

Concordance of genetic and phenotypic characters across a sapsucker hybrid zone

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Hybridization has presented a challenge for taxonomists and conservation biologists, since hybridizing forms could be stable evolutionary entities or ephemeral forms that are blending together. However, hybrid zones also provide a unique opportunity for evolutionary biologists who study the interaction between gene flow and reproductive isolation in speciation. Three forms of woodpeckers (sapsuckers; genus *Sphyrapicus*) in North America that are mostly geographically separated but hybridize with each other where they come into contact present a remarkable system for the study of hybridization. We provide the first comprehensive analysis of phenotypic and genetic variation across a hybrid zone between two of these forms, the red-breasted *Sphyrapicus ruber* and yellow-bellied *S. varius* sapsuckers. The objective was to infer whether selection maintains the differences between forms. Our analysis of eight morphometric and 20 plumage traits, and two molecular markers showed clear differences between the forms and roughly concordant clinal variation across a narrow hybrid zone. Thirty percent of sampled birds in the hybrid zone had mixed west/east genotypes at the genetic markers examined. The center of the genetic cline was located 20 km west of the crest of the Rocky Mountains. The width of the zone was 122 km, narrower than would be expected under neutral blending given reasonable estimates of the age of the zone and individual dispersal distances. Heterozygote deficit and cytonuclear disequilibrium at the centre of the hybrid zone suggested nonrandom mating or limited hybridization. Given these patterns and lack of evidence for habitat segregation we conclude that this hybrid zone is maintained by selection, most likely in the form of hybrid inferiority. This study provides an illustrative example of extensive hybridization between stable entities, providing additional evidence against the historical practice of treating hybridizing forms as members of the same species.

Pleistocene glaciations have resulted in the divergence of a number of animal populations while they were isolated in southern refugia (Weir and Schluter 2004). The subsequent retreat of these ice sheets allowed some of these forms to come into secondary contact. Hybrid zones resulting from such secondary contacts have created confusion in taxonomy (Shunk 2005, McCarthy 2006). In the context of speciation, however, hybrid zones provide an opportunity to study the forces that generate reproductive isolation and lead to the evolution of distinctive groups (Mayr 1963, Grant and Grant 1992, Mallet 1995, Coyne and Orr 2004). Field-based studies of hybrid zones provide insight into the evolutionary dynamics in contact zones, and the levels of reproductive isolation and gene flow between the divergent forms (Jiggins and Mallet 2000, Gompert et al. 2006, Price 2008, Brelsford and Irwin 2009, Mettler and Spellman 2009).

When two interbreeding taxa meet, the relative fitness of parental types and their hybrids determines the dynamics of the hybrid zone (Barton and Hewitt 1985, Jiggins and Mallet 2000). Hybrid fitness can be influenced by morphology (e.g. body size, distinct plumage), behavior, and genetic

incompatibilities acquired during isolation of parental taxa (Barton and Hewitt 1985, Moore and Price 1993, Bronson et al. 2005, Price 2008). When hybrids have reduced fitness relative to their parental types, then a hybrid zone can be maintained by the balance between movement of parental genotypes into the zone and selection against hybrids ('tension zones'; Barton and Hewitt 1985). The environment can also affect fitness and thereby influence species boundaries and hybrid zone dynamics (Moore and Price 1993, Case and Taper 2000, Gill 2004, Price 2008). Social selection such as sexual selection and assortative mating can contribute to the dynamics of hybrid zones (Jiggins and Mallet 2000, McDonald et al. 2001, Stein and Uy 2006). For example, locally common phenotypes can have a selective advantage compared to a foreign intruder (Moore and Price 1993, Price 2008), and assortative mating can improve the fitness of parental types (Price 2008). Research in hybrid zones can provide insights into fitness differences of the parental forms and hybrids, and can elucidate patterns of trait divergence and reproductive isolation between parental forms.

A unique system for the study of hybridization is presented by the woodpecker genus *Sphyrapicus*, which is

endemic to North America (Walters et al. 2002a, b). It consists of four species including a well-separated basal species, *S. thyroides* (Williamson's sapsucker), and three peripheral species confined to a recently diverged superspecies (AOU 1983, Cicero and Johnson 1995). Two of the three species in this group are found in western North America, with the red-breasted sapsucker *S. ruber* in coastal regions and the red-naped sapsucker *S. nuchalis* in dry, interior western highlands. The third species, the yellow-bellied sapsucker *S. varius* is found east of the Rocky Mountains across the boreal forest to the Atlantic coast (Fig. 1). While the biology of these three sapsuckers is quite similar, the extent of red pigmentation, sexual dichromatism and body size vary from east to west (Walters et al. 2002a, b). *Sphyrapicus varius* is sexually dichromatic and *ruber* is sexually nearly monochromatic.

Sphyrapicus nuchalis is intermediate in size and sexual dichromatism to the other two species (Walters et al. 2002a, b). Phylogenetic relationships derived from allozyme and cytochrome b data suggested that within this superspecies, the two western species are most related (Cicero and Johnson 1995, Weir and Schluter 2004).

The breeding ranges of these species include several narrow zones of sympatry (Walters et al. 2002a, b). Here they hybridize and produce viable hybrids, and so were long considered as three subspecies within a single species, then designated *S. varius* (Ridgeway 1914, Howell 1952, Scott et al. 1976). However, evidence from their ecology, distribution, assortative mating at the hybrid zone (between *ruber* and *nuchalis*) and phylogeny based on allozymes and mitochondrial markers supported the more recent designation as

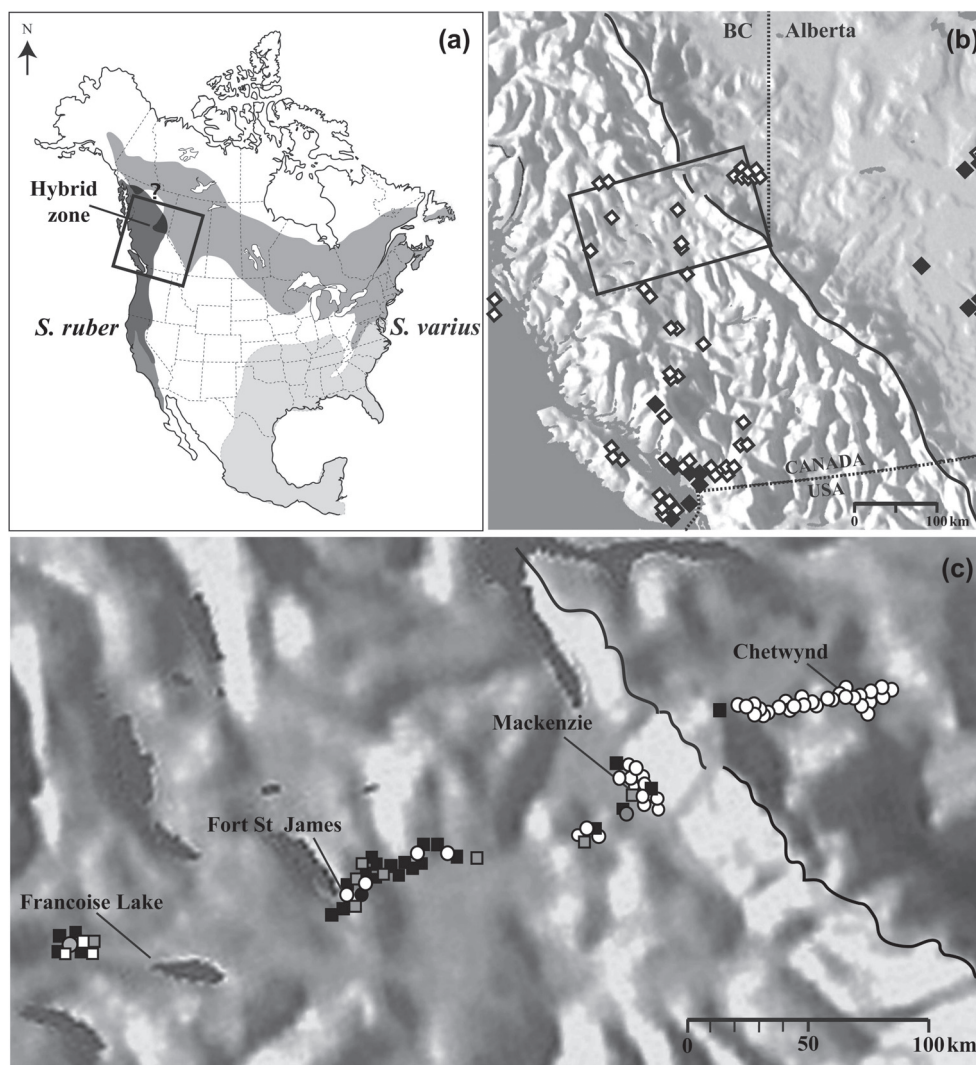


Figure 1. (a) Breeding and wintering ranges of *S. ruber* and *S. varius* (after Walters et al. 2002a, b). The darker colour indicates the breeding range of the corresponding species. The hybrid zone is indicated with black shading. The boxed area is expanded in the next panel. (b) Original collecting locations of the skins studied for historic clines (white diamonds) and allopatric sampling locations for the contemporary sampling (filled diamonds). The black line indicates the crest of the Rocky Mountains. The center of the hybrid zone is boxed and is expanded in the next panel. (c) Subset of individuals (85) sampled across the contact zone. Each point indicates a capture location of a live bird and its corresponding genome at two genetic markers: western COI (squares); eastern COI (circles); homozygous western CHD1Z (black centre); eastern CHD1Z (white centre), and heterozygotes for CHD1Z (grey centre). Some symbols have been moved slightly to avoid overlap. Sapsuckers were absent in the coniferous dominated high elevation forests between the areas of high density in the eastern and western foothills.

three distinct species (AOU 1983, Johnson and Zink 1983, Walters et al. 2002a, b). The two western *Sphyrapicus* species (*nuchalis* and *ruber*) form a better-known hybrid zone from southern British Columbia (BC) to California (Howell 1952, Campbell et al. 1990, Johnson and Johnson 1985). Previous studies suggest that this *nuchalis*–*ruber* hybrid zone is a tension-zone locked in an ecotone with low hybrid fitness (Johnson and Johnson 1985). The *nuchalis* and *varius* hybrid zone in southwestern Alberta (Canada) has been described as a dynamic zone in which the zone centre is moving back and forth (Hudon 2001).

The only occurrence of contact between the most western and most eastern taxa, *ruber* and *varius*, is found in northern BC (Howell 1952, 1953, Scott et al. 1976, Campbell et al. 1990, Shunk 2005; Fig. 1). However this hybrid zone has not been studied previously in any detail. Reports of this contact zone dated back to 1919 where Campbell et al. (1990) reported skins of both pure forms from sympatric zones. Other noteworthy reports of range overlap include Howell's (1952) study of skins collected in the 1920s with hybrid origin, McTaggart-Cowan's report in 1939 of nesting *ruber* near Dawson Creek at the eastern edge of the contact zone (cited in Campbell et al. 1990); Godfrey's note in 1969 of an abrupt change from *ruber* to *varius* in this region (cited in Campbell et al. 1990), and Scott's et al. (1976) collection of a hybrid (*S. ruber* × *S. varius*) from ~150 km south of Mackenzie and ~100 km west of the crest of Rockies.

Here we provide the first detailed characterization of this hybrid zone of western *ruber* and eastern *varius* using a suite of morphometric, plumage and genetic characters. In addition to providing a qualitative and quantitative description of the hybrid zone, we test two major hypotheses. First, we test whether phenotypic variation, which informs the current taxonomic status, is a good predictor of genetic variation, a pattern observed in several other hybrid zones in the same region (Brelsford and Irwin 2009, Irwin et al. 2009, Toews et al. 2011). Second, we test whether the differences between these forms are maintained by selection. A selection-maintained hybrid zone would display a pattern in which phenotypic and genetic clines are concordant and narrower than predicted based on models of neutral diffusion (Barton and Hewitt 1985, Brelsford and Irwin 2009).

Methods

Field sampling

During summer 2009 we used mist nets and dip nets to capture 97 sapsuckers along a 400 km transect line extending from the range of *S. varius* into the range of *S. ruber* (Campbell et al. 1990, Walters et al. 2002a, b). The transect spanned from ~10 km east of Chetwynd (55.68N, 121.54W) to west of Francoise Lake (54.07N, 126.66W; Fig. 1), along highways, forestry roads and water bodies. Mist netting involved 12 and 6 m nets, call playbacks and movable decoys (male life-like sapsucker mount attached to a black cord and pulley system for vertical movement). We scouted for sapsuckers in intervals of ~10 km along the transect line using visual and acoustic cues and playback of drumming calls. When a bird was located, drumming playbacks and

moving decoys were used with aggression calls to encourage the bird to fly close to the decoy (territorial sapsuckers tried to either attack or land close to the decoy). The nets were placed to intercept the flight path. Occasionally we used a dip net attached to a telescopic handle to catch adults at the nest cavities. We collected morphometric and plumage measurements, a blood sample (~50 µl) and photographs from each captured bird. A numbered metal leg band was attached to birds before release. We georeferenced the capture site of each bird (Supplementary material Appendix 1, Table A1).

Sampling from museum collections

Tissue samples of pectoral muscle were collected from allopatric *ruber* specimens temporarily stored at –20 °C at the Cowan Tetrapod Collection at the Beaty Museum, Univ. of British Columbia. These specimens had been retrieved as window kills from the Vancouver area (Supplementary material Appendix 1, Table A1). Similarly, allopatric *varius* were obtained from the Royal Alberta museum as freshly preserved samples of blood in Queen's lysis buffer (Seutin et al. 1991).

We undertook an exhaustive survey to locate museum study skins collected from the same sympatric and allopatric regions as above (Fig. 1). We examined 72 skins collected during 1900 to 1955 from the Beaty Museum, Royal British Columbia Museum and Royal Alberta Museum. This historic sampling demonstrated that *ruber* had been seen up to Mackenzie (Fig. 1), whereas *varius* was found in sites east of the Rockies. However due to poor sample size at the centre of the hybrid zone in the historic dataset (Fig. 1b), we did not carry out detailed comparisons of historic and contemporary hybrid zone.

Morphology and plumage measurements

We measured eight morphometric characters to produce six variables (following Baldwin et al. 1931, Pyle 1997, Miller et al. 1999): culmen length (base of the upper mandible to the distal tip); bill height (measured at the distal end of the nares) and bill width (width of the upper mandible measured at the distal end of the nares) to get the bill breadth (the average of bill height and width); head length (length of the head from the base of the beak to posterior-most point of the skull) and head width (widest point of the head just behind the eye at the squamosal regions) to get the size of the head (the average of head length and width); flattened wing; tail length, and tarsus length. We further measured five plumage characters; width of the black nape band (average width of three measurements, taken from the middle and each end of the nape band), length of the white moustache (white feathers from the base of the beak to the posterior end), moustache width (measured ventral to the eye), width of the black breast band (average width of three measurements as above), and the wing bar (the length of white colour patch in the folded wing). All measurements were determined using a dial caliper (± 0.01 mm). We visually scored (as 0, 1 and 2) the amount of red (carotenoid pigments), white and black (melanin pigments) color in different parts of the body: dorsal head – the amount of red plumes on the forehead (black 0; red 2), black nape-band (b 0; r 2) and beyond the black nape-band (white 0; r 2); sides of the head – the amount

of red on white supercilium (absent 0; r 2), on black band above the supercilium (ab 0; r 2), on eyeline below the supercilium (ab 0; r 2), on white moustachial stripe (ab 0; r 2), and on black submoustachial stripe (ab 0; r 2); ventral head – amount of red on chin (ab 0; r 2), on throat (ab 0; r 2), on black breast band (ab 0; r 2) and beyond the breast band (ab 0; r 2); in other parts of the body – amount (%) of white on the mantle and on the dorsal mid-rectrix. A single observer (SSS) performed all measurements and scoring.

To summarize patterns of covariation in the morphometric and plumage variables, we constructed two principal component analyses (PCA), first for the six morphometric characters, and second for the 17 plumage characters. The principal axis method was used to extract the components followed by an orthogonal rotation. In each PCA, the majority of variation was captured by the first component (Table 1). Because the first two components together accounted for a large amount of variance (75.7% morphometric; 78.3% plumage), we used only the first two principal components from each PCA for the rest of the analysis. PCA analysis was conducted with JMP (ver. 7.0.1, SAS Inst., Cary, NC).

DNA analysis

Blood and muscle tissue samples were stored in Queen's lysis buffer (Seutin et al. 1991). Standard phenol-chloroform

Table 1. Eigenvalues, variance explained and factor loadings of the first two principal components (PC) produced in two principal components analyses (PCAs) of phenotypic traits: A) morphometric traits and B) plumage traits.

	PC1	PC2
A) Morphometric traits		
Eigenvalue	3.79	0.76
Variance explained	63.10%	12.61%
Factor loadings:		
culmen length	-0.43	-0.10
bill depth	-0.43	0.22
head size	-0.39	0.40
flattened wing	-0.41	-0.47
tail length	-0.41	-0.53
tarsus length	-0.38	-0.43
B) Plumage traits		
Eigenvalue	11.41	1.89
Variance explained	67.11%	11.14%
Factor loadings:		
forehead colour	-0.08	0.44
nape colour	-0.28	-0.07
below nape colour	-0.28	0.02
supercilium	-0.28	-0.05
above supercilium	-0.28	-0.07
below supercilium	-0.27	-0.04
moustache	-0.28	-0.01
sub moustache	-0.27	-0.02
moustache length	-0.23	-0.10
moustache width	-0.27	-0.08
chin colour	-0.14	0.59
throat colour	-0.12	0.62
breast colour	-0.26	-0.12
below breast colour	-0.28	-0.02
% white in mantle	0.28	0.00
% white in rectrices	0.24	0.17
wingbar length	-0.01	-0.18

DNA extraction was used to extract DNA. We followed the same procedure of Irwin et al. (2009) for the analysis with several modifications as described below.

Mitochondrial DNA

A section of mitochondrial gene COI of *ruber* and *varius* was downloaded from Genbank, and NEBcutter (<http://tools.neb.com>) was used to design a PCR-RFLP assay (Avisé 2004). We used partial sequences of COI downloaded from Genbank to construct a consensus sequence for each species and identify species-diagnostic variation (DeFilippis and Moore 2000, Hebert et al. 2004, Webb and Moore 2005, Kerr et al. 2007).

We amplified a 749 base pair region of COI for 113 individuals using the primer combination BirdF1 (TTCTC CAACCACAAAGACATTGGCAC) and BirdR1 (ACGT GGGAGATAATTCCAAATCCTG; Hebert et al. 2004). A single-nucleotide polymorphism (SNP), a C to T substitution in the mitochondrial ancestor of *ruber* and *nuchalis*, eliminated a cut site present in *varius* for the restriction enzyme BsrFI (New England Biolabs) at 351 bp from the BirdF1 end of the sequence. Directionality of the substitution was inferred from sequences of *S. thyroides* and more distantly related woodpeckers. The PCR reaction procedure was as follows: 1X PCR buffer (Invitrogen), 1.5 M MgCl₂ (Invitrogen), 0.2 M dNTP mix (New England Biolabs), 0.5 M BirdF1 and BirdR1 primers, 0.04 units l⁻¹ Taq DNA polymerase (Applied Biosystems), and 2.5 ng l⁻¹ template DNA, in a total volume of 10 l. The thermal cycling profile was 3 min at 94 C followed by 35 cycles of 30 s at 94 C, 30 s at 50.5 C, 30 s at 72 C, and ending with 10 min at 72 C. The PCR procedure was followed by a restriction enzyme digestion of 2 l of the PCR product with 2 units of the BsrFI enzyme in a total volume of 6 l. Digestion was performed at 37 C for 2 h and digested DNA was visualized by gel electrophoresis. Our PCR-RFLP procedure produced two bands (351 and 398 bp) for *varius* in the gel, and a single band of 749 bp for *ruber*. The RFLP profile was scored by eye.

CHD1Z; Z-linked nuclear DNA

We screened an intron of the Z-linked nuclear CHD1Z gene following Fridolfsson and Ellegren (1999) and Irwin et al. (2009). Eight males were sequenced, four from representative allopatric regions for each species (Supplementary material Appendix 1, Table A1, for the locations of the samples). A similar PCR reaction recipe described above was used for the primers 2550F (GTTACTGATTCGTCTAC GAGA) and 2718R (ATTGAAATGATCCAGTGCTTG), with 45.5°C annealing temperature (Fridolfsson and Ellegren 1999). Amplified fragments were sequenced bidirectionally using a 3730XL DNA Analyzer (Applied Biosystems) at the McGill Univ. and Génome Québec Innovation Centre, Montréal, Canada.

The sequences of eight males produced an informative SNP at 526 bp from the 2550F end of the ~660 bp fragment of the intron of CHD1Z. Three of the four *ruber* were homozygous for an insertion of a G at 526 bp, while all four *varius* males were homozygous for an allele that lacked

the contact zone (Asmussen et al. 1987, Szymura and Barton 1991, Orive and Barton 2002). We examined the relationship of R_{ij} and the geometric mean of allele frequencies $((pq)^{1/2})$. A positive trend would indicate a greater disequilibrium, hence possibly a lower hybrid fitness at the centre of the hybrid zone (Kawakami et al. 2009). To determine R_{ij} , we measured the cytonuclear disequilibrium (D) between the cytoplasmic locus (COI; cytotype M_w/M_e) and the nuclear locus (CHD1Z, nucleotype A/T; Table 2), $D_i = X_i - m_i p_i$ (Hedrick 2000); where X_i is the frequency of $M_1 A_1$, m_i is the frequencies of cytotype M_1 , and p_i is the observed frequency of nucleotype A (M_1 is either M_w or M_e , A_1 is either A or T; Hedrick 2000). We considered that parental *ruber* and *varius* carry respective genotypes $M_w AA$ and $M_e TT$. Hybrid genotypes were $M_w AT$, $M_e AT$, $M_w TT$, and $M_e AA$.

Level of aggression towards decoys

While luring birds for capture we recorded the behavior of captured birds towards the decoys presented (see 'Field sampling' for the description of decoys). We performed a post-hoc analysis with this dataset to examine whether levels of aggression depended on the traits of responding birds and the decoy. Aggression scores were defined as: 0 = bird flew across the net, no aggression; 1 = bird landed within ~2 m radius of decoy; 2 = bird landed next to the decoy in the same branch/trunk; 3 = active physical attack on the decoy. In allopatric zones, we did not offer heterospecific decoys to targeted birds since we anticipated that the territorial aggression would be highest with conspecific intruders (decoy), but in the hybrid zone we provided both decoys as the likelihood of encounter of both species were fairly high in any given mistnetting session. The dataset involves information on captured birds only; hence it could be biased towards aggressive males due to the fact that they were the ones more often attracted to the decoy. However, we did not catch all the birds that attacked the decoys. We used one-way ANOVA design and t-test (conducted in JMP ver. 7.0.1) to compare the levels of conspecific and heterospecific aggression.

Results

Character separation between *S. ruber* and *S. varius*

The expression of red, white, and black colour patterns varies between the two species (Fig. 2). White and black colour patches are prominent in *S. varius*; some patches are replaced by red in *S. ruber*, especially in the head and breast (Fig. 2). Table 1 summarizes the eigenvalues, the proportion of variance captured by first two PCs of each PCA and factor loadings for phenotypic traits. In the morphometric PCA, PC1 explained 63.1% of the variation and described size differences between the species, with each standardized morphometric variable being roughly equally and inversely correlated with PC1. Morphometric PC2 captured a comparatively minor amount of variation (12.6%), describing differences in shape (increasing with tarsus length and head size, and decreasing with wing and tail length). There was a clear separation of PC1 scores of *varius* and *ruber* in morphometric

characters (ANOVA: $F_{1,86} = 187.92$, $p < 0.001$). We did not observe sexual dimorphism in morphometric traits in either species ($F_{1,86} = 0.04$, $p = 0.837$). The PC1 of plumage captured 67.1% of the variation, correlating roughly with all characters that tend to differ between *varius* and *ruber*, with high values of PC1 indicating *varius*-like characters such as black nape, white supercilium, and black submoustachial stripe, and low values indicating red for those characters (Table 1; see Methods for scoring). PC2 is correlated primarily with forehead, chin and throat color, but explains little variation (11.1%). The PC1 of plumage characters separated the two species ($F_{1,86} = 1339.17$, $p < 0.001$) even more clearly than PC1 of morphometrics (Fig. 2, Table 1). The PC1 of plumage characters slightly separated sexually nearly monochromatic *ruber* between sexes ($F_{1,43} = 5.23$, $p = 0.027$); *varius* on the other hand exhibited a strong sexual dichromatism ($F_{1,42} = 56.64$, $p > 0.001$), which is captured in PC2 (Table 1, Fig. 2).

Clinal variation

In sympatry, some birds showed intermediate measurements in all six measured morphometric characters between *ruber* and *varius* (Fig. 3c; Supplementary material Appendix 1, Table A1). Plumage reflected a similar trend across the sympatric zone (Fig. 3d). Both plumage and morphometric characters had *varius*-like values in the region east of the Rockies except a single *ruber* caught in the eastern foothills (IE16S01: Supplementary material Appendix 1, Table A1). Characters west of the Rockies, however, from Mackenzie to Fort St James, consisted of a broad range of values representing pure *varius*, *ruber* and values ranging in between. This pattern was also reflected in genetic markers (Table 2, Fig. 1–3). Birds with mixed characters consisted of, either red patches in the facial plumage and breast, with less white on the rest of the body in *varius*-type birds, or white and black in the facial plumage in *ruber*-type birds (Fig. 2). Phenotypic characters reflected pure *varius* moving into the Fort St James area (Fig. 3).

We genotyped contemporary birds for both COI ($n = 113$) and CHD1Z ($n = 101$). The western COI haplotype was confined primarily to populations west of the Rockies ($n = 41$). All birds but one phenotypic *ruber* caught east of the Rockies ($n = 44$) had the eastern COI haplotype (Table 2). Similarly all individuals sampled for CHD1Z east of the Rockies and west of Francoise Lake were homozygous for the corresponding CHD1Z genotype except the single pure *ruber* caught in the eastern foothills of the Rockies and a heterozygous *ruber* sampled near Vancouver (Table 2). A mixture of COI haplotypes, and homozygous and heterozygous individuals for CHD1Z were found in the region between Mackenzie and Francoise Lake (Table 2, Fig. 1). In the hybrid zone, 32% (18 out of 56) of our sample had mixed COI/CHD1Z genotypes, providing a lower-bound estimate for the frequency of admixed individuals (Table 2, Fig. 1).

Clinal analysis

Our estimates of contemporary genetic cline centre ranged from 18 to 23 km west of the crest of Rockies (mean centre

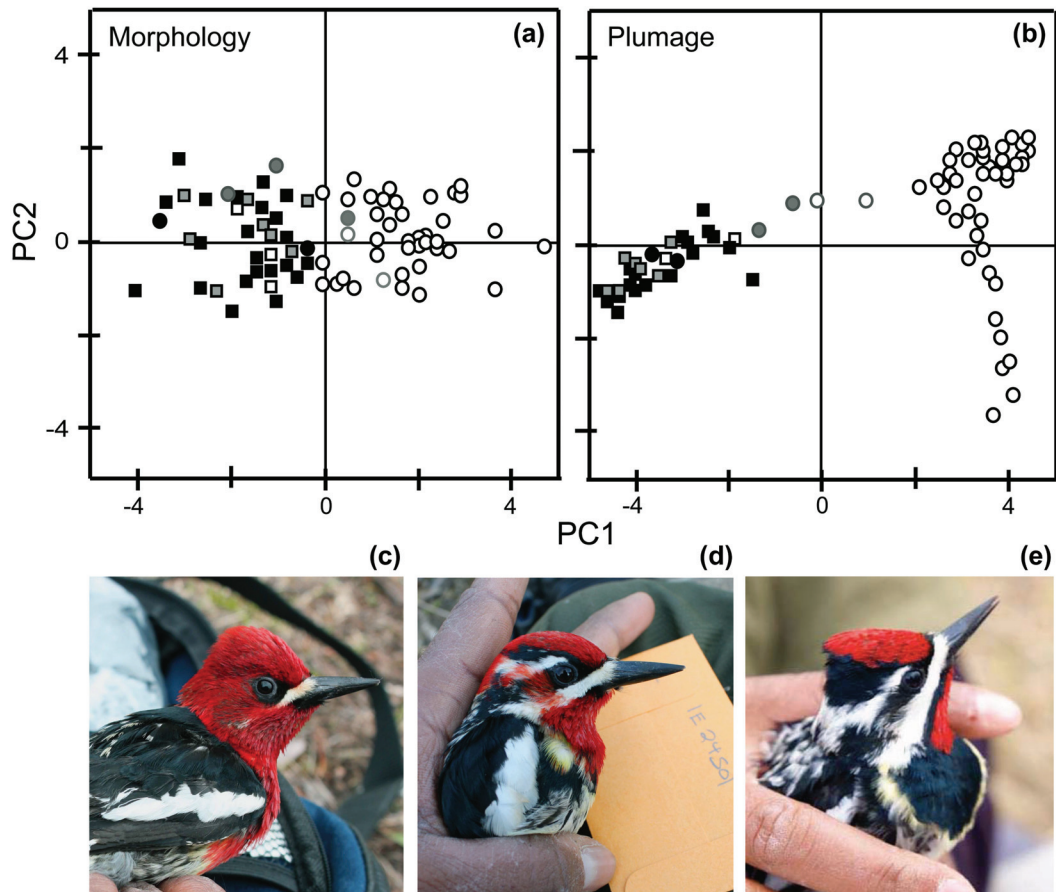


Figure 2. PC1 and PC2 of morphometric (a) and plumage characters (b) in adult *S. ruber* and *S. varius* of both sexes from Francoise Lake to Chetwynd, BC. Each point indicates a PC score with corresponding genotype at two genetic markers for an individual bird (see Fig. 1c for the description of symbols). In the morphometric PCA (a), PC1 represents body size and PC2 represents a complex shape measure (Results and Table 1). In the plumage PCA (b), PC1 represents the expression of carotenoid and melanin-based color and PC2 represents forehead, chin, and throat color (Table 1). Some symbols have been moved slightly to avoid overlap. Examples of male parental phenotypes; (c) *S. ruber* (bird number IE16S01; Supplementary material Appendix 1, Table A1), (d) a hybrid between the two species (IE24S01) and (e) *S. varius* (IE25S01).

20 km west of the crest) with the cline widths ranging from 118 to 128 km (mean width 122 km, Table 3, Fig. 3). These differences are not statistically significant (Table 3). The unconstrained model had the best fit to the data (log-likelihood = -111.98, AIC = 235.96, Table 3) and a model that fits all traits to a single cline had the lowest AIC value (log-likelihood = -112.00, AIC = 231.99), but none of these models were significantly different (Table 3). Cubic splines of both morphometric and plumage traits showed clinal variation in the same area of both COI and CHD1Z clines; ~20 km west of the crest of Rockies (area between Fort St James and Mackenzie, Fig. 3). All these four measured traits are generally concordant in their centers and widths.

Independent comparisons of phenotypic clines and a comparison of genetic clines with phenotypic clines in CFit-7 did not yield any significant differences in cline width or location (Supplementary material Appendix 1, Table A2A). A preliminary comparison of contemporary and historic clines suggested no difference between clines (Supplementary material Appendix 1, Table A2B). We did not carry out further comparisons between contemporary and historic clines due to poor sample size at the centre of the hybrid zone in the historic dataset (Fig. 1b).

Testing whether selection maintains the hybrid zone

We compared neutral cline widths generated by different estimates of dispersal and time since secondary contact. The range of these estimates varied from less cautious to highly cautious. No estimates of dispersal distance are available for *Sphyrapicus*. Therefore we used estimates of generation time and dispersal of the northern flicker *Colaptes auratus*, based on its close phylogenetic relatedness and similarity in ecology, migratory behavior and biogeography to *Sphyrapicus*. Generation time was estimated as 1.9 yr, calculated as in Milá et al. (2007) based on the annual survival rate (0.41–0.53, Wiebe 2006), geometric population growth (1, Milá et al. 2007) and the age of first breeding (1, Walters et al. 2002a, b). Root mean square dispersal (σ) was estimated as 100 km per generation (Moore and Buchanan 1985). Under these parameters, and assuming the secondary contact of *ruber* and *varius* took place just after the reforestation (after the retreat of Pleistocene glaciers) of Pine Pass area near Mackenzie, BC, 7500 yr ago (Barrowclough 1980), a neutrally diffusing cline would have now reached a width of 10 600 km. However, the biogeographic history of other similar boreal species

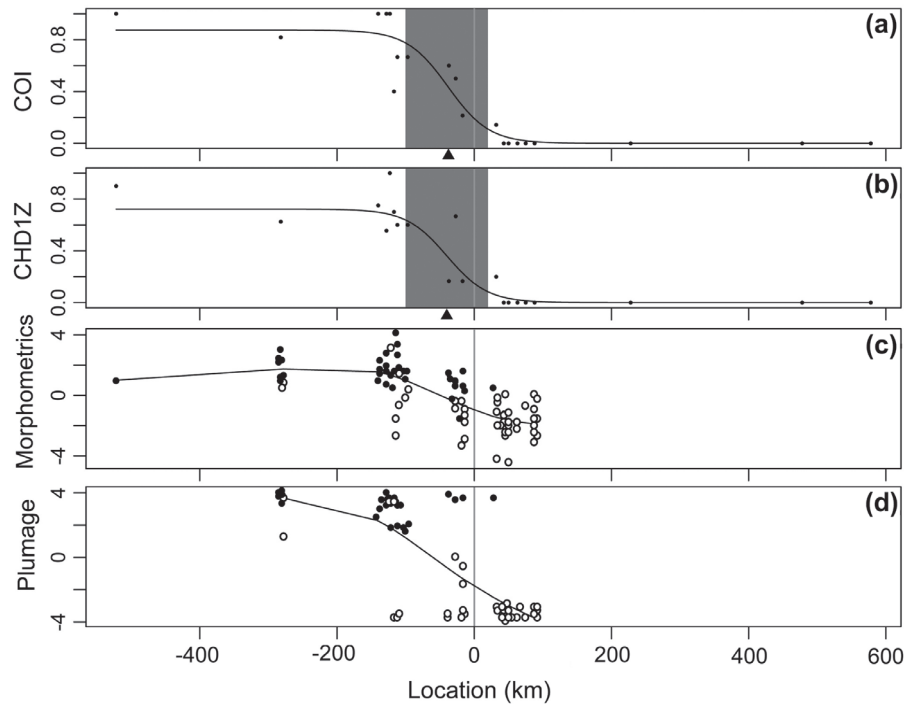


Figure 3. Clines in genetic and phenotypic traits across a hybrid zone of *S. ruber* and *S. varius*. Zero km represents the crest of the Rocky Mountains (Fig. 1b). Points represent allele frequencies (COI and CHD1Z) in small populations (a–b) or PC1 scores (morphometric and plumage characters) of individuals (c–d). The circles in panel (c–d) represent eastern COI (open circles) and western COI (closed circles). The lines represent the best fitting clines generated by CFit-6 (a–b), and fitted cubic splines in R (c–d). Arrows and shared area indicate the centre and the width of genetic clines. (a) COI, (b) CHD1Z, (c) morphometrics, and (d) male plumage.

pairs suggests that the secondary contact may be more recent (Rohwer et al. 2001, Irwin et al. 2009, Toews et al. 2011). A conservative estimate of time since recent contact between taxa (200 yr; based on sight records and historical reports of *Sphyrapicus* in the region) would give a width of 1700 km, a much wider cline compared to the observed clines. A highly cautious estimate of dispersal, based on migratory songbirds (10 km per generation; Paradis et al. 1998) gave a width of 172 km for a neutrally diffusing cline for our most conservative estimate of recent contact (200 yr). All of these widths of assumed neutral clines are wider than the best estimate of the cline widths (122 km), suggesting some form of selection constrains the width of the hybrid zone.

When deviations from HWE were plotted against the distance, a slight peak was observed near the centre of the contact zone (Supplementary material Appendix 1, Fig. A1A). A positive but not significant trend was observed when HWE deviation was plotted against geometric mean

of allele frequencies ($(pq)^{1/2}$; $y = 11.10x + 0.82$, $R^2 = 0.29$, $p = 0.45$). Similarly, we observed high R_{ij} values near the centre of the hybrid zone relative to sites away from the centre (Supplementary material Appendix 1, Fig. A1B). The R_{ij} values and $(pq)^{1/2}$ (Szymura and Barton 1991, Kawakami et al. 2009) showed a positive, but non-significant trend ($y = 6.45x - 0.84$, $R^2 = 0.30$, $p = 0.34$; Supplementary material Appendix 1, Fig. A1C). Although small sample size ($n = 6$) restricts statistical power, these values are consistent with an excess of pure types relative to neutral expectations, suggesting assortative mating and/or selection against hybrids near the centre of the hybrid zone.

Aggression towards a conspecific and heterospecific decoys

Male *varius* was more aggressive towards the heterospecific *ruber* decoy than towards its own conspecific decoy in

Table 3. Comparison of genetic clines based on maximum log-likelihood estimates obtained from Cfit-6 (Gay et al. 2008). Cline centers are in km west from the crest of the Rocky Mountains.

	COI/CHD1Z		Log-likelihood	AIC	Comparison	χ^2 (DF)	p
	Centre (km)	Width (km)					
1. No constraint	23/18	128/118	-111.98	235.96			
2. Centre constrained	20	124/121	-112.00	233.99	1 and 2	0.03 (1)	0.86
3. Slope constrained	21/19	122	-111.99	233.99	1 and 3	0.03 (1)	0.87
4. Centre and slope constrained	20	122	-112.00	231.99	1 and 4	0.03 (2)	0.98
					2 and 4	0.01 (1)	0.96
					3 and 4	0.01 (1)	0.93

sympatry (t -test: $t_{(DF=8)} = 3.75$, $p = 0.005$; Fig. 4). Thirteen individuals of *ruber* chose to attack conspecific decoy in sympatry over the heterospecific decoy (Fig. 4). Therefore none of the captured *ruber* showed heterospecific aggression in the contact zone, even though they were aggressive toward their conspecific decoys (t -test: $t_{(DF=12)} = 8.40$, $p < 0.001$). There were no significant differences between the aggression of each species towards their conspecific decoys in allopatry ($t_{(DF=19)} = 1.32$, $p = 0.20$; Fig. 4). Similarly none of them showed significant differences in the level of aggression for their conspecific decoys in sympatry and in allopatry (One-way ANOVA: $F_{3,31} = 1.24$, $p = 0.31$; Fig. 4). We did not offer heterospecific decoys to the allopatric birds (see Methods). Overall, these results indicate that *varius* males are more aggressive towards *ruber* than the *ruber* males on *varius* in the hybrid zone.

Discussion

We have shown that phenotypic and genetic data reveal similar patterns of extensive hybridization between *S. ruber* and *S. varius* in a hybrid zone 122 km in width, spanning from the western slopes of the Rocky Mountains in northeastern BC to Fort St James' area in interior BC (Fig. 1–3). About 30% of the sampled birds in the hybrid zone were genetic hybrids based on two markers (Table 2), an estimate that would be higher if it were based on more markers. Evidence of genetic introgression extends 100 km further west, up to the western edge of Francoise Lake (Fig. 1). The centers of phenotypic and genetic clines are not different and are

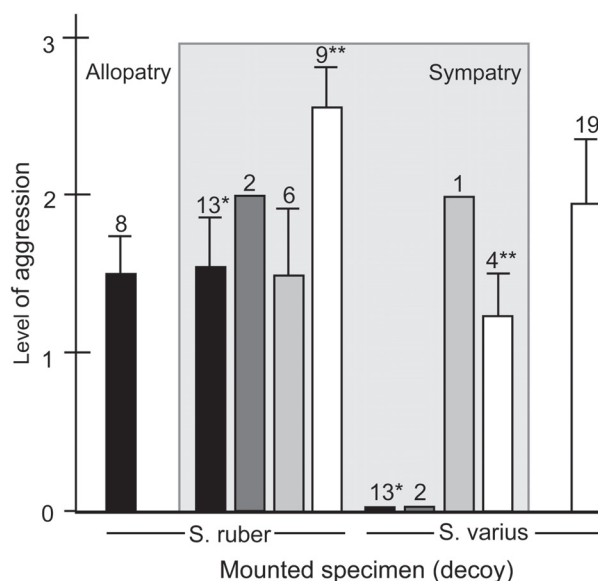


Figure 4. Aggression of male sapsuckers towards a moving decoy (a mounted sapsucker skin). Each decoy was presented with allopatric drumming and aggression calls to stimulate territoriality. Black columns represent *S. ruber*, white columns represent *S. varius* and grey columns represent genetic hybrids (dark grey, *ruber*-like hybrids; light grey, *varius*-like hybrids). *Sphyrapicus ruber* and *ruber*-like hybrids did not attack heterospecific *S. varius* decoys in sympatry. Numbers above each column indicate the number of birds exposed (* $p < 0.001$, ** $p = 0.005$).

located ~20 km west of the crest of the Rocky Mountains (Fig. 3, Table 3, Supplementary material Appendix 1, Table A2A). The concordance of genetic and phenotypic characters across the hybrid zone indicates that the two forms are distinct entities in both genes and phenotypes and that each character can usually predict the other. The width of the hybrid zone is narrower than would be expected under neutral blending under a variety of estimates of time since contact and dispersal distance.

Selection acting on zone maintenance

The concordance of phenotypic and genetic markers, narrow width of the hybrid zone compared to neutral expectations, and apparent rarity of hybrids (although not statistically significant) compared to expectations under random mating and no selection suggest that some form of selection is acting on this hybrid zone (Barton and Hewitt 1989, Jiggins and Mallet 2000). The patterns are consistent with the 'tension zone' model for the maintenance of hybrid zones, in which selection against hybrids is balanced by dispersal of parental types into the zone (Barton and Hewitt 1985). In the hybrid zone, we sampled 18 individuals with hybrid genotypes and observed breeding heterospecific pairs in the wild ($n = 5$), however very few F1-like hybrids ($n = 3$, hybrids with nearly 50% of phenotypic characters of each species) were observed in the dataset. Higher proportion of backcrosses and fewer F1-like hybrids indicate that the zone might be a bimodal hybrid zone (Jiggins and Mallet 2000). The types of selection maintaining this hybrid zone are not known. Detailed future studies incorporating behaviour (e.g. migration; Rohwer and Irwin 2011), environmental factors and a greater coverage of genetic markers might identify contributing factors for relative fitness of hybrids and adults.

Are *S. ruber* and *S. varius* 'good species'?

Hybridization has created debate over the taxonomic position of *Sphyrapicus* (Ridgeway 1914, Howell 1952, Scott et al. 1976, Johnson and Zink 1983, but see Cicero and Johnson 1995). Are they good species under the biological species concept and/or other species concepts? We argue that despite extensive hybridization in sympatry, there are several key factors that suggest the two forms are distinct 'species'. They are phenotypically (Fig. 2, Howell 1952, Johnson and Zink 1983, Walters et al. 2002a, b), genetically (Fig. 3, Johnson and Zink 1983, Cicero and Johnson 1995, Weir and Schluter 2004) and behaviorally (Fig. 4) distinct entities. Except for hybrids in this narrow contact zone, they do not show intermediate phenotypes in the rest of their range (Walters et al. 2002a, b), and both breeding and wintering areas of *ruber* and *varius* remain largely separated from each other (Fig. 1).

Phylogenies based on allozymes and cytochrome b (Cicero and Johnson 1995, Weir and Schluter 2004) suggested that the two forms diverged around 1.0 to 1.2 million yr ago. This fits well with the age of other boreal taxa in North America that are considered distinct species with a similar east-west distribution and migratory pattern (Weir and Schluter 2004). Therefore this hybrid zone of *ruber* and *varius* match the taxonomic criterion for full species

status in North American birds that hybridize (AOU 1998), and support AOU's notion on hybridizing taxa in general; 'hybridization in a narrow and stable hybrid zone is viewed as evidence for lack of free interbreeding' (AOU 1998). *Sphyrapicus ruber* and *varius* are genetically and phenotypically distinct entities despite hybridization in a narrow contact zone, with selection limiting the admixture.

Barriers maintaining the species boundaries

Well-differentiated plumage patterns, especially the amount of red in the head and breast, could facilitate assortative mating and function as an intrinsic pre-zygotic barrier (Brumfield et al. 2001, Stein and Uy 2006, Price 2008). However, observations suggest that rather than serving as a mating barrier, red plumage may encourage hybridization. The redder mate is the male in the majority of observed mixed-species pairs in the *nuchalis*–*ruber* hybrid zone (Howell 1952, Johnson and Johnson 1985). Based on this observation, Johnson and Johnson (1985) suggested that female preference for red males facilitates asymmetric hybridization. A similar preference for red could have resulted in *varius* females mating with redder *ruber* in the *ruber*–*varius* hybrid zone. Red head and facial plumes are predominant in males of all four *Sphyrapicus* species (Campbell et al. 1990, Walters et al. 2002a, b), therefore the red crest, nape and throat could be sexual signals. No information, however, is available on whether this selective advantage translates across species in sympatry and provides a mating advantage to *ruber* males.

No quantitative information is available on the species-specific differences of acoustic behavior in sapsuckers (Walters et al. 2002a, b). Our observations during playback experiments suggest that both species respond equally well to heterospecific drumming and aggression calls. Therefore acoustic differences might not play a major role in reproductive isolation.

Previous studies of sapsucker hybrid zones have focused on the southern part of their ranges where *nuchalis* and *ruber* overlap with a narrow (~20 km wide) hybrid zone in the middle of a steep environmental gradient from warm-wet to cool-dry habitat (Howell 1952, Johnson and Johnson 1985). In contrast, the Pine Pass area of the northeastern BC provides several shallow northeast to southwest passages across the Rocky Mountains. The habitat in this region is in a broad transition from dry forests dominated by spruce (*Picea*) and broadleaf aspen (*Populus*) to the east of the Rockies to moist, coniferous-dominated forest (again mainly *Picea*) in the western interior (Hebda 1997). The 'ecotone' model suggested for the southern hybrid zone (Johnson and Johnson 1985) might not explain this *ruber*–*varius* hybrid zone, which is placed west of the more obvious habitat transition. Nevertheless, the differences in melanin-based plumage in *ruber* and *varius* could be adaptations to separate habitats. *Sphyrapicus varius* is found in mixed conifer forests (Eberhardt 1997, Walters et al. 2002a), which have open canopy and better light penetration. *Sphyrapicus ruber*, which is much darker in dorsal plumage compared to *varius*, is found in disturbed and old-growth conifer-dominated forests (Walters et al. 2002b) that are humid and darker than eastern broadleaf forests.

The two species have different migration routes and wintering areas (Fig. 1), raising the possibility that a migratory divide (Helbig 1991, Rohwer and Irwin 2011) could maintain this hybrid zone. The migratory behavior of hybrids is not known, but hybridization and resulting intermediate migratory behavior could lead hybrids into unfavorable migratory routes and wintering grounds. This could lower survival of F1 hybrids and act as a post-zygotic barrier, promoting speciation through reducing gene flow between the two groups (Helbig 1991, Bensch et al. 1999, Ruegg 2008, Irwin 2009, Rohwer and Irwin 2011).

Is the hybrid zone moving?

Museum skins from this region (Fig. 1b) indicate that only *ruber* was found near the western foothills, and *varius* was found only to east of the Rocky Mountains. Other historical records (see Introduction) indicate breeding *ruber* in Dawson Creek near BC – Alberta border (McTaggart-Cowan 1939), ~150 km east of the easternmost *ruber* collected in the present study. Banding and point count data collected from a banding station in Mackenzie, BC, near the center of the present hybrid zone shows a gradual decline of *ruber* and increase of *varius* and hybrid phenotypes from 1994 to 2009 (Supplementary material Appendix 1, Fig. A2). These observations suggest a possibility of westward movement of this hybrid zone.

Social dominance could be a driver of hybrid zone movement (Pearson and Rohwer 2000, Brumfield et al. 2001, McDonald et al. 2001, Rohwer et al. 2001, Bronson et al. 2005, Curry 2005). In sympatry, *varius* is much more aggressive towards *ruber* (Fig. 4) than vice versa. The aggressive *varius* males (Fig. 4) may be able to hold territories better than sympatric *ruber*. The territory holders would have a better chance to find a mate, hence in sympatry such heterospecific aggression can be favored (Duckworth and Badyaev 2007). Further studies should explore the possibility that this aggression asymmetry is causing westward movement of the hybrid zone.

Conclusion

Here we have provided the first comprehensive analysis incorporating both phenotypic and genetic variation across a hybrid zone of sapsuckers. The concordant phenotypic and genetic characters across the hybrid zone support the present taxonomic status of this species pair. Hybrids and heterozygotes were more rare at the centre of the hybrid zone than expected under random mating with no selection, although this result was not statistically significant. The width of the zone is 122 km, narrower than would be expected under neutral blending. Therefore some form of selection maintains this hybrid zone. This study further highlights the importance of northeastern BC for studies of avian biogeography in North America by adding a hybridizing pair of non-passerines to the list of contact zones in this region that have been analyzed genetically and phenotypically (Toews and Irwin 2008, Brelsford and Irwin 2009, Irwin et al. 2009, Toews et al. 2011). In a number of cases, extensive hybridization occurs in narrow zones of contact between otherwise highly genetically and phenotypically divergent entities (e.g. Audubon's/myrtle warblers, Brelsford and Irwin

2009; MacGillivray's/mourning warblers, Irwin et al. 2009; Townsend's/black-throated green warblers, Toews et al. 2011). These common patterns lead us to question the wisdom of the common taxonomic practice of merging hybridizing forms into a single species, as motivated by a fairly strict application of the biological species concept. Rather, the patterns suggest that extensively hybridizing forms can be remarkably stable over long periods of time, and that stable hybrid zones could be a common phenomenon during the divergence of a single species into two species that are completely reproductively isolated.

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Supplementary material (Appendix J5516 at <www.oikosoffice.lu.se/appendix>). Appendix 1.