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Discord reigns among nuclear, mitochondrial and phenotypic estimates of divergence in nine lineages of trans-Beringian birds

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Abstract

Proposals for genetic thresholds for species delimitation assume that simple genetic data sets (e.g. mitochondrial sequence data) are correlated with speciation; i.e. such data sets accurately reflect organismal lineage divergence. We used taxonomically stratified phenotypic levels of differentiation (populations, subspecies and species) among nine avian lineages using paired, trans-Beringian samples from three lineages each in three orders (Anseriformes, Charadriiformes, and Passeriformes) to test this assumption. Using mitochondrial DNA sequence data and nuclear genomic data (amplified fragment length polymorphisms), we found a lack of concordance between these two genomes in their respective estimates of divergence and little or no relationship between phenotype (taxonomic relatedness) and genetic differentiation between taxon pairs. There are several possible reasons for the discord observed (e.g. selection on one of the genomes or perhaps lineage sorting), but the implications are that genetic estimates of lineage divergence may not be correlated with estimates from other parts of the genome, are not well correlated with the speciation process and are thus not reliable indicators of species limits.

Keywords: birds, genomics, population genetics, speciation

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Introduction

Estimating genetic divergence between populations has been a mainstay of population genetics and systematics (e.g. Wright 1943; Nei 1978, Jost 2008). The genetic distance between humans and chimpanzees, for example, has been estimated using mitochondrial sequences (Horai *et al.* 1995), sex chromosomes (Kaessmann *et al.* 1999, Bohossian *et al.* 2000; Nachman & Crowell 2000), and autosomal nuclear loci (Chen & Li 2001, Chen *et al.* 2001). Some researchers have tried to define a divergence threshold that indicates speciation (König *et al.* 1999; Hebert *et al.* 2004; Papadopoulou *et al.* 2008), which could then be used for species identification and conservation efforts.

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While it is generally recognized that individual gene trees may differ between diverging lineages (Chen *et al.* 2009; Hoffman *et al.* 2009; Melo-Ferreira *et al.* 2009; Sala-Bozano *et al.* 2009), it is reasonable to assume that diverging lineages gradually accrue genomic differences through time. Thus, the concept that a divergence threshold may be used as an indicator of speciation is attractive. If simple measures of genomic divergence are to be used as effective 'speciation indicators', they must be rather tightly correlated with the speciation process and its products. However, Zuckerkandl & Pauling (1962, 1965) predicted that rates of morphological and molecular evolution would not be correlated, because molecular evolution is the result of mostly neutral changes, while morphological evolution can be driven by selection. Omland (1997) tested this prediction by examining whether the number of morphological changes (phenotype) was correlated with the number of

nucleotide changes (genotype) along the branches of trees. He studied eight taxa, ranging from genus comparisons to order comparisons, and found that morphological and molecular evolution appeared to be coupled. Bromham *et al.* (2002) argued that Omland (1997) failed to properly correct for the confounding variable of time; they reanalysed three of his data sets and 13 additional vertebrate data sets by counting morphological and nucleotide changes only on terminal branches. Their results suggested no correlation between morphological and molecular evolution. Davies & Savolainen (2006) largely confirmed Bromham *et al.*'s (2002) findings using both vertebrates and vascular plants. All of these studies examined morphological and molecular evolution in well-established taxa that have already undergone speciation. The relationship between genotype and phenotype below the species level has been relatively neglected, although a correlation would arguably have to exist at these levels if genetic distance thresholds were to be useful indicators of speciation.

Mitochondrial DNA (mtDNA) has an effective population size that is one-fourth that of nuclear DNA (nuDNA; Avise 2004). As a consequence, many scientists studying animals view mtDNA as the 'leading' indicator of divergence (Zink & Barrowclough 2008), although Larsson *et al.* (2009) suggested that the relationship between the two cannot be characterized so simply and that neither genome can really be considered to have a higher resolving power of evolutionary relationships and population structure (or be the 'leading' indicator). Nevertheless, some scientists argue that mtDNA is representative of an organism as a whole and that it can be used to identify new species (e.g. Lane 2009). Others consider that mtDNA data have some weaknesses (e.g. Ballard & Whitlock 2004) and that integrating these data with other data sets will be required for a robust understanding of lineage relationships and species recognition (Rubinoff & Holland 2005; Edwards & Bensch 2009). In birds, for example, intraspecific and interspecific mtDNA divergence estimates can overlap considerably below, e.g. 5% (Johns & Avise 1998; Hebert *et al.* 2004; Winker 2009).

At shallow levels of divergence, especially between groups that are not reciprocally monophyletic in mtDNA, nuDNA sequence data present two problems: (i) on average, nuDNA mutates more slowly than mtDNA, so nuclear genes generally show less variation than mtDNA (Brown 1983); and (ii) nuDNA has a larger effective population size than mtDNA, so variation takes longer to sort among structured populations (Zink & Barrowclough 2008), causing any given nuclear marker to be less likely than mtDNA to track lineage divergence at shallow levels. Fragment analyses such as amplified fragment length polymorphisms (AFLPs; Vos *et al.* 1995) compare the sizes of fragments among samples, and a single nucleotide change can provide phylogenetic signal. When many presumably unlinked loci are examined, a broad assessment of the nuclear genome is obtained, and these techniques are often able to differentiate between taxa at very shallow levels of divergence, even when mtDNA is uninformative (e.g. African cichlids, Allender *et al.* 2003; *Laupala* crickets, Mendelson *et al.* 2004; and snow and McKay's buntings, Maley & Winker 2010). The main assumption of AFLP data is that bands of the same length are homologous; this assumption appears to be increasingly violated as taxonomic distance increases (Bremer 1991; Mechanda *et al.* 2004), but, thus far, these data appear to be robust at shallow levels of divergence (e.g. Nosil *et al.* 2009; Maley & Winker 2010).

Because it is reasonable to assume that diverging lineages gradually accrue genomic differences through time, it is fair to expect that a comparative study will find correlated mtDNA and nuDNA divergences. These genomes have different effective population sizes, so we would probably not expect a one-to-one relationship in divergence between them, but this does not prevent the expectation of a positive linear correlation. However, several processes are known to decouple the evolutionary history of the mitochondrial and nuclear genomes, such as selection, fluctuating population sizes (drift), demography (e.g. sex-biased gene flow) and mtDNA capture (introgression followed by a selective sweep). It is thus possible that simple measures of lineage divergence may not track the divergence process particularly tightly and that signals from the two genomes could differ. In addition to these factors, the time needed to attain reproductive isolation between diverging populations (the speciation process itself) can be quite variable among lineages (Price 2008), which might impart additional variation to an among-lineage comparison of divergence between the mtDNA and nuDNA genomes.

We examined genetic divergences (mitochondrial and nuclear) within avian lineages at three taxonomically stratified levels of phenotypic divergence (populations, subspecies and species) to encompass the complete process of speciation, using taxonomy as a categorical measure of phenotypic divergence. Patterns of genetic divergence were determined using paired samples from three lineages each in three avian orders (Anseriformes, Charadriiformes, and Passeriformes). We estimated the probable number of divergence events that occurred among these nine paired samples, and we also examined whether there were correlations between phenotypic divergence (taxonomic status) and genetic divergence, or between mtDNA and nuDNA estimates of divergence.

Materials and methods

The study system

During the Pleistocene Era (2 million years ago to 10 000 years ago), Beringia was a large, ice-free land mass that experienced occasional inundations during interglacial periods (Hopkins 1967), stretching 1600 km north to south at its narrowest point (Hopkins 1959; Hopkins *et al.* 1965). At the height of the glaciations, Beringia was isolated from North America and western Asia by ice sheets; as a result, it functioned as an important Pleistocene refugium (Pielou 1991).

The cycle of glacial and interglacial periods during the Pleistocene caused a matching cycle of exposure and submergence of the Beringian land mass (Hewitt 1996, 2004). During the Pleistocene, the Bering-Chukchi platform, which connects the North American and Asian continents, was exposed at least nine and possibly as many as 20 times (Hopkins 1967; Pielou 1991). When exposed, this land bridge was covered with vegetation and supported a variety of animal life (Pielou 1991). As the climate warmed and the glaciers melted, the Bering-Chukchi platform would flood, severing the terrestrial connection between North America and Asia (Hopkins 1959, 1967). Eventually, the climate cooled and glaciers reformed, exposing the Bering Land Bridge again.

Each time the cycle of exposure and submergence of the Bering-Chukchi platform repeated itself, the same basic cycle (separation and reuniting of taxa) could have occurred. The biotic effects of this cycle of events should be shared among bird lineages that occur on both sides of Beringia. Additionally, taxa that were split during an early flooding event (if they did not regain contact and freely interbreed) should be more genetically divergent than taxa that were split during one of the more recent interglacial periods. The repetitive vicariance barrier that the Bering Land Bridge emersion and immersion cycle has produced among terrestrial organisms may have caused multiple, coherent, and detectable 'ripple' effects in the genetic divergences of these organisms presently occurring on both sides of the Bering Sea.

Current North American avian taxonomy follows the biological species concept, which emphasizes attainment of reproductive isolation between diverging lineages and recognizes subspecies (AOU 1998). Because reproductive isolation generally occurs rather later than attainment of lineage diagnosability under the phylogenetic species concept, we consider that our study design covers the entire process of speciation under both of these species concepts. We use taxonomic level as a surrogate for phenotypic divergence in our study. Although it is categorical, it is based on phenotype using widely accepted standards (e.g. Mayr & Ashlock 1991), and in birds the results are repeatedly revisited and reviewed (e.g. AOU 1998). Furthermore, this remains the most practical assessment possible because as yet we have no reliable among-lineage measure of speciation-related phenotypic divergence comparable to those commonly used in genetics (Winker 2009).

Mitochondrial DNA (mtDNA)

DNA was extracted from muscle tissue of 162 birds collected in Alaska and Russia using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA). Species from three different orders at three different taxonomic levels were sampled (Appendix I; see Fig. 1 for range maps and haplotype networks). We used currently accepted taxonomic designations (Gibson & Kessel 1997; Dickinson 2003) to choose sister pairs. Individuals were broadly sampled from throughout the known distribution whenever possible (collection localities are given in Appendix I).We amplified the mtDNA NADH dehydrogenase subunit 2 (ND2) gene using primers L5215 (Hackett 1996) and H6313 (Sorenson *et al.* 1999). Amplification was performed following standard PCR protocols, using an annealing temperature of 50 °C. Cycle-sequencing was performed using ABI (Applied Biosystems Inc., USA) Big-Dye Terminator mix and an annealing temperature of 50 °C. Samples were bidirectionally sequenced using a combination of the primers used for amplification and the internal primers L5758 and H5776 (Sorenson *et al.* 1999). Products were sequenced on an ABI 3130 automated sequencer. We aligned sequences by eye without gaps using Sequencher (Genecodes Corporation Inc., USA). To aid in the visualization of these data, we constructed unrooted parsimony trees using PAUP* (Swofford 2002).

Amplified fragment length polymorphisms (AFLPs)

Samples used for mtDNA data were also used for AFLP data generation, although we were not able to obtain AFLP data from all individuals or all primer pairs (Table 1). Sample size effects did not affect our conclusions (Data S1, Supporting information). We followed a modified ABI plant-mapping (large genome) protocol to generate loci (Parchman *et al.* 2006). Restriction of extracted DNA using enzymes *Mse*I and *Eco*RI was performed concurrently with ligation of *Mse*I and *Eco*RI adaptors. Preselective amplification was performed using ABI's preselective primers. We used six primer pair combinations for selective amplification (Appendix II). Genotyping was performed on an ABI 3100 automated sequencer (Applied Biosystems Inc.). We scored the chromatograms using GeneMapper ver. 3.7 (Applied Biosystems Inc.). Only loci that could be

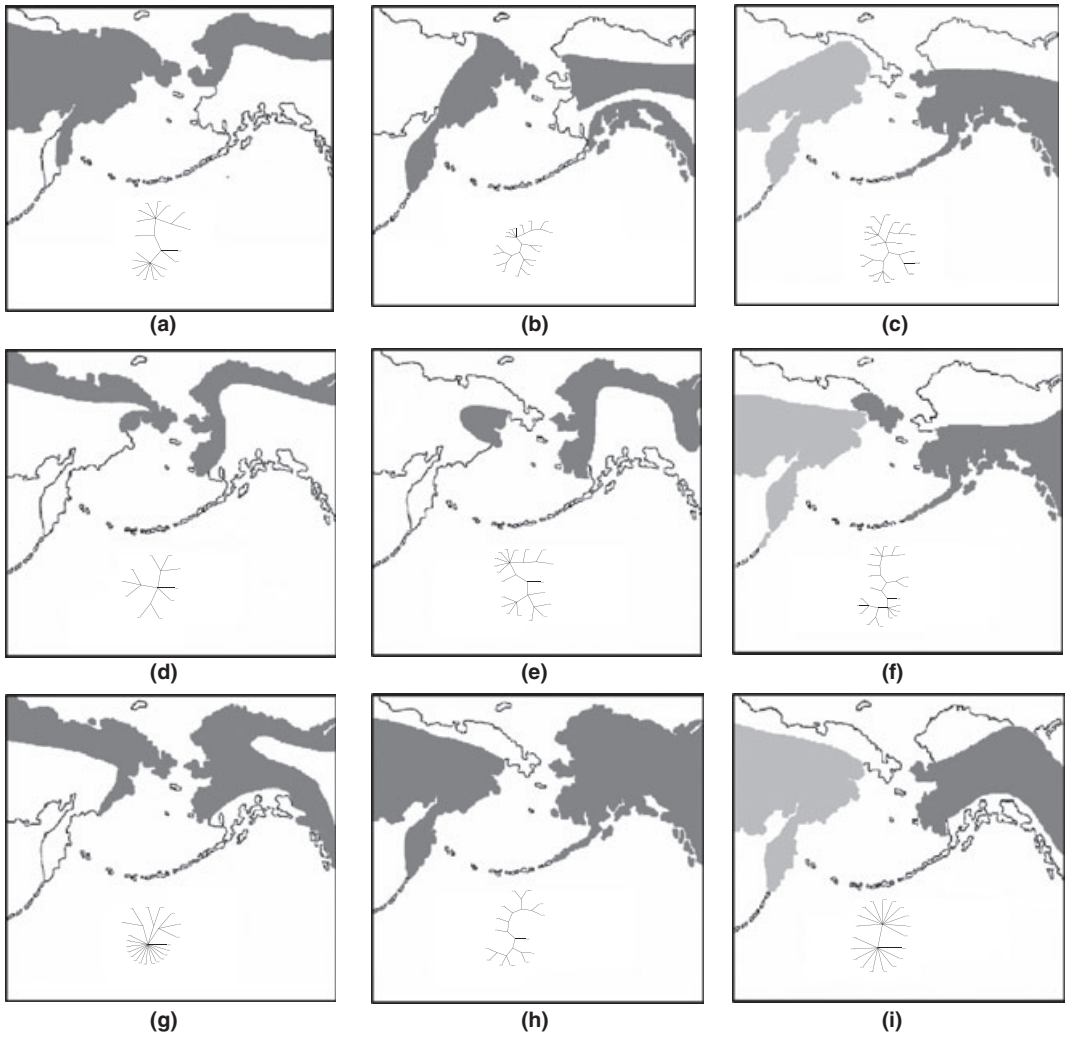

Fig. 1 Distributions and mitochondrial DNA networks of taxa. Operational taxonomic units (OTUs) are unlabelled. Networks were inferred using parsimony. For the species-level lineages, the range of the Alaskan species is in dark grey and that of the Russian species is in light grey. (a) *Luscinia svecica*; (b) *Pinicola enucleator kamtschatkensis/Pinicola enucleator flammula*; (c) *Pica pica/Pica hudsonia*; (d) *Pluvialis squatarola*; (e) *Numenius phaeopus variegatus/Numenius phaeopus hudsonicus*; (f) *Tringa brevipes/Tringa incana*; (g) *Clangula hyemalis*; (h) *Anas crecca crecca/Anas crecca carolinensis*; (i) *Anas penelope/Anas americana*.

determined unambiguously as present or absent in each individual (i.e. clean, well-defined peaks) were scored. We used a minimum peak width of 1.5 bp and a minimum peak height of 100 as a starting point, but then examined each peak individually to maximize the amount of phylogenetic information obtained (Holland *et al.* 2008). Scored data sets have been deposited in the Dryad digital repository (11696338MEC-10-1079.R1).

Comparison of divergence estimates from mtDNA and AFLPs

For mtDNA sequence data, we estimated net nucleotide difference (D_A, the average number of substitutions per site between populations minus the average number of substitutions per site within populations; Nei 1978) between population samples using DnaSP ver. 5 (Librado & Rozas 2009). We also calculated F_{ST} (and *P*-values) for each taxon comparison for our mtDNA data using Arlequin (Excoffier *et al.* 2005). We calculated F_{ST} (and *P*-values) following Lynch & Milligan (1994) for our AFLP data using AFLP-SURV 1.0 (Vekemans *et al.* 2002) with the Bayesian method with uniform priors and 10 000 random permutations to test for significant levels of differentiation.

To examine the potential influence of effective population size on lineage divergence, we tested the relationship between mtDNA-based estimates of effective population size (N_e) and estimates of genetic distance (both mtDNA and nuDNA). We used MIGRATE-n v.3.0.3

Table 1 N_e, D_A, and F_{ST} values. F_{ST} statistics with $P \leq 0.05$ are in bold. The Russian taxon is listed first; the Alaskan taxon is listed second. Orders are listed in taxonomic order and within orders sister taxa are presented with increasing taxonomic divergence, as populations, subspecies and then species. Negative F_{ST} values represent program idiosyncracies and are effectively 0.0

Taxonomic comparison	# AFLP loci*	N_e estimates (Russia)	N_e estimates (Alaska)	D_A (mtDNA)†	F_{ST} (mtDNA)†	F_{ST} (AFLP)†
Clangula hyemalis/Clangula hyemalis	460 (8:12)	6637	546	−0.0001	−0.04081	−0.0004
Anas crecca crecca/Anas crecca carolinensis	420 (8:5)	14 001	3728	0.0532	**0.7450**	−0.0186
Anas penelope/Anas americana	509 (10:8)	877	339	0.0199	**0.9772**	**0.0255**
Pluvialis squatarola/Pluvialis squatarola	379 (5:5)	79 500	121	−0.0002	0.3425	−0.0052
Numenius phaeopus variegatus/Numenius phaeopus hudsonicus	529 (7:9)	455	862	0.0326	**0.8395**	0.0197
Tringa brevipes/Tringa incana	476 (10:10)	45	32	0.0548	**0.8437**	**0.0518**
Luscinia svecica/Luscinia svecica	510 (10:9)	8006	2821	0.0000	0.0079	0.0280
Pinicola enucleator kamtschatkensis/Pinicola enucleator flammula	280 (6:7)	513	1307	0.0302	**0.8055**	−0.0019
Pica pica/Pica hudsonia	413 (9:10)	1991	607	0.0527	**0.6989**	**0.0065**

*Values in parentheses represent Russian:Alaska sample sizes.
†AFLP, amplified fragment length polymorphism; mDNA, mitochondrial DNA.

(Beerli & Felsenstein 1999, 2001) to estimate N_e. We converted the θ estimates using Weir and Schluter's (2008) mutation rate estimates and generation time estimates from the Birds of North America online species accounts (Cornell Laboratory of Ornithology 2005). We performed four linear correlations using STATISTICA (StatSoft Inc., Tulsa, OK), comparing both the Russian and the Alaskan population N_e estimates with D_A (mtDNA) and F_{ST} (AFLPs). To test for a relationship between D_A (mtDNA) and F_{ST} (AFLPs), we also did a linear correlation using Statistica, and we plotted these values to visualize this relationship. Additionally, we did a MANOVA to test for relationships among taxonomic status, mtDNA estimates of divergence and AFLP estimates of divergence.

F_{ST} has known limitations as a measure of population divergence (Jost 2008, 2009; but see also Heller & Siegismund 2009; Ryman & Leimar 2009). In addition to calculating F_{ST} for our AFLP data sets, we therefore also calculated Jost's D using the program Spade (Chao & Shen 2003) and compared Jost's D values for each locus to the calculated F_{ST} value. We did not find any large deviations between the two estimates (data not shown) and so have continued to report all AFLP divergences as F_{ST} values.

Estimation of number of divergence events

We estimated the most probable number of vicariant events that caused mtDNA divergence among our nine comparisons using msBayes (Hickerson *et al.* 2006, 2007). This test enabled us to estimate whether all lineages at the same taxonomic level originated during the same vicariant event. We ran 3 million simulations, bounding the prior distribution for θ between 0.5 and 100. We set the upper bound for the prior distributions of τ at 40.0, migration rate at 10 and ancestral population size at 0.5. All other parameters were set to 0. We analysed our simulations using a tolerance (proportion of posterior distribution analysed) of 0.0005 (as recommended by the authors). We analysed both the full data set (all nine lineages) and a truncated data set containing only the six lineages that represent subspecies- and species-level divergences.

Results

Comparison of divergence estimates from mtDNA and AFLPs

Net nucleotide differences (D_A) ranged from −0.0002 to 0.0548 for our nine pairwise mtDNA comparisons; F_{ST} estimates ranged from −0.0186 (effectively 0.0) to 0.0518 (Table 1). In mtDNA, the *Anas crecca crecca/Anas crecca carolinensis*, the *Tringa incana/Tringa brevipes* and the *Pinicola enucleator kamschatkensis/Pinicola enucleator flammula* comparisons showed the highest divergences, whereas the highest AFLP divergence was found in the *Tringa incana/Tringa brevipes* comparison. The lowest mtDNA divergence values (D_A) occurred in the three population-level comparisons (*Pluvialis squatarola/Pluvialis squatarola*, *Luscinia svecica/Luscinia svecica* and *Clangula hyemalis/Clangula hyemalis*), whereas four pairs had F_{ST} values of effectively 0 based on AFLP estimates: *Clangula hyemalis/Clangula hyemalis*, *Anas crecca crecca/Anas crecca carolinensis*, *Pluvialis squatarola/Pluvialis squatarola* and *Pinicola enucleator kamschatkensis/Pinicola enucleator flammula* (Table 1).

Level of taxonomic divergence was not an accurate predictor of the degree of genetic differentiation

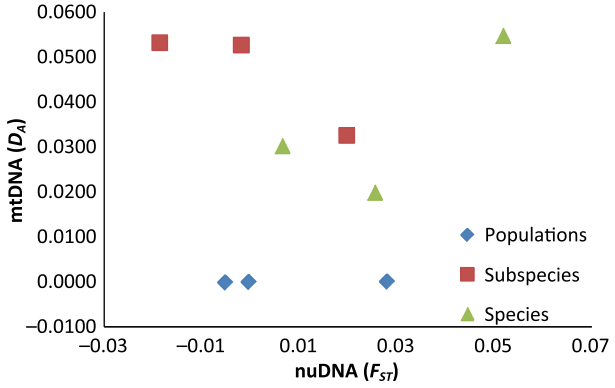

Fig. 2 D_A plotted against corresponding F_{ST}. D_A estimates were calculated using mitochondrial DNA sequence data, whereas F_{ST} values were calculated using amplified fragment length polymorphism data.

between two taxa (Fig. 2). Our three species-level comparisons (*Anas americana/Anas penelope*, *Tringa brevipes/Tringa incana* and *Pica pica/Pica hudsonia*) had both mtDNA and AFLP estimates of population differentiation that were significantly different from 0 ($P \leq 0.05$, from F_{ST} estimates; Table 1), although in only one case (*Tringa*) was this significance coupled with high divergence estimates of D_A and F_{ST} (Table 1). An additional three pairs (*Anas crecca carolinensis/Anas crecca crecca*, *Numenius phaeopus hudsonicus/Numenius phaeopus variegatus*, and *Pinicola enucleator flammula/Pinicola enucleator kamtschatkensis*) had only mtDNA estimates that were significantly different from 0 (Table 1). No additional AFLP-based F_{ST} estimates were significantly different from 0 (Table 1).

We found no significant relationships between N_e estimates (Table 1) and D_A (two linear correlation tests for mtDNA; $P > 0.21$) or F_{ST} (two similar tests for AFLPs; $P > 0.30$). Linear correlation also showed no significant relationship between our two measures of genetic divergence, D_A and F_{ST} ($P = 0.88$, $r = 0.062$; Fig. 2). Although the lowest D_A estimates all occurred in our population-level comparisons, on average the highest levels occurred in subspecies-level comparisons (Table 1). And although on average the highest values of F_{ST} occurred among the species-level comparisons, the lowest average occurred among subspecies (Table 1).

Estimation of number of divergence events

The posterior distribution of ψ (the number of possible vicariant events that caused diversification among the nine lineage pairs) showed low density values from 3 to 8 divergence events and peaked at nine divergence events. The 95% probability density was 2.7 to 9.0; we thus conclude that 3–9 separate vicariant events affected the nine lineages. When the truncated 6-lineage data set was analysed, the 95% probability density became 2.3 to 6.0 vicariant events.

Discussion

We found a remarkable level of discord among all three measures of divergence. Discord between phenotype and genotype is expected; phenotype is often controlled by a few genes and can also be the result of environmental induction (West-Eberhard 2003). Taxonomy in birds has been based on external morphology (mostly plumage), which is likely to be under both natural and sexual selection (e.g. Mayr & Ashlock 1991). Plumage patterns can show substantial differentiation either in the absence of evident genetic differentiation (e.g. Haas *et al.* 2009) or due apparently to drift (Lehtonen *et al.* 2009). Ricklefs (2004) assumed that speciation rate was a measure of genetic change and found that lineages in species-rich phylogroups (inferred using DNA–DNA hybridization) show greater morphological variation than their counterparts in less species-rich clades in passerine birds. This suggested that greater morphological variation is correlated with increased speciation rates, although this relationship could be the result of time and clade age (Ricklefs 2006). When Adams *et al.* (2009) corrected for the effects of clade age (inferred using both mitochondrial and nuclear DNA), they found no relationship between speciation rates and morphological variation in plethodontid salamanders.

Noncorrelation between mitochondrial and nuclear measures of divergence has been previously demonstrated in frogs (Chen *et al.* 2009), lizards (Hoffman *et al.* 2009), and mammals (Godinho *et al.* 2008; Yang & Kenagy 2009). While introgression of the mitochondrial genome is usually suggested as the source of the discrepancy (Irwin *et al.* 2009; Nevado *et al.* 2009; Renoult *et al.* 2009), other factors such as selection, demography and drift owing to fluctuating population sizes could also be responsible.

Selection can affect our results through occurrence in either one or both genomes. Although usually treated as a putatively neutral marker, mtDNA is known to evolve neither neutrally nor in a clock-like manner (Galtier *et al.* 2009); additionally, Nosil *et al.* (2009) suggested that between 5% and 10% of the nuclear genome is under selection. Recent studies have suggested that genetic draft (or hitchhiking of noncoding regions) is so pervasive in nuDNA that there is actually a 'shortage' of neutrally evolving nuclear loci (Wright & Andolfatto 2008).

Demography (specifically, life history traits that do not equally affect the inheritance of mtDNA and

nuDNA) is an important factor that can cause different evolutionary histories for mitochondrial and nuclear genomes. In particular, the dispersal ability and behaviour of a species can greatly affect mtDNA and nuDNA divergence and structuring (Steele *et al*. 2009; Melo-Ferreira *et al*. 2009). Three different dispersal patterns can occur: male-biased dispersal (also known as female-biased philopatry), female-biased dispersal or unbiased dispersal. Both male-biased and female-biased dispersal can result in unequal gene flow of mtDNA and nuDNA; unbiased dispersal will not decouple the evolutionary history of the two genomes between diverging populations. Among animal species, male-biased and female-biased dispersal are common. In birds, dispersal tends to be female-biased except in Anseriformes (which exhibit male-biased dispersal; Temple 2004). If demography played a major role in decoupling the rate of divergence in the genomes of the lineages in our study, we would expect significant correlations between estimates of mitochondrial and nuclear divergence when the Anseriformes lineages are analysed separately from all other lineages. There was no relationship between mtDNA (D_A) and nuDNA (F_{ST}) divergence values among the female-biased dispersers ($P = 0.57$, $r = 0.30$) nor among the three *Anseriformes* ($P = 0.64$, $r = 0.54$). Demography alone does not explain the discord we observed, but it does bear consideration in future studies.

Fluctuating population sizes can also decouple the evolutionary relationships between mtDNA and nuDNA, primarily through stochastic events. Because mtDNA has a smaller effective population size than nuDNA, it is more sensitive to population bottlenecks and any subsequent expansion events (Avise 2004). A decline in population size could cause random fixation of one mtDNA haplotype, which would then spread as the population expanded. In two separated populations, this could result in fixed haplotypes that are either very different (which would increase the estimated divergence) or similar (which would decrease the estimated divergence). The nuclear genome is less likely to be affected by bottlenecks and rapid expansions, and this scenario would presumably result in a nuDNA population divergence estimate that is closer to the true value. Our finding that effective population size estimates (N_e) did not correlate with mitochondrial or nuclear divergence estimates suggests that this factor may not be very important in causing the discord observed.

Using genetic distance as a speciation indicator is attractive, but it is only viable if certain relationships exist, such as a correlation between phenotypic and genotypic divergence, or correlation between different genotypic distance estimates. Our results suggest that using a genetic divergence estimate from part of an organism's genome does not accurately represent organismal divergence and that commonly used measures are not strongly correlated with the speciation process.

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E.M.H. and K.W. are evolutionary biologists interested in the processes of organismal divergence and speciation.

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 Examination of how sample sizes affected estimates of F_{ST} among our AFLP data sets.

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Appendix I

Specimens used in this study, with identifiers [University of Alaska Museum (UAM and REW) and Burke Museum (UWBM) voucher numbers, and GenBank accession numbers]. Collection localities are also included. The side of the Bering Strait each taxon represents is listed in parentheses following the taxon name.

Species	Voucher no.	Collecting localities	GenBank accession no.
Anas crecca carolinensis (Alaskan population)	UAM 11920, UAM 14961, UAM 11251, UAM 11340, UAM 11339, UAM 11338	Fairbanks (5), Izembek (1)	HM640785–HM640790
Anas crecca crecca (Russian population)	UWBM 56971, UWBM 71261, UWBM 44476, UWBM 43947, UWBM 71265, UAM 9255, UAM 14100	Noyabr'sk (1), Magadanskaya Oblast' (3), Anadyr' (1), Shemya Is. (2)	HM640791–HM640797
Anas americana (Alaskan population)	UAM 11908, UAM 11909, UAM 11916, UAM 11919, UAM 11922, UAM 11923, UAM 11924, UAM 11927, UAM 11928	Fairbanks (9)	HM640861–HM640869
Anas penelope (Russian population)	UAM 9359, UAM 11803, UAM 11804, UAM 14529, UAM 17756, UAM 14595, UAM 14538	Buldir Is. (1), Shemya Is. (5), Midway Atoll (1), Attu Is. (1)	HM640870–HM640876
Clangula hyemalis (Alaskan population)	UAM 9395, UAM 11468, UAM 11602, UAM 13154, UAMX 3357, REW 620, REW 583, REW 532, REW 619, REW 531, REW 584, UAM21883	Barrow (2), Montague Is. (2), Ice Cut (4), Y-K Delta (1), Grayling Lake (2), Dalton Hwy mile 71 (3)	HM640808–HM640818
Clangula hyemalis (Russian population)	UWBM 43893, UWBM 43894, UWBM 43895, UWBM 43913, UWBM 43916, UWBM 43917, UWBM 43918, UWBM 43919, UWBM 4370	Anadyr' (9)	HM640819–HM640827
Luscinia svecica (Alaskan population)	UAM 8584, UAM 8585, UAM 8622, UAM 8944, UAM 8945, UAM 8946, UAM 13718, UAM 17727, UAM 15419	Taylor Hwy mile 73 (4), Taylor Hwy mile 47 (2), Ice Cut (3)	HM640910–HM640918
Luscinia svecica (Russian population)	UWBM 44629, UWBM 44630, UWBM 44233, UWBM 44242, UWBM 44243, UWBM 44246, UWBM 44360, UWBM 44361, UWBM 44363	Milkovo (5), Cherskiy (4)	HM640919–HM640927
Numenius phaeopushudsonicus (Alaskan population)	UAM 11044, UAM 13349, UAM 13423, UAM 13925, UAM 9328, UAM 9260, UAM 11760, UAM 20642	Fairbanks (1), Dalton Hwy (2), Taylor Hwy (3), Nome (1), Good News Bay (1)	HM640828–HM640835
Numenius phaeopusvariegatus (Russian population)	UAM 9426, UAM 10049, UAM 14230, UAM 14225, UAM 14229, UAM 14227, UAM 14223	Adak Is. (2), Olango Is. (5)	HM640836–HM640842
Pica hudsonia (Alaskan population)	UAM 10139, UAM 10140, UAM 10141, UAM 10142, UAM 12453, UAM 13049, UAM 13052, UAM 13053, UAM 14665	Kodiak Is. (4), Izembek NWR (1), Richardson Hwy mile 60 (1)	HM640843–HM640851
Pica pica (Russian population)	UWBM 44584, UWBM 44585, UWBM 47197, UWBM 72084, UWBM 72091, UWBM 74569, UWBM 74697, UWBM 74872, UWBM 44581	Sokhoch (3), Khabarovskiy Kray (1), Ussuriysk (2), Gayvoron (3)	HM640852–HM640860
Pinicola enucleatorflammula (Alaskan population)	UAM 11285, UAM 11287, UAM 11286, UAM 10157, UAM 10158, UAM 8794, UAM 7362, UAM 8563, UAM 11831	Revillagigedo Is. (3), Kodiak Is. (4), Homer (1), Dillingham (1)	HM640893–HM640901
Pinicola enucleator kamschatkensis (Russian population)	UWBM 44628, UWBM 47313, UWBM 47314, UWBM 47315, UWBM 47316, UWBM 51628, UWBM 51642, UWBM 51643	Milkovo (1), Sakhalinskaya Oblast' (3), Snezhnaya Dolina (1)	HM640902–HM640909
Pluvialis squatarola (Alaskan population)	UAM 14238, UAM 13347, UAM 13389, UAM 13488, UAM 13390	Mactan Is. (1), Deadhorse (1), Dalton Hwy (3)	HM6409798–HM640802

Appendix I (Continued)

Species	Voucher no.	Collecting localities	GenBank accession no.
Pluvialis squatarola (Russian population)	UWBM 51608, UWBM 43931, UWBM 43963, UWBM 43964, UWBM 44500	Zaliv Odyan (1), Anadyr' (3), Cherskiy (1)	HM640803–HM640804
Tringa brevipes (Russian population)	UAM 7534, UAM 7535, UAM 8521, UAM 8805, UAM 9398, UAM 9399, UAM 9402, UAM 9404, UAM 10112	Attu Is. (8), Shemya Is. (1)	HM640884–HM640893
Tringa incana (Alaskan population)	UAM 8420, UAM 10101, UAM 10135, UAM 10176, UAM 10496, UAM 13434, UAM 21813, UAM 15181	Attu Is. (5), Nunivak Is. (1), Amlia Is. (1), Thompson Pass (1)	HM640877–HM640883

Appendix II

*Mse*I and *Eco*RI selective amplification primers used for this study. Only the last three base pairs of the primers are reported. Primers are from Applied Biosystems Inc.

Primer combination	*Mse*I primer	*Eco*RI primer
1	AGG	CTA
2	AAG	CAG
3	ACT	CTT
4	ACA	CAA
5	AAC	CTA
6	ACC	CTC

Supporting Information.

Examination of how sample sizes affected estimates of F_{ST} among our AFLP datasets.

Estimates of within- and between-population genetic variation improve with increasing sample size. Among our datasets we found that the lowest population sample size (N_{low}) in a taxon-pair comparison (from Table 1) was correlated with estimates of F_{ST} (Figure S1). Lower sample sizes caused a depressed value of F_{ST}; the linear correlation was significant (F = 13.3, P < 0.008) and the best-fit linear model was F_{ST} = -0.0599 + 0.00962 × N_{low}. This general relationship was verified among the three strongest datasets by randomly sampling them to create 10 datasets of each that compared 5 individuals from each population in a pair. The average F_{ST} values for these smaller, randomly generated datasets were all smaller than F_{ST} values for the full datasets; a linear correlation on these values standardized so that the F_{ST} of the full dataset equaled 1.0 (Figure S2) was significant (F = 43.4, P < 0.003), and the best-fit linear model for standardized F_{ST} was F_{ST} = 0.935 + 0.0071 × N_{low} (these models were generated to estimate the slope, m).

Although N_{low} had a significant effect on our estimates of F_{ST}, replication among taxon levels and the distribution of smaller sample sizes resulted in these effects biasing our analyses *towards* finding a positive relationship with D_A. This bias occurred because our three species-level comparisons had an average N_{low} of 9.3, whereas the subspecies- and population-level comparisons had average N_{low} values of 6.0 and 7.3, respectively (effectively making F_{ST}–based divergence estimates for the lower taxonomic divisions lower). Therefore, as expected given these distributions, when estimates of F_{ST} were corrected for N_{low}, there was still no significant relationship with D_A (Figure S3). These results were equivalent when using both of the values of m in the linear models above for applying the corrections to F_{ST} (Figure S3 uses m = 0.0071).

Figure legends:

Figure S1. AFLP-based estimates of F_{ST} in relation to the lowest population size in each pairwise comparison. Negative values of F_{ST} are due to program algorithms and are effectively zero, although they have been used here at their calculated values.

Figure S2. A decline in F_{ST} estimates in relation to sample size is demonstrated here within the three strongest datasets by randomly subsampling each to produce 10 datasets of N = 5 from each population. Here, values have been standardized to examine the slopes of the relationships by setting each full dataset F_{ST} value to 1.0 (upper right) and adjusting the mean F_{ST} of each of the 10 randomized pairwise comparisons accordingly (lower left).

Figure S3. Mitochondrial divergence (D_A) in relation to AFLP-based F_{ST} corrected for sample size (N_{low}).

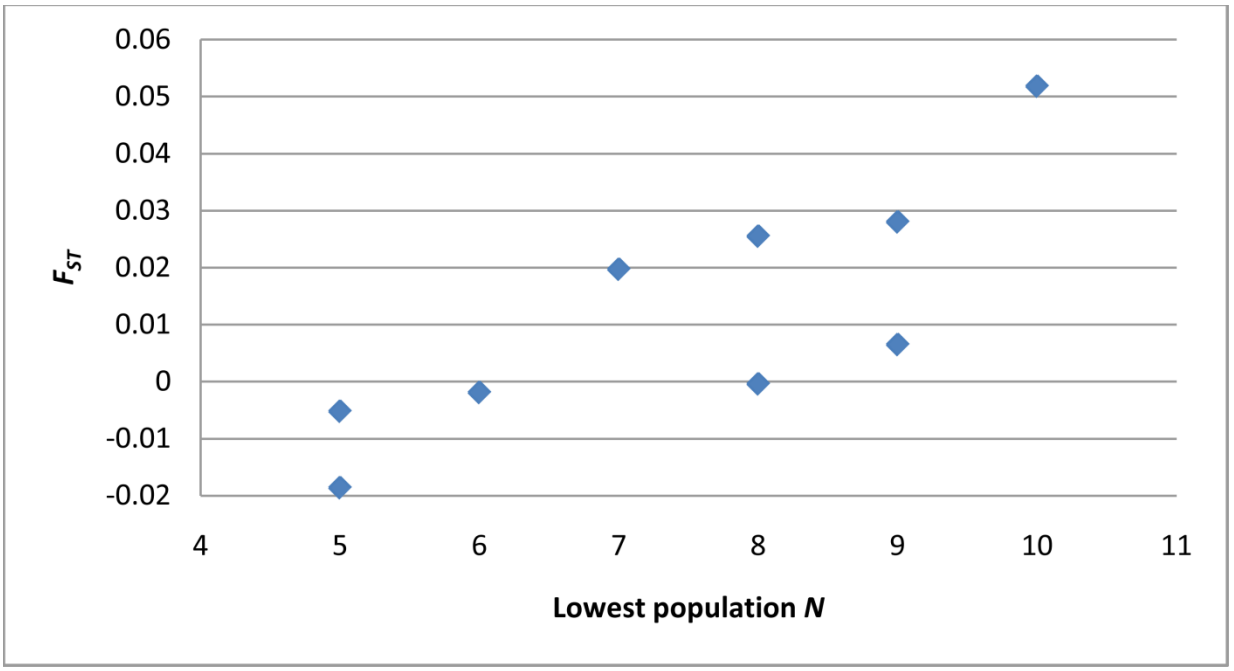

Figure S1

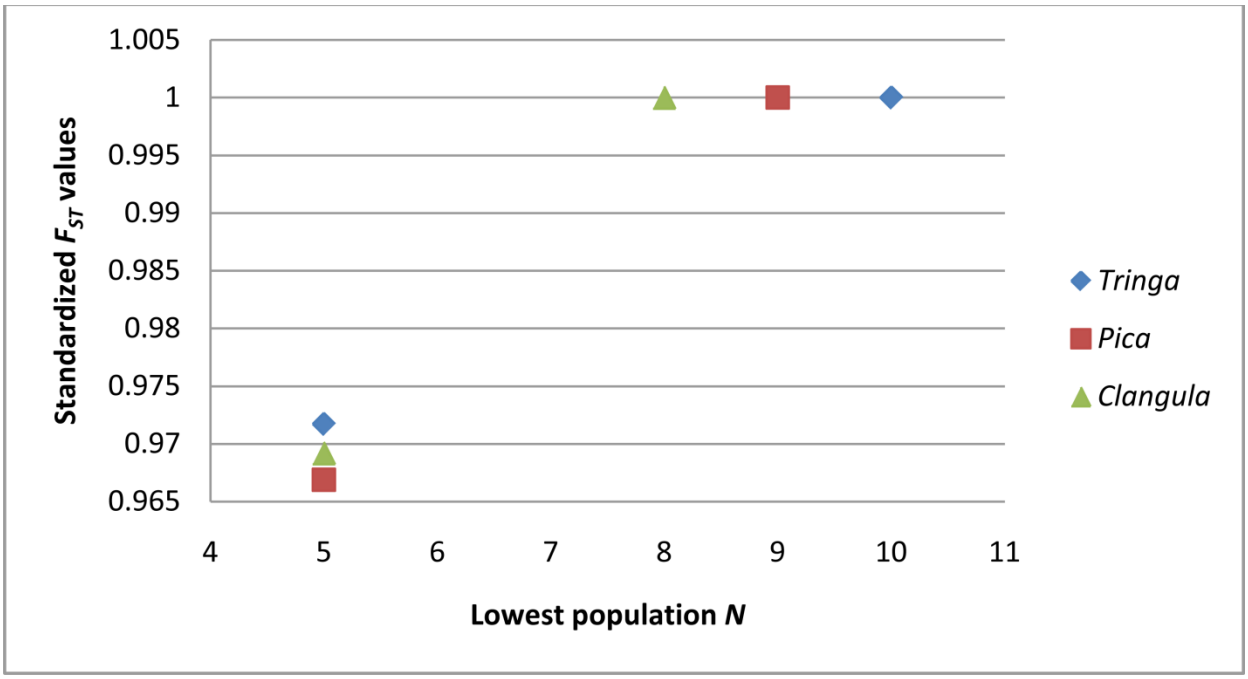

Figure S2

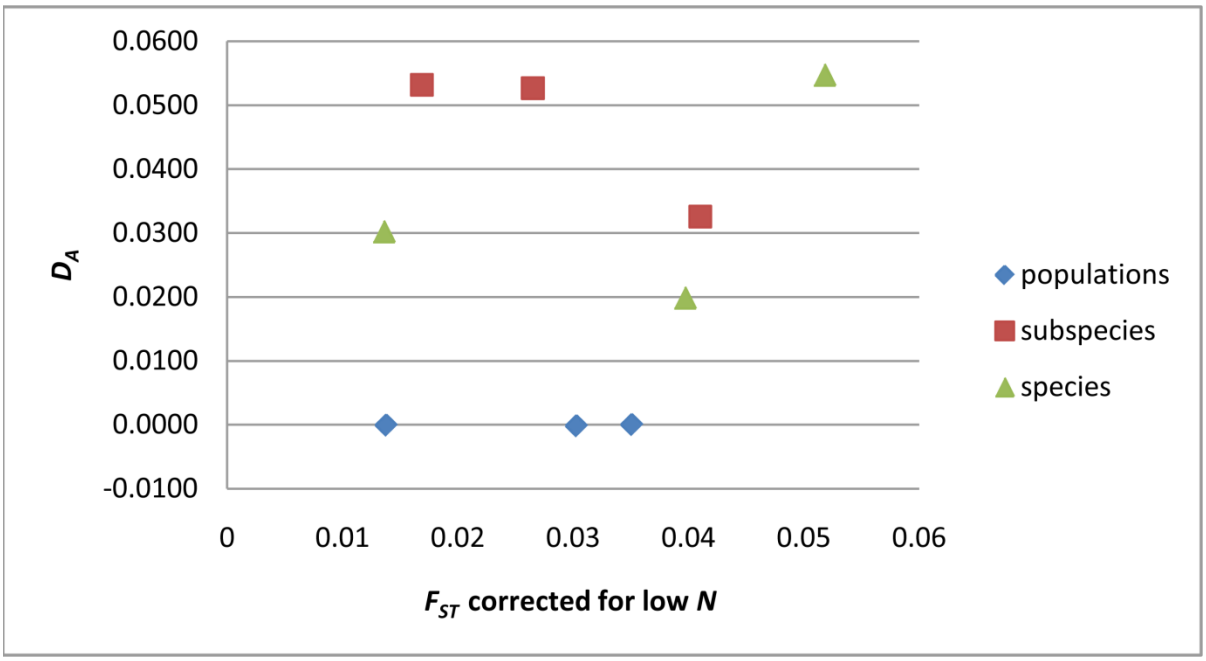
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Figure S3