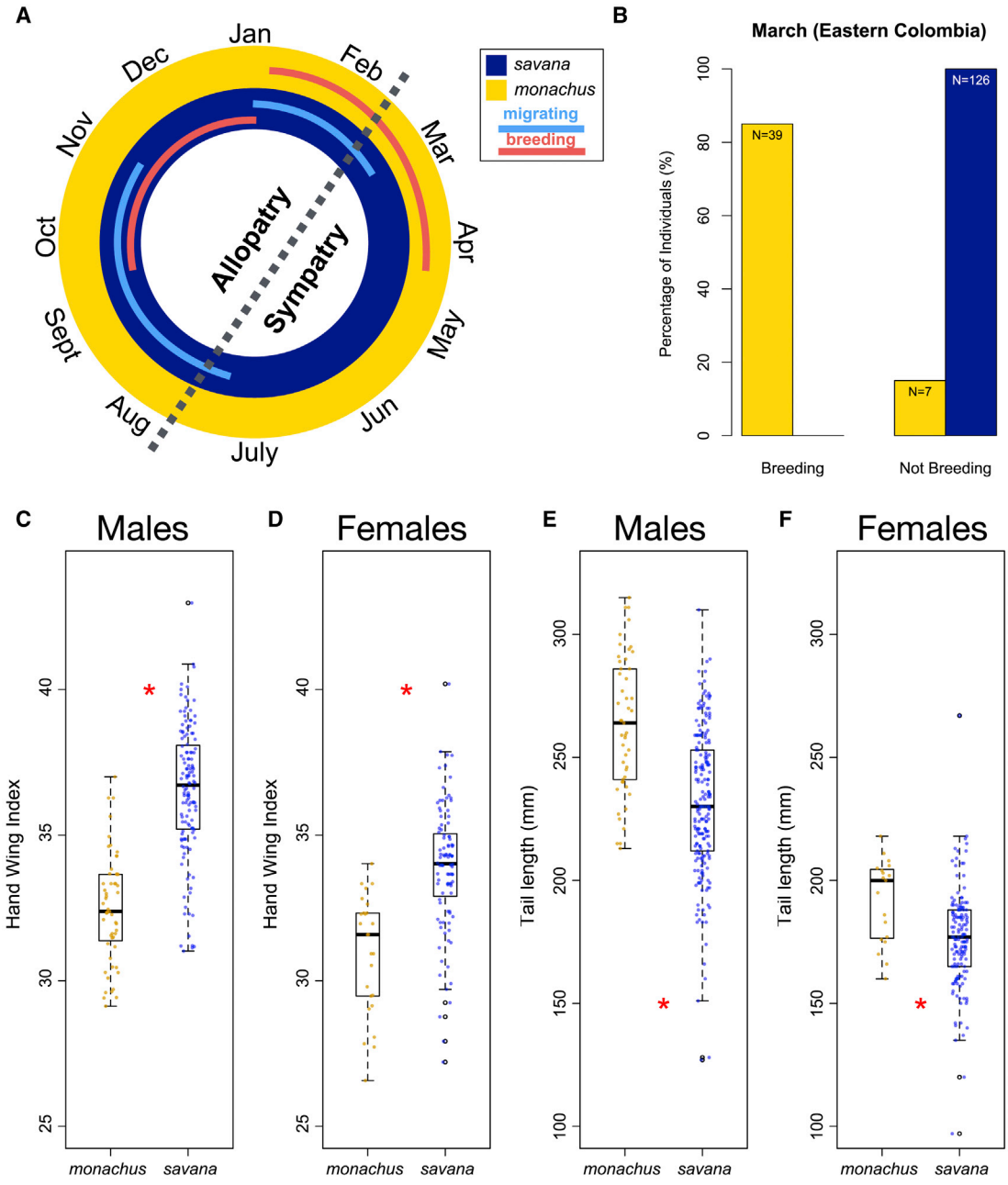

Figure 3. Breeding Condition and Morphology of Coexisting Sedentary and Migratory Forked-Tailed Flycatchers

(A) Annual cycle for sedentary *T. s. monachus* (yellow) and migratory *T. s. savana* (blue). Sedentary and migratory fork-tailed flycatchers are sympatric during part of the year but are at different stages of their reproductive phenology; sedentary birds breed while sympatric with migratory birds, which in turn breed when populations are allopatric. The approximate duration of migration of *T. s. savana* in fall and spring is indicated with light blue lines, and breeding seasons of both subspecies with red lines. The dotted gray line separates times of the year when the two subspecies are sympatric and when they are allopatric.

(B) Percentage of individuals captured in breeding condition (males with a cloacal protuberance and females with an incubation patch) in eastern Colombia. No individuals of *T. s. savana* were in breeding condition, whereas most *T. s. monachus* were.

(C–F) Morphology plots of the fork-tailed flycatcher. Hand wing index for males (C) and females (D). Note that wings of migratory males and females are pointier than those of their sedentary counterparts. Tail length of males (E) and females (F). Note that tails are significantly longer in sedentary males and females than in migratory birds.

Asterisks indicate $p < 0.05$ in ANOVAs.

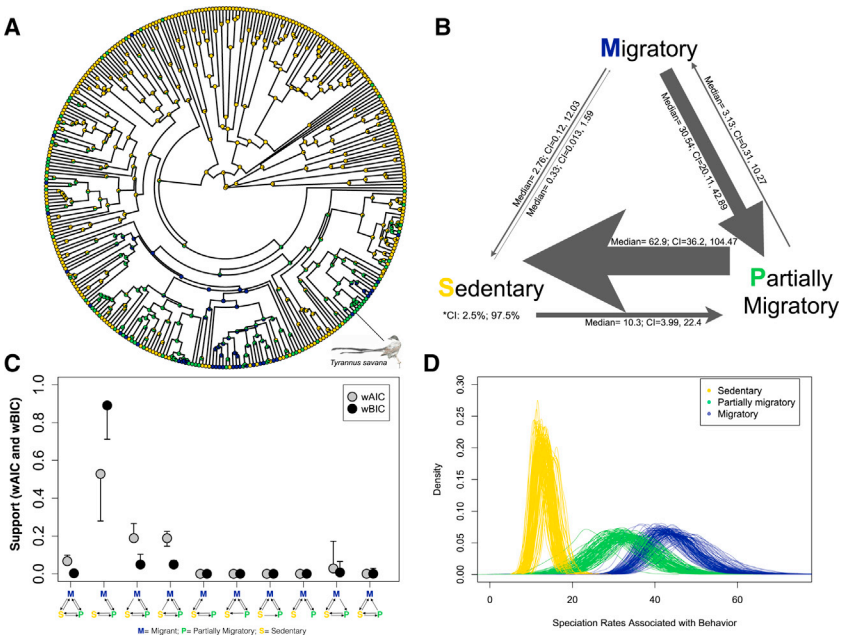

Figure 4. Evolution of Migration and Speciation Rates across New World Flycatchers

Full migration, partial migration, and sedentary behavior have evolved multiple times in the Tyrannidae yet not with the same frequency, and lineages which migrate have higher speciation rates than those which do not.

(A) Maximum clade credibility tree of Tyrannidae (sedentary behavior in yellow, migratory in blue, and partially migratory in green) with ancestral state probabilities at the nodes reconstructed under the threshold model of trait evolution. Note the lability of migratory behavior: full migration, partial migration, and sedentary behavior have evolved multiple times. The fork-tailed flycatcher is indicated by the arrow pointing to the illustration (illustrated by Andrés Montes).

(B) Median transition rates estimated over the posterior tree distribution by using multi-state speciation and extinction (MuSSE) models while allowing all possible transitions. Arrows indicate the direction of transitions between character states (width is proportional to transition rates; confidence intervals represent the lowest 2.5% and highest 97.5% of the distribution). Note that migration has been more often lost than gained.

(C) Support for 10 MuSSE models with different character-state transition matrices calculated over the posterior tree distribution. The model with highest support involves partial migration as an intermediate step in evolution between sedentary and migratory strategies. Dots represent the median support and error bars correspond to the 95% highest posterior density (HPD).

(D) Speciation rates of lineages with full migration, partial migration, and sedentary behaviors. Probability densities indicate values obtained from Markov chain Monte Carlo (MCMC) runs and each line corresponds to one of 100 random trees sampled from the posterior distribution to account for phylogenetic uncertainty. Full migration and partial migration have significantly higher rates than those associated with a sedentary state.

See also Figures S2–S4 and Tables S2 and S3.

migratory behavior leading to alternative strategies. Because they are independent evolutionary lineages retaining their phenotypic integrity and experiencing no gene flow in sympatry, sedentary, and migratory populations of fork-tailed flycatchers can be considered different species under most species definitions [45].

Switches in Migratory Behavior and Macroevolution

How general is speciation via loss of migratory behavior and what are the consequences of such changes for patterns of clade diversification? To address these questions, we first asked whether speciation via loss of migration as evidenced in the fork-tailed flycatcher is observable at a macroevolutionary level across the Tyrannidae, a clade with >400 species. Ancestral state reconstructions using a threshold model of trait evolution [46, 47] and comparisons of alternative threshold and Markovian models describing transitions among character states [48] revealed that changes in migratory behavior have occurred repeatedly across a phylogeny of 332 flycatcher species (Figure 4A). Furthermore, model comparison analyses strongly indicated that partial migration (species having both migratory and sedentary populations) is an intermediate evolutionary step between migratory and sedentary character states (and vice versa; Figures 4B and 4C), with significantly asymmetric transition rates toward loss of migration (Figure 4B).

Classical hypotheses proposed to explain the origin of migratory behavior postulate that partial migration is a stepping stone [49, 50] or an evolutionary precursor [51] of obligate long-distance migration to temperate breeding areas, originating from sedentary tropical ancestors. This view has been recently challenged with the argument that migration likely evolves as an adaptation to persistence of breeding sites [52] as opposed to a consequence of escaping from competition in tropical environments [53]. Regardless of the specific sequence of events involved in changes in migratory behavior, the multiple independent transitions to and from migration in the Tyrannidae allow an assessment of whether migration is associated with macroevolutionary diversification. Indeed, speciation rates estimated by fitting multi-state speciation and extinction models [48] were higher for migratory and partially migratory lineages than those of sedentary lineages (Figures 4D, S2, and S3). Results of comparative analyses were robust to using alternative phylogenies as the basis for analysis to account for missing species (Figure S2A), to analyzing sub-clades with fewer sedentary species (Figure S2B), to alternative ways of treating data (Figures S2 and S4), and to conducting analyses separately for tropical and temperate environments to account for potentially confounding effects of latitude on speciation [54] (Figure S3).

Across the evolutionary history of birds, global cooling appears to promote the evolution of migration due to increased seasonality in temperate areas [55], whereas loss of migratory behavior or reduction in migratory distance is associated with global warming [11, 56–59]. We hypothesize that a combination of warm periods in Earth's history, such as those experienced during interglacial periods of the Pleistocene [60], together with ecological opportunity, such as changes in resource availability, might have favored loss of migration in the fork-tailed flycatcher. Similar events might have driven repeated loss of migration

across the Tyrannidae, with important consequences for diversification.

Partial migration exists in a variety of forms [59]: migrants and residents might breed in sympatry but overwinter separately, overwinter together and breed in allopatry, or migrate facultatively (i.e., individuals changing behavior across years [61]). Different kinds of partial migration arguably represent stages in a behavioral and geographic continuum [16, 58]. Evolution across this continuum might be rapid when populations are exposed to strong environmental changes [62], as evidenced by a shortening of migratory distance promoted by recent global warming [58]. Thus, evolutionary lability of migratory behavior [61] might allow individuals to switch between strategies given variation in the environment [7, 60].

Loss of migration is not the only mechanism via which changes in migratory behavior might promote speciation. Even when populations differing in migratory strategy breed in the same areas, they might diverge if hybrids between migratory and sedentary individuals experience reduced fitness [63, 64], for example by flying toward inhospitable areas [65]. Incipient prezygotic reproductive isolation might also arise if migratory populations breed in sympatry yet overwinter in different locations; in such cases, individuals might arrive at different times at breeding grounds and therefore mate assortatively, which results in divergence associated with allochronic breeding [15]. Moreover, in migratory species in which populations migrate to different locations, hybrids might migrate in intermediate yet unfavorable directions when orientation is genetically determined, resulting in postzygotic isolation via reduced hybrid fitness [9, 63, 64, 66]. Finally, switches in breeding hemisphere and inverted migratory patterns (e.g., populations that originally breed in the north temperate zone and migrate southward switching to breed in the south temperate zone and migrating northward) also have the potential to promote divergence and eventually speciation [67, 68].

Conclusions

Our results provide evidence that loss of migration has promoted speciation in the fork-tailed flycatcher and likely across the Tyrannidae, revealing a potentially important yet often underappreciated role for changes in migratory behavior in driving breeding isolation and thereby the origin of new species. Furthermore, we suggest that this mechanism of divergence should be examined in other avian clades as well as in other migratory animals to establish its generality. How is population divergence via changes in migratory behavior initiated? We surmise that loss of migration in a formerly migratory population is unlikely to be caused by novel mutations of large phenotypic effects. Because migratory behavior is a polygenic trait, which is expressed under different environmental triggers [69, 70], we speculate that diversification driven by evolutionary changes in migratory strategy is initiated via plasticity. Behavioral differences reflecting plasticity and resulting in establishment might then be followed by selection, leading to the accumulation of adaptive genetic differences between migratory and sedentary populations [7, 71]. Behavioral plasticity might be subsequently lost because of selective pressures canalizing migratory behavior [52]. Under this view [52, 72, 73], plastic expression of alternative behaviors allows populations to enter new adaptive zones, where selection on correlated life-history and morphological traits might further promote population divergence and diversification [7, 74, 75]. Speciation via alternative migratory behaviors illustrates the dual role of factors intrinsic and extrinsic to organisms acting in concert to promote diversification [76].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Speciation in the Fork-tailed Flycatcher
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- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.01.064.

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AUTHOR CONTRIBUTIONS

V.G.-B., C.D.C., and A.E.J. designed the project. V.G.-B. and C.D.C. wrote the manuscript. V.G.-B. and R.M. analyzed the data. A.E.J., D.T.T., and V.G.-B. collected blood samples in the field. V.G.-B. and A.E.J. conducted behavioral observations. O.L.-R generated a preliminary phylogenetic hypothesis of the Tyrannidae. A.E.J., C.Y.M., S.R., D.T.T., and R.M. contributed ideas to different stages of the project and edited the manuscript. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Frozen tissues for DNA of *Tyrannus savana*	Wild (Archived at natural history collections)	See Table S1
Blood extractions for DNA of *Tyrannus savana*	Wild	See Table S1
Specimens of *Tyrannus savana* for morphology	The Field Museum	figshare https://figshare.com/projects/Speciation_and_loss_of_migration_in_New_world_flycatchers_Tyrannidae_/73719
Chemicals, Peptides, and Recombinant Proteins		
Phenol:Chlorophorm:Isoamyl Alcohol	Sigma-Aldrich	P2069
*Pst*I Endonuclease	Cornell University Institute for Genomic Diversity	
Taq DNA Polymerase and buffers	Invitrogen	11615-036
dNTPs	Invitrogen	10297-018
Critical Commercial Assays		
DNeasy Blood and Tissue Kit	QIAGEN	69506
Genotyping-by-sequencing library construction	Cornell University Institute for Genomic Diversity	GBS plate "TYSA" run on lane 2 of flowcell C5EMPACXX on a HiSeq2500 instrument
Deposited Data		
mtDNA sequences	This Paper	GenBank accessions MN895933-MN895996
GBS Illumina reads	This Paper	Project accesion number SRA: PRJNA600207, sample accession numbers BioSample: SAMN13824682-SAMN13824776
Morphometric and Natural History data	This Paper	Figshare https://figshare.com/projects/Speciation_and_loss_of_migration_in_New_world_flycatchers_Tyrannidae_/73719
Tyrannidae phylogenetic trees	This Paper	Figshare https://figshare.com/projects/Speciation_and_loss_of_migration_in_New_world_flycatchers_Tyrannidae_/73719
Experimental Models: Organisms/Strains		
Tyrannus savana	Wild	N/A
Oligonucleotides		
ND2 Primer (light strand): TATCGGGCCCAT ACCCCGAAAAT	[77]	L5215
ND2 Primer (heavy strand): CTCTTATTTAA GGCTTTGAAGGC	[78]	H6313
Software and Algorithms		
MUSCLE 3.8.31	[79]	http://www.drive5.com/muscle
PopART 1.6	[80]	http://popart.otago.ac.nz/index.shtml
pyRAD v. 3.0.2	[81]	https://github.com/dereneaton/pyrad
Skewer v. 0.2.2	[82]	https://github.com/relipmoc/skewer
Trimmomatic v. 0.36	[83]	
Bowtie2 v. 2.1.0	[84]	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools v. 1.0	[85]	https://github.com/samtools/samtools
Picard Tools v. 1.84	Broad Institute	https://github.com/broadinstitute/picard
GATK v. 3.3-0	[86]	https://software.broadinstitute.org/gatk/
Angsd v. 0.917	[87]	http://www.popgen.dk/angsd/index.php/ANGSD

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
ngsTools 1.0.1	[88]	https://github.com/mfumagalli/ngsTools
ngsDist v.1.0.2	[89]	https://github.com/fgvieira/ngsDist/
ngsAdmix v. 32	[90]	http://www.popgen.dk/software/index.php/NgsAdmix
FastME v. 2.1.5.1	[91]	https://gite.lirmm.fr/atgc/FastME/
Plink v. 1.9	[92]	https://www.cog-genomics.org/plink2/
Treemix v. 1.12	[28]	https://bitbucket.org/nygcresearch/treemix
RAxML v. 7.2.6	[93]	https://cme.h-its.org/exelixis/web/software/raxml/index.html
FastSimcoal2 v. 2.6.0.2	[25]	http://cmpg.unibe.ch/software/fastsimcoal2/
PartitionFinder v. 1.1.1	[94]	https://github.com/brettc/partitionfinder
jModelTest v. 2.1.7	[95]	https://github.com/ddarriba/jmodeltest2
BEAST v. 1.8	[96]	https://beast.community/
R	R Core Team	https://www.r-project.org/

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and code should be directed to and will be fulfilled by the Lead Contact, Valentina Gómez-Bahamón (vgbahamon@gmail.com). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We captured wild Fork-tailed Flycatchers using mist nets and extracted blood samples from the brachial vein [97, 98] at South American localities, which we supplemented with tissues from museum specimens and samples of two Tropical Kingbirds (*T. melancholicus*) employed as outgroup (see Table S1).

METHOD DETAILS

Speciation in the Fork-tailed Flycatcher

DNA extraction and mtDNA sequencing

DNA was extracted using QIAGEN DNeasy Blood and Tissue kits or phenol-chloroform extraction [99]. We amplified and sequenced the complete NADH dehydrogenase subunit 2 gene (1,036 bp) using primers L5215 [77] and H6313 [78] following published protocols [100]. Chromatograms were edited and aligned using *Geneious v5.4* [101], and sequences were aligned with *MUSCLE* [79]. Our final ingroup alignment consisted of 33 sequences of *T. s. savana*, 21 of *T. s. monachus,* 5 of *T. s. sanctamartae* and 3 of *T. s.circumdatus*.

Genotyping by sequencing and bioinformatic data preparation

Genotyping by Sequencing (GBS) libraries were built at the Cornell University Institute for Genomic Diversity. This approach consists of cutting genomic DNA into short fragments with a restriction enzyme and sequencing a subset of similarly sized fragments to obtain genotypes at markers spread across the genome [102]. The *Pst*I restriction enzyme was used to generate fragments from 94 samples individually barcoded and single-end sequenced on a HiSeq 2500 Genome Analyzer (Illumina Inc., San Diego California). Raw single-end reads were demultiplexed using the first step of the *pyRAD* pipeline [81, 103], allowing for a maximum of one mismatch in barcode identity. Five individuals were removed from the dataset due to low read counts (< 400,000 reads) and high levels of mismatched barcodes (> 8%). Demultiplexed reads were quality-trimmed and filtered using *skewer* [82] and we used *Trimmomatic* [83] to mask sites and trim read ends with phred base quality below 20, remove adaptor contamination, and discard post-trimming reads shorter than 36 bp. We then mapped our filtered reads to an unpublished draft genome of *T. s. savana*, kindly provided by the B10K consortium project (Guojie Zhang and B10K Consortium, personal communication B10K ID: B10K-DU-001-37). Given the fragmented nature of the draft genome (128,031 scaffolds; 1.07 Gbp; N50 = 428.2 kbp), we only used scaffolds longer than 1000 bp (10,780 scaffolds; 1.04 Gbp; N50 = 442.2 kbp) from the reference. We aligned reads using *Bowtie2* [84], sorted alignments with *Samtools* [85], added read groups using *Picard* (http://broadinstitute.github.io/picard), and realigned around indels using *GATK* [86]. Finally, for each subspecies we identified sites with average per-individual coverage below 30X, that were genotyped for at least 25% of individuals of that subspecies and which did not deviate significantly from Hardy-Weinberg equilibrium (α = 0.05) using *ANGSD* [87]. For further analyses we used only sites that passed all filters in all populations. In addition, we restricted analyses to sites with phred base qualities above 30 and reads with mapping qualities above 20.

Natural history

We searched for roosting and nesting sites, made behavioral observations, and captured Fork-tailed Flycatchers to band them, take morphological measurements, and examine breeding condition during field seasons at various sites in the Llanos of Eastern

Colombia. We captured birds by placing mist nets at a distance of 2-4 m from active nests and using a conspecific call played at 2 m from the net before dawn [104], and at a roosting site before dusk. We banded birds with a unique metal band. Subspecies, sex and age were determined based on plumage and other traits [105]. At the sites where migratory and sedentary Fork-tailed Flycatchers coexist, we distinguished subspecies by the primary feathers if they were males, and for all birds by the color of the back which is darker in migratory individuals. To determine breeding status we noted the presence or absence of incubation patch and cloacal protuberance [106].

Morphology

We measured all the birds we captured, as well as museum specimens in the collection of The Field Museum. The measurements taken were as follows: wing length (distance from the carpal joint to the longest primary feather), Kipp's distance *Kd*(the distance from the tip of longest primary feather to the tip of the first secondary feather [107], tarsus length, bill depth, length and width, and tail length. We standardized Kipp's distance by calculating the hand wing index *HWI*, which gives an estimation of wing shape; larger values indicate more pointed wings and smaller values indicate rounder wings: $HWI = (100 * Kd / WI)$, where *WI* is wing length. Because of limited sample sizes for other subspecies, we focused on comparisons between *T. s. monachus* (sedentary) and *T. s. savana* (migratory) based on a total of 518 birds. Our analyses employed data for 46 *T. s monachus* (26 males, 19 females) and 426 *T. s. savana* (236 males, 184 females) measured in the field, as well as 46 *T. s. monachus* specimens (32 males, 14 females).

Evolution of migration and speciation rates across New World flycatchers

Phylogenetic inference

We compiled published data available for Tyrannidae in GenBank (downloaded using PhyLoTa Browser [108]) and used them to reconstruct a comprehensive phylogeny of the family based on concatenated sequences of two mitochondrial and six nuclear loci totaling 8,518 bp for 329 species in the Tyrannidae (77% of the species in the family; Table S3). Because not all species of Tyrannidae had sequences for all genes, our matrix had a total of 59.4% missing bases; on average, each species had 3,536 bp (41.6%; range 284-8507 bp [3%- 100%]). When more than one sequence was available for a given species, we aligned them in using *MUSCLE* and obtained a consensus, resulting in a single sequence per species in our final alignment. We chose the best-fit model of sequence evolution and partitioning strategy for each gene in *PartitionFinder* [94] for coding sequences and in *jModeltest* [95] for non-coding sequences (i.e., introns). The resulting supermatrix was used to estimate the posterior distribution of phylogenetic trees under a Bayesian framework using *BEAST* [96] v. 1.8. We performed four separate runs, each for 50,000,000 generations, sampling every 5,000, using a relaxed log-normal molecular clock. The clock rate of one of the partitions was set to one, and the rest were estimated relative to that fixed one. A Yule process was used to model cladogenesis, and priors were set using default values, except for estimated clock-rates for which we used uniform priors between 0 and 20. Run logs were visually inspected in *Tracer* [109] to determine burnin values which were 15,000,000 in three of the runs and 20,000,000 in the fourth (resulting in 27,000 post-burnin trees). To obtain a maximum clade-credibility tree, we ran the combined post-burnin trees in *TreeAnnotator*.

Character-state classification

We defined migratory birds as those species that, after their breeding season, move annually to overwinter in a different location and then return to the same breeding area the following year. Partial migrants are species in which at least one subspecies or population is migratory and at least another subspecies or population is sedentary. This category includes cases in which migratory and resident populations breed in the same geographic region, as well as those in which they breed in different regions, although the former situation is rare. Sedentary lineages are those that stay in the same area year-round. Based on published data [22], we assigned character states (migratory, partial migrant, sedentary) to every species of Tyrannidae in our phylogeny. We focused on latitudinal migration (i.e., boreal or austral migration) and did not consider longitudinal or altitudinal migration.

QUANTIFICATION AND STATISTICAL ANALYSIS

Speciation in the Fork-tailed Flycatcher

Population genetic analyses using mtDNA

We constructed a median-joining haplotype network of mtDNA sequences [110] using *PopART* [80] and assessed population differentiation using the ϕ_{ST} statistic: $\phi_{ST} = ((\pi_T - \pi_S) / \pi_T)$, where π_T is the overall nucleotide diversity of the dataset and π_S is the average within-population nucleotide diversity. We also examined partitioning of genetic diversity within and among populations conducting an AMOVA [111] in the R package *pegas* [88] to evaluate the extent of genetic structure at this locus among populations.

Population genetic analyses using GBS

Whenever possible we performed analyses directly on genotype likelihoods estimated in *ANGSD* [87] to account for statistical uncertainty in genotyping. To evaluate the extent of genetic structure and admixture between *T. savana* subspecies, we conducted a principal components analysis (PCA) based on a genetic covariance matrix estimated with *ngsTools* [90], and estimated admixture proportions using *ngsAdmix* [112] assuming k = 2-7 ancestral populations. Both analyses were performed on sites with a SNP p value below 0.01 and minor allele frequencies above 0.05. To describe genetic variability within sedentary and migratory subspecies, we estimated the proportion of heterozygous sites for each individual $(Hom)(Het)H = (Het / Hom + Het)$ in *ANGSD* by generating folded site frequency spectra for each sample and dividing the number of sites with two distinct alleles over the total number of genotyped sites. To assess whether sedentary populations potentially resulted from a small founder population, we first calculated Tajima's D [91] based on folded site-frequency spectra for each subspecies (or all sedentary individuals) estimated in *ANGSD* using *thetaStat*

(distributed with *ANGSD*). F_{ST} values were estimated in *ANGSD* for all populations with two or more individuals. To test for isolation by distance we performed a Mantel test using the R package *vegan* (https://github.com/vegandevs/vegan) with 10000 permutations, using Spearman's ρ as the test statistic. Geographic distances between breeding populations were estimated with the R package *raster* (https://CRAN.R-project.org/package=raster).

A BioNJ tree was built in *FastME* [89] based on genetic distances estimated with *ngsDist* [93]. The tree was rooted using *T. melancholicus* as an outgroup, and nodal support was evaluated by generating 500 bootstrapped distance matrices in *ngsDist*, sampling blocks of 10 variable sites (SNP p value < 0.01) to account for LD. In addition, we built a maximum-likelihood tree of concatenated SNPs (see below for SNP calling details) using *RAxML* [113] under the GTR+Gamma model, using Lewis's acquisition bias correction (–asc-corr lewis) [92]. Support was evaluated using 1000 fast bootstrap pseudoreplicates. We also used *Treemix* [28] to estimate a subspecies tree while incorporating gene flow among them. We called SNPs in *ANGSD* using the filters described above (including SNP p value < 0.01) for sites with a genotype posterior probability above 0.99, and used *plink* [114] to produce allele counts. *Treemix* was run for m = 0-7 migration edges. The reported value of *m* was selected based on the increase in pseudolikelihood values as migration edges were added. The best value of *m* was the one after which adding an additional edge resulted in a log-likelihood increase below 1.92 (the critical value of a likelihood ratio test with one degree of freedom and $\alpha = 0.05$). Based on this criterion, we chose 2 as *m* irrespective of the number of SNPs binned together during covariance estimation (-k flag). Based on PCA, BioNJ, and admixture analyses individuals of *T. s. monachus* from Central America were treated as a separate lineage during data filtering as well as in genetic diversity, Tajima's D, and *Treemix* analyses.

For demographic analyses in *fastsimcoal* [25] aimed at estimating divergence times and gene flow among populations, we only used data from *T. s. savana* and the Central and South American *T. s. monachus*, which were recovered as separate lineages in our genetic structure and phylogenetic analyses. This allowed us to maintain a tractable number of parameters in our demographic model. Parameter estimations were based on a minor allele (i.e., folded) 3D SFS, which we obtained by generating an unfolded 3D SFS in *ANGSD* [87], and using *dadi* [115] to fold it to a minor allele spectrum. Parameters were optimized using 150 expectation/conditional maximization (ECM) cycles, each consisting of $2.5e^6$ simulations. We ran 200 independent optimizations and selected the one with the highest estimated likelihood as the maximum-likelihood estimate. Our simulations assumed a mutation rate of 4.6×10^{-9} per site per generation, following an estimate for the Collared Flycatcher (*Ficedula albicolis*, family Muscicapidae) [116], and divergence times in years were obtained assuming a generation every 2 years.

Morphological analyses

We conducted separate single factor ANOVAs for traits associated with aerodynamics (wing shape and tail length) comparing *T. s. savana* (migratory) and *T. s. monachus* (sedentary). Females and males were analyzed separately.

Evolution of migration and speciation rates across New World flycatchers

Partial migration as an intermediate step in the evolution of full migration and sedentariness

We examined trait evolution using two kinds of comparative phylogenetic models to ensure that our results were robust to different statistical assumptions. First, we estimated character-state transition rates under multi-state speciation and extinction models (*MuSSE*) to account for variation in diversification rates associated to each character state [48]. To determine whether a model with partial migration as an intermediate state between being sedentary and migratory is supported in the Tyrannidae, we compared the support of our data all possible models of character-state transition across 20,000 posterior trees (i.e., including models with all the possible transitions among the three character-states). Models were built by allowing and disallowing transitions and optimized using maximum likelihood in the R package *diversitree* [48]. Akaike information criterion weights (AICw) and Bayesian information criterion weights (BICw) were estimated in the R package *paleoTS* [117]. Finally, we parametrized the full MuSSE model using Markov chain Monte Carlo sampling (hereafter MCMC) on the maximum clade-credibility tree. We ran the chains for 10,000 generations, with a burnin of 1,000 (both chosen based on visual inspection of trace logs), with an exponential prior of 1/20, and a random starting point.

Second, we used a threshold model adopted from quantitative genetics in which discrete character states change gradually depending on an underlying continuous trait [46, 47, 118]. The threshold model was proposed to describe the evolution of discrete character states with a polygenic basis [119]. To determine whether partial migration is an intermediate step between migratory and sedentary states assuming the threshold model, we tested all possible transition sequences under a Brownian-motion model of trait evolution in the R package *phytools* using MCMC sampling [120]. There are three possible sequences by which transitions can occur: (1) from migratory to partially migratory to sedentary, or the reverse ($M \leftrightarrow P \leftrightarrow S$), (2) from partially migratory to sedentary to migratory, or the reverse ($P \leftrightarrow S \leftrightarrow M$), and (3) from partially migratory to migratory to sedentary, or the reverse ($P \leftrightarrow M \leftrightarrow S$), results are reported in Figure S2. We ran MCMC chains for 10,000,000 generations with a burnin of 1,000,000 generations, and estimated the effective sample size of parameters to evaluate convergence and visually inspected chains to assess proper mixing and determine the burnin in Tracer. Statistical support was assessed using deviance information criterion (DIC) values estimated in *phytools*.

Speciation rates in association with migratory strategy

We tested for associations between migratory strategy and evolutionary diversification in the Tyrannidae by fitting multi-state speciation and extinction models (*MuSSE*). This method analyzes the evolution of traits with multiple character states and estimates speciation (λ) and extinction (μ) rates associated to each state assuming that changes in state occur following a continuous-time discrete-state Markov process along the branches of a tree [48]. We first examined statistical support (based on AICw and BICw values) for various models describing diversification and extinction rates in 2,000 posterior trees as described above. The models tested consisted of: 1. allowing all speciation rates and extinction rates associated with a character state (i.e., migratory, partially

migratory, sedentary) to be different, 2. constraining speciation and extinction rates associated with a character state to be equal, 3. constraining speciation rates associated with a character state to be equal and allowing extinction rates to differ, and 4. allowing speciation rates associated to a character state to differ and constraining extinction rates associated to a character state to be equal. We selected the model with highest support as the basis to estimate speciation and extinction rates associated to each character state and to estimate net diversification rates (speciation-extinction). We found that the model with highest support was the one allowing speciation rates associated to a character state to differ and constraining extinction rates associated to a character state to be equal. Because extinction was unrealistically estimated as ~0, we only focused on speciation rates. We ran *MCMC* on 100 random trees from the posterior distribution for 10,000 generations, with a burnin of 1,000 (both chosen based on visual inspection of trace logs), with an exponential prior of 1/20, and a random starting point.

Accounting for missing species

Because the phylogeny of the Tyrannidae we used as a basis for comparative analyses was incomplete, we also performed model-fitting using the set of phylogenetic trees generated in [121], which assembled a global phylogeny of birds using DNA sequences and added taxa lacking sequence data based on taxonomy and a Yule pure-birth model. We downloaded 10,000 trees (Hackett backbone) including all known species of Tyrannidae from http://birdtree.org/subsets/ and built a maximum clade-credibility tree. We performed *MuSSE* model fitting using *MCMC* as described above.

Subclade analysis

Our phylogeny of the Tyrannidae recovered two main sister clades: one containing 60 sedentary species, and the other containing a mix of sedentary, migratory and partially migratory species. To determine to what extent the relatively large sedentary clade influenced our estimates of speciation rates, we excluded this clade from analysis and fitted *MuSSE* only on its sister clade using *MCMC* sampling (10,000 generations, burnin 0.1, exponential prior 1/20, random starting point).

Treating migratory and partially migratory species in a single category

We grouped partial migrants and fully migratory species in a single migratory category to determine whether this produced the same results in terms of the direction of shifts between character states (i.e., migration, no migration) than in analyses in which we treated partially migratory species separately. We used the binary state speciation and extinction model *BiSSE* [122] implemented in the R package *diversitree* to estimate transition rates and the associated speciation and extinction rates. We used *MCMC* sampling (10,000 generations, burnin 0.1, exponential prior 1/20, random starting point). We also trimmed the phylogeny as described above and estimated parameters aiming to determine if including the early diverging clade of mostly sedentary species affects our conclusions even when treating migratory and partially migratory species in a single category.

Accounting for distribution of missing species

Most comparative methods assume that missing taxa in incomplete phylogenies are randomly distributed along the tree. Our analyses included sequence and behavioral data for 77% of the species of Tyrannidae. To address whether our dataset conforms to the assumption of randomly distributed missing taxa, we used the MCC tree built using data from [121]. http://birdtree.org/subsets/. We coded whether a taxon was represented in our study as a binary character, and tested for phylogenetic signal on this character. Briefly, the approach we followed [123] consists on counting the minimum number of evolutionary steps leading to the observed character distribution on a tree using maximum parsimony, and comparing it to a null distribution, generated through permutation of the character states while keeping the tree fixed; a trait is considered to exhibit phylogenetic signal if the number of observed steps is smaller than the null expectation [123]. To implement this test, we modified the phylo.signal.disc R function (phyloint package; https://github.com/stoufferlab/phyloint/), http://www.mail-archive.com/r-sig-phylo@r-project.org/msg00922.html and performed 1,000 permutations to generate a null distribution. In addition to the MCC tree, we ran this test on 1,000 of the trees from [120], to account for topological uncertainty.

Simulating character states to evaluate spurious correlations

BiSSE and MuSSE models have been shown to detect spurious correlations between character states and diversification rates [124]. To evaluate whether this was the case with our data, we simulated discrete traits with three states on the Tyrannidae maximum-clade credibility tree using the *sim.char()* function of the Geiger R package [125] under a classic Markov model of discrete trait evolution, which does not incorporate state-dependent rate variation. We then fit a MuSSE model with equal extinction and different speciation rates (which was the best fit to our data), and one with equal speciation and extinction rates across states, to the simulated data using diversitree [48], and estimated the likelihood ratio statistic (LRS) between the two models. This allowed us to generate a null distribution of LRS given our tree, which we compared to the LRS between these two models obtained when using our migration data.

Simulations were performed using four different rate matrices (999 simulations per matrix): three symmetric matrices with all transition rates equal to 1, 10, and 50, and one with the maximum-likelihood transition rates obtained by fitting the best MuSSE model (one extinction and three speciation rates) to our migration data and the MCC tree. Likelihoods were optimized using the subplex method and run until either the likelihood increase between two iterations was lower than 2e-16, or 20,000 iterations were performed. Optimizations that ran for longer than eight days were interrupted and not included in analyses.

Speciation rates associated with migration and breeding latitude

Because recent speciation rates of birds are higher in temperate areas [54], we evaluated whether the pattern we observed (i.e., greater speciation rates in migratory lineages, which are concentrated at higher latitudes) reflect effects of breeding latitude as opposed to behavior. Because the Tyrannidae has intra-tropical as well as temperate-tropical migrants we tested whether speciation rates of migratory birds were similar and higher than those estimated for sedentary birds that breed in temperate and tropical environments separately. We tested for associations between migratory strategy and breeding latitude in the Tyrannidae by fitting

MuSSE models to estimate speciation and extinction rates associated to each of the following character states: migratory-temperate breeders, sedentary-temperate breeders, migratory tropical-breeders, sedentary topical-breeders, migratory temperate and tropical breeders, and sedentary temperate and tropical breeders. We ran an *MCMC* analysis on the MCC tree as described above, but starting from the maximum likelihood estimates for all parameters.

DATA AND CODE AVAILABILITY

The mtDNA sequences generated during this study are available at GenBank (accession numbers GenBank: MN895933-MN895996), and GBS sequences at NCBI's SRA (BioProject number SRA: PRJNA600207; accession numbers BioSample: SAMN13824682-SAMN13824776). Code, raw morphological data, phylogenetic trees, and simulation data are available at Figshare repository under project Figshare: 73719 (https://figshare.com/projects/Speciation_and_loss_of_migration_in_New_world_flycatchers_Tyrannidae_/73719).