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The Importance of Recent Ice Ages in Speciation: A Failed Paradigm

John Klicka and Robert M. Zink

the contact area while avoiding steric clashes. Although VRC is distant from VRA and VRB within an Fr-RBD monomer, VRC from one subunit is close to VRB of the adjacent subunit of the trimer model, such that there are only three variable lobes per trimer, with each being composed of sequences from two Fr-RBD subunits (D. Fass, thesis, Massachusetts Institute of Technology, Cambridge, 1997).

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40. We thank S. C. Harrison, S. J. Gamblin, and D. C. Wiley for helpful discussions and N. Azubine for media preparation. J.M.B. is a Whitehead Fellow and acknowledges support from the W. M. Keck Foundation. This work was funded by the Howard Hughes Medical Institute and utilized the W. M. Keck Foundation X-ray Crystallography Facility at the Whitehead Institute. The coordinates have been deposited in the Protein Data Bank with accession number 1AOL.

Late Pleistocene glaciations have been ascribed a dominant role in sculpting present-day diversity and distributions of North American vertebrates. Molecular comparisons of recently diverged sister species now permit a test of this assertion. The Late Pleistocene Origins model predicts a mitochondrial DNA divergence value of less than 0.5 percent for avian sister species of Late Pleistocene origin. Instead, the average mitochondrial DNA sequence divergence for 35 such songbird species pairs is 5.1 percent, which exceeds the predicted value by a factor of 10. Molecular data suggest a relatively protracted history of speciation events among North American songbirds over the past 5 million years.

Evidence from molecular systematics has provided fresh insights into several long-standing controversies regarding avian evolution, including the origin of birds (1), the origin and distribution of modern-day avian orders (2), and the survival of avian lineages across the Cretaceous-Tertiary boundary (3). The timing of the origin of modern bird species remains unclear. Many authors (4, 5) postulate a recent origin for North American songbird species (Order Passeriformes) and species complexes.

These origins are typically associated with Late Pleistocene glacial cycles (6–8) involving (i) fragmentation of a widespread ancestral species into refugia during periods of glacial advance and (ii) subsequent genetic divergence among small isolated populations, followed by (iii) range expansion during interglacials. Typically, one [beginning circa (ca.) 100,000 years ago] or two (ca. 250,000 years ago) such cycles are invoked. This model, here termed the Late Pleistocene Origins (LPO) model, is widely accepted today [see (9) for example].

If mitochondrial DNA (mtDNA) evolves at a clocklike (10) rate of 2% per million years (My) (11), then a plot of divergence values for sister species of Late Pleistocene origin should be strongly left-skewed and leptokurtic. Invoking either one or two glacial cycles, mtDNA sequences of species pairs should differ on average by 0.2 to 0.5%. The LPO model also predicts that phylogenetic analyses of mtDNA sequences (haplotypes) from recently separated species will result in trees that do not reflect recognized species (taxonomic) limits. That is, haplotypes in one species can be more closely related to haplotypes in the sister species than to those in their own (12). We tested the LPO paradigm directly by analyzing most of the

songbird taxa used to construct it. Table 1 depicts all North American songbird species (13) for which Late Pleistocene origins have been postulated (4, 5) and for which comparative mtDNA data (14) are now available (16–19). These comparisons represent the best estimates of divergence times among what are presumed to be the most recently evolved songbird species. The plot of observed divergence values (Fig. 1) is neither left-skewed nor leptokurtic. The average percent divergence for the 35 taxon pairs is 5.1% (SD \pm 3.0), which suggests an average Late Pleistocene divergence time of 2.45 million years ago (Ma) (20). This estimated divergence time and a divergence time consistent with a Late Pleistocene origin differ by an order of magnitude. Alternatively, if the molecular clock is improperly calibrated and our average percent divergence does correctly reflect genetic change occurring since the beginning of the last glacial advance (assume 5.1% change per ca. 100,000 years), two taxa isolated for 1 My would differ by 50%. Multiple substitutions at the same nucleotide position and eventual DNA saturation make a figure this high improbable, and no such saturation effects were detected in the taxon pairs examined.

An additional test of the LPO model derives from comparing the 35 “Late Pleistocene” species pairs with 13 pairs of species not specifically theorized to have evolved in the Late Pleistocene (21). The mean mtDNA pairwise distance for these presumably older songbird pairs is 5.2% (SD \pm 2.3), a value not significantly different from that of the 35 “Late Pleistocene” pairs (Mann-Whitney U test, $P = 0.48$). Furthermore, the distributions of mtDNA distances for the two groups are not different (Kolmogorov-Smirnov two-group test; $\chi^2 = 1.4$, df = 2, $P = 0.50$). These results contradict the expectations of the LPO model. Overall, these data

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reflect a protracted history of speciation throughout the Pleistocene and Pliocene. Inspection of Fig. 1, however, reveals evidence of regional and temporal pulses of diversification. For example, the North American Great Plains is a region where many morphologically and ecologically similar pairs of species that “seemingly differentiated in allopatry during the

Table 1. Estimates of mtDNA percent sequence divergence and estimated ages for pairs of North American songbird species (13) that have been postulated to have differentiated during most recent Pleistocene glacial advances (4, 5). Standard errors are shown when available. ND2, NADH subunit 2; ND6, NADH subunit 6.

Taxon pair	Sequence divergence (%)	Estimated age (2% divergence/My)	Data type	Source [see (18)]
<i>Parus bicolor</i> versus <i>P. inornatus</i> (Tufted and Plain titmice)	7.4	3,700,000	RFLP, 19 endonucleases, 134 restriction sites	h
<i>Parus b. bicolor</i> versus <i>P. b. atricristatus</i> (Tufted and Black-crested titmice)	0.4	200,000	RFLP, 19 endonucleases, 83 restriction sites	a
<i>Quiscalus major</i> versus <i>Q. mexicanus</i> (Boat-tailed and Great-tailed grackles)	1.6	800,000	RFLP, 19 endonucleases, 80 restriction sites	a
<i>Parus atricapillus</i> versus <i>P. carolinensis</i> (Black-capped and Carolina chickadees)	4.6	2,300,000	RFLP, 15 endonucleases, 48 restriction sites	b
<i>Sturnella magna</i> versus <i>S. neglecta</i> (Eastern and Western meadowlarks)	5.3	2,650,000	RFLP, 9 endonucleases, 171 restriction sites	c
<i>Poliophtila melanura</i> versus <i>P. nigriceps</i> (Black-tailed and Black-capped gnatcatchers)	4.8	2,400,000	Sequence, cytochrome (cyt) b, ND2, ND6, 922 nucleotides	d
<i>Poliophtila melanura</i> versus <i>P. californica</i> (Blacktailed and California gnatcatchers)	4.0	2,000,000	Sequence, cyt b, ND2, ND6, 922 nucleotides	d
<i>Pipilo erythrophthalmus</i> versus <i>P. Maculatus</i> (Rufous-sided and Spotted towhees)	0.8	400,000	RFLP, 16 endonucleases	f
<i>Dendroica c. coronata</i> versus <i>D. c. auduboni</i> (Myrtle and Audubon's warblers)	0.6	300,000	RFLP, 14 endonucleases, 49 restriction sites	g
<i>Dendroica townsendi</i> versus <i>D. occidentalis</i> (Townsend's and Hermit warblers)	0.7	350,000	RFLP, 14 endonucleases, 49 restriction sites	g
<i>Dendroica townsendi</i> versus <i>D. virens</i> (Townsend's and Black-throated Green warblers)	2.5	1,250,000	RFLP, 14 endonucleases, 49 restriction sites	g
<i>Ammodramus caudacutus</i> versus <i>A. nelsoni</i> (Saltmarsh and Nelson's Sharp-tailed sparrows)	1.3	650,000	RFLP, 17 endonucleases, 82 restriction sites	i
<i>Ammodramus maritimus</i> ssp. (Gulf and Atlantic coastal forms)	1.0	500,000	RFLP, 18 endonucleases, 89 restriction sites	j
<i>Piranga olivacea</i> versus <i>P. ludoviciana</i> (Scarlet and Western tanagers)	6.4 (±0.75)	3,200,000	Sequence, cyt b, 1050 nucleotides	k
<i>Passerina cyanea</i> versus <i>P. amoena</i> (Indigo and Lazuli buntings)	6.6 (±0.78)	3,300,000	Sequence, cyt b, 1050 nucleotides	k
<i>Passerina cyanea</i> versus <i>P. versicolor</i> (Indigo and Varied buntings)	6.4 (±0.77)	3,200,000	Sequence, cyt b, 1050 nucleotides	k
<i>Sialia sialis</i> versus <i>S. mexicana</i> (Eastern and Western bluebirds)	5.0 (±0.67)	2,500,000	Sequence, cyt b, 1050 nucleotides	k
<i>Cardinalis cardinalis</i> versus <i>C. sinuatus</i> (Northern Cardinal and Pyrrhuloxia)	8.7 (±0.87)	4,350,000	Sequence, cyt b, 1050 nucleotides	k
<i>Calcarius lapponicus</i> versus <i>C. mccownii</i> (Lapland and McCown's longspurs)	8.7 (±0.87)	4,350,000	Sequence, cyt b, 1050 nucleotides	k
<i>Calcarius lapponicus</i> versus <i>C. ornatus</i> (Lapland and Chestnut-collared longspurs)	9.2 (±0.89)	4,600,000	Sequence, cyt b, 1050 nucleotides	k
<i>Oporornis philadelphia</i> versus <i>O. tolmiei</i> (Mourning and McGillivray's warblers)	2.1 (±0.44)	1,050,000	Sequence, cyt b, 1050 nucleotides	k
<i>Oporornis philadelphia</i> versus <i>O. agilis</i> (Mourning and Connecticut warblers)	7.3 (±0.80)	3,650,000	Sequence, cyt b, 1050 nucleotides	k
<i>Spizella pallida</i> versus <i>S. breweri</i> (Clay-colored and Brewer's sparrows)	6.1 (±0.74)	3,050,000	Sequence, cyt b, 1050 nucleotides	k
<i>Pheucticus ludovicianus</i> versus <i>P. melanocephalus</i> (Rose-breasted and Black-headed grosbeaks)	4.4 (±0.63)	2,200,000	Sequence, cyt b, 1050 nucleotides	k
<i>Cyanocitta cristata</i> versus <i>C. stelleri</i> (Blue and Steller's jays)	10.7	5,350,000	Sequence, cyt b, 1032 nucleotides	k
<i>Amphispiza belli</i> versus <i>A. bilineata</i> (Sage and Black-throated sparrows)	10.9	5,450,000	Sequence, cyt b, 288 nucleotides	e
<i>Pipilo aberti</i> versus <i>P. crissalis</i> (Abert's and California towhees)	2.1	1,050,000	Sequence, cyt b, ND2, 744 nucleotides	l
<i>Pipilo aberti</i> versus <i>P. fuscus</i> (Abert's and Canyon towhees)	5.7	2,850,000	Sequence, cyt b, ND2, 744 nucleotides	l
<i>Toxostoma rufum</i> versus <i>T. longirostre</i> (Brown and Long-billed thrashers)	6.3	3,150,000	Sequence, cyt b, ND2, ND6, 944 nucleotides	l
<i>Toxostoma lecontei</i> versus <i>T. redivivum</i> (Leconte's and California thrashers)	8.1	4,025,000	Sequence, cyt b, ND2, ND6, 944 nucleotides	l
<i>Toxostoma lecontei</i> versus <i>T. crissale</i> (Leconte's and Crissal thrashers)	8.3	4,125,000	Sequence, cyt b, ND2, ND6, 944 nucleotides	l
<i>Toxostoma redivivum</i> versus <i>T. crissale</i> (California and Crissal thrashers)	7.1	3,550,000	Sequence, cyt b, ND2, ND6, 944 nucleotides	l
<i>Toxostoma bendirei</i> versus <i>T. cinereum</i> (Bendire's and Gray thrashers)	1.7	825,000	Sequence, cyt b, ND2, ND6, 944 nucleotides	l
<i>Icterus galbula</i> versus <i>I. bullockii</i> (Baltimore and Bullock's orioles)	4.7	2,350,000	Sequence, cyt b, 530 nucleotides	m
<i>Agelaius phoeniceus</i> versus <i>A. tricolor</i> (Red-winged and Tricolored blackbirds)	6.7	3,350,000	Sequence, cyt b, 890 nucleotides	m
Mean values (n = 35)	5.1 (±3.0)	2,550,000		

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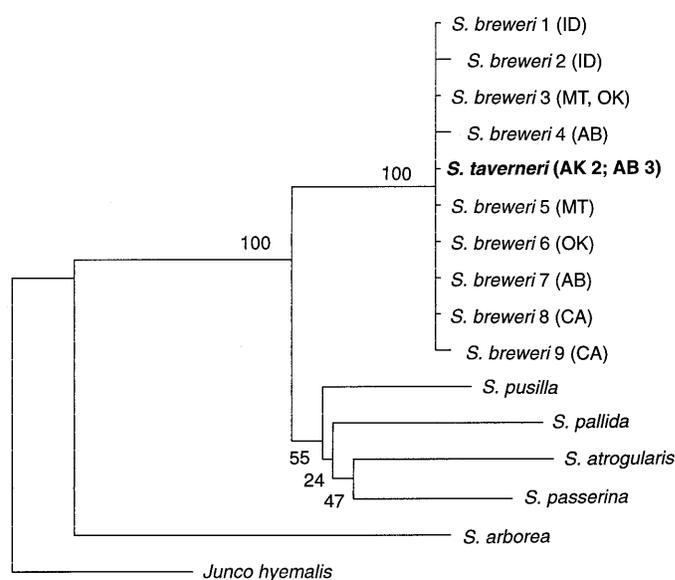
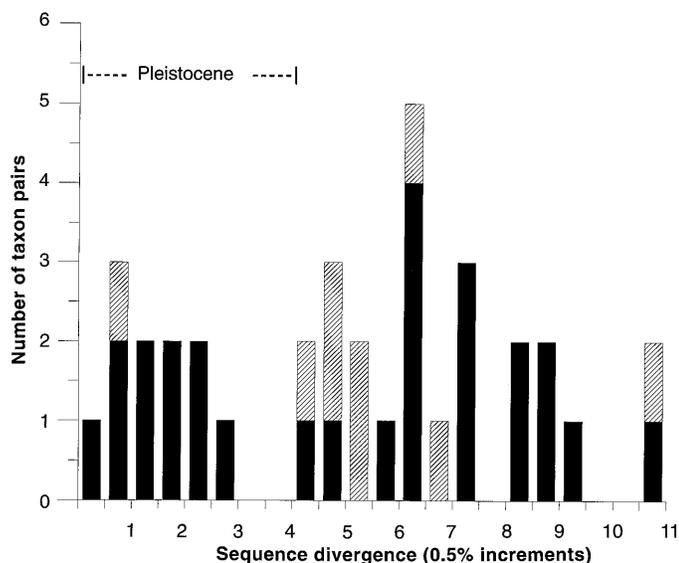


Fig. 1 (left). Plot of estimated percent sequence divergence for haplotypes representing 35 pairs of North American songbird taxa (see Table 1). This distribution is not significantly non-normal ($P > 0.1$). Cross-hatched portions of bars represent Great Plains species pairs. **Fig. 2 (right).** A neighbor-joining tree based on a Kimura two-parameter (14, 15) distance matrix for all

members of the avian genus *Spizella*. The tree is rooted at *Junco*, with numbers indicating node strength over 1000 bootstrap replications. The average pairwise distance among *pusilla*, *pallida*, *atrogularis*, and *passerina* is 5.7% (SD \pm 0.5). U.S. state and Canadian province abbreviations are given in parentheses.

Pleistocene" (22) are in secondary contact. The mean sequence divergence among these species pairs is 5.3% (SD \pm 1.8), and only one value is less than 4%. The clustering of several values (Fig. 1) near the Pliocene-Pleistocene boundary reveals a probable regional pulse of speciation, but at a time much earlier than is generally assumed.

We know of only one songbird species pair whose genetic characteristics are wholly consistent with a Late Pleistocene origin. The Timberline Sparrow [*Spizella (breweri) taverneri* (23)] is similar genetically [$<0.1\%$ sequence divergence (24)] to its sister taxon, the Brewer's Sparrow [*Spizella (breweri) breweri*]. Over a 1450-base pair (bp) span of mtDNA, 12 *breweri* specimens differed from 7 of *taverneri* at a single fixed nucleotide position (24), yielding an estimated divergence time of 35,000 years ago. Extremely low divergence values, however, are not the only expected genetic signature of recent speciation. The lack of reciprocal monophyly (12) reflected in the topology of a *breweri* and *taverneri* haplotype tree (Fig. 2) confirms recent divergence (25). That no other such topologies are known for songbird species pairs is further evidence against the LPO model.

Differing interpretations of the avian fossil record have led to controversy over the timing of the origin of songbirds. Wetmore (26) advocated Late Pliocene origins, whereas others [for example, see (7)] subsequently postulated a Late Pleistocene

radiation. Recent molecular studies (16, 17) of single avian genera again point to somewhat earlier origins. Our comprehensive analyses of mtDNA divergence values and haplotype trees now establish that the majority of North America's "youngest" species have Early Pleistocene or Late Pliocene origins.

The LPO model follows from the premise that glacial cycles provided conditions conducive to speciation (27). However, ice sheets grew large in the Northern Hemisphere beginning 2.4 Ma (28), and climatic oscillations sufficient to produce major changes in flora and fauna are now dated to the Tertiary (8). Thus, large-scale geographic shuffling, splitting, and bottlenecks of populations have been occurring since at least that time. The data presented here (Fig. 1) are congruent with this view in suggesting a relatively continuous history of speciation events. Periodic glacial cycles may have strongly influenced the diversification of the North American songbird fauna, but if so, these events occurred much earlier than is typically proposed (4, 5). Our results show that evidence of Late Pleistocene diversification for songbirds will more likely be found among geographically segregated conspecific populations and subspecies (8) but not among traditionally recognized sister species. The most recent glaciations were not, it seems, the force driving songbird diversification so much as they functioned as an ecological obstacle course through which only some spe-

cies were able to persist (29). The entrenched paradigm proclaiming that many North American songbird species originated as a consequence of these glaciations is flawed.

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10. Our conclusions rely on a molecular "clock" that is presumed to "tick" at a constant rate. The topic of molecular clocks is contentious [J. C. Avise, *Molecular Markers, Natural History and Evolution* (Chapman & Hall, New York, 1994)]. Caveats include (i) nucleotide substitution rate heterogeneity, presumably caused by variation in metabolic rate, body size, and generation time [A. P. Martin and S. R. Palumbi, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4087 (1993)]; (ii) population history (frequency of bottlenecks, for example); and (iii) among-taxon variation in constraints on mutation and fixation [D. P. Mindell and C. E. Thacker, *Annu. Rev. Ecol. Syst.* **27**, 279 (1996)]. Within-genome rate heterogeneity ("hypervariable" noncoding regions, for example) may also influence

- rate calculations. To mitigate these sources of bias, we confined comparisons to a single group of birds, the oscine passerines. We compare taxa only when the overall mtDNA genome was sampled with restriction fragments [restriction fragment length polymorphisms (RFLPs)] or sequence data were available for coding genes. Differences between closely related species are primarily third base position transitions, and such changes probably evolve in a clocklike manner.
11. Independent clock calibrations for a diverse array of avian taxonomic orders support a mtDNA substitution rate of 2%/My. These include: geese {Anseriformes [G. F. Shields and A. C. Wilson, *J. Mol. Evol.* **24**, 212 (1987)], 2.0%/My}; Old-world partridges and fowl [Galliformes [E. Randi, *Mol. Phylogenet. Evol.* **6**, 214 (1996)], 2.0%/My}; cranes {Gruiformes [C. Krajewski and D. G. King, *Mol. Biol. Evol.* **13**, 21 (1996)], 0.7 to 1.7%/My}; albatrosses [Procellariiformes [G. B. Nunn, J. Cooper, P. Jouventin, C. J. R. Robertson, G. G. Robertson, *Auk* **113**, 784 (1996)], 0.65%/My}; Hawaiian honeycreepers [Passeriformes [C. L. Tarr and R. C. Fleischer, *ibid.* **110**, 825 (1993)], 2.0%/My}, and New-world quail [Galliformes (R. M. Zink, unpublished data), 2.0%/My].
 12. This phenomenon is termed lineage sorting. Alternatively, haplotype trees from species isolated for a sufficient period do reflect species (taxonomic) limits (termed reciprocal monophyly).
 13. Species studied traditionally have been considered sisters, although recent work has shown that some are not sisters but rather are members of species complexes. All pairs and complexes are thought to have Late Pleistocene origins (5). Three subspecies pairs are included because they qualify as phylogenetic species; their inclusion makes the tests more conservative. Nomenclature follows the most recent AOU Check-list [American Ornithologists' Union, *Check-list of North American Birds* (Allen Press, Lawrence, KS, ed. 6, 1983)] and subsequent supplements.
 14. We compared both mtDNA coding gene sequences and RFLPs, which provide comparable estimates of songbird sequence divergence (15). A subset ($n = 12$; those shown in Fig. 1 with standard errors) of sequence data having an uncorrected average percent divergence of 6.3 (± 0.76) has a Kimura two-parameter [S. Kumar, K. Tamura, M. Nei, *MEGA: Molecular Evolutionary Genetic Analysis* (Pennsylvania State Univ., University Park, PA, 1993), version 1.01] corrected distance of 6.7 (± 0.86). The similarity of these values indicates little saturation.
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 18. In the following, letters a through m correspond with the molecular study references shown in Table 1: a [J. C. Avise and R. M. Zink, *Auk* **105**, 516 (1988)]; b (16); c (19); d [R. M. Zink and R. C. Blackwell, *Mol. Phylogenet. Evol.*, in press]; e [N. K. Johnson and C. Cicero, in *Acta XX Congressus Internationalis Ornithologici* (New Zealand Ornithological Congress Trust Board, Wellington, New Zealand, 1990), pp. 601–610]; f [R. M. Ball Jr. and J. C. Avise, *Auk* **109**, 626 (1992)]; g (17); h [F. B. Gill and B. Silkas, *Condor* **94**, 20 (1992)]; i [J. D. Rising and J. C. Avise, *Auk* **110**, 844 (1993)]; j [J. C. Avise and W. S. Nelson, *Science* **243**, 646 (1989)]; k [J. Klicka, this study]; l [R. M. Zink, unpublished data]; and m [K. Omland and S. Lanyon, unpublished data].
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 20. Species are younger than the ages suggested by the coalescence times of their respective haplotypes, but this bias is minimal [W. S. Moore, *Evolution* **49**, 718 (1995)]. Edwards [in *Avian Molecular Systematics and Evolution*, D. P. Mindell, Ed. (Academic Press, New York, 1997), pp. 251–278] estimated an interspecific distance correction of 350,000 years, which would have the maximum effect of shifting values in Fig. 1 one column to the left.
 21. Those 13 pairs are as follows: Mexican and Chestnut-backed chickadees (*Parus sclateri* versus *P. rufescens*, 4.75%), Black-capped and Mountain chickadees (*Parus atricapillus* versus *P. gambeli*, 4.1%) (16); Rusty and Brewer's blackbirds (*Euphagus carolinus* versus *E. cyanocephalus*, 5.4%), Hooded and Orchard orioles (*Icterus cucullatus* versus *I. spurius*, 4.6%), Bronzed and Shiny cowbirds (*Molothrus aeneus* versus *M. bonariensis*, 2.0%) (19), Baird's and Henslow's sparrows (*Ammodramus bairdii* versus *A. henslowii*, 4.9%), Seaside and Sharp-tailed sparrows (*Ammodramus maritimus* versus *A. caudacutus*, 2.15%) [R. M. Zink and J. C. Avise, *Syst. Zool.* **39**, 148 (1990)], Bell's and White-eyed vireos (*Vireo bellii* versus *V. griseus*, 5.7%), Philadelphia and Warbling vireos (*Vireo philadelphicus* versus *V. gilvus*, 7.85%), Black-capped and Solitary vireos (*Vireo atricapillus* versus *V. solitarius*, 10.8%), Red-eyed and Black-whiskered vireos (*Vireo olivaceus* versus *V. altiloquus*, 4.5%) [B. W. Murray, W. B. McGillivray, J. C. Barlow, R. N. Beech, C. Strobeck, *Condor* **96**, 1037 (1994)], Swamp and Lincoln's sparrows (*Melospiza georgiana* versus *M. lincolni*, 3.6%), and Black-chinned and Field sparrows (*Spizella atrogularis* versus *S. pusilla*, 6.5%) (R. M. Zink, unpublished data).
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 24. DNA methods followed standard protocols (15). Primers for polymerase chain reaction included L14841 [T. D. Kocher *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6196 (1989)] and H4A [J. Harshman, thesis, University of Chicago (1996)] for cytochrome b (1050 bp), and LGL2 and H417 [C. L. Tarr, *Mol. Ecol.* **4**, 527 (1995)] for control region I (400 bp).
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 30. We thank D. Alstad, J. Curtsinger, R. Shaw, G. Hewitt, J. Avise, F. McKinney, S. Lanyon, S. Weller, C. Tarr, R. Fleischer, R. Sikes, and A. Kessen for comments. K. Omland and S. Lanyon provided unpublished data. We thank Louisiana State University (F. Sheldon) and the University of Washington (S. Edwards) for tissue samples. This work was funded in part by the Dayton and Wilkie Natural History Funds and NSF.

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Regulation of Human Placental Development by Oxygen Tension

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Cytotrophoblasts, specialized placental cells, proliferate early in pregnancy and then differentiate into tumor-like cells that establish blood flow to the placenta by invading the uterus and its vasculature. In this study, cytotrophoblasts cultured under hypoxic conditions (2 percent oxygen), mimicking the environment near the uterine surface before 10 weeks of gestation, continued proliferating and differentiated poorly. When cultured in 20 percent oxygen, mimicking the environment near uterine arterioles, the cells stopped proliferating and differentiated normally. Thus, oxygen tension determines whether cytotrophoblasts proliferate or invade, thereby regulating placental growth and cellular architecture.

The human placenta's unique anatomy (Fig. 1) is due in large part to differentiation of its epithelial stem cells, termed cytotrophoblasts (1). How these cells differentiate determines whether chorionic villi, the placenta's functional units, float in maternal blood or anchor the conceptus to the uterine wall. In floating villi, cytotrophoblasts differentiate by fusing to form multinucle-

ate syncytiotrophoblasts whose primary function—transport—is ideally suited to their location at the villus surface. In anchoring villi, cytotrophoblasts also fuse, but many remain as single cells that detach from their basement membrane and aggregate to form cell columns (Fig. 1A). Cytotrophoblasts at the distal ends of these columns attach to and then deeply invade the uterus [interstitial invasion (Fig. 1, A and C)] and its arterioles (endovascular invasion). As a result of endovascular invasion, the cells replace the endothelial and muscular linings of uterine arterioles, a process that initiates maternal blood flow to the placenta and greatly enlarges the vessel diameter. Paradoxically, the cells invade only the superficial portions of uterine venules. How this unusual behavior is regulated is unknown.

Our laboratory is studying the differen-

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