ARTICLES

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A "Trilling" Case of Mistaken Identity: Call Playbacks and Mitochondrial DNA Identify Chorus Frogs in Southern Québec (Canada) as *Pseudacris maculata* and Not *P. triseriata*

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Cryptic species are often difficult to study and recognize, especially when little morphological criteria are available to help distinguish between closely related species. Although congeners may be very similar in appearance, combining genetic analyses with differences in mating calls and breeding phenology may allow for new distinct species to be recognized and properly identified (Newman et al. 2012). Frogs within the genus *Pseudacris* are a good example of this. The Boreal Chorus Frog (*Pseudacris maculata*) and the Western Chorus Frog (*P. triseriata*) are two North American species that are extremely similar in size and coloration, which has created taxonomic confusion in the past (Platz 1989; Klaus and Lougheed 2013). To distinguish the two species, the ratio of the tibia length to the snout-to-vent length (SVL) has been used as a criterion of discrimination. *Pseudacris triseriata* has a slightly larger ratio of 42.6, whereas *P. maculata* has a ratio of roughly 39.3 (Smith 1956). With the two species so similar in size and shape however, tibia/SVL ratios could overlap and lead to species misidentification. An alternate identification criterion often used in the field is the playback of each species' display call during the breeding season. Although each species' song may initially sound very similar, *P. maculata* has been found to have a longer call with a lower pulse rate, whereas *P. triseriata*'s call is much shorter and has double the pulse rate (Platz 1989).

The first studies that delineated the range limits of *P. maculata* and *P. triseriata* were largely based on the morphometric measures of the tibia/SVL ratio, and did not include any song playback analyses (Smith and Smith 1952; Smith 1956). These studies determined *P. maculata*'s range to extend in latitude from the central US into Canada, and in longitude from the Rocky Mountains to northwestern Ontario. *Pseudacris triseriata*'s range included Ontario's extreme southern end, southwestern Québec, and several US states surrounding the Great Lakes (Smith and Smith 1952; Smith 1956) (Fig. 1A). However, recent studies based on species identification through breeding call and molecular-based analyses indicate that *P. maculata*'s range may extend further east than once believed, and raises some important questions about the true range of both species (Fortin et al. 2003; Lemmon et al. 2007a; Ouellet et al. 2009). Furthermore, although contact zones between the two species have not yet been identified, Lemmon et al. (2007b) determined that range overlap is highly likely to occur in southeastern Ontario, and stressed the importance of considering this geographic area when conducting *Pseudacris* biodiversity studies.

Initial range delineation studies did not extend *P. maculata*'s range into southern Ontario nor into Québec. The two species were believed to be allopatric until *P. maculata* populations were identified through song analysis along northern Québec's James Bay area (Fortin et al. 2003; Ouellet et al. 2009). Lemmon et al.'s (2007a) phylogenetic analysis of North American *Pseudacris* species using two mtDNA genes (12S and 16S), also proposed a new distribution of *Pseudacris* tree frogs in the United States and southern Ontario (Canada). According to this study, the distribution of *P. triseriata* would be limited to the United States and to Ontario's extreme southern end, but *P. maculata* would have a larger distribution in southeastern Canada (Fig. 1B). This recent molecular work raises questions as to whether previous range map studies using tibial bone measures may have misidentified *P. maculata* for *P. triseriata* in eastern Canada.

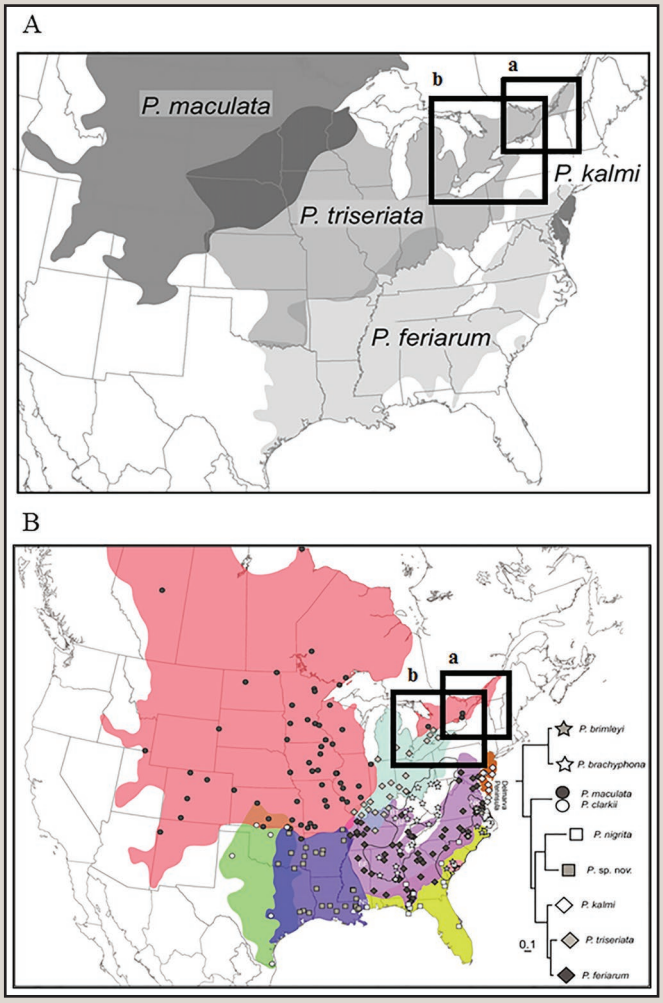

FIG. 1. A) Original *Pseudacris* range maps delineated by Smith and Smith (1952) and Smith (1956) predominantly using tibia/snout-to-vent length ratios. B) New phylogeny-based range delineation suggested by Lemmon et al. (2007a) using mitochondrial DNA. Source of maps: Lemmon et al. (2007a). Reproduced with permission by the copyright owner. Areas labeled as "a" and "b" delineate the location of our study sites within Québec and Ontario, respectively.

In light of Lemmon et al.'s (2007a) work, NatureServe (2014) now identifies chorus frog populations in eastern Canada to be composed of *P. maculata* instead of *P. triseriata*, but thorough revisions of the two species' range boundaries are necessary, especially considering the rapid decline of chorus frogs in Québec and Ontario (Seburn et al. 2008).

As of March 2000, the Québec government classified what was always believed to be *P. triseriata* frog populations as Vulnerable. A recovery plan and several habitat conservation plans have since been created and partially implemented to help conserve remnant populations (Équipe de rétablissement de la rainette faux-grillon de l'ouest 2000; Angers et al. 2007, 2008a, 2008b). Due to the major population declines that have been documented in Québec and Ontario since 2002, the Great Lakes-St. Lawrence-Canadian Shield chorus frog population has been classified as Threatened by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC), and is listed in Schedule 1 of the Species at Risk Act (SARA) (COSEWIC 2008). The status of *P. maculata* populations has not yet been evaluated by COSEWIC. They are however, listed as endangered in Vermont (USA) (Ferguson 2013) and critically imperiled in Michigan (NatureServe 2014). Populations have also been found to be declining in New York (USA) (Corser et al. 2012), in Ontario and along James Bay in northern Québec (Desroches et al. 2010).

The objective of this study was to help establish the true eastern distribution of *Pseudacris* chorus frogs by conducting a distance-based clustering analysis of individuals from southeastern Ontario, from along the Québec/Ontario border, and from several sites located in southwestern Québec. Individuals from various parts of the USA were also included in the analysis for comparison purposes. Species identification was further confirmed at each site using a non-genetic method of anuran identification of breeding call playback analysis. Shedding light on the true range of *Pseudacris* in southeastern Canada will help federal and provincial management practices protect the remaining fragile chorus frog populations found at the limit of their distribution, and will help determine the true identity of chorus frogs in southeastern Canada.

METHODS

Sites and sampling.—We sampled various localities within several major regions of Québec and Ontario (Canada). In southern Québec, four sites were sampled in the Outaouais region: two in 2006 near the municipality of Chelsey (U12) and in eastern Gatineau (U3), and two in 2009 in LuskVille (A11) and near Chatham (A12). In southern Québec's Montérégie region, frogs from five sites were collected in the spring of 2007: Boucherville (BOU), Brossard (BRO), Boisé du Tremblay (BT), La Prairie (LP), and Île Perrot (IP). Individuals from four sites in Ontario were also collected in 2009: Almonte (DW), Arnprior (ARN), Lake Opinicon (OP), and Cataraqui Park near Kingston (CA) (Fig. 2). An additional eight sequences of both *P. triseriata* and *P. maculata* individuals were retrieved from GenBank and were included in the dataset for comparison purposes (Table 1).

We played breeding call recordings of both *P. maculata* and *P. triseriata* at each site and noted which species responded. Sites were originally selected by listening to breeding calls from a distance, yet upon arrival at each site, the frogs were disturbed and their calls were silenced. Sitting silently for approximately thirty minutes allowed the frogs to resume their normal activities. We had two different breeding call recordings for each species, stored and played from an Apple iPod touch (©2007 Apple Inc.). The recordings were obtained from the Canadian Amphibian and Reptile Conservation Network, both online (FrogWatch) and on CD (EMAN/RESE). We proceeded to play one *P. triseriata* breeding call recording for three minutes, followed by an additional three minutes of silence used to note whether a response occurred. This was then repeated using the breeding call of *P. maculata*. The entire procedure was subsequently carried out again using the second breeding call recording we had available. To avoid introducing bias, the procedure was immediately repeated playing *P. maculata* recordings first, followed by *P. triseriata* recordings. Additionally, we alternated the order of which species' playback recordings were first played in successive sites. Recordings were played at each site in 2007 and 2009, and were carried out at approximately 1500 h.

A varying number of individuals were caught by hand from each site (N = 19–25); those samples are currently undergoing a population genetics analysis as well (N. Tessier, pers. obs.). For each individual captured, a toe clip was removed with disinfected scissors and stored in 95% ethanol at room temperature. The

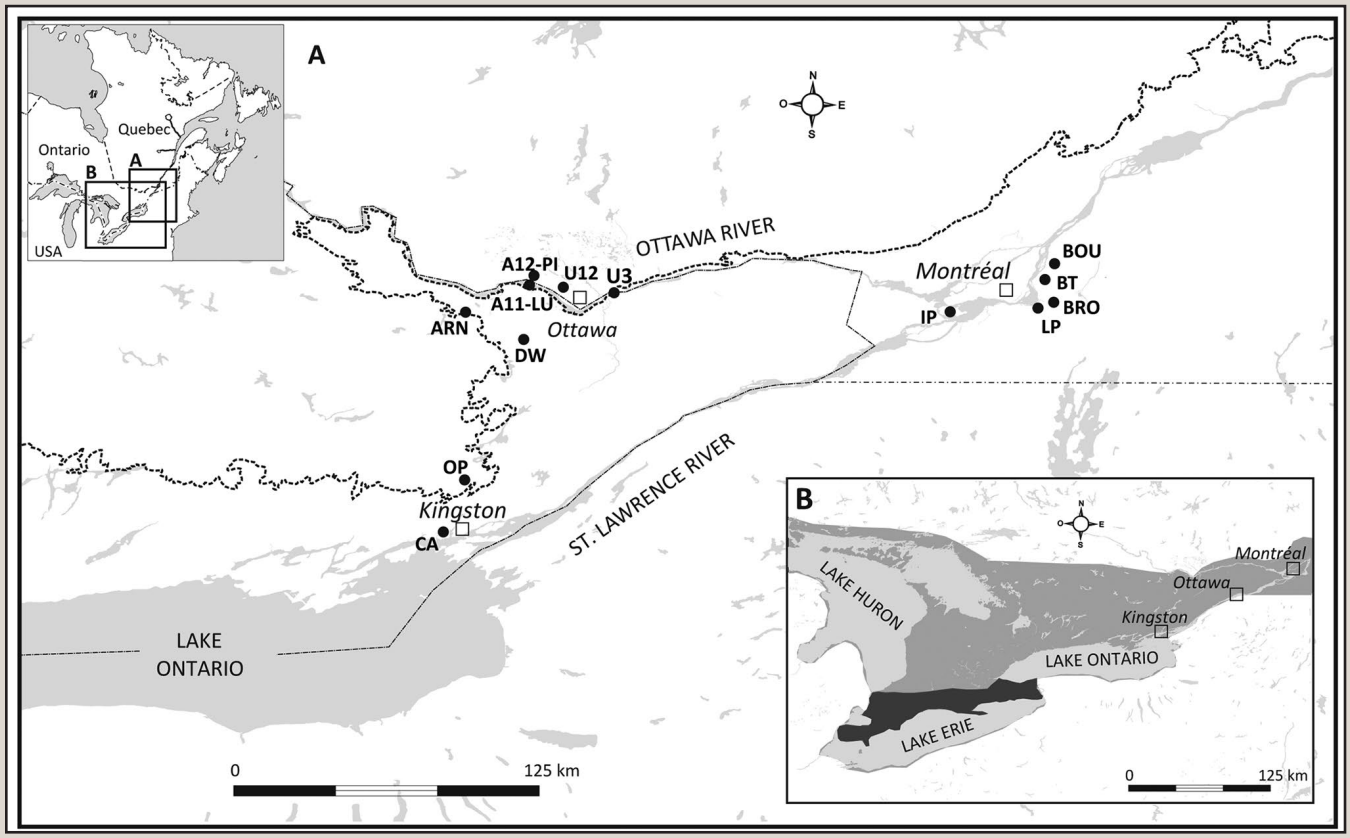

FIG. 2. Geographic location of all chorus frog sampling sites included in this study. In Québec's Outaouais region: Chelsey (U12), Gatineau (U3), LucksVille (A11), and Chawtham (A12). In Québec's Montérégie region: Boucherville (BOU), Brossard (BRO), Boisé du Tremblay (BT), La Prairie (LP), and Île Perrot (IP). Individuals from Ontario: Almonte (DW), Arnprior (ARN), Lake Opinicon (OP), and Cataraqui Park near Kingston (CA). The Carolinian and Great Lakes - St. Lawrence - Canadian Shield faunal provinces are depicted in dark and medium gray, respectively.

TABLE 1. Location and GenBank accession numbers for the 16 reference *Pseudacris* individuals included in this study.

Species	Accession Number	Location	State/Province & Country
Pseudacris maculata	AY291089	McKinley	New Mexico, USA
Pseudacris maculata	AY291090	Douglas	Kansas, USA
Pseudacris maculata	AY291083	Archuleta	Colorado, USA
Pseudacris maculata	AY291082	Lac Seul	Ontario, Canada
Pseudacris maculata	AY291080	Gunnison	Colorado, USA
Pseudacris maculata	EF472135	Christian	Missouri, USA
Pseudacris maculata	EF472122	Jersey	Illinois, USA
Pseudacris maculata	EF472100	Bayfield	Wisconsin, USA
Pseudacris triseriata	AY291091	Berrien	Michigan, USA
Pseudacris triseriata	EF472157	Breckinridge	Kentucky, USA
Pseudacris triseriata	EF472156	Niagara	New York, USA
Pseudacris triseriata	EF472151	Logan	Ohio, USA
Pseudacris triseriata	EF472145	Niagara R.M.	Ontario, Canada
Pseudacris triseriata	EF472139	Daviess	Kentucky, USA
Pseudacris triseriata	EF472138	Perry	Illinois, USA
Pseudacris triseriata	EF472137	Montgomery	Tennessee, USA

scissors were disinfected with ethanol between each individual clipping. All frogs were then immediately released at the exact site of capture and were temporarily monitored to ensure that normal behavior ensued. Subsets of three to four individuals per site were then selected at random and used for further molecular identification.

Molecular analysis.—DNA was extracted using the Invitrogen™ PureLink™ Genomic DNA kit (Version A, February 2007) (Invitrogen™ Inc., Burlington, Canada). To verify the maternal lineage of the chorus frogs in our dataset, a 335-base pair (bp) portion of the 16S mitochondrial gene was sequenced for each individual using the 16S-1F and 16S-4R primers (Johnson et al. 1998). The 16S region is less conserved than the 12S region, thus has greater variability and is usually used for phylogenetic studies at the genus level (Hwang and Kim 1999). PCR amplifications were carried out in 50-mL volumes and included 100–200 ng of genomic template DNA per sample, 1.4X reaction buffer, 2.5 mM of $MgCl_2$, 0.35 mM of each dNTP, 0.22 µM of each primer, 0.5 units of *Taq* DNA polymerase (1 U ≈ 16.67 nkat), and 35.7 µL of sterile H_2O. Thermal cycling was carried out on a GeneAmp® PCR System 9700 (Applied Biosystems) under the following

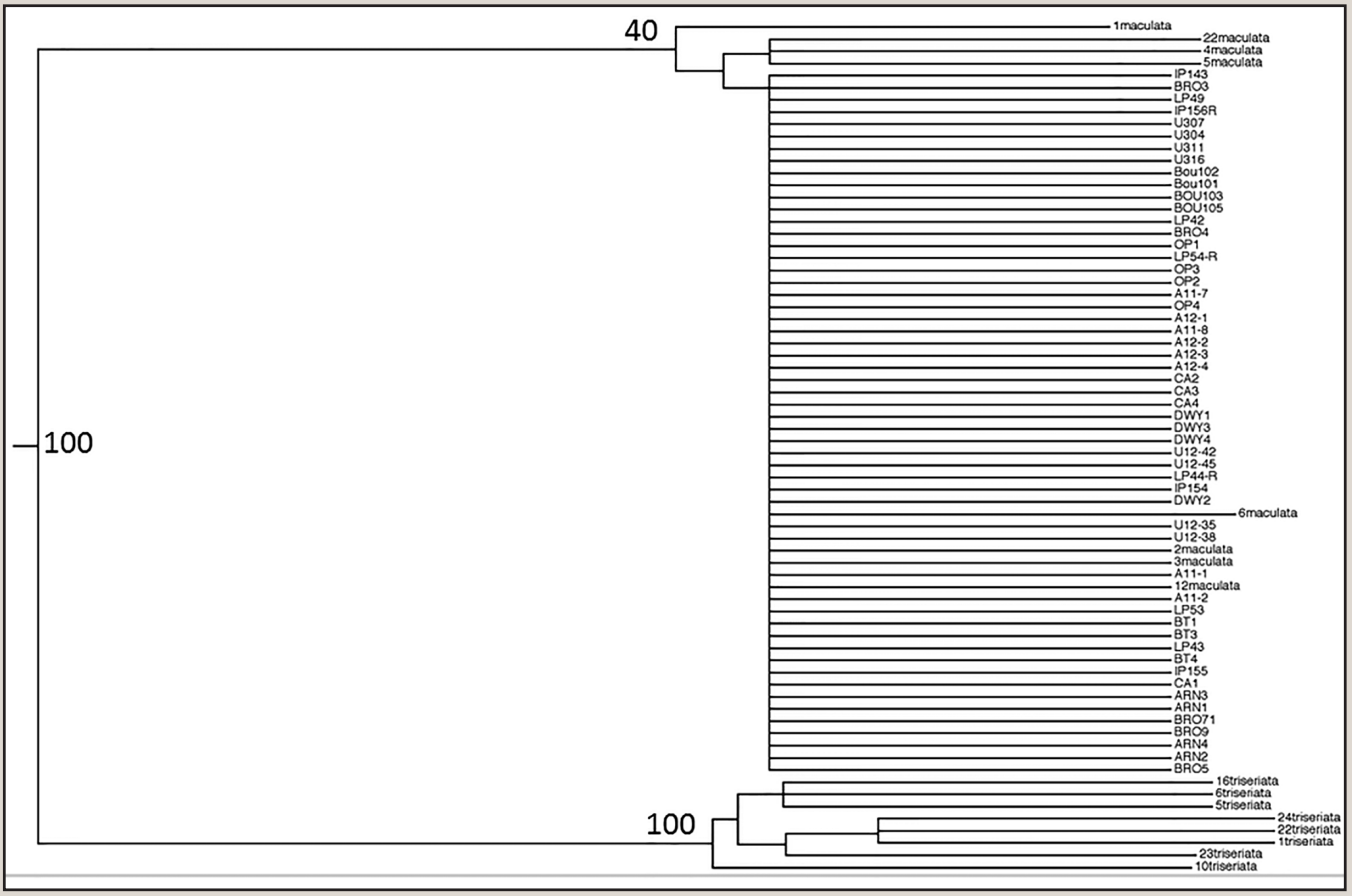

FIG. 3. Neighbor-joining tree constructed from the 335 bp 16S mtDNA gene sequence (strict consensus supported by 1000 bootstrap replicates and distances corrected with a Jukes-Cantor model). All 54 individuals found in Québec and Ontario are grouped with the eight *P. maculata* reference sequences, whereas the eight reference sequences for *P. triseriata* define a distinct group with 100% bootstrap support.

conditions: an initial denaturation of 94°C for 1 minute; 31–38 cycles of 94°C for 45 seconds, 50°C for 1 minute and 45 seconds, and 72°C for 1 minute and 30 seconds; and a final extension step of 72°C for 10 minutes. Amplification success was determined by staining the PCR products with SYBR® Green I nucleic acid gel stain (Invitrogen™ Inc., Burlington, Canada), running them on a 2% agarose gel, and revealing the bands under UV light. All PCR products were sent to the McGill University Génome Québec Innovation Centre for purification and sequencing using an ABI-3730XL DNA Analyzer (Applied Biosystems). Sequences were then visualized with 4PEAKS 1.7.2 (Mekentosj B.V., Aalsmeer, The Netherlands) and aligned with ClustalX (Thompson et al. 1997). All sequences were deposited in the GenBank public repository (accession numbers KM669659-KM669712).

Distance-based clustering analysis and species identification.—Aligned sequences were evaluated with jModel Test 2 v 2.1.5 (Darriba et al. 2012) to compare different DNA substitution models, and the Jukes-Cantor (JC) model (Jukes and Cantor 1969) was determined to be the best fit for our data. Accordingly, a neighbour-joining tree was then estimated from JC-corrected distances with 1000 bootstrap replicates to determine branch support. This tree was then used to determine the identification of the Québec samples with respect to the reference sequences of known identity. All clustering analyses were performed in R (R Development Core Team 2013) using the "ape" package (Paradis et al. 2004).

RESULTS

At every sampled site, chorus frogs responded to the playback recordings of *P. maculata* rather than those of *P. triseriata*. Frogs remained silent after *P. triseriata* recordings were played.

Mitochondrial gene.—The 16 reference sequences (8 *P. maculata* and 8 *P. triseriata* individuals) produced four haplotypes per species, and all Québec (N = 39) and Ontario (N = 15) individuals matched the *P. maculata* 1 haplotype (Table 2). A total of 14 variable sites were used to discriminate the two *Pseudacris* species, and the corresponding neighbor-joining tree is presented in Fig. 3. In this tree, all 54 individuals found in Québec and Ontario were grouped with the eight known *P. maculata* sequences. The eight *P. triseriata* reference sequences produced their own distinct group with 100% bootstrap support, therefore separation between the two species was highly supported.

DISCUSSION

Although the identification of *P. maculata* in Québec has only been observed in recent history, their populations were already established within the province, yet misidentified as *P. triseriata*. A number of studies based on breeding calls have found *P. maculata*'s northeastern range limit to extend 100 km eastward into northwestern Québec (Fortin et al. 2003; Ouellet et al. 2009), and genetic analyses have also confirmed its presence

in southeastern Ontario (Lemmon et al. 2007a). In our present study, 16S mtDNA sequence analysis, as well as chorus frog breeding call inventories in the peri-urban areas of the Greater Montréal and Outaouais regions of Québec, established for the first time that remnant chorus frog populations in southern Québec are actually comprised of *P. maculata* individuals rather than *P. triseriata*.

The aforementioned studies (along with the present one) strongly suggest that tibia/SVL ratios, on which the identification of *P. triseriata* and *P. maculata* individuals were based during the original delineation of their respective ranges, should be reconsidered as a reliable diagnostic criterion for species identification. Tibia/SVL ratios have long been used in taxonomy and systematics, yet little is known in terms of this measure's precision and accuracy. Rogers (2009) found that Boreal Toads required aggressive and awkward handling to take proper tibial and SVL length measurements, but errors in measurement still varied up to 30% within the same individual. He suggested that tibial and SVL measurements should be used to construct rough size indices rather than for precision, and that the gape width measurement method yielded better results. Hayek et al. (2001) also recommend employing anuran morphometrics for rough rather than precise measurements, and to use caution when drawing biological inferences from these data. Climatic conditions may also play a role in intraspecific SVL measurements. *Pseudacris regilla* was found to significantly fluctuate in SVL measurements when exposed to differing weather conditions (e.g., temperature, altitude, and rainfall) (Calhoon and Jameson 1970). With climate change becoming increasingly important to consider in amphibian genotypic/phenotypic studies (Spear et al. 2012), it is a variable that must be taken into account when employing tibia/SVL ratios for species identification purposes.

In the very first studies to delineate *Pseudacris* range limits (Smith and Smith 1952; Smith 1956), all measurements were taken on preserved specimens. However, Bleakney (1959) found that even the most carefully preserved chorus frog specimens will exhibit a body length decrease of 1–3 mm, but whose tibia length will only decrease between 0–1 mm. Furthermore, the initial delineation studies did not incorporate song playback recordings when identifying chorus frogs. Playback methods were first pioneered in the mid-1960s by Capranica (1965; 1966). Breeding call playbacks have shown to successfully identify several different anuran species within the same habitat (Sung et al. 2005; Acevedo and Villanueva-Rivera 2006), however these methods were not as robust for population size and abundance estimates (Corn et al. 2000), even for *P. maculata* (Corn et al. 2011). Call playbacks of a single mating call (rather than a chorus of breeding calls) have proven most efficient in obtaining responses from resident frogs, and allow researchers to easily access a wide range of habitats that otherwise might be physically difficult to work in when using capture methods of identification (Sung et al. 2005). On the other hand, the use of recording devices and speakers may be costly; however, the methods and devices employed in this study proved to be cost-efficient without undermining the effectiveness of the breeding call recordings and responses.

Our findings have several implications for the conservation and research of Canadian *P. maculata* and *P. triseriata* populations. First, they suggest that the Great Lakes-St. Lawrence-Canadian Shield chorus frog population should be renamed a *P. maculata* population. This population was originally named as a *P. triseriata* frog population by the COSEWIC, listed under

TABLE 2. Polymorphic nucleotide positions for the Western Chorus Frog (*Pseudacris triseriata*) compared to its congener species, the Boreal Chorus Frog (*P. maculata*) using the Genbank 16S mtDNA sequences (8 *P. maculata* and 8 *P. triseriata* individuals). The sequences produced four haplotypes per species, but 14 variable sites helped distinguish *P. maculata* from *P. triseriata*. All Québec and Ontario specimens (N = 54) matched the *P. maculata*1 haplotype. Nucleotides identical to the *P. maculata*1 haplotype are indicated with a period (.).

16S Haplotypes	N	17bp	22bp	24bp	74bp	75bp	76bp	77bp	82bp	85bp	97bp	103bp	111bp	118bp	182bp	184bp	186bp	203bp	248bp	292bp	293bp
P. maculata 1	4	G	A	T	T	T	A	T	A	C	G	T	T	C	T	T	G	T	T	C	G
P. maculata 2	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
P. maculata 3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.
P. maculata 4	1	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. triseriata 1	3	A	.	C	A	C	.	C	T	T	A	A	C	T	C	A	A	C	C	.	.
P. triseriata 1	3	A	G	C	A	C	.	C	T	T	A	A	C	T	C	A	A	C	C	.	.
P. triseriata 1	1	A	G	C	A	C	.	.	T	T	A	A	C	T	C	A	A	C	C	.	.
P. triseriata 1	1	A	G	C	A	C	.	C	T	T	A	A	C	T	C	A	A	C	C	.	A

SARA, and represents an Evolutionary Significant Unit (ESU) for conservation. Considering the scenarios of dispersal routes for *P. maculata* (Lemmon et al. 2007b) and their disjunct population distribution in Canada, the Great Lakes-St. Lawrence-Canadian Shield population may have evolved in isolation, but this assertion requires further investigation. Consequently, this population's status should be re-evaluated separately from that of *P. maculata* populations further west into Canada. Moreover, comparative studies between chorus frogs from Québec and Ontario are needed to determine if locally adapted phenotypic traits (e.g., difference in advertisement call structure), or if differences on a finer genetic scale (e.g., population genetic analyses) have evolved. In their phylogenetic study of the trilling chorus frogs, Lemmon et al. (2007a) found *P. maculata* and *P. clarkii* to show significant differences in morphology and behavior, but were undifferentiated with respect to their mitochondrial DNA. In terms of a fine scale genetic analysis, we are currently employing a population genetics analysis to help determine the level of genetic differentiation and isolation of this important ESU (N. Tessier, pers. obs.).

Although not much is known about *P. maculata*'s overall population stability, individuals located at the edge of their range limits are often found in sub-optimal habitats and experience greater environmental fluctuations, which increases their vulnerability (Gaston 1990; Mott 2010). Therefore, Québec chorus frogs in particular, whose populations have been disappearing and whose situation has become precarious, should be monitored closely. Multiple studies have tracked the rigorous population decline of chorus frogs in the Montérégie and Outaouais regions of Québec, and argued that urgent conservation actions need to be taken to save them from extinction (Bonin and Galois 1996; Picard and Desroches 2004; Angers et al. 2008a, b; COSEWIC 2008; Seburn et al. 2008; Équipe de rétablissement de la rainette faux-grillon de l'ouest 2010). Since the 1950s, Québec has seen a 37% decrease in chorus frog population size every ten years, and the decline is expected to continue (COSEWIC 2008; Équipe de rétablissement de la rainette faux-grillon de l'ouest 2010). Chorus frog population surveys in Ontario indicate a 42.6% decline in abundance over the last decade despite hosting areas where chorus frogs seem relatively abundant (COSEWIC 2008).

The greatest threat to these chorus frog populations is the progressive destruction and modification of their habitat for land use, development, and urbanization—an endless process primarily driven by economic and population growth (Trauger et al. 2003). Currently, less than 10% of viable chorus frog habitats are located in federally or provincially protected parks and refuges, leaving most populations vulnerable to the increased anthropogenic pressures (Environment Canada 2010). The long-term protection of remnant habitats is key for ensuring their continued survival.

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