

PHYLOGEOGRAPHIC PATTERNS IN *MOTACILLA FLAVA* AND *MOTACILLA CITREOLA*: SPECIES LIMITS AND POPULATION HISTORY

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ABSTRACT.—We conducted phylogeographic analyses of *Motacilla flava* (Yellow Wagtail) and *M. citreola* (Citrine Wagtail). We analyzed mitochondrial DNA sequences from 167 *M. flava* specimens obtained from 17 localities throughout Eurasia and Alaska, and 38 specimens of *M. citreola* obtained from 7 Eurasian localities. Phylogenetic analysis revealed three clades within traditionally recognized *M. flava*: Europe and southwestern Asia, northeastern Eurasia, and southeastern Asia. Those groups should be considered species, because together they are not monophyletic, and are interspersed with *M. citreola*, *M. cinerea*, and *M. alba*. *Motacilla citreola* also is paraphyletic, consisting of two species-level groups. Northeastern and southeastern groups of *M. flava* each appear to be sister taxa to eastern and western groups of *M. citreola*, respectively. Together those four groups form a clade, whereas the western *M. flava* group is considerably more distant. Within each of the three groups of *M. flava*, and the two groups of *M. citreola*, little phylogeographic structure was detected. Signatures of past population expansion are evident for some populations of *M. flava*; expansion is more recent in Moscow, Kursk (western group), Yamal, and Anabar (northeastern group), and older in Tyva and Vyatka (western group). A history of population stability is inferred for the Yamal population of *M. citreola*. Nested-clade analyses detected contiguous range expansion for southeastern *M. flava* and restricted gene flow with isolation by distance for northeastern *M. flava* and eastern *M. citreola*. Received 15 October 2001, accepted 2 February 2003.

Резюме.—Нами проведен филогеографический анализ нуклеотидных последовательностей митохондриальных генов желтой (*Motacilla flava*) и желтоголовой (*M. citreola*) трясогузок. Образцы тканей были взяты от 167 *M. flava* из 17 популяций Евразии и Аляски, и 38 *M. citreola* из 7 Евразийских популяций. В рамках традиционно распознаваемого вида *M. flava* филогенетический анализ обнаружил существование трех линий, распространенных в Европе и Юго-западной Азии, в Северо-восточной Евразии и в Юго-восточной Азии. Поскольку эти линии не являются монофилетической группой, и на филогенетическом дереве разделены линиями желтоголовой, горной и белой трясогузок, целесообразно их выделение в отдельные виды. Парафилетическим также оказался вид *M. citreola*, западная и восточная линии которого заслуживают присвоения им видового статуса. На филогенетическом дереве северо-восточная линия желтой и восточная линия желтоголовой трясогузок являются сестринскими группами, также как и юго-восточная линия желтой и западная линия желтоголовой трясогузок. Западная линия желтой трясогузки оказалась значительно более удаленной от этих четырех линий, вместе образующих монофилетическую группу. Географическая структура распределения гаплотипов в каждой из пяти линий прослеживаются слабо. Признаки быстрого роста численности очевидны для некоторых популяций желтой трясогузки. Рост численности произошел раньше в популяциях из Тывы и Вятки (западная линия) и позже в популяциях из Москвы, Курска (западная линия), Ямала и Анабара (северо-восточная линия). Численность ямальской популяции желтоголовой трясогузки была стабильной на протяжении долгого времени. Анализ “иерархических монофилетических групп” (Nested Clade Analysis) показал расширение ареала юго-восточной желтой трясогузки и ограниченный обмен генов с изоляцией расстоянием в популяциях северо-восточной линии желтой и восточной линии желтоголовой трясогузок.

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MANY ASPECTS OF population history can be inferred from DNA sequences (Avice 2000, Nee et al. 1996). Phylogenetic analysis of DNA sequences yields a tree, which when superimposed over the geographic distribution of populations, reveals whether the history of populations has been one of isolation, panmixia, or some combination thereof. For example, haplotypes from different localities or regions might be reciprocally monophyletic, which suggests a history of isolation. Such a finding often challenges current species limits. Coalescence analyses contribute further information by revealing population increases and the magnitude and direction of gene flow (Hewitt 1996, Templeton 1998). Collectively, those analyses constitute phylogeography (Avice 2000).

Phylogeographic studies are valuable in showing how populations responded to the late Pleistocene cycles of glaciation, especially in the northern hemisphere. For example, Merila et al. (1997) compared mtDNA sequences from populations of Greenfinch (*Carduelis chloris*) distributed along a north-south transect in Europe. An unstructured haplotype phylogeny revealed no pattern of historical isolation. However, northward decrease in nucleotide diversity (π) was consistent with postglacial recolonization, a phenomenon termed "leading edge expansion" (Hewitt 2000). In another European species, the noctule bat (*Nyctalus noctula*), no genetic evidence was found that could be interpreted

as directional colonization and glacial-induced bottlenecks (Petit et al. 1999). In North America, the few studies available suggest that species have had idiosyncratic Pleistocene histories (Zink 1997, Fry and Zink 1998, Barrowclough et al. 1999). Unfortunately, little is known about broad-scale phylogeographic patterns of vertebrates distributed across Eurasia (Fedorov et al. 1999, Kryukov and Suzuki 2000, Salzburger et al. 2002), the largest expanse of land in the northern hemisphere. Eurasia was not extensively glaciated during the last glacial advance (Würm), unlike North America. However, permafrost covered large northern areas and many habitats were fragmented and displaced southward (Andersen and Borns 1994). We set out to characterize mtDNA phylogeography in a diverse set of Eurasian birds to document patterns of genetic differentiation and to discover genetic consequences of late Pleistocene climate changes (see Koblik et al. 2001; Rohwer et al. 2001; Zink et al. 2002a, b).

The Yellow Wagtail (*Motacilla flava*) is widely distributed throughout the Old World and has colonized Alaska in the New World (Cramp 1988). Variation within that species has been recognized by the description of two subspecies groups, *lutea* and *flava*, each containing numerous subspecies and sometimes treated as separate species (Badyaev et al. 1998, Koblik et al. 2001; Table 1). There are several hybrid zones (Czikeli 1985), and often mixtures of pheno-

TABLE 1. Traditional taxonomy and scoring of 6 morphological characters for 13 taxa of *Motacilla* males. Codes: ol = olive, olgn = olive-green, ygn = yellow-green, yol = yellow-olive, lgy = light gray, gy = gray, dgy = dark gray, dgn = dark green, bl = black, y = yellow, w = white, - absent, + present. Taxonomic codes for *M. flava*: L = *M. f. lutea* complex, F = *M. f. flava* complex (1 - *flava* group, 2 - *feldegg* group, 3 - *thunbergi* group; from Cramp [1988]). MtDNA groupings from Figure 2; WE = west, EA = east, NE = northeast, SE = southeast.

	MtDNA group	Taxonomic code	Crown	Back color	Breast spots	Auricular patch color	Super-cilium	Chin color
<i>flava lutea</i>	WE	L	y	ygn	-	y	y	y
<i>flava flava</i>	WE	F-1	gy	yol	-	dgy	w	w
<i>flava beema</i>	WE	F-1	lgy	yol	-	lgy	w	w
<i>flava leucocephala</i>	WE	F-1	w	ygn	-	w	-	w
<i>flava feldegg</i>	WE	F-2	bl	olgn	-	bl	-	y
<i>flava thunbergi</i>	WE	F-3	dgy	olgn	+	bl	-	y
<i>flava plexa</i>	NE	F-3	dgy	ol	+	bl	-	y
<i>flava tschutschensis</i>	NE	F-3	gy	ol	+	dgy	w	w
<i>flava taiwana</i>	SE	L	ygn	olgn	-	dgn	y	y
<i>flava macronyx</i>	SE	F-3	dgy	olgn	-	dgy	-	w
<i>citreola werae</i>	WE	-	y	gy	-	y	-	y
<i>citreola citreola</i>	EA	-	y	dgy	-	y	-	y
<i>citreola quassatrix</i>	EA	-	y	gy	-	y	-	y

types associated with different subspecies exist over relatively broad geographic areas.

We sequenced parts of three mitochondrial gene regions in 167 individuals of *M. flava* representing 10 subspecies taken from 16 localities throughout Eurasia and one site in Alaska (Fig. 1, Table 2). Phylogenetic analysis of haplotypes revealed three groups of *M. flava* that were not monophyletic, and which were interspersed with two groups of *M. citreola* and some outgroups. Therefore we also report here a phylogeographic survey of *M. citreola* (38 individuals, seven localities). In addition to exploring discrepancies between the mtDNA gene tree and taxonomic limits, we determined whether groups of populations showed genetic signatures of population and range expansions similar to those documented for other European species (Hewitt 2000).

METHODS

Motacilla flava and *M. citreola* specimens were collected during the breeding season. Outgroup taxa (*M. clara*, *M. capensis*, *M. aguimp*, *M. cinerea*, *M. alba*, and *M. lugens*) were represented by up to two individu-

als. From all specimens, a study skin was preserved and deposited at the Burke Museum, University of Washington, Seattle; the Moscow State University Zoological Museum, Moscow, Russia; or the Bell Museum, University of Minnesota, St. Paul.

Tissue samples were stored in lysis buffer or liquid nitrogen. Genomic DNA isolation and purification followed either a modified Chelex (Zink et al. 1998) or phenol-chloroform protocol (Hillis et al. 1996). Mitochondrial gene regions (Table 3) were amplified via polymerase chain reaction (PCR) (Saiki et al. 1988). Polymerase chain reaction products were cleaned using a Qiaquick PCR Purification Kit (Qiagen, Valencia, California). Sequencing reactions were cleaned using standard Sephadex columns and sequenced on an ABI 310 automated sequencer using Dideoxy Terminator kit protocol. One-stranded automated sequencing was performed for *M. flava*; 15 sequences were compared in both directions with minimal correction needed (0.4% of uncertain bases were resolved). For *M. citreola* and outgroups, we sequenced both strands of ND3 and cytochrome *b* (*cyt b*). Sequences were aligned and edited using SEQUENCHER 3.1.1 (Gene Codes Corporation, Ann Arbor, Michigan). Sequence data have been deposited in GenBank (AF443466–AF443561, AF445463–445558, AF446393–446860). Mitochondrial origin of DNA was supported by sequencing disparate regions,

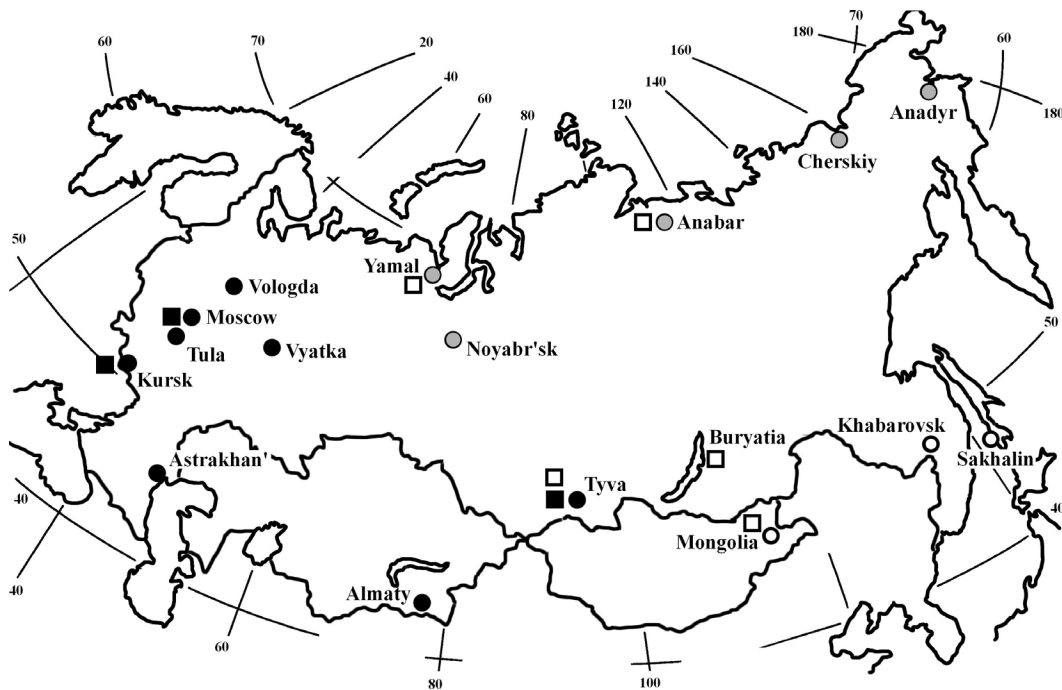


FIG. 1. Map of Eurasia showing general location of collecting sites. Black circles indicate western clade of *M. flava*, gray circles indicate northeastern *flava*, open circles indicate southeastern *flava*, black squares indicate western *M. citreola*, and open squares indicate eastern *citreola*.

TABLE 2. Genetic characteristics of *Motacilla flava* and *M. citreola* samples; see Figure 1 for locality information. Fu's F -values presented only if significant ($P < 0.05$).

Locality	Subspecies	N	Number of haplotypes	π	Fu's F
Western samples of <i>M. flava</i>					
Almaty	<i>feldegg</i>	6	6	0.0045	-2.90
Astrakhan'	<i>feldegg, flava</i>	7	7	0.0044	-
Kursk	<i>flava, beema</i>	12	12	0.0033	-8.54
Moscow	<i>flava, thunbergi</i>	18	14	0.0025	-7.29
Tula	<i>flava</i>	5	5	0.0041	-1.90
Vologda	<i>flava</i>	6	5	0.0027	-
Vyatka	<i>beema, flava, lutea</i>	19	19	0.0047	-13.02
Tyva	<i>beema, leucocephala</i>	17	12	0.0042	-3.53
Southeastern samples of <i>M. flava</i>					
Khabarovsk	<i>taivana</i>	5	5	0.0024	-
Sakhalin	<i>taivana</i>	6	4	0.0029	-
Mongolia	<i>macronyx</i>	9	8	0.0065	-
Northeastern samples of <i>M. flava</i>					
Alaska	<i>tschutschensis</i>	6	5	0.0022	-2.26
Anabar	<i>plexa</i>	10	8	0.0016	-2.97
Anadyr	<i>tschutschensis</i>	7	7	0.0022	-2.02
Noyabr'sk	<i>plexa</i>	8	7	0.0024	-3.00
Cherskiy	<i>plexa</i>	8	2	0.0005	-
Yamal	<i>plexa</i>	18	9	0.0016	-3.32
Western samples of <i>M. citreola</i>					
Kursk	<i>werae</i>	3	3	0.0024	-
Moscow	<i>werae</i>	2	2	0.0052	-
Tyva	<i>werae, werae × quassatrix</i>	8	5	0.0015	-
Eastern samples of <i>M. citreola</i>					
Tyva	<i>quassatrix</i>	5	4	0.0014	-
Mongolia	<i>quassatrix</i>	6	3	0.0016	-
Buryatia	<i>citreola</i>	2	2	0.0022	-
Anabar	?	1	1	0	-
Yamal	<i>citreola</i>	11	7	0.0020	-3.12

absence of stop codons, and the existence of a large number of haplotypes, all of which are inconsistent with nuclear copies (Zhang and Hewitt 1996).

We used ARLEQUIN software (Schneider et al. 2000) to compute π , F_{st} , Fu's (1997) test of selective neutrality, mismatch distributions (for localities with >10 individuals), the number of haplotypes (allowing in ARLEQUIN up to 20% missing data per site, and with gaps included), and to perform a Mantel's (1967) test of pairwise F_{st} values versus geographic distances. Nucleotide diversity provides an index of genetic variability and can reveal patterns of population expansion. We regressed nucleotide diversity against latitude, expecting smaller values to be in the north because of leading edge expansion (Hewitt 2000). The value of F_{st} measures the extent of population subdivision taking into account degree of haplotype differentiation. Fu's (1997) F value tests for departure from neutral expectation assuming an infinite-site model without recombination; it is sensitive to population expansions, which generate negative values owing to excess rare haplotypes.

The mismatch distribution is the distribution of pairwise base pair differences among individual haplotypes, and its shape provides information about recent changes in population size (Rogers and Harpending 1992). We used Schneider and Excoffier's (1999) finite-site method, which involves a parametric bootstrap estimate of confidence for the fit of the mismatch distribution to the distribution expected from sudden population expansion. The average number of individuals exchanged per generation among populations (Nm) was computed from pairwise population F_{st} values; those values are used as indications of relative magnitudes of gene flow, not the actual number of individuals moving between populations. Mantel's tests were used as an approximation of the magnitude of genetic diversity resulting from isolation by distance. Those tests compared a matrix of pairwise F_{st} values with straight-line geographic distances between samples; the null hypothesis is that the matrices are independent, so that rejection would indicate an isolation-by-distance effect.

TABLE 3. Number of base pairs sequenced for various gene regions. LCR4 and H1248 (Tarr 1995) amplified the control region (CR), L5215/H5578 (Hackett 1996) amplified ND2, L10702/H11289 (J. G. Groth pers. comm.) amplified ND3, and L14841 (Kocher et al. 1989)/H15299 (Hackett 1996) amplified *cyt b*. NA = not amplified.

Taxa	No. of individuals	CR	ND2	ND3	Cyt <i>b</i>	Total base pairs
<i>M. flava</i>	167	387	352	254	NA	993
<i>M. citreola</i>	38	458	388	561	NA	1407
<i>Motacilla</i> species	22	458	388	561	425	1832

Using *F* statistics or an algorithm that assumes that all geographical associations are due to gene flow can yield an estimator of *Nm* that is biologically misleading (Templeton 1998). We used nested-clade analysis (NCA; Templeton et al. 1995) because of its potential to distinguish between gene flow, past fragmentation, and range expansion. Nested-clade analysis uses a haplotype network to test the null hypothesis of no geographical association of haplotypes. Program TCS version 1.13 (Clement et al. 2000) was used to construct haplotype networks. Minor ambiguities (closed loops) were resolved using parsimony (by favoring associations of haplotypes from the same locality). Clades were nested according to rules described in Templeton et al. (1987). Program GEODIS version 2.0 (Posada et al. 2000) with 10,000 resampling events was used to calculate the clade distance (D_c , average geographic distance of haplotypes from the clade to the geographical center of that clade), nested-clade distance (D_n , average distance of haplotypes from the clade to the geographic center of all haplotypes at next nesting level), and difference between interior and tip clades ($I-T$). We used the inference key of Templeton (1998) to estimate causes of geographical associations of haplotypes.

For phylogenetic inference within major geographic groupings, we generated a neighbor-joining tree in PAUP* (Swofford 2000) and estimated the percentage of invariant sites, transition-transversion ratio, base frequencies, and shape parameter (γ) on that topology. Those values were then input into PAUP* and a heuristic maximum-likelihood search was performed. We re-estimated the input parameters on the resultant topology and used them in a second heuristic search to arrive at a maximum-likelihood topology. We used PAUP* to generate maximum-parsimony trees from equally weighted characters, and we constructed strict and 50% majority rule consensus trees. Tree topologies were independent of whether gaps were included.

To test species limits, we analyzed a subset of 22 haplotypes including divergent individuals within each taxon, which had been sequenced for additional base pairs (Table 3). We performed branch and bound parsimony searches on those haplotypes with characters equally weighted, excluding missing and ambiguous sites ($n = 208$; results did not differ if those sites were included). We conducted a maxi-

mum-likelihood search following the protocol outlined above and performed 100 bootstrap replicates. Alternative tree topologies were evaluated with the Shimodaira-Hasegawa (1999) test. We summarized taxonomic history and states of characters used by taxonomists to classify subspecies (Table 1). Those characters, equally weighted, were subjected to maximum parsimony analysis (exhaustive search).

RESULTS

TAXONOMIC FINDINGS

Both maximum-parsimony and maximum-likelihood analyses (not shown) for all individuals of all taxa resolved three groups of haplotypes currently classified as *M. flava*, corresponding to Europe and southwestern Asia, northeastern Asia, and southeastern Asia (Fig. 1). Those three groups did not form a clade. We also found two groups (eastern, western) of *M. citreola*, which were not sister groups. To explore that result further, we conducted phylogenetic analysis of the restricted set of 22 haplotypes (for 1,832 bp), which resolved 46 equally parsimonious trees, the consensus of which (not shown) matched a maximum-likelihood tree (Fig. 2) in showing lack of monophyly for the three groups of *M. flava* and two groups of *M. citreola*. A maximum-parsimony analysis including gaps revealed 8 trees, which were a subset of the 46 found without gaps. The western clade of *M. flava* was separated from the other *M. flava* and *M. citreola* clades by *M. cinerea* and *M. alba/lugens*. Furthermore, none of the trees recovered monophyly of the northeastern and southeastern clades of *M. flava* because different *M. citreola* clades were each sister to them (western *citreola* with southeastern *flava*, and eastern *citreola* with northeastern *flava*).

We tested alternative topologies, starting with one of the 46 equally parsimonious trees, which was similar to that shown in Figure 2. Making the two *M. citreola* groups monophyletic did

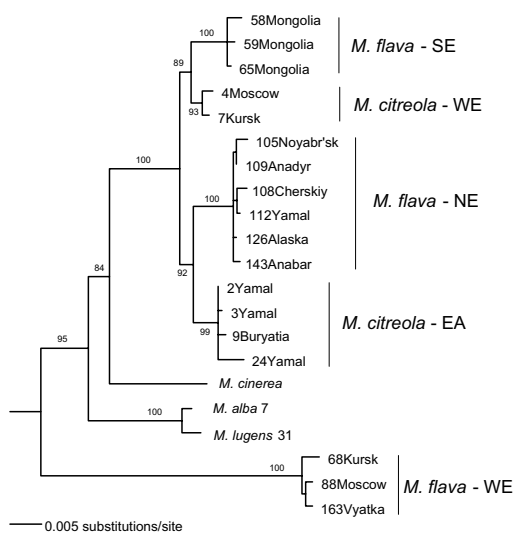


FIG. 2. Maximum likelihood tree showing relationships among major groups of *M. flava*, *M. citreola*, and near relatives, rooted with outgroups. Lab numbers precede locality names. Numbers above branches are percentages of 100 maximum likelihood bootstrap replicates supporting that branch. SE = southeast, WE = west, NE = northeast, EA = east.

not result in a significantly worse log likelihood score (Shimodaira–Hasegawa test; $P = 0.08$), nor did forcing the monophyly of the northeastern and southeastern *M. flava* groups (Shimodaira–Hasegawa test, $P = 0.08$). Rearranging the topology to make western *M. flava* sister to eastern *M. flava* plus *M. citreola* was significantly worse ($P = 0.01$). A tree with a monophyletic *M. flava* was significantly worse ($P < 0.001$). We subsequently treated each of the three groups of *M. flava* and two groups of *M. citreola* independently. Lastly, removing *M. alba/lugens*, primarily white-and-black-plumaged taxa, outside of western *M. flava*, thereby creating a “yellow” clade (all *M. flava*, *M. citreola* and *M. cinerea*), was not significantly worse ($P = 0.61$).

A maximum-likelihood test did not reject ($\chi^2 = 29.8$, $df = 20$, $P > 0.05$) the assumption of a molecular clock.

PHYLOGEOGRAPHY OF GEOGRAPHIC CLADES

Haplotype trees.—Among 90 individuals of *M. flava* from western Eurasia, there were 69 haplotypes. The most common haplotype was found in six individuals representing six localities. The data set contained 41 parsimony uninformative

and 31 informative base positions (including gaps but excluding outgroup sequence). We evaluated over 20,000 equally parsimonious trees (length 117, consistency index [CI] 0.67, rescaled consistency index [RC] 0.45), the consensus of which (not shown) was unstructured; however, 15 of the 17 haplotypes from Vyatka formed a clade (93% of trees) in a 50% majority rule consensus tree (and in the maximum-likelihood tree). One haplotype found in Vyatka was also found in Kursk, Moscow, and Vologda. Therefore, that locality apparently exhibits the early stages of evolutionary isolation. Because of the latter result, analyses below were done with and without the Vyatka sample. Subspecies designations did not predict clusters of haplotypes.

There were 31 haplotypes among the 57 individuals of *M. flava* from northeastern Eurasia. The most common haplotype was found in 6 localities and in 16 individuals. The data set contained 17 parsimony uninformative and 9 informative base positions (including gaps but excluding outgroup sequence; 993 bp total). We evaluated over 12,500 equally parsimonious trees (length = 32, CI = 0.88, RC = 0.72), the consensus of which (not shown) was unstructured; however, ~50% of haplotypes from Anabar clustered together. Subspecies designations did not predict clusters of haplotypes.

There were 17 haplotypes among 20 individuals of *M. flava* from three localities in southeastern Eurasia. The data set contained 13 parsimony uninformative and 13 informative base positions (including gaps but excluding outgroup sequence; 993 bp total). We evaluated 54 equally parsimonious trees (length 35, CI = 0.74, RC = 0.50), the consensus of which was unstructured (not shown), although several basal haplotypes were from Mongolia. Subspecies designations did not predict clusters of haplotypes.

Twenty-three haplotypes were found among 38 individuals of *M. citreola*. The data set of 1,407 bp (including gaps) contained 22 parsimony uninformative and 28 informative characters. We obtained over 20,500 equally parsimonious trees (length 62, CI = 0.81, RC = 0.76), the consensus of which (not shown) showed two distinct regional groupings, western (Moscow, Kursk, Tyva; subspecies *M. c. werae*) and eastern (Mongolia, Anabar, Buryatia, Yamal, Tyva; subspecies *M. c. citreola* and *M. c. quassatrix*) (Fig. 1). There were 9 haplotypes among 13 western and 14 haplotypes among 25 eastern

TABLE 4. Population pairwise F_{st} values for western *Motacilla flava* samples. An asterisk indicates $P < 0.05$.

	Kursk	Almaty	Moscow	Astrakhan'	Tula	Vologda	Vyatka
Kursk	0.0						
Almaty	0.049	0.0					
Moscow	0.0	0.06	0.0				
Astrakhan'	0.117*	0.0	0.157*	0.0			
Tula	-0.004	0.051	0.016	0.134*	0.0		
Vologda	0.037	0.125*	0.021	0.163*	0.006	0.0	
Vyatka	0.151*	0.163*	0.186*	0.172*	0.127*	0.171*	0.0
Tyva	0.090*	0.093	0.102*	0.137*	0.098	0.110*	0.184*

individuals. The most common haplotype was found in eight individuals representing four eastern localities. There was no phylogeographic structure in haplotype trees (not shown) for the eastern or western groups of *M. citreola*, but two individuals (31 Tyva and 11 Mong) were basal to a polytomy of eastern haplotypes.

Genetic diversity.—Values of F_{st} for the western region of *M. flava* was 0.13 ($P < 0.05$) with the sample from Vyatka included, and 0.08 ($P < 0.05$) without it. Pairwise F_{st} values (Table 4) ranged from 0.0 to 0.19, and most comparisons involving Astrakhan' and Vyatka were significant. Values of F_{st} for the northeastern group of *M. flava* was 0.11 ($P < 0.05$). Pairwise F_{st} values (Table 5) ranged from 0.02 to 0.27, and all comparisons involving Anabar were significant. Also, all values between northeastern and southeastern samples were significant. Values of F_{st} for the southeastern region of *M. flava* was 0.11 ($P < 0.05$). Pairwise F_{st} values (Table 5) ranged from 0.062 to 0.13.

For the eastern *M. citreola* clade F_{st} was 0.14 ($P < 0.05$) and pairwise F_{st} values ranged from 0.04 to 0.17. For the western clade F_{st} was 0.19 ($P < 0.05$) and pairwise F_{st} values ranged from 0.005 to 0.30 (Table 6).

Genetic variability.—Nucleotide diversity

(Table 2) for western samples of *M. flava* ranged from 0.0025 to 0.0047. Several F_u 's F values (Table 2) were significantly negative, which suggests either population expansion or deviation from selective neutrality. Nucleotide diversity among northeastern samples of *M. flava* ranged from 0.0005 to 0.0024, with lower values being found in the northeastern most localities (except Alaska). F_u 's F values were generally negative and significant. Nucleotide diversity for the three southeastern samples of *M. flava* ranged from 0.0024 to 0.0069 (Table 2); the southernmost sample (from Mongolia) had the highest value. F_u 's F values were not significant. For *M. citreola*, nucleotide diversity ranged from 0.0014 to 0.0022 for the eastern clade and 0.0015 to 0.0052 for the western clade. F_u 's F values were not significant except for the sample from Yamal. No significant associations between nucleotide diversity and latitude were found for each of five studied groups (Fig. 3). An overall test was not performed because our samples were taken from five independently evolving groups.

Population expansion and gene flow.—For our largest samples of *M. flava* (Moscow, Vyatka, Tyva, Kursk, Yamal, Anabar), mismatch distributions (Fig. 4 A–E, Kursk not shown) were

TABLE 5. Pairwise F_{st} values for eastern *Motacilla flava* samples. Note that all values between northeastern and southeastern samples are significant. An asterisk indicates $P < 0.05$.

	Sakhalin	Khabarovsk	Mongolia	Cherskiy	Anadyr	Yamal	Noyabr'sk	Alaska
Sakhalin	0.0							
Khabarovsk	0.128	0.0						
Mongolia	0.133*	0.062	0.0					
Cherskiy	0.930*	0.939*	0.810*	0.0				
Anadyr	0.885*	0.886*	0.767*	0.035	0.0			
Yamal	0.914*	0.915*	0.836*	-0.020	0.047	0.0		
Noyabr'sk	0.876*	0.875*	0.766*	0.075	0.025	0.056	0.0	
Alaska	0.885*	0.888*	0.763*	0.049	0.030	0.032	0.017	0.0
Anabar	0.897*	0.898*	0.797*	0.265*	0.129*	0.23*	0.195*	0.212*

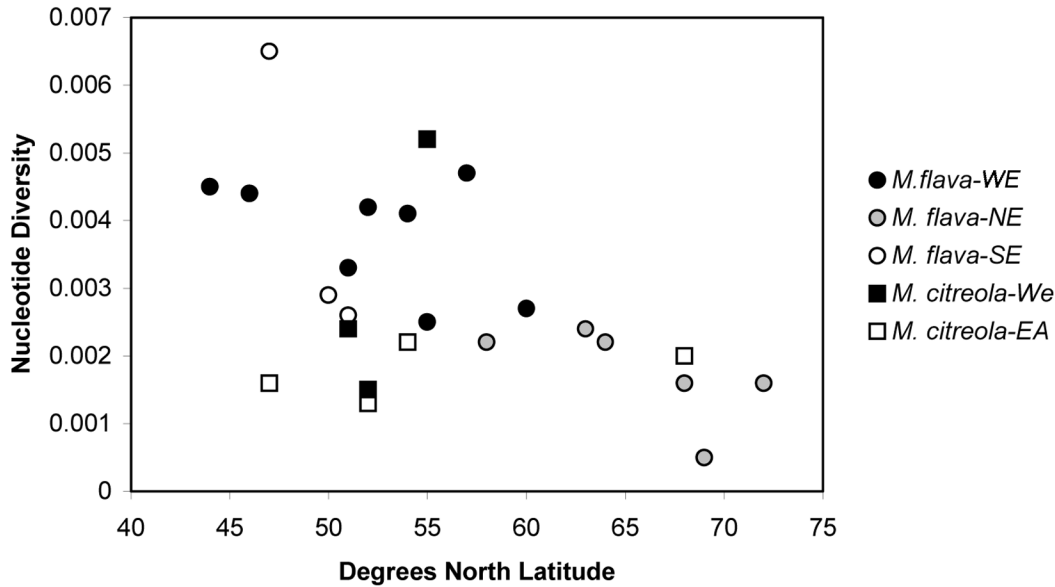


FIG. 3. Plot of nucleotide diversity and latitude in three groups of *M. flava* and two groups of *M. citreola*. Symbols correspond to those in Figure 1.

consistent with past population expansion. Values of Nm for western *M. flava* (not shown) ranged from 2.2 to very high values indicating gene flow between localities.

For northeastern samples of *M. flava*, Nm values (not shown) ranged from 1.4 to 29 indicating generally high gene flow. No mismatch distributions were computed for southeastern samples; Nm values were large.

The mismatch distribution for Yamal population of *M. citreola* (Fig. 4F) differed from the expectation for sudden population expansion ($P < 0.05$) and was bimodal, which indicates a history of population stability. Values of Nm within both *M. citreola* clades were >3.0 .

Mantel's tests were not significant for western and northeastern groups of *M. flava* (tests were

not performed on *M. citreola* and southeastern *M. flava* because too few sampling localities were available).

Nested-clade analyses.—Program TCS detected more than 10000 loops in the network of western *M. flava* haplotypes. It was not feasible to resolve those loops because of too many homoplasious characters (Posada and Crandall 2001).

For northeastern *M. flava* a single haplotype network was constructed. The final nested cladogram comprised thirty-three 0 step clades (haplotypes), thirteen 1 step clades, four 2 step clades, and the entire cladogram (Fig. 5). Haplotype 142Anab could have been grouped with one of three clades, 1-2, 1-12 or 1-11. We tested all alternatives and found that although nesting does affect the significance of clades

TABLE 6. Pairwise F_{st} values for *Motacilla citreola* samples. Note that western and eastern samples are significant. An asterisk indicates $P < 0.05$.

	Yamal	Buryatia	Mongolia	Tyva-quassatrix	Tyva-werae	Moscow
Yamal	0.0					
Buryatia	0.043	0.0				
Mongolia	0.048*	0.125	0.0			
Tyva-quassatrix	0.148*	0.174	0.104	0.0		
Tyva-werae	0.885*	0.908*	0.900*	0.909*	0.0	
Moscow	0.861*	0.826	0.869	0.884*	0.296	0.0
Kursk	0.867*	0.859	0.877*	0.891*	0.175	0.005

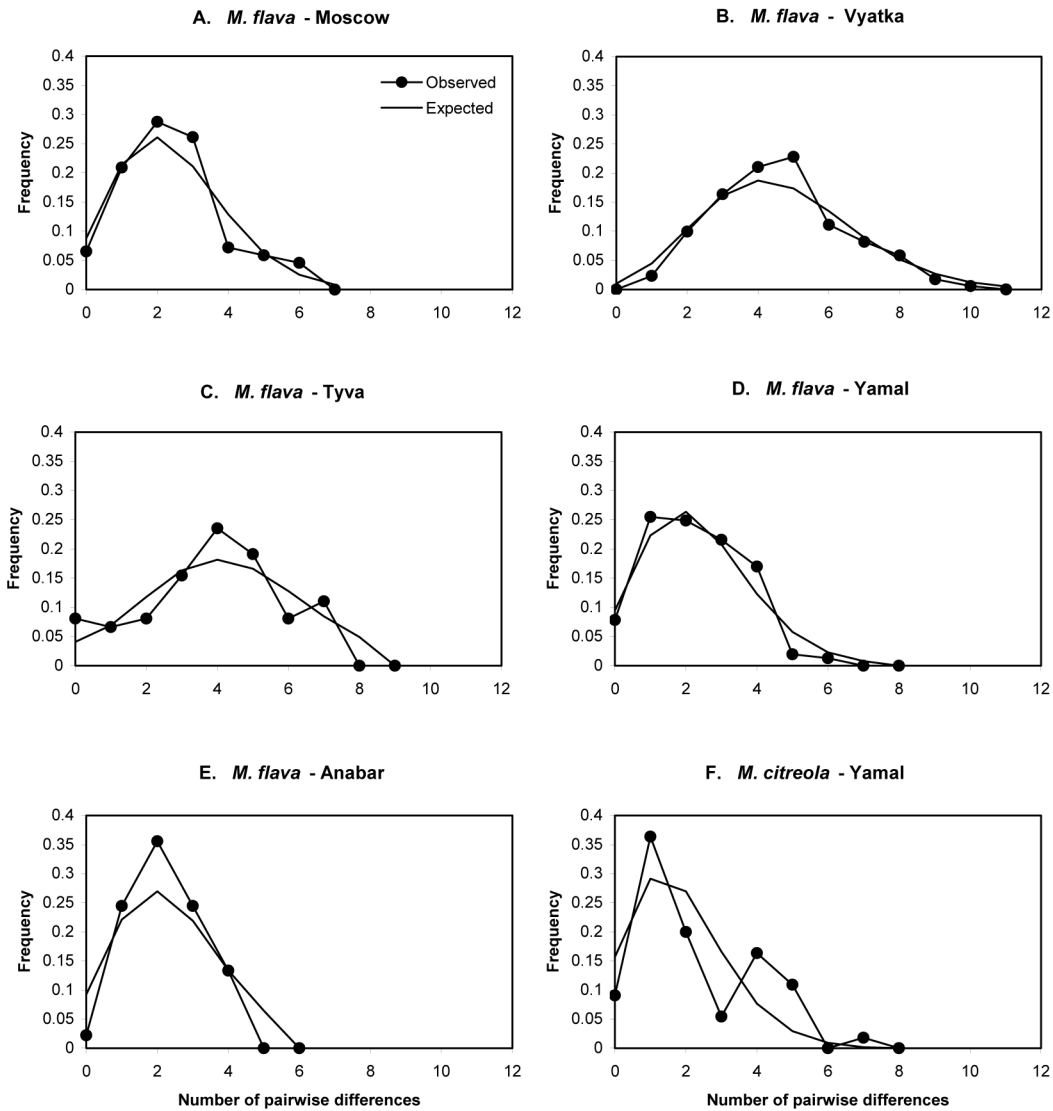


FIG. 4. Observed and expected mismatch distributions for *M. flava* (A = Moscow, B = Vyatka, C = Tyva, D = Yamal, E = Anabar), and *M. citreola* (F = Yamal).

it did not affect the inferences. Clade 1-2 displayed one significant value (D_n is large for 126,129 Alas) but that outcome was inconclusive. Clade 2-0 had significantly structured subclade 1-8, which consisted of three haplotypes from Yamal (D_c and D_n are significantly small), leading to an inference of restricted gene flow with isolation by distance (inference key 1-2-3-4no). The entire cladogram also displayed significant values (D_n is large for 2-0, D_c is small

for 2-3, the clade comprising five individuals from Anabar), also suggesting restricted gene flow with isolation by distance (inference key 1-2-3-4no). According to the assumptions of NCA, clade 1-2 is considered ancestral, because its geographical distribution covers all sampled localities (Templeton 1998). Many of tip clades in our analysis of northeastern *M. flava* have restricted ranges (Fig. 5), which is consistent with restricted gene flow.

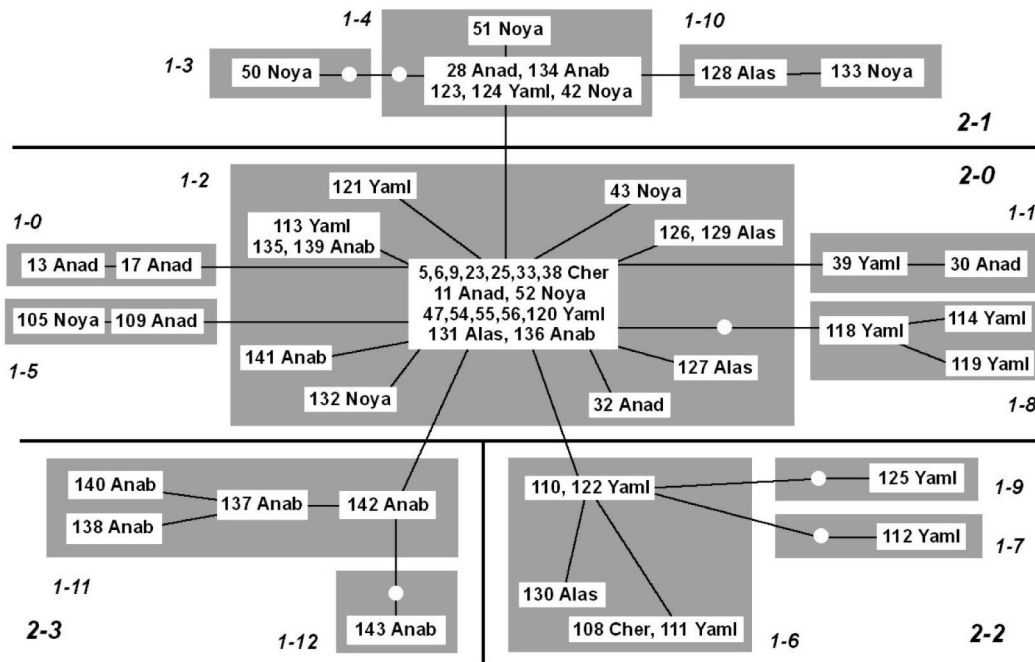


FIG. 5. Haplotype network for northeastern *M. flava*. Each line in the network represents a single mutation, white circles represent unsampled haplotypes, white rectangles represent 0 step clades (haplotypes), gray rectangles indicate 1 step clades, thick lines indicate 2 step clades. Haplotype numbers followed by the locality (see Fig. 1): Noya = Noyabr'sk, Anad = Anadyr, Alas = Alaska, Yaml = Yamal, Anab = Anabar, Cher = Cherskiy.

The cladogram (not shown) for southeastern *M. flava* was nested to a four-step level and consisted of 17 haplotypes, 15 one-step clades, 7 two-step clades, and 3 three-step clades. Statistically significant distance values were detected only at two higher level 3 step clades, both of which had haplotypes from all three sampling sites. From those, only one inference of contiguous range expansion could be made (inference key 1-2-11-12no). For southeastern *M. flava*, a clade consisting of six individuals from Khabarovsk and Sakhalin followed the pattern predicted for ancestral haplotypes. There was no widespread common haplotype in that group, but haplotype 29Khab had the most mutational connections and was considered the root by TCS, which suggests that Khabarovsk was the place from where southeastern *M. flava* haplotypes expanded (to Mongolia and Sakhalin). However, on the basis of parsimony (tree not shown) five out of eight haplotypes from Mongolia were basal on a strict consensus tree, which suggests expansion from Mongolia.

For *M. citreola* two cladograms were constructed by TCS. Out of 10 haplotypes only 3 were terminal on the network for western *M. citreola*. Resolving the relationships between haplotypes would be arbitrary, so we did not perform nested-clade analysis on that group.

There were 14 haplotypes, 9 one-step clades, 4 two-step clades in a network (not shown) for eastern *M. citreola* nested to the three step level. Out of all clades just one 1 step clade showed significant distance values. That internal clade consisted of the most common haplotype (eight individuals from four localities), and six haplotypes one mutational step apart from it. The inference key (1-2-11-17-4no) led to restricted gene flow with isolation by distance. The range of that clade covers all sampled localities, but it is most frequent in Tyva (80% of sampled individuals) and Yamal (72%). According to the strict consensus parsimony tree (not shown), haplotypes from Tyva and Mongolia are basal. Thus, either Mongolia or Tyva are the most likely sites of recent origin for that group. Hence, colonization of eastern part of Eurasia by *M. citreola* went from south to north.

DISCUSSION

TAXONOMIC CONCLUSIONS

The Yellow Wagtail is a common, well-studied Palearctic species. Our results were surprising in showing that the current taxonomies of *M. flava* and *M. citreola* do not reflect the evolutionary history of the mtDNA gene tree. We provided our initial result to G. Voelker, who subsequently confirmed our results (Fig. 2) with all relevant outgroups (Voelker 2002). The geographically coherent distribution of the reciprocally monophyletic groups of haplotypes suggests that the mtDNA gene tree is an accurate portrayal of the organismal history of those taxa, as has been shown for many other avian taxa (Moore 1995, see Degnan 1993 for an exception). That is, although the mtDNA tree can misrepresent the organismal tree, it would be illogical to expect such a biased tree to be as geographically structured as that in Figure 2. Therefore, on the basis of the findings of our comprehensive sampling of each taxon, and the analysis by Voelker (2002), taxonomic changes can be inferred from the mtDNA tree. The traditionally classified *M. flava* consists of more than one species. The western and eastern components should be reclassified as separate species. On the basis of reciprocal monophyly of mtDNA haplotypes, it is warranted to recognize the northeastern and southeastern taxa as evolutionarily significant units (Moritz 1994) and probably phylogenetic species. If recognized as species, the name *Motacilla flava* applies to western forms, the northeastern group becomes *Motacilla tschutschensis* (Gmelin 1789), and the southeastern group is *Motacilla taivana* (Swinhoe 1863). More rigorous sampling and analyses of all forms are needed to ascertain complete species limits and ranges of each taxon.

The taxonomy of *M. citreola* also requires revision. Our two clades correspond to the eastern subspecies *M. c. citreola* and *M. c. quasatrix* (Portenko 1973) and a western form *M. c. werae*. Where the two forms meet is unclear (Cramp 1988, Dement'ev 1954). However, our sample from Tyva includes individuals from both clades, indicating that that site is situated in a contact zone. Given that the two taxa probably are not sisters (Fig. 2), it is likely that that contact is secondary (Cracraft 1989). Our tree (Fig. 2) and the high F_{st} value (0.88) between the

eastern and western groups of *M. citreola* suggest that two phylogenetic species are involved; those taxa are certainly evolutionarily significant units (Moritz 1994). The western group becomes *M. werae* (Buturlin 1907) whereas *M. citreola* remains for eastern populations.

Phylogenetic results (Fig. 2; Voelker 2002) illustrate that the species-level taxonomy of at least some Palearctic taxa is unreliable. The previous classification of wagtails (Table 1) clearly conflicts with the mtDNA divisions. For example, two members of the *lutea* complex (*M. f. lutea*, *M. f. taivana*) are in different mtDNA groups. Inspection of the plumage characters (Table 1) used to construct the traditional taxonomy reveals why it conflicts with the mtDNA gene tree. Previous taxonomic groupings were based largely on single characters. When morphological characters (Table 1) are considered simultaneously (parsimony analysis not shown), character conflicts result in an unresolved strict consensus tree. Thus, in fact, the morphological characters on which taxonomists based their subspecies assignments do not support the current taxonomy when analyzed in concert. It follows that the subspecies schemes (Table 1) resulted from differential weighting of morphological features by individual taxonomists. Of interest is the observation that each of the major mtDNA groupings contains similar mixtures of phenotypes. Assuming that the mtDNA tree reflects evolutionary history, that suggests considerable convergence in the external phenotypic characters used by taxonomists in classifying taxa in that group.

POPULATION HISTORY AND GENETIC-PHENOTYPIC EVOLUTION

At the time when the Laurentide Ice sheet extended over much of North America (18,000 years before present), the landscape of Eurasia differed considerably (see Hewitt 2000). In Eurasia, glaciers covered most of Europe, but east of $\sim 120^\circ\text{E}$, glaciation was essentially absent. However, permafrost occurred over the nonglaciated landscape and extended south to 40°N , and much farther in central Asia. Thus, although Eurasia was not as extensively glaciated as North America, habitats were strongly displaced southwards (Frenzel et al. 1992). Depending on the degree to which wagtails bred in areas of permafrost, one might posit that

the species have recently recolonized the northern reaches of their current Eurasian ranges, following climatic amelioration. Such a recent history leads one to predict the genetic signatures of range and population expansions.

As a result of our discovery of multiple independently evolving groups, the sample sizes for each of five studied taxa limited inferences about history of each taxon. Phylogenetic analyses of haplotypes within each of the major clades of *M. flava* and *M. citreola* did not recover reciprocally monophyletic groups. Unstructured haplotype trees are an expected consequence of populations or groups of populations having been isolated for less than $2N_{ef}$ generations (N_{ef} is the inbreeding effective size of the female population), the time required for evolution of reciprocal monophyly (Avice 2000). However, it is possible that either populations were historically stable and connected by gene flow, or that the current range was only recently reoccupied.

Most of the mismatch distributions, Nm values, and the negative Fu's F values, are consistent with past population expansions and gene flow. Coalescence theory yields inferences about the relative age of population expansion from the shape of a mismatch distribution (Slatkin and Hudson 1991). Unimodal distributions are indicative of population expansion, and as the mean number of differences between haplotypes increases, the mode of the distribution shifts to the right and the inferred age of population expansion becomes older. If a mismatch distribution is multimodal, a history of population stability can be inferred. For example, the Moscow region was under glacial ice during the last glacial cycle, and a recent range expansion is expected. We found that for *M. flava* populations from Vyatka and Tyva (Fig. 4B, C), expansion does predate that for Moscow (Fig. 4A) and Kursk (not shown). Two northeastern localities Yamal and Anabar (Fig. 4D, E) exhibited mismatch distributions similar to those for Moscow and Kursk, indicating relatively recent past population expansions, as expected. Contrary to expectation, the (far northern) Yamal sample of *M. citreola* showed a signature of stability. Too few samples of *M. citreola* were available to allow inferences about the histories of the two major clades; it is possible that gene flow from unsampled differentiated populations contributed to a multimodal mismatch distribution.

Although the plot in Figure 3 suggests a northward decline in nucleotide diversity, as found for many European animals (Hewitt 2000), statistical inference is complicated because the data come from five independently evolving groups. No evidence of significant northward expansion was found within any of five studied groups (Fig. 3). However, that result could be due to insufficient sampling of the northern Europe and a large part of Siberia (Fig. 1). Thus, although it is possible that nucleotide diversity shows a signature of leading-edge expansion, further sampling is required.

The significant F_{st} values within groups of *M. flava* and *M. citreola*, the tendency for haplotypes from Anabar and Vyatka to cluster together, and limited isolation-by-distance (nested-clade analysis for northeastern *M. flava* and eastern *M. citreola*) suggest that some of those groups of populations are in the early stages of geographic differentiation. That characterization differs from that of widespread bird species found in North America, which are typically less differentiated (Zink 1997). Thus, some but not all (Zink et al. 2002a, b) Eurasian taxa might be more differentiated owing to a less severe glacial history, greater geographic distances, or greater degrees of previous genetic structure having been retained across the last Ice Age. Additional species require study to ascertain which is the general pattern.

Our historical scenario for wagtails raises the question of how phenotypic differentiation originated if there has been limited isolation, gene flow, or both. Evolution of plumage differentiation (Table 1) could be explained by prior isolation of populations for a time sufficient for morphological differentiation, but not for reciprocal monophyly in mtDNA haplotype trees. Because morphological traits are likely polygenic, they can evolve at a faster rate than single-locus traits such as sequences in the mtDNA genome. Thus, the disparity between mtDNA and morphology could reflect differing evolutionary rates. In addition, broad zones of overlap occur in which phenotypes that characterize subspecies occur at single locations (Table 2), which suggests that previously isolated populations have come into contact. Thus, an alternative explanation would involve spread of phenotypes among major groups via hybridization. In any event, phenotypically defined subspecies are not on independent evolutionary

trajectories, as is true for many other birds (Ball and Avise 1992, Zink et al. 2000).

METHODOLOGICAL CONSIDERATIONS

Nested-clade-analysis has been used infrequently in avian phylogeography and it is useful to compare inferences based on it to other more commonly used approaches. Methods such as F_{st} , Mantel's test, parsimony trees, and mismatch distributions suggested a history of isolation of major groups followed by recent range expansion. Although some of our sample sizes were small, nested-clade-analysis indicated some additional trends, such as isolation by distance, which was not detected by Mantel's test. That might result because Mantel's test considers all samples simultaneously, whereas nested-clade-analysis considers (only) significant clades independently. Nonetheless, many assumptions in nested-clade-analysis are tenuous (Knowles and Maddison 2002), which could lead to discrepancies with traditional methods. For example, TCS often identified the most common haplotype as the root haplotype. However, the probability of the most frequent haplotype being oldest is equal to its frequency, which is low in our study (and would decrease with addition of more sequences). In addition, the oldest haplotype is not necessarily the root. An old haplotype might be basal to only one clade of a two-clade haplotype tree, with the real root haplotype having gone extinct. Thus, it remains useful to consider outgroup rooting if possible. In our analysis of southeastern *M. flava*, it seemed that maximum parsimony was more likely to identify the root than statistical parsimony as implemented in TCS. Thus, both nested-clade-analysis and traditional methods should be considered jointly.

Note added in proof: Alstrom et al. (2003) suggested that there were either two or many species of *M. flava*, specifically suggesting that many subspecies studied by us might qualify as species. Our data strongly contradict both suggestions, as with broader taxon sampling, we and Voelker (2002) show that three evolutionarily distinct forms exist. Although Alstrom et al. considered *M. f. plexas* as part of *M. f. thunbergi*, our study showed that this enlarged taxon is distributed between the western and northeastern groups. Therefore, the taxonomic

scheme suggested by Alstrom et al. is inconsistent with our and Voelker's (2002) mtDNA studies.

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