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Discordance between genomic divergence and phenotypic variation in a rapidly evolving avian genus (*Motacilla*)

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We dedicate this work to Anders Ödeen, a dear friend, colleague, and pioneer in wagtail genetics, who passed away during the preparation of this manuscript.

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ABSTRACT

Generally, genotypes and phenotypes are expected to be spatially congruent; however, in widespread species complexes with few barriers to dispersal, multiple contact zones, and limited reproductive isolation, discordance between phenotypes and phylogeographic groups is more probable. Wagtails (*Motacilla*) are a genus of birds with striking plumage pattern variation across the Old World. Up to 13 subspecies are recognized within a single species, yet previous studies using mitochondrial DNA have supported polyphyletic phylogeographic groups that are inconsistent with subspecies plumage characteristics. In this study, we investigate the link between phenotypes and genotype by taking a phylogenetic approach. We use genome-wide SNPs, nuclear introns, and mitochondrial DNA to estimate population structure, isolation by distance, and species relationships. Together, our genetic sampling includes complete species-level sampling and comprehensive coverage of the three most phenotypically diverse Palearctic species. Our study provides strong evidence for species-level patterns of differentiation, however population-level differentiation is less pronounced. SNPs provide a robust estimate of species-level relationships, which are mostly corroborated by a combined analysis of mtDNA and nuclear introns (the first time-calibrated species tree for the genus). However, the mtDNA tree is strongly incongruent and is considered to misrepresent the species phylogeny. The extant wagtail lineages originated during the Pliocene and the Eurasian lineage underwent rapid diversification during the Pleistocene. Three of four widespread Eurasian species exhibit an east-west divide that contradicts both subspecies taxonomy and phenotypic variation. Indeed, SNPs fail to distinguish between phenotypically distinct subspecies within the *M. alba* and *M. flava* complexes, and instead support geographical regions, each of which is home to two or more different looking subspecies. This is a major step towards our understanding of wagtail phylogeny compared to previous analyses of fewer species and considerably less sequence data.

1. Introduction

In birds, male plumage differences among closely related taxa are often believed to be the result of sexual selection, and to play an important role in reproductive isolation (Price, 2008). Plumage differences can evolve rapidly (Olsson et al., 2010; Omland and Lanyon, 2000; Milá et al., 2007), and when populations are geographically structured, may result from spatial variation in selection regimes (Price, 2008). Recent studies have demonstrated that a small number of genes can cause dramatic plumage differences despite limited genetic differentiation throughout the remainder of the genome (Poelstra et al., 2015; Toews et al., 2016; Vijay et al., 2016; Mason and Taylor, 2015). Lack of overall genetic differentiation in taxa with distinct phenotypic differences is likely due to either (1) recent divergence, with strong selection on phenotype, or (2) large-scale introgression, except on pre-existing adaptive genetic differences. In such cases, it is unlikely that phylogenetic relationships gleaned from few loci accurately reflect true species trajectories.

Genera that contain widespread species complexes are useful systems for investigating geographic variation in phenotypes because they offer comparisons between populations and species at different stages of the speciation continuum. Species complexes are often characterized by high frequencies of hybridization and poorly developed isolation barriers, despite being structured geographically (Price, 2008).

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Traditionally, it was thought that sympatric species would respond similarly to environmental factors influencing divergence, and therefore the study of sympatric complexes might reveal important biogeographic barriers. However, a growing body of literature suggests that species specific differences have a direct effect on demography, and spatially concordant genetic breaks should not be the expectation (Zamudio et al., 2016). For example, sexually selected traits, such as plumage, can directly affect genetic diversity via assortative mating or species recognition (Price, 1998). The complex interactions of gene flow, drift, and selection play a large role in determining the outcome of speciation and the rate at which species move along the speciation continuum. While the placement of recently diverged and introgressed lineages may be difficult, these can be contrasted with older, reproductively isolated groups within the same genus.

One bird system that is particularly well suited for such studies is the passerine genus *Motacilla* in the family Motacillidae. *Motacilla* consists of 12 species distributed throughout the Old World (Alström and Mild, 2003; del Hoyo et al., 2004) that have earned the common name wagtails due to their propensity to pump their long tails up and down. Within wagtails, there are multiple examples of taxa at different stages in the speciation process: from barely differentiated parapatric populations, to subspecies/species with distinct plumages that meet in hybrid zones, to fully reproductively isolated species (Alström and Mild, 2003). Previous phylogenetic and phylogeographic studies of *Motacilla* report mitochondrial relationships incongruent with both taxonomy (Pavlova et al., 2005; Voelker, 2002; Pavlova et al., 2003; Li et al., 2016; Alström and Ödeen, 2002; Ödeen and Björklund, 2003; Ödeen & Alström, 2001) and nuclear relationships (Alström and Ödeen, 2002; Ödeen and Björklund, 2003; Ödeen & Alström, 2001), with suggestions that mitochondrial DNA (mtDNA) poorly reflects the true phylogeny (Alström and Mild, 2003; Alström and Ödeen, 2002; Ödeen and Björklund, 2003; Ödeen & Alström, 2001). Several of these studies focused on aspects of wagtails' plumage diversity, some proposing cases of remarkable parallel plumage evolution (Alström and Mild, 2003; Alström and Ödeen, 2002; Ödeen & Alström, 2001) and others implicating the role of selection in rapid plumage evolution (Pavlova et al., 2005).

Of particular interest have been the four sympatric, migratory wagtail species White Wagtail *M. alba*, Grey Wagtail *M. cinerea*, Citrine Wagtail *M. citreola*, and Yellow Wagtail *M. flava* which are widely distributed across the Palearctic during the breeding season. These species represent a striking contrast in spatial variation in male breeding plumage (cf. Fig. 1). Currently, subspecies are defined by differences in both color and pattern of head plumage in the *M. flava* complex (13 subspecies) and by head, back, and wing-covert plumage in *M. alba* (9 subspecies) (Alström and Mild, 2003). On the basis of both genetic and plumage data, many of these subspecies have been treated as separate species (reviewed in Alström and Mild, 2003). Plumage differences are thought to have evolved rapidly and in conflict with phylogeographic structure (Pavlova et al., 2005; Pavlova et al., 2003; Li et al., 2016; Ödeen and Björklund, 2003; Ödeen & Alström, 2001). In contrast, the other two Palearctic breeding species, *M. cinerea* and *M. citreola*, lack this extreme plumage variation (3 subtly different and 2 distinct subspecies, respectively (Alström and Mild, 2003).

Wagtails can be broadly categorized by breeding distribution (i.e. Palearctic, Afrotropical). Whereas only some of the Palearctic species are migratory, all of the Afrotropical species are resident (Alström and Mild, 2003; del Hoyo et al., 2004). Species with Palearctic breeding distributions can be further categorized by plumage color (i.e. "black-and-white" and yellow). Past phylogenetic reconstructions have not fully supported these groupings. *M. cinerea*, *M. citreola*, and the *M. flava* complex all have yellow plumage, but the latter two species have repeatedly been found to be polyphyletic (Voelker, 2002; Pavlova et al., 2003; Alström and Ödeen, 2002; Ödeen & Alström, 2001). Genetic data places the polytypic *M. alba* within the "black-and-white" plumage group, along with three monotypic species with rather restricted allopatric distributions in the Indian subcontinent (White-browed Wagtail *M. maderaspatensis*), Cambodia (Mekong Wagtail *M. samveasnae*), and Japan (Japanese Wagtail *M. grandis*) (Alström and Mild, 2003; Alström and Ödeen, 2002). The black-and-white Afrotropical African Pied Wagtail *M. aguimp* has also been placed within this group (Alström and Ödeen, 2002; Ödeen & Alström, 2001). The other two Afrotropical (Cape Wagtail *M. capensis* and Mountain Wagtail *M. clara*) and the Malagasy (Madagascar Wagtail *M. flaviventris*) species are closely related (Voelker, 2002; Alström and Ödeen, 2002; Alström et al., 2015) and have slight or no geographical variation in plumage (del Hoyo et al., 2004). A recent phylogenetic exploration of the family found the São Tomé endemic São Tomé Shorttail *Amaurocichla bocagii* nested within *Motacilla*, and proposed its inclusion within this genus (Alström et al., 2015). Overall, relationships among species are unclear and are in need of reexamination.

In this study, we utilize genome-wide SNPs, nuclear introns, and mtDNA to analyze phylogenetic relationships and divergence patterns in *Motacilla*, with complete species-level sampling and comprehensive coverage of the three most diverse Palearctic species. We (1) estimate the first complete time-calibrated species tree for this group; (2) use genome-wide SNPs to reconstruct the phylogeny and investigate the agreement between genotype and phenotype in the three most variable wagtail species; and (3) demonstrate conclusively that mtDNA alone is inappropriate for phylogenetic studies of *Motacilla*.

2. Materials and methods

2.1. Sanger data

Throughout the manuscript, we follow the taxonomy of Alström and Mild (2003) and Alström et al. (2015).

To resolve species-level relationships and infer divergence times, we utilize previously published and unpublished sequences from (1) three nuclear introns (CHD1Z, ODC, Mb) for 42 individuals across all 12 *Motacilla* species (Alström and Ödeen, 2002; Ödeen and Björklund, 2003), and (2) two mitochondrial regions (ND2, CR) for 103 individuals across all species, including all subspecies of *M. alba*, *M. flava*, and *M. citreola* (Table S1) (Pavlova et al., 2005; Voelker, 2002; Li et al., 2016; Ödeen and Björklund, 2003). *Dendronanthus indicus*; *Anthus pratensis*, and *Anthus trivialis* were used as outgroups (Alström et al., 2015).

2.2. ddRADseq data

2.2.1. Sampling

If wagtail divergence was recent or shaped by rapid ancestral radiations, then the timing between divergence events may have been too short for the emergence of phylogenetically informative mutations (Gohli et al., 2015; Rokas and Carroll, 2006), potentially leading to the mito-nuclear discordance shown in previous studies. We therefore enhanced our inferential power by collecting thousands of genome-wide SNPs.

We obtained extensive geographic sampling and near-complete taxonomic coverage across Eurasia from samples at the Burke Museum of Natural History and Culture. We augmented these specimens with samples from other natural history museums to provide complete sampling for the genus (Fig. S1, Table S1). A total of 246 birds were sampled from 11 of the 12 recognized *Motacilla* species (Alström and Mild, 2003; Alström et al., 2015) (*M. maderaspatensis* not included due to lack of tissue samples). Our sampling focused on the widespread migratory Eurasian wagtail species (*M. alba, M. flava, M. citreola, M. cinerea*) with multiple described subspecies. We examined museum skins and assigned individuals to subspecies using morphological criteria outlined in Alström and Mild (2003). For rooting phylogenetic trees, we sampled three individuals from the monotypic sister genus (*Dendronanthus* Alström et al., 2015).