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Multi-locus reassessment of a striking discord between mtDNA gene trees and taxonomy across two congeneric species complexes

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ABSTRACT

Resolving relationships among members of the yellow and citrine wagtail species complexes is among the greatest challenges in avian systematics due to arguably the most dramatic disagreements between traditional taxonomy and mtDNA phylogeny. Each species complex is divided into three geographically cohesive mtDNA clades. Each clade from one species complex has a sister from the other complex. Furthermore, one cross-complex pair is more distantly related to the remaining two pairs than are several other wagtail species. To test mtDNA gene tree topology, we sequenced the mtDNA ND2 gene and 11 nuclear introns for seven wagtail species. Our mtDNA gene tree reconstruction supported the results of previous studies, thereby confirming the disagreement between mtDNA phylogeny and taxonomy. However, our multi-locus species tree which used mtDNA clades as "taxa" was consistent with traditional taxonomy regardless of whether mtDNA was included in the analysis or not. Our multi-locus data suggest that despite the presence of strongly supported, geographically structured mtDNA variation, the mtDNA gene tree misrepresents the evolutionary history of the yellow and citrine wagtail complexes. This mito-nuclear discord results from mtDNA representing the biogeographic, but not evolutionary history of these recently radiated Palearctic wagtails.

> "There is perhaps no other group of Palearctic birds the variation of which is as puzzling as that of the Yellow Wagtail (*Motacilla flava*) complex." (Mayr, 1956)

1. Introduction

Mitochondrial DNA (mtDNA) gene tree para- or polyphyly (GTP) results from a discord between taxonomy and phylogenetic signal of the mtDNA variation. Funk and Omland (2003) observed GTP in almost a quarter of the 2319 animal species included in their review. In a later survey of 151 avian studies published from 1990 to 2008, McKay and Zink (2010) found 122 (14%) cases of mtDNA GTP among 856 species. The latter authors attributed the majority of these mtDNA GTP cases (68) to incorrect taxonomy. In other words, McKay and Zink essentially hypothesized (or more accurately, assumed) that in the face of a well-supported mtDNA phylogeny, any conflict between phylogeny and taxonomy were attributable to taxonomic issues, regardless of how thorough previous taxonomic work (almost certainly based on morphology) had been.

However, the assertion of McKay and Zink (2010) that in these cases of GTP the taxonomy was the cause of discord was not supported by independent data (e.g. nuclear DNA (nuDNA)), despite the frequently voiced concerns over potential problems with relying exclusively on mtDNA in phylogenetic reconstruction (Ballard and Whitlock, 2004; Edwards et al., 2005; Bazin et al., 2006; Dowling et al., 2008; Brito and Edwards, 2009; Edwards and Bensch, 2009; Galtier et al., 2009). Rather, the assertion of taxonomic oversight was based on the presumption that geographically structured mtDNA variation better represents the evolutionary history of taxa than does taxonomy (Pavlova et al., 2003; Zink and Barrowclough, 2008).

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To resolve such cases of discord, McKay and Zink (2010) suggested recognizing each mtDNA clade of a paraphyletic taxon as a distinct species. Indeed, most of these 68 avian GTP cases would require a simple splitting of paraphyletic taxa. However, some mtDNA GTP cases would require significant taxonomic rearrangements across multiple species. For example, a little over decade ago, Voelker (2002) and Pavlova et al. (2003) published papers based on mtDNA sequences, which suggested that previous generations of taxonomists had markedly misinterpreted the evolutionary history of wagtails in the genus *Motacilla*. A widely recognized species at that time, the yellow wagtail, which is currently split into the western (*M. flava*) and eastern (*M. [f.] tschutschensis*) yellow wagtails (Clements et al., 2017), was found to consist of three geographically concordant mtDNA clades. Furthermore, it's close relative, the citrine wagtail (*M. citreola*), consisted of two clades. This in itself was not a surprising discovery, as molecular characters frequently reveal divergent taxa embedded within traditionally recognized species. This is especially true for those species with extensive phenotypic variation or large Holarctic or Palearctic ranges, e.g. the former winter wren that is currently treated as three distinct species (*Troglodytes troglodytes, T. hiemalis,* and *T. pacificus;* Drovetski et al., 2004), the Eurasian nuthatch *Sitta europaea* (Zink et al., 2006; Hung et al., 2012), or the horned lark *Eremophila alpestris* (Drovetski et al., 2014). In fact, based on its extensive phenotypic variation (see e.g., deo Hoyo et al., 2004), some taxonomists have suggested that the yellow wagtail complex consists of four (Stepanyan, 2003) or as many as eleven (Sangster et al., 1998; Sangster et al., 1999) closely related species.

What was unexpected in the findings of Voelker (2002) and Pavlova et al. (2003) was the phylogenetic arrangement of the yellow wagtail clades (Fig. 1 in Voelker, 2002 and Fig. 2 in Pavlova et al., 2003). In particular, *M. flava* was placed as sister to a large clade of wagtail species, which included two lineages (northern and southern) of *M. [f.] tschutschensis*. In this larger clade, the white (*M. alba*), gray (*M. cinerea*), and citrine wagtails were portrayed as being more closely related to *M. [f.] tschutschensis*, than the latter was to *M. flava*; this despite *M. [f.] tschutschensis* and *M. flava* having traditionally been considered conspecific. Further confusing traditional taxonomic wisdom, the western Palearctic citrine wagtail (*M. c. werae*) and eastern Palearctic citrine wagtail (*M. c. citreola*) were not sisters despite their considerable morphological similarity. Instead, *M. c. werae* and *M. c. citreola* were more closely related to the southern and northern clades of *M. [f.] tschutschensis*, respectively.

Yet another study of yellow and citrine wagtails (Ödeen and Björklund, 2003), although limited in sampling of *Motacilla* species and geographic coverage, included two individuals of the south-central Palearctic *M. citreola calcarata*, a lineage not included in the other two studies. Adding to the incongruities between traditional taxonomy and mtDNA tree topology, *M. c. calcarata* was placed as the sister to *M. flava*.

Therefore, from an overall perspective gained from the three studies, each of the three yellow wagtail mtDNA clades (*M. flava*, northern *M. [f.] tschutschensis*, and southern *M [f.] tschutschensis*) had a sister citrine wagtail clade (*M. c. calcarata*, *M. c. citreola*, and *M. c. werae*, respectively), with two other wagtail species (*M. alba* and *M. cinerea*) falling between the *M. flava/M. c. calcarata* and the other two sister pairs (northern *M [f.] tschutschensis/citreola* and southern *M. [f.] tschutschensis/werae*). Further, these cross species complex sister clades tend to be parapatric or partially sympatric, and their divergences can largely be explained by glacial cycles in Palearctic (Voelker, 2002; Pavlova et al., 2003; Voelker, 2010). Thus, in no small part due to the geographically coherent structure of relationships, the mtDNA conclusion of multiple "species" existing within both the yellow and citrine wagtail complexes seemed a reasonable conclusion.

However, the unexpected polyphyletic arrangement of the yellow and citrine wagtails could also be explained by the failure of the mtDNA gene tree to capture wagtail evolutionary history (Ballard and Whitlock, 2004; Edwards et al., 2005; Bazin et al., 2006; Dowling et al., 2008; Brito and Edwards, 2009; Edwards and Bensch, 2009; Galtier et al., 2009). After all, generations of taxonomists have studied phenotypic variation and concluded that there was extensive taxonomic variation among yellow wagtails, but no suspicion of polyphyly was expressed. Thus, the results of wagtail mtDNA studies provide one of the most marked conflicts between traditional taxonomic arrangements and mtDNA gene trees in birds reported to date.

From a phenotypic perspective, the most likely explanation for conflict is simply that the significant plumage variation inhibited recognition of species polyphyly of the magnitude shown in the mtDNA tree. Alternatively, from a mtDNA perspective, the most likely explanation for the conflict is that the mtDNA tree represents the evolutionary history of a single locus, which can misrepresent the "species tree" because of stochastic lineage sorting or the effects of natural selection. In addition, mtDNA introgression followed by a selective sweep may have played a role in generating the observed relationships (Weckstein et al., 2001; Drovetski et al., 2015).

Striking, compound discords between mtDNA gene tree and taxonomy as in case of yellow and citrine wagtail species complexes demand assessment with independent data. An obvious recourse to evaluate such a discord is to determine the phylogenetic pattern of genetic variation at other, independently segregating loci. Unfortunately, for the case of *Motacilla*, only a single study (Ödeen and Björklund, 2003) has included a single Z-chromosome linked intron obtained from just a few individuals representing *M. flava*, northern *M. [f.] tschutschensis,* southern *M. [f.] tschutschensis*, *M. c. calcarata*, and *M. cinerea*. The topology of this tree appears consistent, to the extent possible, with that of the mtDNA gene trees: *M. flava* and *M. c. calcarata* were sisters, and both *M. [f.] tschutschensis* clades were more closely related to *M. cinerea* than to the *M. flava/M. c. calcarata* pair. However, this study falls short of providing a sufficient testing of the mtDNA tree topology due to low sample sizes and just one nuclear marker being included.

To assess the findings of the existing *Motacilla* mtDNA studies (Voelker, 2002; Ödeen and Björklund, 2003; Pavlova et al., 2003) and to test whether conflict between traditional taxonomy of yellow and citrine wagtails and the mtDNA gene tree is consistent across multiple loci, we sequenced the mtDNA ND2 gene and 11 nuclear introns for seven of 12 wagtail species and analyzed these data using a multi-locus species tree approach. We hypothesize that if the mtDNA phylogeny is correct, then the multi-locus trees based on "taxa" representing mtDNA clades should be similar to the mtDNA phylogeny where each yellow wagtail taxon is more closely related to a citrine wagtail taxon (i.e., taxonomy is incorrect). Alternatively, if traditional taxonomy is correct, then "taxa" representing mtDNA clades will group by traditional species on the multi-locus tree, such that the three yellow wagtail taxa will be others' closest relatives, as will the three citrine wagtail taxa (i.e., mtDNA is incorrect).

2. Material and methods

2.1. Taxon sampling

In this study we follow the eBird/Clements checklist of birds of the world: v2016 (Clements et al., 2017) as our taxonomic reference. We obtained a total of 279 tissue samples of seven species of *Motacilla* wagtails and three samples of the Pechora pipit (*Anthus gustavi*) from museum collections (Appendix S1, Supporting information). All birds represent locally breeding populations. We focused our sampling on the three currently recognized wagtail species in which the geographic variation of mtDNA contradicts traditional taxonomy (Voelker, 2002; Ödeen and Björklund, 2003; Pavlova et al., 2003): *M. [f.] tschutschensis* (n = 173), *M. flava* (n = 49), and citrine wagtail (*M. c. werae* n = 7, *M. c. calcarata* n = 5, *M. c. citreola* n = 9; Fig. 1). Other species of wagtails included the African pied (*M. aguimp*), White (*M. alba*), Cape (*M. capensis)*, and Gray (*M. cinerea*). These latter four species where included

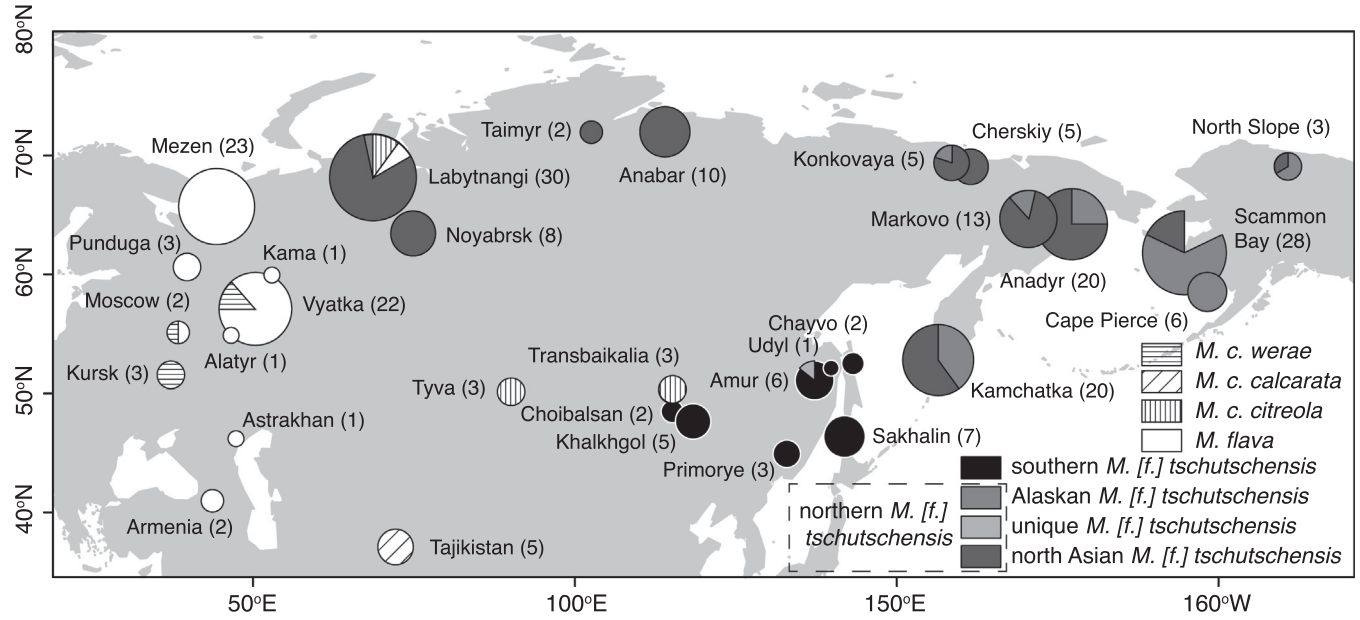

Fig. 1. Sampling localities, sample sizes, and distribution of mtDNA clades of the citrine (*Motacilla citreola*), western yellow (*M. flava*), and eastern yellow (*M. [f.] tschutschensis*) wagtails. The north Asian, Alaskan, and the unique haplotypes of *M. [f.] tschutschensis* were combined into the northern *M. [f] tschutschensis* taxon in phylogenetic analyses. Circle area is proportional to the sample size. Incomplete circle at Scammon Bay, Alaska indicates that mtDNA ND2 gene was not sequenced for 5 of 28 wagtails sampled there but at least one other locus was.

primarily to test the monophyly of the three focal species and as such were sampled less extensively (n = 2–21). The Pechora pipit was used as an outgroup for all wagtail species.

2.2. DNA sequencing

Total genomic DNA was extracted from tissue samples using the JETQUICK Tissue DNA Spin Kit (Genomed, Loöhne, Germany) according to the manufacturer's instructions. For all 282 samples, we attempted to sequence the complete mtDNA ND2 gene (1041 base pairs (bp)) but failed for five *M. [f.] tschutschensis* and one *M. cinerea* (Appendix S1, Supporting information). Also, for three Pechora pipits, two *M. aguimp*, nine *M. alba*, two *M. capensis*, 20 *M. cinerea*, seven *M. c. werae*, four *M. c. calcarata*, seven *M. c. citreola*, 26 *M. flava*, and 110 *M. [f.] tschutschensis* we sequenced intron 9 of the Z-chromosome specific Aconitase 1 gene (ACO1I9). Finally, for a subset of three Pechora pipits, two *M. aguimp*, three *M. alba*, two *M. capensis*, three *M. cinerea*, three *M. c. werae*, five *M. c. calcarata*, six *M. c. citreola*, three *M. flava*, and 10 *M. [f.] tschutschensis* we sequenced 10 autosomal introns from different chromosomes (Appendix S2, Supporting information).

2.3. Phylogenetic analyses, molecular clock and tests of neutrality

PCR fragments were sequenced in both directions on an ABI 3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) at the Macrogen Europe facility. The sequences were aligned automatically in Sequencher 5.0.1 (Gene Codes Corporation, Ann Arbor, MI) and verified manually to ensure consistent alignment of indels. Sequences are available on GenBank; see Appendix S1 for accession numbers (pending acceptance).

Alleles of heterogametic individuals that differed in length due to the presence of indel(s) were identified manually as described previously (Drovetski et al., 2013, 2015). This phasing method utilizes the complementary strand on the other side of an indel as a reference for alignment of shifted sequences that allows one to identify which nucleotide in the double peaks of the complementary strand belongs to the shorter allele and which belongs to the longer allele. Alleles of heterogametic individuals with equal-length alleles and multiple nucleotide differences were resolved using PHASE 2.1.1 (Stephens et al., 2001) and utilizing known allele sequences.

We used *BEAST 2.1.3 (Drummond et al., 2012) to reconstruct ND2 and ACO1I9 gene trees and a multi-locus species tree based on 10 autosomal introns and on all 12 loci. We used the Bayesian information criterion (BIC) implemented in jModelTest 2.1.7 (Posada, 2008) to select evolutionary models for each locus (Appendix S2, Supporting information). To choose a molecular clock prior, we conducted two independent analyses for each locus. In one, we used a strict clock prior and in the other, a relaxed lognormal clock prior. Subsequently we used a maximum likelihood ratio test (Huelsenbeck and Crandall, 1997) to compare the strict clock tree likelihood with that of the relaxed clock tree. Since MLRT P-values were ≥ 0.726 for all 12 loci, we only report the results of our *BEAST analyses with the strict molecular clock prior.

Two MCMC analyses were run for 10^9 generations with a 10^4-generation burn-in and parameters sampled every 10^4 steps to insure their convergence, where all effective sample size (ESS) parameters are > 200 (our lowest ESS score was 233). Runs were combined using LogCombiner 2.0.2 (Drummond et al., 2012). Tracer 1.5 (http://beast.bio.ed.ac.uk/Tracer) was used to determine the effective sample size of each parameter and calculate its mean and 95% highest posterior density (95% HPD) interval. Trees were constructed using TreeAnnotator 2.0.2 (Drummond et al., 2012) and visualized in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

We conducted the McDonald–Kreitman (MK) test (McDonald and Kreitman, 1991) implemented on the following website: http://mkt.uab.es (Egea et al., 2008) to determine whether mtDNA ND2 gene evolution was consistent with neutrality. The neutrality of nuclear intron evolution was tested using the Hudson-Kreitman-Aguade (HKA) test (Hudson et al., 1987) implemented in HKA software (https://bio.cst.temple.edu/~hey/software/software.htm#HKA) for the citrine wagtail/*flava* pair and for the two clades of *tschutschensis*. These pairs were sister clades in the species tree based on all 12 loci (see below).

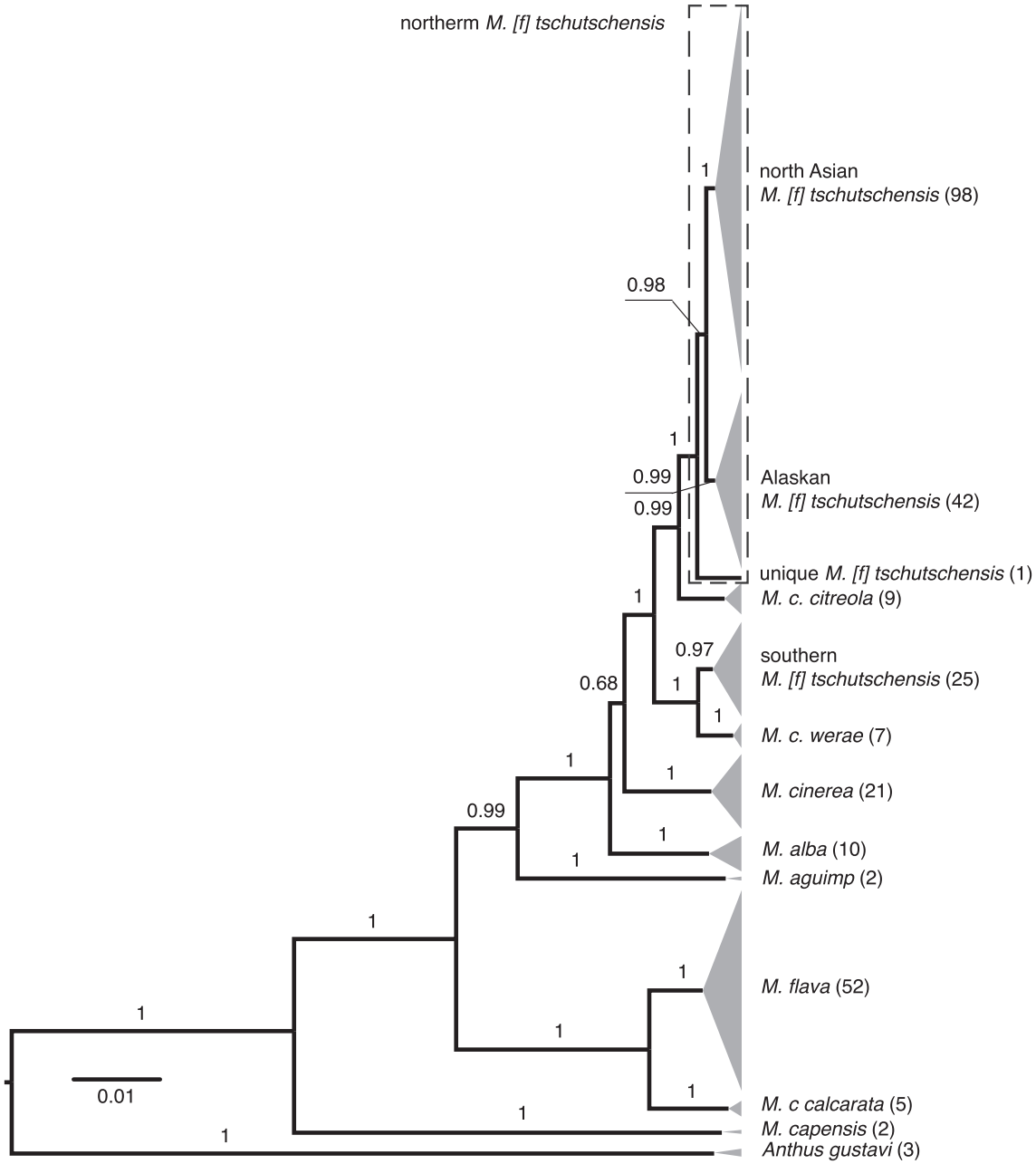

Fig. 2. MtDNA ND2 gene tree. Numbers next to branches indicate posterior probability values. Clades indicated by gray triangles were used as "taxa" in our ACO1I9 gene tree and multilocus species tree reconstructions. The only exceptions were *M. [f.] tschutschensis* clades enclosed in the dashed box identified as northern *M. [f.] tschutschensis*. These clades were treated as a single "taxon" (northern *M. [f.] tschutschensis*) because they were monophyletic, closely related, and were present together in many localities (Fig. 1).

3. Results

3.1. mtDNA ND2 gene tree

The genus *Motacilla* and most of its constituent species (*M. capensis*, *M. aguimp*, *M. alba*, *M. cinerea,* and *M. flava*) were monophyletic in the mtDNA ND2 gene tree (all supported with posterior probability values PP = 1) whereas two species, *M. [f.] tschutschensis* and the citrine wagtail, appeared polyphyletic (Fig. 2). One *M. [f.] tschutschensis* clade (PP = 1) dominated northernmost Asia from Labytnangi to Anadyr and Kamchatka and was present in low frequency in Alaska (Fig. 1). The sister to the North-Asian *M. [f.] tschutschensis* clade was another *M. [f.] tschutschensis* clade (PP = 0.99) that was predominantly present in Alaska, but was also found in nine of 20 individuals from Kamchatka, and was present in low frequency in Anadyr, Markovo, Cherskiy, and Labytnangi (Fig. 1). The monophyly of the North-Asian and Alaskan clades of *M. [f.] tschutschensis* was strongly supported (PP = 0.98) and a single haplotype from Amur was their sister (PP = 1; Fig. 1). These three clades combined correspond to the northern *M. [f.] tschutschensis* clade identified in earlier studies (Voelker, 2002; Pavlova et al., 2003).

The closest relative (PP = 0.99) of the northern *M. [f.] tschutschensis* clade was *M. c. citreola* that included individuals from Labytnangi, Tyva, and Transbaikalia (Fig. 2). The second citrine wagtail clade *werae* (PP = 1) included birds from Kursk, Moscow, and Vyatka and was sister to the southern *M. [f.] tschutschensis* clade that included individuals from Dornod, Amur, Primorye, and Sakhalin. The two clade pairs representing northern *M. [f.] tschutschensis/M. c. citreola* and southern *M. [f.] tschutschensis/M. c. werae* were sisters (PP = 1).

The third clade of the citrine wagtail (*M. c. calcarata* (PP = 1; Fig. 2)) that combined birds from Tajikistan was the sister (PP = 1) to

M. flava (PP = 1). The *M. c. calcarata/M. flava* clade was distantly related to *M. [f.] tschutschensis* and other citrine wagtail clades. In fact, with the exception of *M. capensis,* which was sister to all other wagtail lineages in our study, the *M. c. calcarata/M. flava* was more distantly related to *M. [f.] tschutschensis* and other citrine wagtail clades than were *M. aguimp, M. alba,* and *M. cinerea*. Therefore, our expanded sampling confirms that as currently recognized, both the citrine wagtail and *M. [f.] tschutschensis* are polyphyletic in their mtDNA, and that *M. flava* together with *M. calcarata* are more distantly related to *M. [f.] tschutschensis, M. c. citreola,* and *M. c. werae* than are several other wagtail species as was suggested by earlier studies (Voelker, 2002; Pavlova et al., 2003).

Haplotypes from different citrine wagtail clades were not observed together in any of the sampled localities, whereas different *M. [f.] tschutschensis* clades were observed together in several areas (Fig. 1). Most of these localities (North Slope and Scammon Bay of Alaska, Kamchatka, Anadyr, Markovo, Cherskiy, and Labytnangi) harbored haplotypes from two sister northern *M. [f.] tschutschensis* clades: Alaskan and North-Asian. However, in Amur we found haplotypes from two distantly related southern *M. [f.] tschutschensis* and northern *M. [f.] tschutschensis* clades. Yet, the most intriguing result was the discovery of two individuals carrying *M. flava* haplotypes together with 24 North-Asian and one Alaskan *M. [f.] tschutschensis* haplotypes in Labytnangi (Fig. 1), where a previous study (Pavlova et al., 2003) reported only *M. [f.] tschutschensis* haplotypes. Unfortunately, mtDNA alone cannot distinguish between alternative explanations for this discovery: sympatry of two genetic lineages or their hybridization.

3.2. Z-chromosome ACO1I9 gene tree

The ACO1I9 gene tree (Fig. 3) differed from the mtDNA ND2 gene tree (Fig. 2). The most significant disagreement between these trees was the monophyly of all sampled citrine wagtails (*M. c. werae, M. c. calcarata,* and *M. c. citreola*; PP = 1) and *M. [f.] tschutschensis* (PP = 0.88) in the ACO1I9 tree whereas both species appeared polyphyletic in the ND2 tree. Also *M. alba* and *M. aguimp*, which appeared distantly related in the ND2 tree, formed a single clade that was the sister to the *M. [f.] tschutschensis*/citrine wagtail pair (PP = 0.96; Fig. 3). Although the monophyly of all wagtails was strongly supported in the ACO1I9 tree (PP = 1), the relationships among *M. flava*, *M. cinerea*, *M. capensis* and the ((*M. [f.] tschutschensis* + *M.* citrine), *M. alba* + *M. aguimp*) clade were poorly supported (PP ≤ 0.53; Fig. 3).

The ACO1I9 tree identified the presence of hybridization between *M. flava* and northern *tschutschensis* in both West Siberian localities (Labytnangi and Noyabrsk). Only two of 27 birds sampled in Labytnangi had *M. flava* mtDNA (Fig. 2). Unfortunately, we were able to sequence ACO1I9 for just one male which had both *M. flava* ACO1I9 alleles (i.e., was pure *M. flava* in both loci). In contrast, among 13 birds with northern *M. [f.] tschutschensis* mtDNA and available ACO1I9 data, nine appear to have had a hybrid ancestry: one female had a *M. flava* ACO1I9 allele and eight males had one *M. flava* and one *M. [f.] tschutschensis* ACO1I9 allele. One male appears to be a backcross of a hybrid female carrying northern *M. [f.] tschutschensis* mtDNA with a *M. flava* male, as both of its ACO1I9 alleles were from the *M. flava* clade. Only three of the 13 Labytnangi birds had mtDNA and two ACO1I9 alleles that were consistent with pure *M. [f.] tschutschensis*. In Noyabrsk, all eight sampled birds had northern *M. [f.] tschutschensis* mtDNA haplotypes (Fig. 1). We sequenced ACO1I9 for two males from Noyabrsk. One male had both *M. flava* ACO1I9 alleles (backcross) and the other was a pure *M. [f.] tschutschensis* in both loci. Therefore, our data suggest that hybridization between *M. flava* and *M. [f.] tschutschensis* in northwestern Siberia is frequent and asymmetric, and involves *M. flava* males and *M. [f.] tschutschensis* females.

3.3. Multi-locus species trees

In the multi-locus species tree reconstruction we used mtDNA clades (Fig. 2) as "taxa". The only exception was *M. [f.] tschutschensis* for which we combined individuals from two sister mtDNA clades (Alaskan and North-Asian) into a single northern *M. [f.] tschutschensis* clade because geographic sorting of these closely related mtDNA lineages was incomplete (Fig. 1).

Despite the widespread incomplete lineage sorting across individual autosomal gene trees (Appendix S3, Supporting information), all but two nodes of the multi-locus species tree based on 10 autosomal introns were strongly supported (Fig. 4a). The topology of the autosomal gene tree differed from that of both ND2 and ACO1I9 gene trees. The main differences concerned the relationships among *M. flava*, *M. [f.] tschutschensis* and citrine wagtails. Similar to the ACO1I9 tree but in contrast to the ND2 tree, all three citrine wagtail clades (*M. c. werae, M. c. calcatrata,* and *M. c. citreola)* were grouped together (PP = 1; Fig. 4a). However, in contrast to the ACO1I9 gene tree the three citrine wagtail clades were differentiated and geographically structured, where *M. c. werae* and *M. c. calcarata* appeared to be more closely related to each other (PP = 1), than either was to *M. c. citreola.*

In contrast to both ND2 and ACO1I9 gene trees, *M. flava* appeared to be closely related to the *M. [f.] tschutschensis* and citrine wagtail clades (PP = 1). Citrine wagtails and *M. flava* were recovered as sisters, with northern *M. [f.] tschutschensis* more closely related to them than it was to southern *M. [f.] tschutschensis*. However, neither relationship was sufficiently supported (PP = 0.72 and 0.45, respectively) and thus we cannot reject the monophyly of *M. [f.] flava,* northern and southern *M. [f.] tschutschensis*.

The addition of ND2 and ACO1I9 sequences to our multi-locus analysis did not significantly affect the species tree topology, but did increase the tree depth by over an order of magnitude (Fig. 4b). In the 12 loci tree, *M. flava* remained more closely related to citrine wagtails than to either of *M. [f.] tschutschensis* clades that became each other's sisters. However, neither node was sufficiently supported (PP = 0.76 and 0.58, respectively).

3.4. Neutrality tests

The McDonald–Kreitman test found no evidence for selection on the mtDNA ND2 gene in any of the three sister-clade comparisons (*M. c. calcarata* vs. *M. flava*, *M. c. werae* vs. southern *M. [f.] tschutschensis* , and *M. c. citreola* vs. northern *M. [f.] tschutschensis*; Fisher's exact *p*-values of 0.724, 0.091, and 1.000 respectively) or between the distantly related clades (*M. c. calcarata* + *M. flava* vs. *M. c. werae* + southern *M. [f.] tschutschensis* + *M. c. citreola* + northern *M. [f.] tschutschensis*; P = 0.453). Likewise, HKA test results for our 10 autosomal and one Z-specific loci found no significant deviations of intraspecific polymorphism and mean pairwise divergence in either comparison (citrine wagtail + *M. flava*, and northern *M. [f.] tschutschensis* + southern *M. [f.] tschutschensis*) from those expected under neutrality (both $p \geq .974$). Therefore, neutral evolution of the autosomal and Z-specific introns used in our study could not be rejected.

4. Discussion

4.1. Mito-nuclear discord in phylogenetic signal

Our mtDNA analysis, which substantially expanded the geographic coverage and sample size of earlier studies (Voelker, 2002; Ödeen and Björklund, 2003; Pavlova et al., 2003), confirmed that three geographically cohesive polyphyletic clades exist within both the citrine and yellow wagtail species complexes. Each citrine wagtail mtDNA clade was strongly supported as the sister to a different yellow wagtail clade: *M. c. calcarata* was the sister to *M. flava*, *M. c. werae* was sister to the southern *M. [f.] tschutschensis*, and *M. c. citreola* was sister to

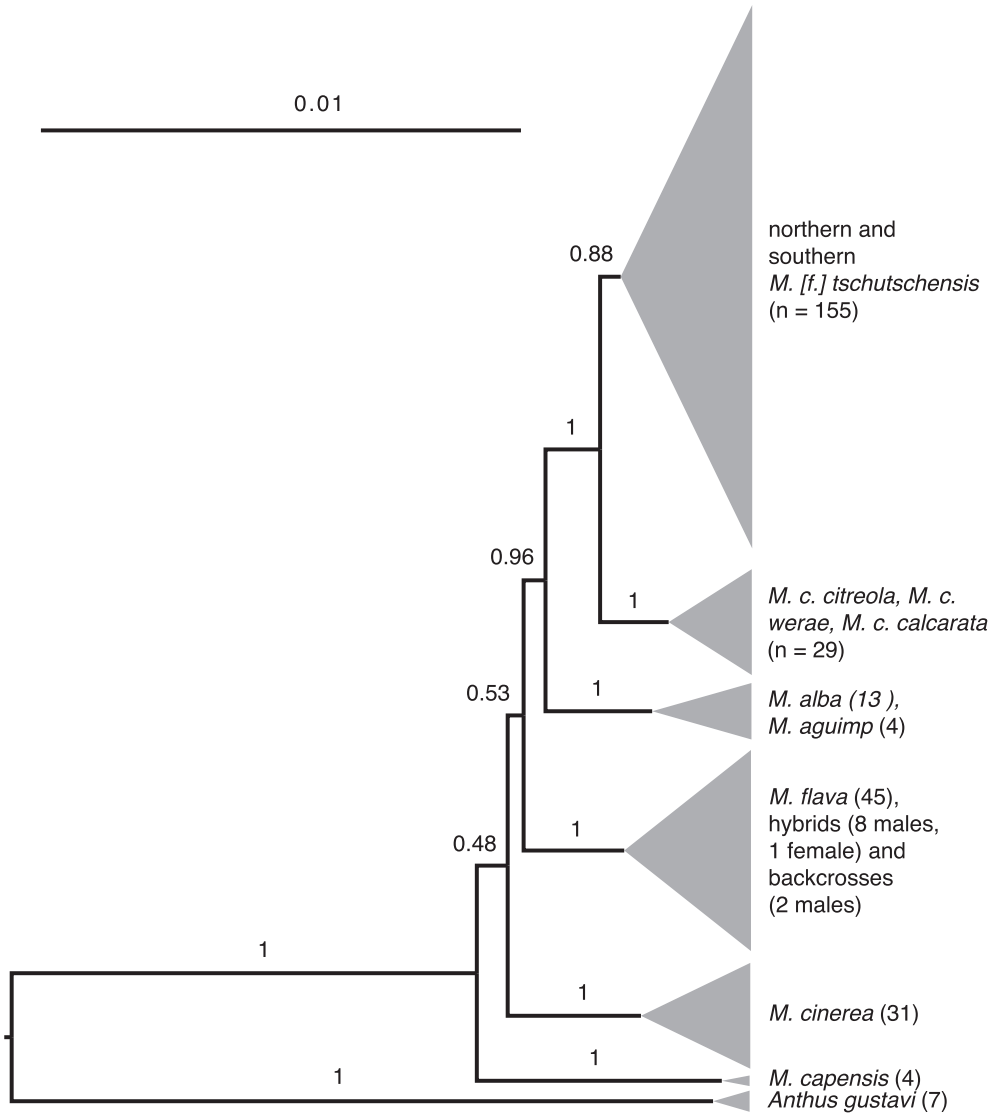

Fig. 3. ACO1I9 gene tree. Numbers next to branches indicate posterior probability values. A male hybrid refers to a male that has mtDNA and one Z-chromosome from *M. [f.] tschutschensis* and one Z-chromosome from *M. flava*. A female hybrid refers to a female that has mtDNA from *M. [f.] tschutschensis* and Z-chromosome from *M. flava*. A backcross male refers to a male that has mtDNA from *M. [f.] tschutschensis* and both Z-chromosomes from *M. flava*.

northern *M. [f.] tschutschensis*. Furthermore, only the *M. c. werae* + southern *M. [f.] tschutschensis* and *M. c. citreola* + northern *M. [f.] tschutschensis* pairs were each other's sisters, whereas the *M. c. calcarata* + *M. flava* pair was more distantly related to the first two pairs than were three of the four other wagtail species we included: *M. c. cinerea*, *M. alba*, and *M. aguimp*. Thus, yellow and citrine wagtail species complexes represent arguably the most striking example of discord between mtDNA gene tree and traditional taxonomy in an avian genus. It is also worth noting here that similar discord seems to be apparent in the white wagtail complex. While we did not extensively sample this complex, *M. alba* and *M. aguimp* are not sisters in the mtDNA gene tree, but are grouped together in the multi-locus tree.

The MK test identified no significant selection effect on the ratio of synonymous to non-synonymous substitutions in any of the three yellow/citrine wagtail clade pairs or in the comparison of the *M. c. calcarata* + *M. flava* pair versus the other two pairs combined. Therefore, neither the divergence of sister clades nor the divergence of distantly related groups of clades was affected by significant selection pressure on mtDNA.

This striking discord between mtDNA and taxonomy was only partially supported by our ACO1I9 gene tree. In contrast to mtDNA, all citrine wagtails were combined into a single clade that was the sister to a single *M. [f.] tschutschensis* clade and *M. alba* was grouped with *M. aguimp*. Only *M. flava* remained distantly related to *M. [f.] tschutschensis* and citrine wagtails defying traditional taxonomy. The sister relationship of all citrine wagtails and *M. [f.] tschutschensis* conflicts with the results of an earlier study (Ödeen and Björklund, 2003) that used the Z chromosome specific CHD1Z intron, and indicated a close relationship between *M. c. calcarata* and *M. flava*. This disagreement between ACO1I9 and CHD1Z trees may result from differences in the genetic composition of *M. c. calcarata* populations wintering in India (Ödeen and Björklund, 2003) and breeding in Tajikistan (this study) or the idiosyncratic evolutionary histories of the two loci despite their location on the same chromosome.

Our autosomal data contradicted the mtDNA gene tree to an even greater extent than did the Z-chromosome gene tree. At the same time, the autosomal tree was the most consistent with the traditional taxonomic treatment of yellow and citrine wagtails. Despite having used our mtDNA clades as “taxa” in our autosomal species tree reconstruction, the phylogenetic positions of these taxa differed significantly from those recovered on the mtDNA gene tree. In accordance with traditional taxonomy, the monophyly and close relationship of all yellow and citrine wagtails was strongly supported (PP = 1; Fig. 4a), and indeed citrine wagtail monophyly (*M. c. werae*, *M. c. calcarata*, and *M. c. citreola* as closest relatives) was equally strongly supported (PP = 1). Our autosomal tree also suggested the presence of geographically cohesive structuring of the citrine wagtail subspecies: *M. c. werae* was sister to its geographic neighbor *M. c. calcarata*, and *M. c. citreola* was more

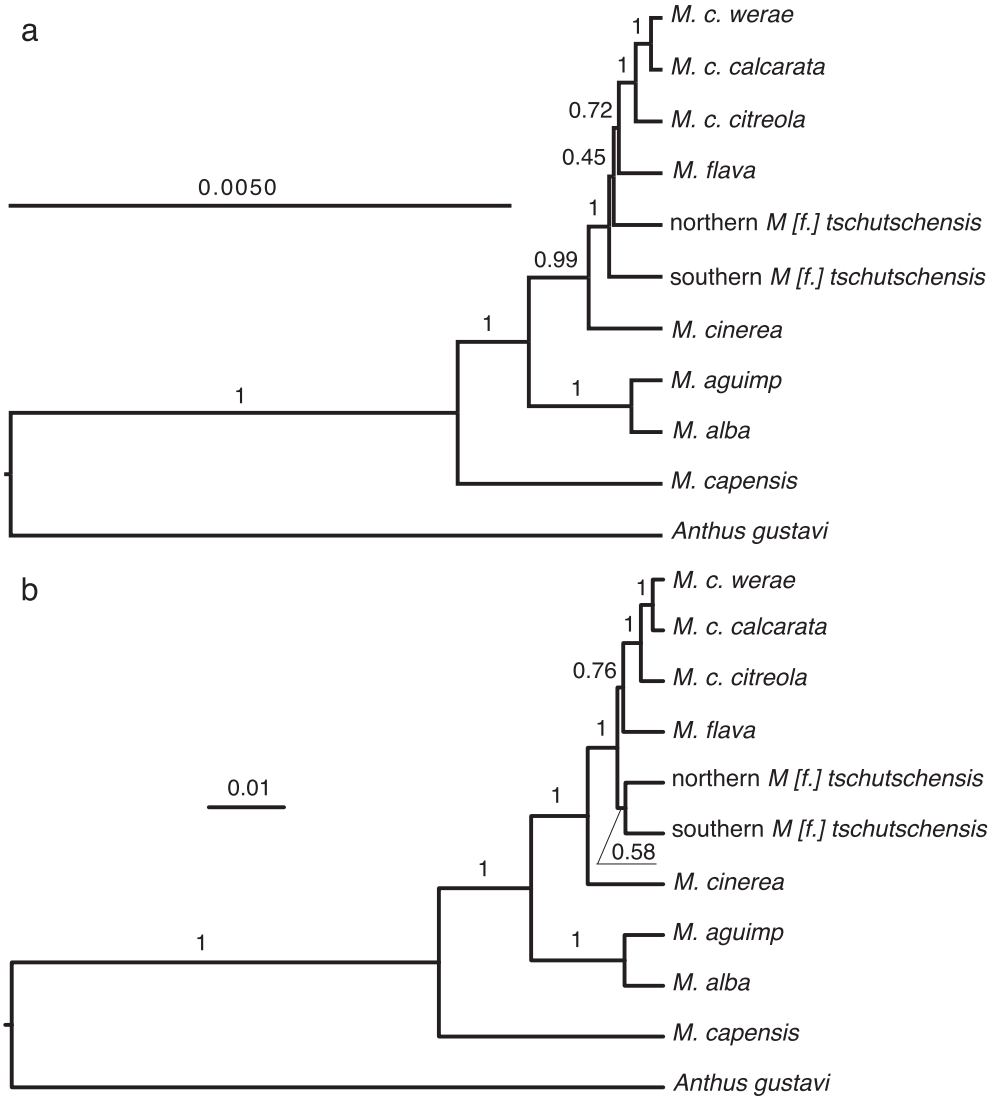

Fig. 4. Multi-locus species trees based on 10 autosomal introns (a) and on mtDNA ND2 gene, z-chromosome linked ACO1I9, and 10 autosomal introns (b). Numbers next to branches indicate posterior probability values.

distantly related to them than they were to each other. Therefore, these subspecies may be candidates for elevation to species status pending more thorough geographic sampling. Although relationships among *M. flava* and the two *M. [f.] tschutschensis* lineages were unresolved, their monophyly as suggested by traditional taxonomy could not be rejected. *M. flava* and both *M. [f.] tschutschensis* appear to have the greatest amount of variation and the least amount of lineage sorting across autosomal loci (Appendix S3, supplementary information) and may require sampling of more loci and individuals to resolve their relationship.

Despite the strong disagreement among the mtDNA, Z-chromosome and autosomal multi-locus tree topologies, combining all 12 loci recovered a tree topology that was virtually identical to that of the 10 autosomal loci species tree (Fig. 4). The only difference was in the apparent monophyly of the two *M. [f.] tschutschensis* clades in the 12 loci tree, but it was poorly supported (Fig. 4b). The similarity of the autosomal and all-loci tree topologies suggests that the multi-locus species tree reconstruction is robust to strong conflicts in the phylogenetic signal and significant heterogeneity of evolutionary rates across loci.

McKay and Zink (2010) specifically used the case of the yellow and citrine wagtails (Voelker, 2002; Pavlova et al., 2003) as an example of subspecies or groups of subspecies that were erroneously placed into the wrong species complex based on phenotype, and suggested that mtDNA correctly identified historical lineages. They proposed that recognizing each mtDNA clade as a distinct species would resolve this case of GTP (McKay and Zink, 2010). This conclusion was rooted in the view that the statistically supported geographic structure of mtDNA variation in these wagtails must be a reliable indicator of the evolutionary history of study taxa (Pavlova et al., 2003; Zink and Barrowclough, 2008). Our multi-locus data suggest that in the case of yellow and citrine wagtail complexes, mtDNA structure does not represent the evolutionary history of the taxa despite strong statistical support of geographically structured clades.

Another explanation frequently invoked in the case of mtDNA GTP is an introgressive sweep of mtDNA of one species by that of another (Rheindt and Edwards, 2011). In a recent study of grasshopper warblers (Drovetski et al., 2015), mtDNA of the Middendorf's grasshopper-warbler (*Locustella ochotensis*) appeared to have been replaced by mtDNA of the Pleske's grasshopper-warbler (*Locustella pleskei*). The mtDNA clade of the Middendorf's grasshopper-warbler had little variation and was imbedded within the much greater Pleske's grasshopper-warbler variation despite the much smaller population size of the latter.

Although the variation within all three citrine wagtail mtDNA clades appears to be smaller than the variation within their respective sister *M. flava* or *M. [f.] tschutschensis* clades (which also corresponds to the differences in population sizes), the presence of three pairs of clades requires invoking independent mtDNA introgressive sweeps in each of the three pairs. However, the greater divergence of *M. flava* + *M. c. calcarata* pair from the southern *M. [f.] tschutschensis* + *M. c. werae* and

northern *M. [f.] tschutschensis* + *M. c. citreola* pairs than some other wagtail species remains unexplained. Two additional phenomena are also inconsistent with a selective sweep hypothesis. First, the selective sweep did not happen after secondary contact between *M. c. werae* and *M. c. citreola* in Tyva (Pavlova et al., 2003), or between *M. flava* and *M. [f.] tschutschensis* in northwestern Siberia (this study), or between *M. c. werae* and *M. flava* which are sympatric in Europe (Fig. 1). If mtDNA haplotypes were under the strong positive selection required for a selective sweep, this selection should affect all populations experiencing secondary contact. Second, there are three cross complex pairs of clades with only two species complexes involved. Strong positive selection resulting in selective sweeps should reduce the number of divergent clades, not increase it.

4.2. Hybridization of *M. flava* and *M. [f.] tschutschensis* in northwestern Siberia

The distant relationship between parapatric *M. flava* and *M. [f.] tschutschensis* in both mtDNA and Z-chromosome loci allowed us to discover the presence of asymmetric hybridization between these two currently recognized species in northwestern Siberia (Labytnangi and Noyabrsk, Fig. 1). The majority of birds from this region were of mixed ancestry and in all cases hybridization involved *M. [f.] tschutschensis* females or hybrid females carrying *M. [f.] tschutschensis* mtDNA, and *M. flava* males. However, we found no evidence of hybridization between more closely related (according to mtDNA and Z-chromosome loci) *M. c. citreola* and *M. [f.] tschutschensis* despite their overlapping ranges.

Parapatric species are more prone to hybridization than are sympatric species (Mayr, 1942; Randler, 2002, 2006). Unfortunately, the underlying drivers of this pattern have yet to be sufficiently explained. A scarcity of mating partners at the edges of the distribution forcing females to choose hetero-specific males, or imperfect development of species recognition by females, have been suggested as possible explanations for more frequent hybridization between parapatric as compared to sympatric species (Randler, 2002, 2006). The scarcity of mating partners does not seem relevant to *M. flava* and *M. [f.] tschutschensis*. Our data showed that birds carrying *M. [f.] tschutschensis* mtDNA and Z-chromosomes are more common in Labytnangi and Noyabrsk than birds with *M. flava* haplotypes in those two loci. This suggests that *M. flava* females should be more likely to mate with *M. [f.] tschutschensis* males, which is the reverse of what we found.

In contrast, the imperfect development of species recognition by females could explain the presence of hybridization between *M. flava* and *M. [f.] tschutschensis* and its absence between the *M. c. citreola* and *M. [f.] tschutschensis*. All yellow wagtails breeding in Labytnangi and Noyabrsk phenotypically belong to the same subspecies of *Motacilla flava thunbergi*; (Clements et al., 2017) regardless of which mtDNA and Z-chromosome haplotypes they carry. Therefore, females may not be able to choose a genetically conspecific mate on the basis of the phenotype, whereas avoiding *M. c. citreola* males that have a significantly different plumage should be easier. However, the apparent strong asymmetry of the hybridization between *M. flava* and *M. [f.] tschutschensis* cannot be explained by the imperfect development of species recognition by females because it should affect both species equally.

Sympatric distribution of closely related species indicates the presence of sufficient ecological or behavioral divergence between them to avoid interspecific competition, whereas a parapatric distribution indicates the presence of interspecific competition that results in the formation of a hybrid zone. Our studies of historical biogeography and geographic modes of speciation in grouse (Tetraoninae), accentors (Prunellidae), and redstarts (*Phoenicurus*) showed that recently divergent taxa had similar ecology and were allopatric or parapatric (Drovetski, 2003; Drovetski et al., 2013; Voelker et al., 2015). Any level of sympatry among taxa was achieved after sufficient time in allopatry and ecological divergence.

The asymmetric introgression of genetic material from a local taxon into that of a range-expanding species (invader) is expected under selective neutrality, and has been supported by both simulation and empirical studies (Currat et al., 2008; Excoffier et al., 2009; Drovetski et al., 2015). A neutral hybrid zone should be moving in the direction opposite to the direction of gene flow between populations due to demic diffusion (Cavalli-Sforza et al., 1993), i.e. presence of local alleles at the leading edge of the invader's population, and due to the higher probability of persistence of introgressed alleles in the expanding invader's population than in the declining local population (Excoffier et al., 2009). The cryptic hybridization we discovered between *M. flava* and *M. [f.] tschutschensis* may provide a unique opportunity for advancing our understanding of speciation and the evolution of isolating mechanisms, irrespective of species concept assumed.

4.3. Potential causes of mito-nuclear discord

The divergence of Palearctic *Motacilla* appears to be very recent and Pleistocene climatic oscillations could have resulted in the observed complex pattern of their mtDNA variation. The age of divergence of widespread Palearctic wagtails is estimated to have begun in the Middle Pleistocene, approximately 0.79 (range 0.50–1.11) Ma ago (Drovetski et al., 2013). This estimate is an order of magnitude more recent than the start of divergence within the closely related to Motacillidae genus *Prunella* (Prunellidae) that was estimated at 7.31 (5.5–9.32) Ma ago on the same tree (Drovetski et al., 2013). The paleontological record agrees with a very recent divergence of yellow and citrine wagtails, where the oldest records are dated to the middle Pleistocene from 0.78 to 0.128 and 0.47 to 0.128 Ma before present, respectively. The earliest records of the gray wagtail are even younger at 0.25–0.128 Ma, and only white wagtail records date to earlier times (1.4–1.1 Ma before present; Tyrberg, 1998).

The rapid radiation of the Palearctic wagtails from a single common ancestor might have resulted in geographic isolation into glacial refugia of incompletely sorted, but geographically structured (e.g., due to isolation by distance) ancestral polymorphisms. In this case, lineage divergence (as reflected by mtDNA gene trees in particular) could follow geographic patterns. At the same time, the isolation and decrease of effective population size could accelerate divergence of incipient species genomes within each refugium (stages $t_1 - t_3$ and $t_4 - t_6$ in Appendix S4, Supporting information).

During interstadials, populations of incipient species can expand from refugia and come into contact. Upon secondary contact, nuclear genomes of ecologically similar forms (i.e. populations of the same incipient species) may introgress asymmetrically thereby significantly increasing the frequency of nuclear alleles of one population, across both previously isolated populations. Introgression between hybridizing avian species is often asymmetric and extensive (Currat et al., 2008; Excoffier et al., 2009; Petit and Excoffier, 2009; Rheindt and Edwards, 2011; Toews and Brelsford, 2012). No nuclear introgression occurs between populations of widely sympatric but ecologically different incipient species (e.g., our Citrine and eastern yellow wagtails), as their members do not compete for the same resources.

In contrast to nuDNA, mtDNA introgression is much slower (Currat et al., 2008; Excoffier et al., 2009; Petit and Excoffier, 2009; Rheindt and Edwards, 2011) which allows populations of the same incipient species to retain polyphyletic variation as reflected in the geographic structuring we recovered (stages t_4 and t_7 in Appendix S4, Supporting information). Lower levels of mtDNA introgression result from its association with females, which are the most dispersive sex in birds (Clarke et al., 1997). Under selective neutrality, the greater flow of loci associated with the most dispersive sex decreases the frequency of haplotypes introgressing from a “local” taxon into “invader” in the contact zone, whereas, the lower flow of loci associated with the least dispersive sex allows the introgressing “local” haplotypes to reach higher frequency in the “invader” (Excoffier et al., 2009; Petit and Excoffier, 2009). In all 16 studies of birds and insects with female-

biased dispersal examined by Excoffier et al. (2009), mtDNA had lower introgression than nuDNA. In the recent study of Pacific grasshopper-warblers the estimated introgression of nuDNA was an order of magnitude higher than introgression of mtDNA and the absence of introgression could not be rejected for mtDNA in all five pairwise estimates, except the inferred introgressive sweep of mtDNA of the Middendorf's grasshopper-warbler by that of the Pleske's grasshopper warbler (Drovetski et al., 2015).

Ecologically divergent populations do not hybridize, even those expanding from the same refugium despite their genomes sharing the similar part of the ancestral polymorphism. Lack of competition allows ecologically divergent forms to co-exist in sympatry without hybridization (Appendix S4, Supporting information).

This pattern is illustrated by our samples from Labytnangi, where we found *M. c. citreola*, *M. flava*, *M. [f.] tschutschensis*, and *M. alba* co-occurring in a narrow band of riparian habitat. However, only the ecologically similar, but most divergent in their mtDNA and Z-chromosomes *M. flava* and *M. [f.] tschutschensis* hybridize. This hybridization is asymmetric and shows only nuDNA introgression. This suggests that in the future (under current conditions), the nuclear genome of one lineage may be replaced by that of another, but both divergent mtDNA lineages will remain. Therefore, multiple cycles of glacial oscillations could have preserved divergent ancestral mtDNA lineages initially sorted geographically, while also allowing nuDNA to sort into ecologically divergent lineages, and phenotypes, that were recognized by traditional taxonomy. This hypothesis is further supported by the geographic distribution of wagtail mtDNA lineages, which fit a common geographic pattern described for many Palearctic avian taxa whose ranges have been affected by Pleistocene glaciations (Voelker, 2010).

5. Conclusions

In conclusion, our multi-locus data support the traditional taxonomy that recognizes close relationship between yellow and citrine wagtail species complexes, and among taxa within them. Put another way, “old school” taxonomy should still be considered as having potential relevance in the DNA age. Therefore, we caution against the out-of-hand dismissal of traditional taxonomy in cases when mtDNA appears to contradict it, regardless of how strong the support of geographically coherent clades in the mtDNA gene tree might be. Rather, obvious discords between phenotype, taxonomy and mtDNA demand more thorough investigation through the application of multi-locus approaches.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2017.11.023.

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