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TECHNICAL NOTE

Development of 10 novel SNP-RFLP markers for quick genotyping within the black-capped (*Poecile atricapillus*) and Carolina (*P. carolinensis*) chickadee hybrid zone

M. A. McQuillan[1] · A. V. Huynh[1] · S. A. Taylor[2,3] · A. M. Rice[1]

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Abstract As species ranges shift due to anthropogenic change, accurate detection of hybridization between species will become increasingly important for conservation biologists. The black-capped (*Poecile atricapillus*) and Carolina (*Poecile carolinensis*) chickadee hybrid zone is difficult to study because the parental species possess similar morphologies and song is an unreliable species identifier. Further, the hybrid zone is moving northward rapidly due to environmental change. Here, we present 10 single nucleotide polymorphism markers developed from black-capped and Carolina chickadee transcriptome sequences. This marker set coupled with species-specific restriction enzyme digestion allows fast, easy genotyping of pure species and hybrid individuals within the hybrid zone.

Keywords *Poecile atricapillus* · *Poecile carolinensis* · SNP-RFLP · Hybridization · Chickadee

Hybridization—where distinct species come into contact and mate—is a frequent natural phenomenon with implications for speciation, extinction, and conservation (Coyne and Orr 2004; Mallet 2005; Abbott et al. 2013). Rates of hybridization are expected to rise as anthropogenic changes cause species ranges to shift towards higher latitudes and elevations, bringing previously isolated species into contact (Parmesan 2006; Chen et al. 2011). One potential result is a reduction in biodiversity (Rhymer and Simberloff 1996; Chunco 2014). Therefore, accurately detecting hybridization events is increasingly important for conservation biologists.

Here, we present a rapid genetic method for diagnosing hybrids between two North American songbird species: black-capped (*Poecile atricapillus*) and Carolina (*Poecile carolinensis*) chickadees. These species form a long hybrid zone stretching from Kansas to New Jersey, which is rapidly moving northward due to climate warming (Taylor et al. 2014a; McQuillan and Rice 2015). Moving hybrid zones are increasingly common (Buggs 2007), and are sensitive bioindicators of environmental change (Taylor et al. 2015). Because morphology and song are not reliable species identifiers in or near this hybrid zone (Kroodsma et al. 1995), we developed a small number of nuclear SNP markers amenable to PCR amplification and restriction enzyme digestion, to allow quick and accurate genotyping.

We developed SNP markers from a personal transcriptome database. Briefly, we isolated total RNA from pectoral muscle sampled from 5 black-capped and 5 Carolina chickadees collected from allopatry (Ithaca, New York and Baton Rouge, Louisiana, respectively) using Tri-Reagent (Sigma-Aldrich, St. Louis MO, USA). We used isolated RNA as templates to prepare Illumina sequencing libraries using the TruSeq RNA Sample Preparation Kit v. 2 (Illumina, San Diego CA, USA) following manufacturer

McQuillan and Huynh have contributed equally to this work.

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✉ M. A. McQuillan
mam612@lehigh.edu

[1] Department of Biological Sciences, Lehigh University, Bethlehem, Pennsylvania 18015, USA

[2] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14850, USA

[3] Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, Colorado, 80309, USA

Table 1 Ten SNP markers for detecting hybridization between the black-capped and Carolina chickadee. PCR products for each primer pair can be differentially digested by their respective restriction enzyme, allowing for species-specific genotyping at each locus

Locus ID	PCR product length (bp)	Forward primer (5′–3′)	Reverse primer (5′–3′)	BCCH allele	CACH allele	Restriction enzyme	BCCH banding pattern	CACH banding pattern	Annealing Temp (°C)	Digestion Temp (°C)	Zebra finch chromosome number
c0p171	211	TCACACAAC AGCCATTTG TACG	TTCCACATG GGATATCTT GC	G	A	AluI (New England Biolabs)	119 bp; 92 bp	211 bp	56.4	37	1
c0p183	301	TCTTCTGCA GGAAGTAAA CACTG	AAGTTCAGG ACTCCATTT TGCG	T	G	TaaI (Thermo Fisher Scientific)	152 bp; 85 bp; 64 bp	237 bp; 64 bp	57	65	1
c0p184	221	ATCCATGCA GAAAGACTG GG	TCACTCTCA GTGCATAAG CTCC	T	G	TaaI (Thermo Fisher Scientific)	150 bp; 71 bp	221 bp	57.7	65	3
c0p238	267	TGGAGATGC AGTTTTTCT CCC	AAGAGGAGA TGGCTTTTC GC	T	A	NspI (New England Biolabs)	120 bp; 111 bp; 36 bp	231 bp; 36 bp	53.5	37	1
c0p251	183	AACCAACAG CTCTGTTCC CTG	AATGCAGAA GGCAGGAAC CTC	C	T	BccI (New England Biolabs)	116 bp; 67 bp	183 bp	58	37	10
c0p283	279	TTTCTTTCC TGCAAAGCC CC	TAAGGAGGG CCGTCCAAC AC	A	T	NcoI (New England Biolabs)	279 bp	153 bp; 126 bp	56.4	37	21
c0p303	291	TAGAGGCAG CAGTGGTAC AG	ATGAGCGGT GTTTGTTCC TTG	G	A	AccI (New England Biolabs)	150 bp; 141 bp	291 bp	55.8	37	1
c0p356	200	AGGAATTCC ACTGGTAAC TTACTG	TGAAGAACA GTCATTAGT TTTGGC	G	A	Hpy188III (New England Biolabs)	129 bp; 71 bp	200 bp	54	37	21
c0p373	274	GACTGCAAT CCTCTTGGC TG	CAGCCTCAA TGCAATCCA CAG	C	T	Hpy188III (New England Biolabs)	274 bp	137 bp; 137 bp	55.8	37	3
c0p628	217	ACACTCCTG CATCTTCCC TTC	AGGCTTTGG GATTCTTTG TGC	C	T	BsaAI (New England Biolabs)	110 bp; 107 bp	217 bp	55	37	21

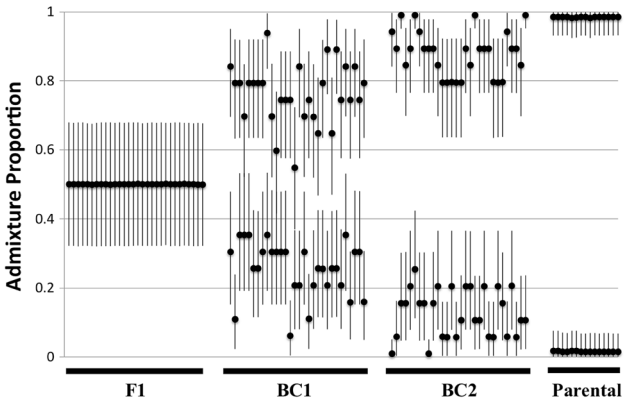

Fig. 1 Admixture proportions and 90 % credible intervals for simulated F1 hybrid, *BC1* first generation backcross hybrid, *BC2* second generation backcross hybrid, and pure-species parental genotypes. An admixture proportion of 1 indicates 'pure' black-capped chickadee ancestry, while 0 indicates 'pure' Carolina ancestry

protocols. Libraries were sequenced as 100 nt single-end reads on the Illumina HighSeq 2500 platform at the Carver Biotechnology Center at the University of Illinois, Urbana-Champaign. From this database, we selected 10 loci (Table S1), each containing a single nucleotide difference divergently fixed between species and lacking introns based on alignment with the zebra finch (*Taeniopygia guttata*) genome. Additionally, we selected loci so that a unique restriction enzyme would digest the variable site in one species' amplified allele fragment, but not the other's, using the web-based software SNP-RFLPing v2.0 (Chang et al. 2010). Therefore, restriction digestion of each amplified locus results in a unique number of fragments depending on whether the individual possesses the black-capped or Carolina chickadee allele (or both; Table 1, Fig. S1).

To confirm within-species fixation of each SNP allele, we Sanger sequenced the 10 loci in an additional 10 black-capped and 10 Carolina chickadee museum samples collected from allopatry (Table S2). PCR reactions were run in 10 µL volumes with the following: 100 ng template DNA, 0.2 µL of both forward and reverse primers (10 µM), 5 µL DreamTaq Master Mix (2X, Thermo Scientific, Waltham, Massachusetts, USA), and 3.6 µL water. Once fixation was confirmed, we tested our markers by genotyping 63 adult chickadees from a hybrid zone population (Lehigh University; 40°36.087′N, 75°21.363′W), using restriction enzyme digestion of amplified PCR products. We extracted genomic DNA from field-collected blood samples using DNeasy kits (Qiagen, Venlo, Netherlands), and prepared PCR reactions as described above. We then added 10U of restriction enzyme to 5 µL of PCR product, and performed all digests overnight. Table 1 lists primer sequences, PCR annealing temperatures, restriction enzymes, and digestion temperatures for all 10 loci. We ran digested PCR products and undigested controls on 2% agarose gels at 150 V for 45 min and verified bands visually (Fig. S1).

We used STRUCTURE v.2.3.4 (Hubisz et al. 2009) to assess the power of our markers to detect hybrids. We generated simulated genotypes of F1 and backcross hybrids using the program HYBRIDLAB (Nielsen et al. 2006). We used genotypes of the 30 pure-species individuals from allopatry as initial inputs. We generated 30 simulated genotypes each of first generation (F1) hybrids, as well as first and second generation backcrosses to each parental species. We then used STRUCTURE with K set equal to 2 to estimate admixture proportions (±90% credible intervals) for the simulated genotypes (Fig. 1). These markers allow confident differentiation between parental individuals and individuals with hybrid ancestry, with decreasing power in advanced generation hybrids (Fig. 1). Finally, we used STRUCTURE to estimate the ancestry of the 63 unknown individuals from our hybrid zone population (see supplemental information for additional methods). We detected 3 pure black-capped chickadees, 35 pure Carolina chickadees, and 25 chickadees of hybrid ancestry (Fig. S2).

In sum, we present a panel of 10 SNP-RFLP markers for reliably detecting hybridization between black-capped and Carolina chickadees. These markers can accurately distinguish between pure-species individuals and hybrids (Fig. 1). In addition, we successfully used these markers to genotype 63 chickadees of unknown ancestry from a hybrid zone population (Fig. S2). This quick and cost-effective method for diagnosing hybridization in these morphologically similar species will be an important tool in understanding when and where hybridization is occurring, and how climate change influences hybridizing populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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