Genetic Monitoring of At-risk Species in Canada: an Overview and a Case Study, the Northern Leopard Frog (*Lithobates pipiens*) in Western Canada.

by

Parinaz Khalilzadeh

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APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. David Lesbarrères
(Co-Supervisor/Co-directeur(trice) de thèse)

Dr. Lea Randall
(Co-Supervisor/Co-directeur(trice) de thèse)

Dr. David Coltman
(Committee member/Membre du comité)

Dr. Damian Picard
(External Examiner/Examinateur externe)

Approved for the Office of Graduate Studies
Approuvé pour le Bureau des études supérieures
Tammy Eger, PhD
Vice-President Research (Office of Graduate Studies)
Vice-rectrice à la recherche (Bureau des études supérieures)
Laurentian University / Université Laurentienne

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"که عشق آسان نمود اول ولی افتاد مشکل ها"

حافظ شیرازی

Abstract

Genetic assessment and monitoring are critical in evaluating the success of conservation efforts for at-risk species. Genetic assessment can provide valuable information, such as identifying conservation management units and revising taxonomy, which can be used to improve population connectivity and perform translocations. Genetic monitoring helps quantify changes in gene frequencies over time, thus providing feedback for conservation management actions, particularly for at-risk species. In the first part of my thesis, I reviewed the status reports of 788 at-risk designatable units (DUs) listed by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC), which were available up until 2019. My objectives were to evaluate how many Canadian at-risk species have 1) been genetically assessed and 2) undergone genetic monitoring. My results show that genetic data are available for 50% of DUs, although only 2% of them underwent genetic monitoring, and there is a significant taxonomic bias in the availability of genetic information, with mammals and fishes being over-represented and mosses being under-represented. In the second chapter, I investigated the population genetics of one such at-risk species, the northern leopard frog, *Lithobates pipiens*, which has undergone a rapid decline in the number of populations in western Canada, particularly in British Columbia and Alberta. The last published genetic research on the populations and DUs of the species in Canada was in 2008, and the Creston Valley population in British Columbia is the last remaining population in the Rocky Mountain DU at the most western limit of the species range. To understand changes in population genetics parameters over time, I compared current genetic diversity from four populations: Creston Valley, Drain K, Prince's Spring, and Cypress Hills, with 1) data from the Wilson et al. study (2008) at the same locations and 2) the extinct population of Fort

Steele, British Columbia. Furthermore, to assess changes in the genetic diversity of the Creston Valley population in British Columbia over time, allelic richness and expected heterozygosity of the population were compared at 3-time points using genotypes from 2000 (Hoffman and Blouin 2004), 2004 (Wilson et al. 2008), and 2019. My results show that the current populations of the species in western Canada are highly genetically differentiated from each other, that genetic diversity declines from east to west, and that the Creston Valley population has likely experienced a population bottleneck. Yet, genetic monitoring of the four northern leopard frog populations shows that both genetic diversity and structure had changed very little between 2004 and 2019. But there was evidence of decline in genetic diversity of Creston Valley population between 2000 and 2019, suggesting that genetic monitoring may need to occur over longer time frames in order to detect meaningful changes in genetic diversity. Overall, my results suggest that management plans and recovery strategies should include genetic data in the conservation of at-risk species. My work also contributes to the recovery efforts of the northern leopard frog, particularly in managing the Creston Valley population.

Keywords: Creston Valley**,** designatable units, genetic monitoring, northern leopard frog

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Introduction

The majority of threats associated with the current biodiversity crisis are due to human activities causing climate change, pollution, habitat fragmentation, and deforestation (Singh 2002, Frankham et al. 2010, Frankham 2019). Among other consequences, wildlife populations are getting smaller, becoming more isolated, and losing their genetic diversity (Frankham et al. 2019). Genetic diversity is critical to populations' long-term survival allowing them to adapt to changing environments. Conservation genetics as a field has been developed to maintain or even improve genetic diversity in at-risk populations and species (Singh 2002, Frankham 2019) by providing conservation managers with critical information to identify conservation management units, revise taxonomy, improve population connectivity, and perform efficient translocations (Green 2005). Additionally, recent advances in genetics/genomics techniques have made genetic monitoring feasible, allowing for the study of changes in population genetics parameters at two or more time points (Schwartz et al. 2007). Therefore, genetic monitoring of at-risk species can help conservation managers understand when the populations of the species need conservation actions such as genetic rescue (Schwartz et al. 2007, De Barba et al. 2010). The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) commissions status reports for at-risk species and proposes conservation units to the government (COSEWIC 2018). Designatable Units are a population or groups of populations whose features make them distinct from other populations (Green 2005, COSEWIC 2018). However, the proportion of genetic data and genetic monitoring among DUs of listed species has never been assessed. Therefore, in the first part of my thesis, I reviewed 788 status reports of at-risk species from COSEWIC to understand how many Canadian species have undergone genetic monitoring. However, there

may have been genetic studies or monitoring that occurred but weren't mentioned in the COSEWIC report, or the results were not published.

For some species whose populations are on the verge of extinction in parts of their range, such as the northern leopard frog (NLF, *Lithobates pipiens*), recovery teams can greatly benefit from genetic data and monitoring in order to inform adequate management in these critical cases. Approximately two million years ago, the eastern and western clades of NLF were separated by the Mississippi River and the North American Great Lakes (Hoffman and Blouin 2004). While the eastern clade populations are secure in Canada, the western populations are at risk (Wilson et al. 2008), including the Rocky Mountains and Western Boreal/Prairie DUs (COSEWIC 2009). Among populations of the western clade that have been studied, the Creston Valley population in British Columbia was the most endangered NLF population with the lowest genetic diversity (COSEWIC 2009; Wilson et al. 2008). The western clade NLF populations would thus benefit from genetic assessment and monitoring to evaluate their current genetic statuses (e.g., genetic diversity, inbreeding, and effective population size) and provide conservation managers with the necessary information to conduct recovery actions such as conservation translocations. Therefore, I assessed the current NLF population genetic structure in western Canada in the second part of my thesis. I also genotyped 10 individuals from the extinct population of Fort Steele (collected in 1973) in British Columbia to compare its population genetic parameters such as allelic richness, observed heterozygosity, expected heterozygosity, inbreeding coefficient, and effective population size to the Creston Valley population's parameters. Both of these populations belong to the Rocky Mountain DU and were only separated by 88.3 km, making them valuable populations for temporal comparison. Additionally, I used genetic data from the

Wilson et al. (2008) study to conduct genetic monitoring across four populations assessed in 2004 and 2019. Furthermore, to assess changes in the genetic diversity of the Creston Valley population in British Columbia over time, I compared allelic richness and expected heterozygosity of the population at 3-time points using genotypes from 2000 (Hoffman and Blouin 2004), 2004 (Wilson et al. 2008), and 2019 (genotyped in this study).

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Chapter 1 - How Are Genetic Data used in the Assessment of at-risk Species in Canada?

Abstract

To address the challenge of the alarming rate of biodiversity decline, many principles and strategies for monitoring biodiversity have been established by different national and international organizations. The results of these monitoring programs often serve as the basis for governments and conservation managers to propose appropriate conservation measures for at-risk species. Recent advances in genomic technologies can be used to identify meaningful conservation units for wildlife species and to monitor changes in their genetic diversity over time. When resources are finite, the use of genetic data and genetic monitoring are therefore critical to efficiently target species or populations that are the most genetically depauperate and enhance conservation managers' confidence in applying appropriate strategies. The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) assesses candidate at-risk species to identify discrete evolutionary designatable units (DUs) and determine their at-risk status before proposing recovery strategies where needed. In this context, I reviewed all existing COSEWIC status reports to assess how many Canadian at-risk species have 1) been genetically assessed at the species or DU level and 2) undergone genetic monitoring. My results show that genetic data are available for 50% of DUs, although only 2% of them underwent genetic monitoring, and there was a significant taxonomic bias in the availability of genetic information. While vascular plants have the highest number of at-risk species and DUs, the highest number of genetically assessed and monitored species and DUs are among mammals and fishes. Given the increased number of at-risk species, the lack of genetic information in the management of Canadian species is concerning. At a time

when the status quo is not an option to preserve biodiversity, I strongly encourage government and conservation biologists to collaboratively revisit recovery plans and monitoring programs to address this issue. My results provide insights into revisiting management plans and recovery strategies to include genetic studies in the conservation of at-risk species and help their recovery teams.

Keywords: Committee on the Status of Endangered Wildlife in Canada, Designatable Units, Genetic monitoring, Species at Risk

Introduction

Many species are currently endangered or critically endangered at local, regional, and global scales due to habitat loss and degradation, climate change, invasive species and diseases (Singh 2002). Therefore, sustained efforts are needed to protect these species from extinction. The International Union for Conservation of Nature (IUCN) states that retaining the genetic diversity of at-risk species must be one of the key objectives of conservation programs (IUCN/SSC 2013). Genetic diversity plays a fundamental role in conservation biology and enables populations to successfully adapt to constantly changing environments (Frankham et al. 2010, Frankham et al. 2019). Low levels of genetic diversity increase the likelihood of inbreeding depression which is a reduction in the fitness of a population due to high levels of inbreeding, causing an accumulation and/or increased expression of deleterious alleles (Charlesworth and Willis 2009, Kardos et al. 2016, Díez-Del-Molino et al. 2018).

One effective approach to conserving biological diversity is identifying sub-specific biodiversity units that are critical in setting conservation priorities (Green 2005). Designatable Units (DUs) are defined as a population or group of populations that have features making them "discrete" and "evolutionarily significant" relative to other populations (Green 2005, COSEWIC 2018b). Assessing genetic distinctiveness is essential in identifying DUs, as different taxonomic groups have different patterns of genetic variability, life history, and population structure (Green 2005). Although genetic assessment provides an overview of a population's characteristics at a single time point, recent advances in genetics/genomics techniques also allow for more precise monitoring of population genetic changes over time, a critical perspective to evaluate the success of conservation management strategies (Schwartz et al. 2007).

Genetic Monitoring, a Recent Phenomenon in Conservation

Genetic monitoring is defined as the "quantification of temporal changes in population genetic metrics or other population data generated using molecular markers" and can be defined under two categories (Schwartz et al. 2007). Category I uses diagnostic molecular markers for traditional population monitoring and has two sub-categories (Ia and Ib; Figure 1). Sub-category Ia is used to identify individuals. It estimates abundance as well as vital rates such as survival and recruitment (Figure 1; (Schwartz et al. 2007). For instance, in a study by Fisher *et al*. (2013), the abundance of wolverines was estimated using mtDNA genetic tagging via hair sampling over three years and showed wolverine density was 2-3 times lower in areas with increasing urban development as compared to natural habitats (Fisher et al. 2013, COSEWIC 2014). Sub-category Ib focuses on identifying species or other groups (e.g., genetically differentiated populations, subspecies, or genera) using diagnostic genetic markers and estimating hybridization, geographical range, site occupancy, and pathogens or parasites' presence, prevalence, and transmission (Schwartz et al. 2007). An example of this is a five-year monitoring effort on Canada lynx (*Lynx canadensis*) which identified changes in the species' geographic range based on mtDNA (Mckelvey et al. 2006, Schwartz et al. 2007). By contrast, Category II uses genetic markers to monitor changes in population genetic parameters (e.g., effective population size (N_e), genetic diversity, gene flow, and population admixture over time in populations) (Schwartz et al. 2007). For example, in a study of deepwater sculpin (*Myoxocephalus thompsonii*) in Lake Ontario, Canada, genetic diversity decreased over time, and the historical population contained more genetically distinct clusters than today (Welsh et al. 2017).

There are four forces that are responsible for changes in gene diversity, including mutation, migration, natural selection, and genetic drift (Frankham *et al*. 2010). Mutations lead to genetic variants, while genetic drift and natural selection are the evolutionary processes that govern their spread and maintenance (Good et al. 2017). Mutation rates vary in different species, and species with shorter generation times tend to have a higher mutation rate annually due to more frequent copying of their genome and a high probability of errors during DNA replication (Thomas et al. 2010). Generation time is defined as the average age of parents of the existing cohort of newborn individuals in the population (COSEWIC 2015). Careful consideration of generation time is thus essential for interpreting many ecological and evolutionary processes in conservation biology (Frankham et al. 2010, Latta et al. 2013). Additionally, consideration of generation time is critical in conducting Category II monitoring studies. Ideally, sampling will take place over periods equal to or greater than the specific generation time to ensure that the fundamental genetic changes (e.g., allelic frequencies) that occurred in the population(s) over time will be observed.

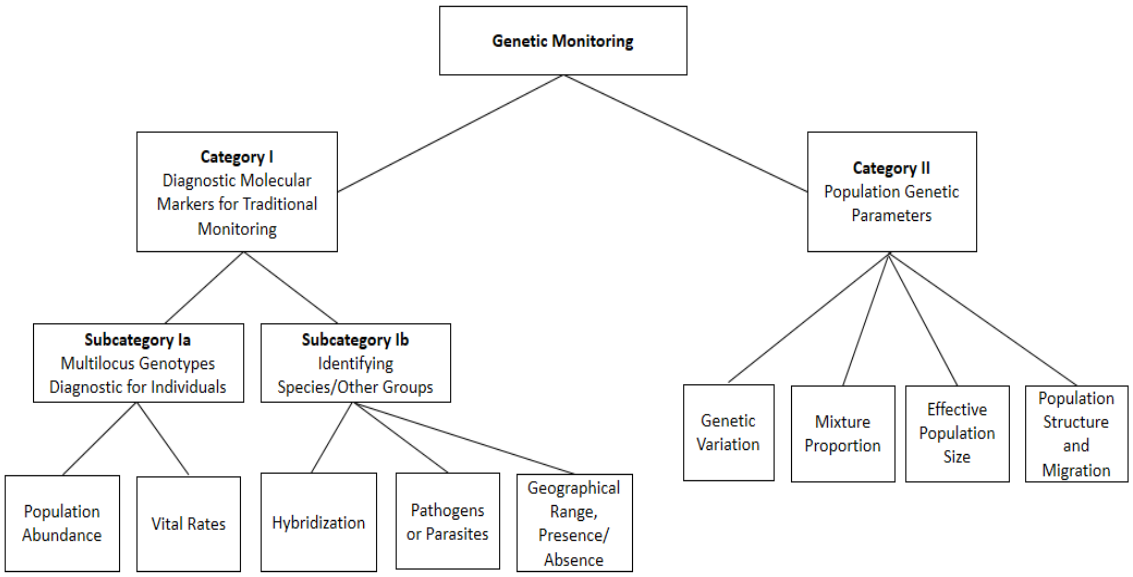

Figure 1. Categories of genetic monitoring which are guidelines to aim designing studies on tracking populations' genetic changes over time (Adapted from Schwartz et al. 2007).

Genetic monitoring is critical in understanding species and population trends, such as their abundance, survival rate, and hybridization. It can support COSEWIC members in revising a species' status and conservation managers to apply appropriate conservation measures (Schwartz et al. 2007, De Barba et al. 2010). Advances in genetic/genomics techniques have increased our ability to quantify temporal changes in the genetic diversity of at-risk species within their DUs (Schwartz et al. 2007, De Barba et al. 2010). Genetic monitoring can also provide useful information on changes in effective population size and genetic diversity (e.g., heterozygosity) before, during, and after translocations (Díez-Del-Molino et al. 2018), revealing founder effects and populations' genetic admixture (La Haye et al. 2017), and ultimately validating a particular management plan or suggesting that strategies should be adjusted accordingly (Griffiths and Pavajeau 2008). The genetic rescue of the inbred Florida Panther (*Puma concolor coryi*) highlights such a successful application

of genetic monitoring in wildlife management (Frankham et al. 2019). Two groups of panthers inhabit southern Florida, including hybridized and un-hybridized panthers. In the early 1990s, the un-hybridized group had approximately 20-25 individuals left in the wild, and the comparison of genetic diversity between modern and historical samples revealed very low genetic diversity and high levels of inbreeding (Johnson et al. 2010, Frankham 2019). Given the geographic and genetic connectedness of the nearby Texan panther's population, eight Texan females were translocated to southern Florida. The augmented Florida population reached 230 individuals within 12 years due to a higher survival rate, increased litter size, and a reduction in inbreeding depression. Genetic monitoring showed that this population had 50% higher heterozygosity than the original inbred population (Hostetler et al. 2010, Johnson et al. 2010, Frankham et al. 2019).

The Committee on the Status of Endangered Wildlife in Canada

The Committee on the Status of Endangered Wildlife in Canada (COSEWIC), consisting of wildlife experts from government, academia, non-governmental organizations, indigenous groups, and the private sector, is an independent advisory panel to the Minister of Environment and Climate Change in Canada. Since 1977, this committee has had the duty of designating wildlife species in danger of disappearing from Canada, and its members meet twice a year to assess the status of wildlife species at risk of extinction (COSEWIC 2018b). COSEWIC has three steps in the assessment process. First, the committee prepares a prioritized candidate list with wildlife species requiring assessment. Second, taxonomic sub-committees determine whether species should be assessed as a single DU or divided into multiple DUs within its Canadian range. Afterward, available evidence from scientific research, traditional ecological knowledge holders, and relevant stakeholders are assembled

in specific status reports. These reports include information such as distribution, the extent of occurrence, occupancy, abundance, population and habitat trends, and factors or threats limiting the wildlife species at the DU level. Several criteria, such as behavioral, morphological, geographic distribution, and genetic distinctiveness, are used to identify DUs. In the final step, COSEWIC members review the information in the status report and apply it to the COSEWIC criteria (following IUCN criteria) (COSEWIC 2018b) to determine whether a DU meets the thresholds for designation as a species at risk, including extinct, extirpated, endangered, threatened, special concern, data deficient, and not-at-risk (Lukey and Crawford 2009, COSEWIC 2018b). Genetic monitoring is critical in assessing the effective management of at-risk species, particularly at the DU level. Therefore, I reviewed COSEWIC status reports and their associated recovery/management plans until 2019 to determine how many Canadian at-risk species have been genetically assessed at the species or DU level and 2) undergone genetic monitoring.

Methods

Under the Species at Risk Act (SARA), a DU is considered equal to "a wildlife species" and a species in a COSEWIC status report is automatically considered a DU. To avoid confusion in this chapter, the word "species" represents its biological definition and may encompass several DUs. Using the COSEWIC database, I collected the status reports of all Canadian endangered, threatened, and special concern species that were available until 2019 (Government of Canada 2020). In cases where multiple status reports were available for a DU, I reviewed the most current one. The data consisted of 788 DUs and included their geographic range, taxonomic group (consisting of mammals (marine and terrestrial), birds, fishes (marine and freshwater), reptiles, amphibians, arthropods, mosses, lichens, and

vascular plants), species' conservation status, COSEWIC last assessment date and the existence of a recovery or management plan. All the status reports were searched for keywords such as "genetic", "molecular", and "genetic monitoring" to estimate the total number of DUs with genetic information per status category. I considered genetic information as the availability of all types of genetic assessment, including taxonomic, population, and conservation genetics studies. I also recorded the number of DUs without genetic information but ongoing or recommended genetic research. For DUs where genetic monitoring existed, I assessed which category was used and if genetic sampling time points were equal to or greater than the known generation time in the category II study designs (Schwartz et al. 2007; Appendix 1). To investigate specific biases, I assessed the availability of genetic assessment and genetic monitoring information per taxonomic group and conservation status. A chi-square test of independence was performed to examine the relationship between genetic assessment studies and taxonomic groups. In cases where a status report was vague, or information was not available, I searched for potential information in publications referenced by the COSEWIC report, recovery plans, and management plans associated with the report.

Results

From a total of 788 DUs representing 640 species-at-risk in Canada, 363 DUs were considered endangered, 190 were threatened, and 235 were of special concern (Figure 2), including 512 which had a recovery or management plan (65%). Half of all DUs (n=394) had available genetic information mentioned in the status report resulting in only 217 DUs (28%) with both recovery/management plan and genetic information available. An additional 133 out of 394 DUs without genetic information available had recommended or ongoing genetic

research. From a conservation status perspective, only 177, 95, and 122 DUs had genetic information available for endangered, threatened, and special concern respectively (Figure 2). Among the 788 DUs listed, only 18 (2%) had undergone genetic monitoring: 2 (11%) under category I, 14 under category II (78%), and only 2 (11%) DUs showing genetic monitoring under both categories.

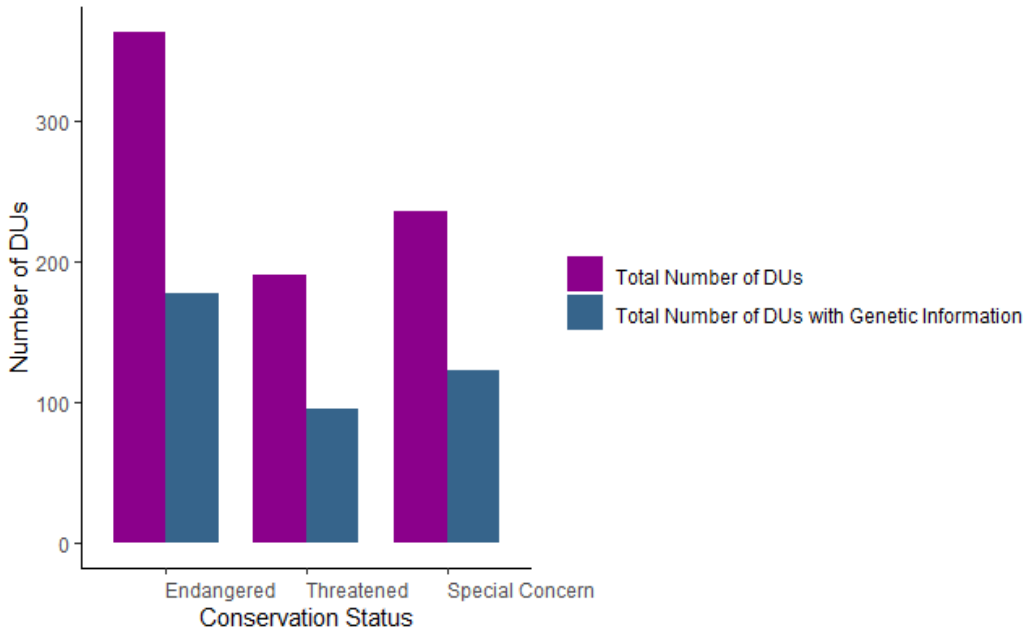

Figure 2. Number of endangered, threatened, and special concern DUs along with the number of DUs including genetic information in each category.

At the taxonomic level, a significant bias was observed, with some groups having significantly more genetic information than the average ($X^2 = 61.49$, df = 9, $p < 0.0001$). Vascular plants presented the highest number of at-risk species and DUs (202 and 206, respectively; Figure 3); however, fishes and mammals presented the highest number of genetically assessed species and DUs, with 76 species (157 DUs) and 36 species (64 DUs),

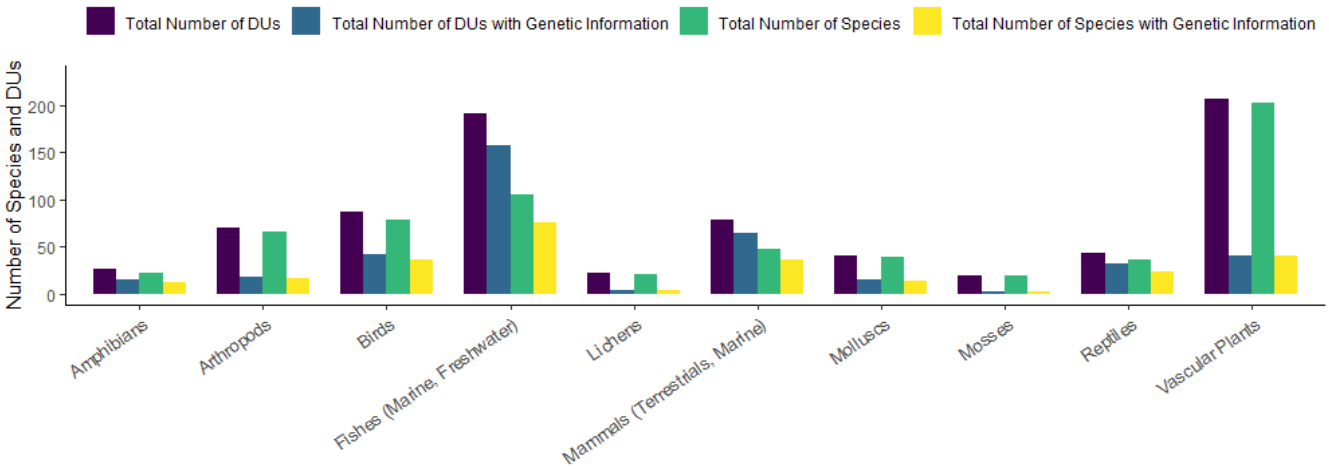

Figure 3. Total number of species and associated DUs, including the ones with genetic information based on the taxonomic group.

respectively. By contrast, mosses and lichens were the taxonomic groups with the least genetic information available through their reports. Among the 20 at-risk species of mosses (20 DUs), only 3 reports presented genetic information. Among the 21 at-risk lichen species (23 DUs), only 5 reports presented genetic information (Figure 3).

Among 18 genetically monitored DUs (Category 1 and 2), 7 were mammals, 7 were fishes, 2 were amphibians, and 2 were birds (Figure 4; Table 1). A majority of these genetically monitored DUs were listed as special concern (61%), while only one DU was listed as a threatened species, and 6 DUs were listed as endangered (Figure 4).

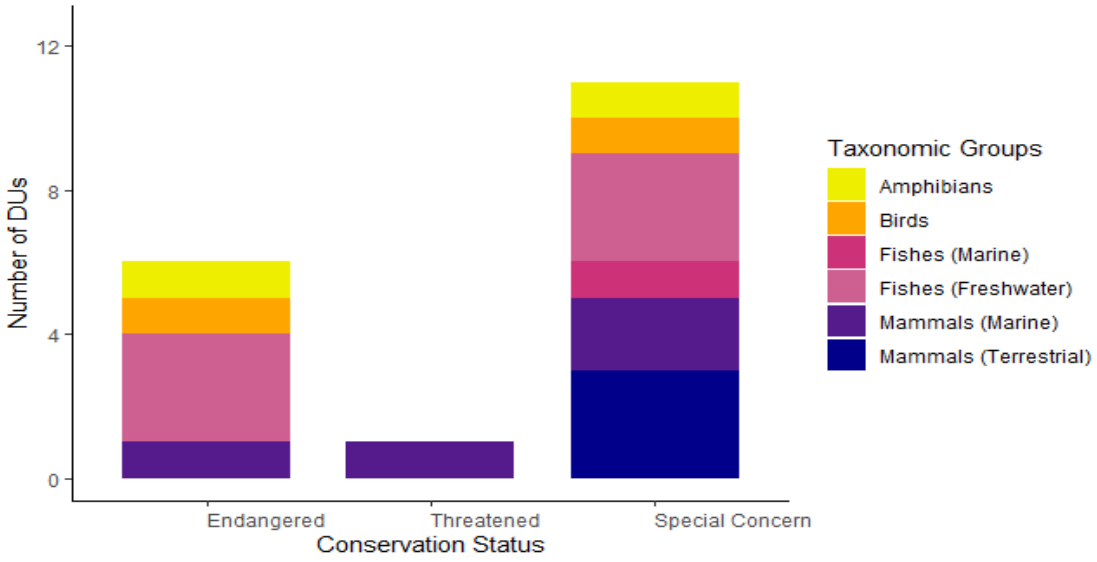

Figure 4. Number of DUs with genetic monitoring per conservation status and taxonomic group.

Table 1. List of genetically monitored species.

Conservation Status	Taxonomic Group	Species	Scientific Name	Number of Genetically Monitored DUs
Endangered	Amphibians	Northern Leopard Frog	*Lithobates pipiens*	2
	Birds	Northern Bobwhite	*Colinus virginianus*	1
	Fishes (freshwater)	Copper Redhorse	*Moxostoma hubbsi*	1
		Enos Lake Benthic/ Limnetic Threespine Stickleback	*Gasterosteus aculeatus*	2
	Mammals (marine)	North Atlantic Right Whale	*Eubalaena glacialis*	1
Threatened	Mammals (marine)	Northern Fur Seal	*Callorhinus ursinus*	1
Special Concern	Bird	Peregrine Falcon pealei subspecies	*Falco peregrinus pealei*	1
	Fishes (freshwater)	Deepwater Sculpin	*Myoxocephalus thompsonii*	1
		Dolly Varden	*Salvelinus malma malma*	1
		West slope Cutthroat Trout	*Oncorhynchus clarkii lewisi*	1
	Fishes (marine)	Sockeye Salmon	*Oncorhynchus nerka*	1
	Mammals (marine)	Atlantic Walrus	*Odobenus rosmarus rosmarus*	2
	Mammals (terrestrial)	Polar Bear	*Ursus maritimus*	1
		Spotted Bat	*Euderma maculatum*	1
		Wolverine	*Gulo gulo*	1

Discussion

Developing comprehensive strategies which include recovery/management plans is essential in conserving at-risk species (McCune et al. 2013, COSEWIC 2018b). Yet, 35% of DUs at risk of extinction (special concern, threatened, or endangered status) are without a recovery/ management plan in Canada. Moreover, half of all at-risk DUs are without genetic information. This lack of genetic information can be detrimental to their effective management by preventing measures to minimize the sources of inbreeding and loss of

genetic diversity (Frankham et al. 2010, Frankham et al. 2019). Furthermore, genetic monitoring has only been conducted for 2% of DUs in Canada and despite the priority of endangered species in conservation, most genetically monitored DUs fall within the special concern category, the least likely category that we assessed to face extinction. While 17% of the total existing DUs of at-risk species have either ongoing or recommended genetic studies suggesting that genetic data may be available in the future, it remains that genetic monitoring of the Canadian at-risk species is extremely rare.

Genetic data can help conservation managers in various ways by providing critical information such as identifying conservation management units, revising taxonomy, improving population connectivity, and performing translocations (Taylor and Kearns 2021). However, even when genetic information is available, it is sometimes insufficient to assess the genetic health of the species and associated DUs due to insufficient sample size and/or sampling limited to a small geographic area or time period (Sinclair and Hobbs 2009; Hale *et al.* 2012). Prairie Skink (*Plestiodon septentrionalis*) is a good example of a species with insufficient genetic knowledge of its population structure. In Canada, the species inhabits a small area of southwestern Manitoba in the Brandon and Lauder sandhills (COSEWIC 2017). Genetic analysis using microsatellites revealed that the skinks in the Brandon sandhills, where the majority of the populations occur, belong to two genetic clusters, one in the north and one in the south, divided by the Assiniboine River. However, no genetic data are available for the Lauder sandhills population(s), and the small sampling size limited the researchers' ability to detect fine-scale genetic differentiation of the populations in the area (McFadden 2010, Siu 2011, COSEWIC 2017). The spatial genetic structure of populations can be affected by habitat fragmentation which can increase genetic isolation of populations,

in turn leading to inbreeding and reduced genetic variation. Therefore, the availability of detailed spatial genetic structure of populations could help conservation managers choose the right scale when deciding on a management strategy to maintain genetic variability across the species range (Pometti et al. 2018).

My results reveal taxonomic biases in the availability and use of genetic information in assessing species status. Vascular plants have the highest number of endangered, threatened, and special concern DUs followed by fishes and mammals. However, the latter two groups show higher proportions of available genetic information and genetic monitoring. Additionally, only three out of twenty moss species had available genetic information. These biases in the genetic investigation can be a result of the economic and charismatic status of fishes and mammals in Canada. Both groups contain species that are critical food and economic resources (Grzimek 2004). Furthermore, several species (e.g., polar bear, and wood bison) are important cultural symbols and subjects of art, books, and movies (Grzimek 2004, COSEWIC 2013, 2018a). Such bias needs to be addressed by conservation managers, researchers, and funding agencies in order to preserve biodiversity as a whole (Titley et al. 2017).

Specific generation time was appropriately considered in all the category II genetic monitoring studies (Appendix 1). For instance, historical samples of the northern leopard frog (*Lithobates pipiens*) from 1975 were compared with the samples from 1999-2000 to assess if remnant populations have low genetic diversity due to a recent bottleneck or to a history of peripheral distribution (Hoffman and Blouin 2004). As the generation time of the species is 2-3 years (Hoffman et al. 2004, COSEWIC 2009, Species at Risk Committee 2013), the authors could confidently conclude that the average expected heterozygosity and

the average number of alleles per locus in historical populations were higher than remnant populations (Hoffman & Blouin 2004).

Conclusion

Using genetics and genomic approaches can help researchers and conservation managers better understand the threats of anthropogenic change and address conservation concerns for species at risk and their associated DUs. Therefore, conservation managers should develop comprehensive recovery strategies incorporating genetic information. My analysis of Canada's at-risk biodiversity provides a unique opportunity to understand the role of genetic assessment and genetic monitoring in determining conservation status and highlight a very limited use of both. My findings also reveal taxonomic bias in the species/DUs in which genetics has been studied. Given the importance of genetic information as a tool in modern conservation (Schwartz et al. 2007, Frankham et al. 2010, Frankham 2019), as well as the total number of at-risk species in Canada, a comprehensive plan and timelines for integrating genetic monitoring need to be a priority for the government, the scientific community, and conservation managers.

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Chapter 2- Genetic Assessment and Monitoring of Wild, Captive, and Reintroduced Northern Leopard Frog (*Lithobates pipiens*) Populations

Abstract

Northern leopard frogs (*Lithobates pipiens*) were once widely distributed throughout North America but have undergone a dramatic population size decline over recent decades in western Canada and the United States. In British Columbia, only a single population remains at the Creston Valley Wildlife Management Area. Yet, the viability of this population is uncertain. In an effort to conserve this unique population, information about the current population genetic structure of the species in western Canada as well as its genetic health is critical. The current genetic structure of northern leopard frog populations in western Canada was assessed using microsatellite markers. Historical samples from the extinct population of Fort Steele in British Columbia were compared with the Creston Valley population to get an overview of changes in population genetic parameters over time. Genotypic data from four populations (Creston Valley, Drain K, Prince Spring, and Cypress Hill) sampled in 2004 and 2019 were compared. Additionally, to evaluate changes in the genetic diversity of the Creston Valley population in British Columbia over time, allelic richness and expected heterozygosity of the population were compared at 3-time points using genotypes from 2000, 2004, and 2019. Northern leopard frog populations in western Canada showed high genetic differentiation, with genetic diversity decreasing from east to west. The westernmost population of the species (Creston Valley), presented the lowest genetic diversity and lowest effective population size. Although there weren't notable changes in genetic parameters between 2004 and 2019, there was evidence of decline between 2000 and 2019. The extinct population of Fort Steele had private alleles, while the current Creston Valley population did

not, suggesting a genetic bottleneck in the Creston Valley population. Therefore, genetic rescue, especially for the endangered Creston Valley population, should be considered. Additionally, continued genetic monitoring will help in the effective management of the species by providing information on the success of conservation actions.

Keywords: Creston Valley, Genetic assessment, Genetic monitoring, Northern leopard frog, Population genetic structure

Introduction

Conservation translocation refers to the human-mediated, wild-wild, and captive-wild intentional transfer of living organisms from one area to another, either inside or outside their natural range (Armstrong and Seddon 2008, IUCN/SSC 2013, Germano et al. 2015). Conservation translocation outside of a species' natural range is labeled introduction/assisted colonization, and within their indigenous range is labeled restoration. Reintroduction (transferring individuals to habitats within the indigenous range) and reinforcement (releasing individuals into an existing population of conspecifics) are two types of population restoration (Conant 1988, IUCN/SSC 2013). Conservation translocation is an important wildlife management tool with the main purpose of establishing viable and self-sustaining populations and plays a key role in conserving rare species (Dodd and Seigel 1991, Griffiths and Pavajeau 2008). The number of translocation events has recently burgeoned (Germano et al. 2015, Taylor et al. 2017) and is predicted to greatly increase if assisted colonization is adopted as a tool for climate change mitigation (IUCN 2013; Germano et al. 2015). In assessing the success of reintroduced populations, it is essential to customize the success criteria for rare species by considering their own unique set of issues that have contributed to their rarity (Haskins 2015). Indeed, the important criteria to assess the success of reintroduced populations are linked to abundance (i.e., population size), extent (i.e., distribution of populations, dispersal), persistence (i.e., self-sustainability), and resilience (i.e., genetic diversity; Pavlik 1996). Maintaining genetic diversity is critical to the realization of reintroduction programs as it provides the means for populations to adapt to altered environments and ensure their long-term survival (Frankham 2019).

Genetic monitoring consists of quantifying the changes in genetic parameters over a time period that can be estimated using genetic markers (Schwartz et al. 2007). Therefore, genetic monitoring of translocated populations can be useful as it offers conservation managers an overview of the success of their programs (Griffiths and Pavajeau 2008) by providing valuable information such as changes in effective population size and genetic diversity (e.g., heterozygosity) over time (Díez-Del-Molino et al. 2018). Genetic monitoring can also reveal founder effects and changes in population genetic admixture (La Haye et al. 2017). Genetic admixture, which is the presence of DNA in an individual from a distantly-related population in translocated populations with different sources, can cause dilution of locally adapted genes and hybrid breakdown (Lynch 1991). Moreover, identifying genetic differences between current individuals and those from the past is an important monitoring tool to measure recent changes such as inbreeding (F), and heterozygosity (H), in at-risk species (Bi et al. 2013, Der Sarkissian et al. 2015).

The northern leopard frog (NLF; *Lithobates pipiens*, Figure 1) is an example of a species that would benefit from the insights gained from genetic monitoring. This species is one of the most widely distributed amphibians in North America (Alberta Sustainable Resource Development 2003, Werner 2003, Wilson et al. 2008). Although the species is secure in many American states and Canadian provinces, it has experienced declines in abundance and has disappeared from many parts of its western range (Leonard et al. 1999, Werner 2003, Wilson et al. 2008, COSEWIC 2009). These declines are likely due to a combination of factors such as habitat loss and fragmentation, disease outbreaks (e.g., chytridiomycosis and ranavirus), invasive species, pollution, and climate change (Kendell and Prescott 2007, Wilson et al. 2008, Green et al. 2020).

The two main eastern and western clades of the northern leopard frog have been separated by the Mississippi River and the great lakes of North America for approximately two million years (Hoffman and Blouin 2004a, O'Donnell and Mock 2012). Furthermore, there is also a major split between east and west in North Dakota, divided by the Missouri River. These two clusters differentiated ~13–18 kya, during a period of glacial retreat in the northern Great Plains (Waraniak et al. 2019), with prehistoric climate and landscape features such as major rivers and lakes playing a critical role in dividing east-west NLF populations by acting as physical barriers to gene flow (Waraniak et al. 2019).

In western Canada, the western clade of NLF is divided into two distinct designatable units (DUs) based upon their "discreteness" and "evolutionarily significance" (COSEWIC 2009): 1) the Western Boreal/Prairie designatable unit (Alberta, Manitoba, Saskatchewan, and Northwest Territories), which is currently designated as Special Concern under the federal Species at Risk Act (COSEWIC 2009), and 2) the Rocky Mountain designatable unit (including the Creston Valley population and the captive population at the Vancouver Aquarium in British Columbia which is Endangered (COSEWIC 2009) and Critically Imperiled (S1) in British Columbia (B.C. Conservation Data Centre 2019). In the northwestern United States, the NLF is Critically Imperiled (S1) in Washington and is listed as a species of greatest conservation need in Idaho (ID) (Idaho Official Government Website) and Imperiled (S2). In Montana, the populations within the western mountains of the state are Species of Concern (S3), while the populations on the Great Plains are apparently Secure (S4). In North Dakota, the species has no rank, while in South Dakota, the species is secure (S5) (NatureServe 2017).

In a previous study, Wilson et al. (2008) revealed that NLF genetic diversity declines from western Ontario westward, and the only existing wild population in British Columbia, located in the Creston Valley, not only had the lowest genetic variation but was also genetically distinct from other populations in North America that they examined (Wilson et al. 2008). In Washington, the last remnant population inhabits the Potholes Reservoir area of the Columbia Basin Wildlife Area (Germaine and Hays 2009), and recent genetic data discovered the presence of three subpopulations (Seaborn and Goldberg 2020). The average number of alleles per locus and heterozygosity were low for all of the subpopulations, and the entire population had a small effective population size (Seaborn and Goldberg 2020). In Idaho, the range of the NLF is limited to the southeastern part of the state, in the Mid-Snake River area, as populations historically inhabiting northern and southwestern Idaho have been extirpated (Idaho Department of Fish and Game 2017). Like the Canadian NLF populations, the overall genetic diversity of the western clade of NLF in the United States is low (Phillipson et al. 2011, Seaborn and Goldberg 2020).

Figure 1. A Northern Leopard Frog in Captivity, Calgary Zoo. Photo Credit: Lea Randall

Aquariums and zoos (Keulartz 2015) have a key role in conservation translocation programs by providing funding, staff, and expertise as well as equipment, animals for release, and project coordination (Gilbert et al. 2017). However, most captive populations in reintroduction programs are small, which presents challenges such as potentially high levels of inbreeding, genetic drift, loss of genetic diversity, and genetic adaptation to captivity (Gilligan and Frankham 2003, Griffiths and Pavajeau 2008). In Canada, there are currently three NLF captive populations: the Wilder Institute/Calgary Zoo, Vancouver Aquarium, and Edmonton Valley Zoo. The founder individuals of these captive populations all come from the Rocky Mountain population located in the Creston Valley (Ward 2017, Penney 2018, Martin 2019).

In the United States, there are at least five NLF conservation translocation programs that have occurred or are on-going in the states of Arizona, New Mexico, Nevada, Montana, and Washington (Randall et al. 2021). In Canada, there have been several NLF reintroduction efforts that have been undertaken in the provinces of Alberta and British Columbia which have met with variable success. Reintroduction efforts in British Columbia have met with some initial success but have not managed to produce long-term self-sustaining populations (Randall et al. 2021). The individuals used for reintroductions were sourced from the Creston Valley and captive breeding facilities and it is possible that low genetic diversity may be hampering recovery efforts (Wilson et al. 2008, Environment and Climate Change Canada 2017; Randall et al. 2021).

Genetic information is critical for understanding the success of conservation translocation programs (Pavlik 1996) and my aim in this chapter was to assess the population genetic structure of current NLF populations in western Canada. Moreover, I compared the

genetic change of the historical Rocky Mountain population near Fort Steele from the 1970s with the current Creston Valley population. Likewise, I genetically monitored the Creston Valley population and three populations in Alberta (Prince Spring, Drain K, and Cypress Hill) using available genotypes from 2004 and samples collected in 2019 for this study. To determine if there needed to be a longer time period between monitoring events to detect changes in genetic diversity, I also compared changes in allelic richness and expected heterozygosity for the Creston Valley population at 3-time points in 2000 (Hoffman and Blouin 2004b), 2004 (Wilson et al. 2008), and 2019. Given that the Rocky Mountain population is considered endangered, it is important to have a better understanding of changes in genetic diversity and effective population size in order to effectively manage the population.

Material and Methods

Sample/Data Collection

During the spring and summer of 2019-2020, 10 northern leopard frog populations (one captive, one reintroduced, and eight wild) inhabiting western Canada and one reintroduced population in the northwestern United States (Appendix, Table S1; Figure 2) were sampled for genetic material using MW 113 dry buccal swabs which were further stored in 95% ethanol. Adults and juvenile frogs were swabbed rather than young of the year under the assumption that they were less likely to be siblings. However, when this was not possible, young of year frogs were swabbed (Wilson et al. 2008). To swab the inside of the frog's cheeks, the swab was rolled back and forth gently between the index finger and thumb 20 times per side, and an additional 20 times under the tongue, avoiding the upper mouth area.

Buccal swabs of animals from different clutches were collected to avoid sampling siblings at the Vancouver Aquarium. Given the status of the Creston Valley population and to increase the accuracy of effective population size analysis for the population, 5 tadpoles were collected from each of 6 egg masses in 2019 at that site. Tadpoles were euthanized using a buffered 1.0% w/v solution (10 g per L) of MS222 and were stored in 95 % ethanol. Additionally, we obtained 10 tissue samples (Freeze dried) of northern leopard frogs from South Slave (Northwest Territories), which were collected by researchers in 2009 (Schock 2010) and stored in 95% ethanol (Figure 2). We also obtained and analyzed the genotype data from the Wilson et al. study (2008) in order to better understand the current population genetic differentiation of the species and monitor changes in population genetic metrics over time. Furthermore, 10 historical (1973) skins of northern leopard frogs were collected from a now extinct population that was inhabiting Fort Steele, British Columbia (Figure 2). These historical samples were accessible via the Amphibian Collection of the Canadian Museum of Nature (Khidas and Torgersen 2022).

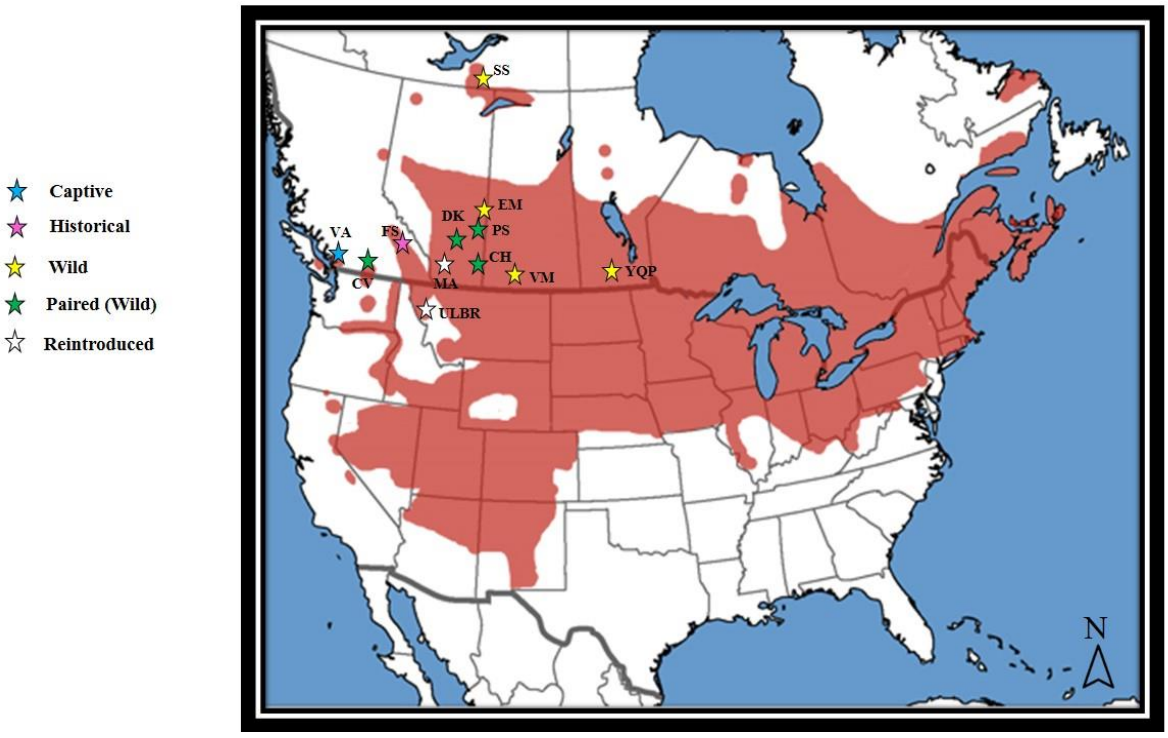

Figure 2. Sampling sites of northern leopard frogs. Codes for population identification are given in Table 1. Colors represent the sampled population's type (yellow = wild; white = reintroduction sites; pink = historical sites; green = wild populations for which genetic monitoring analyses were possible using samples of Wilson et al. (2008)).

Laboratory Methods

DNA from each sample was extracted using DNeasy Blood & Tissue kits (Qiagen). Each set of extracted samples had an extraction negative. Ten microsatellite loci previously published for northern leopard frog Rpi100, Rpi101, Rpi103, Rpi104, Rpi106, Rpi107, Rpi108 (Hoffman et al. 2003), RP193 (Hoffman and Blouin 2004b), Rasp09, and Rasp20 (McKee et al. 2011) were individually amplified. Each 15 µL polymerase chain reaction (PCR) consisted of 0.27 µmol/L each primer, 120 µmol/L dNTPs, 1.5 U of Taq polymerase, 1× PCR buffer (50 mmol/L KCl, 0.1% Triton X-100, 0.16 mg/mL bovine serum albumin, 10 mmol/L Tris buffer, pH 8.8), and ~40 ng of genomic DNA. Each reaction contained 2.5 mmol/L MgCl2, except for the Rpi101 and Rpi193 amplifications, which contained 2.0

mmol/L MgCl2. The amplification protocol consisted of an initial denaturing step of 94 °C for 1 min followed by three cycles of denaturing at 94 °C for 30 s, annealing at 44 °C for 20 s, and extension at 72 °C for 5 s followed by 30 cycles of denaturing at 94 °C for 15 s, annealing at 45 °C for 20 s, and extension at 72 °C for 1 s followed by a final 30 min extension at 72 °C (Hoffman et al. 2003, Hoffman and Blouin 2004b, Wilson et al. 2008, McKee et al. 2011). Amplicons were visualized using an Applied Biosystems 3730 automated sequencer at the University of Alberta's Centennial Center for Interdisciplinary Science (II), and a visual assessment of genotypes was done to ensure the accuracy of the scoring. To guarantee amplification consistency, 2% of samples were randomly rerun. MICRO-CHECKER v. 2.2.3 was used to assess the presence of null alleles. This software can identify genotyping errors due to null alleles, short allele dominance (large allele dropout), and the scoring of stutter peaks, and also detects typographic errors (Van Oosterhout et al. 2004).

Data Analysis

The genotypes of northern leopard frog samples were used to understand the current genetic diversity and population structure of the species in western Canada. The historical samples from 1973, as well as genotypes of 4 populations (Creston Valley, Drain K, Cypress Hill, and Prince's Spring) obtained in 2004 (Wilson et al. 2008), were compared with the current population's genotypes to monitor changes in population genetic metrics of the species over time. Additionally, information on the allelic richness and expected heterozygosity of the Creston Valley population from Hoffman and Blouin (2004b) and Wilson et al. (2008) was used to compare changes in the genetic diversity of the population

at 3-time points using samples collected in 2000 (Hoffman and Blouin 2004b), 2004 (Wilson et al. 2008) and 2019 (this study).

Linkage disequilibrium (LD) and deviations from the Hardy–Weinberg equilibrium (HWE) were estimated by GENEPOP version 4.7.5 (Rousset 2008). Levels of significance for both LD and HWE tests were adjusted using non-sequential Bonferroni corrections (Rice 1989). For all current and historical populations, expected (H_e) and observed heterozygosity (H_o) were calculated using GenAlEx version 6.503 (Peakall and Smouse 2006). Given that allelic richness is influenced by the number of samples per locus, with large samples potentially containing more alleles than small samples, rarefaction allelic richness and rarefaction private alleles' richness were calculated using HP- Rare 1.0 (Kalinowski 2005). Under this method, equal-size subsamples from each population were considered to determine whether there were any alleles present in one population but absent in all others (Kalinowski 2005). Inbreeding coefficients were estimated using FSTAT version 2.9.4 (Goudet 1995). For the genetic diversity and inbreeding measurements of samples from Creston Valley in British Columbia, in addition to the 96 buccal swabs, genotypes of one tadpole from each of the 6 egg masses were chosen randomly and were included in the analysis.

After removing alleles with frequencies lower than 5% (Pcrit = 0.05) and omitting singleton alleles, the effective population size for each sampling site was calculated using the linkage disequilibrium method in the NeEstimator software version 2.1 (Do et al. 2014). In these calculations, all the individuals (e.g., siblings) sampled from Creston Valley were used in order to have the highest number of individuals. The impact of sibling's presence on effective population size is not well investigated (Waples and Anderson 2017, Sládkovičová

et al. 2022), although a small sample size can lead to downward biases in the effective population size (Waples and Yokota 2007, Sved et al. 2013, Sládkovičová et al. 2022). Therefore, samples from endangered species or museums can be biased in the estimation of effective population size due to the relatively low number of available samples. To raise the accuracy of calculations when the sampling size is low, the numbers of loci and alleles per locus need to be increased (Waples and Do 2010).

A nested ANOVA method (Weir and Cockerham 1984) was used to quantify genetic differentiation between current populations using a measure of genetic distance (F_{ST}). The calculation was done via FSTAT version 2.9.4 (Goudet 1995). To test genetic isolation by distance throughout the study area, a regression between pairwise genetic distances (FST/ (1-FST)) and the natural logarithm of geographical distances between non-reintroduction sampling sites (Rousset 1997) was performed in GENEPOP version 4.7.5 (Rousset 2008). For geographical distances, the shortest route between two sampling sites was measured using the distance tool in GoogleEarth version 4.2.1.

To explore population differentiation and the number of genetic clusters in the study area, a Bayesian clustering method was used (STRUCTURE version 2.3.4; Pritchard et al. 2000). 10 independent runs for k = 1 to 12 were performed for the sampling sites of Canada. Each chain was run with a burn-in of 100,000 and an additional 1,000,000 iterations. To identify the most likely number of genetic clusters, the Delta K method (Eavnno et al. 2005) was performed in Structure Harvester (Earl and VonHoldt 2012). The log probability of the data [LnP (D)] was plotted as a function of the numbers of clusters (K) to determine the optimal configuration (Pritchard et al., 2000).

Results

Genetic Diversity and Structure of Current Northern Leopard Frog Populations

Deviations from Hardy–Weinberg equilibrium, or evidence of linkage disequilibrium was not observed for any of the genotyped loci. Null alleles were seen infrequently throughout the dataset using the MICRO-CHECKER v. 2.2.3. Because they were present in 2 out of 10 genotyped loci, all of the data were used for the further analysis. The mean number of alleles in this study was 4.32 ± 0.45 for the non-captive populations (wild and reintroduced) of Canada and 2.24 ± 0.36 for the historical samples from Fort Steele. Rarefied allelic richness for sampling sites ranged from 1.76 ± 0.21 SE to 4.99 ± 0.47 SE, and rarefied private allele richness varied from 0 to 0.88 ± 0.27 SE for the Creston Valley and Yellow Quill Property, respectively (Table 1). The Creston Valley population showed the lowest genetic diversity (rarefied allelic richness and expected heterozygosity), while the Yellow Quill Property population in Manitoba had the highest genetic diversity (Table 1). There was a significant relationship between the genetic diversity measures (allelic richness and H_e) and longitude with an east-to-west decline ($r^2 = 0.64$, $p = 0.017$ for allelic richness and $r^2 = 0.72$, $p = 0.007$ for He; Figure 3A & B). The inbreeding coefficient (F_{IS}) within populations ranged from - 0.04 ± 0.05 SE to 0.10 ± 0.093 SE to for Drain K and South Slave, respectively. However, standard errors overlapped among inbreeding coefficients of sampled populations which makes the comparison between them difficult, but Empress, Creston Valley and South Slave populations show evidence of inbreeding (Table 1).

The effective population size (N_e), the single-sample method based on linkage disequilibrium for alleles with frequencies greater than 5%, ranged from 2.0 to 305.1 with

frogs of Creston Valley and Val-Marie having the lowest and highest N_e, respectively, although the 95% confidence intervals for the measured N_e of the populations overlapped considerably (Table 2).

The genetic distance between all sampled populations in this study varied between moderate (0.05 -0.15), great (0.15 -0.25), and very great differentiation (above 0.25; Wright 1978; Table 3). The lowest F_{st} for the current non-captive Canadian populations (wild and reintroduced, assessment with 10 loci) was 0.097 between Val-Marie and Yellow Quill Property, while the highest F_{st} was 0.625 between Creston Valley and Magrath (Table 3). In fact, Creston Valley was very greatly different from all other wild and reintroduced frog populations in Canada but showed very little genetic differentiation (0.024) with the captive population from the Vancouver Aquarium (Table 3).

Table 1. Measures of genetic diversity and inbreeding in each sampling site. Sampling sites in each section are ordered from east to west.

Population Type	**Sampling Site**	**ID**	**n**	**Rarefied Allelic Richness (AR)**	**Rarefied Private Allelic Richness (PR)**	**Observed heterozygosity (H_o)**	**Expected heterozygosity (H_e)**	**Inbreeding coefficient (F_{is})**
Wild	Yellow Quill Property, Manitoba	YQP	19	4.99	0.88	0.74	0.75	0.05
			SE	0.47	0.27	0.05	0.04	0.05
	Val-Marie, Saskatchewan	VM	9	4.44	0.10	0.65	0.65	0.06
			SE	0.48	0.24	0.08	0.06	0.08
	Empress, Alberta	EM	5	3.30	0.32	0.60	0.59	0.09
			SE	0.30	0.18	0.07	0.04	0.08
	Cypress Hill, Alberta	CH	31	3.86	0.45	0.68	0.68	0.01
			SE	0.30	0.14	0.05	0.04	0.07
	Prince's Spring, Alberta	PS	32	2.90	0.38	0.50	0.50	0.07
			SE	0.38	0.21	0.09	0.09	0.06
	South Slave, Northwest Territories	SS	10	2.9	0.11	0.42	0.44	0.10
			SE	0.47	0.05	0.11	0.09	0.09
	Drain K, Alberta	DK	36	2.76	0.03	0.50	0.47	-0.05
			SE	0.24	0.02	0.07	0.06	0.06
	Creston Valley, British Columbia	CV	96	1.76	0.00	0.23	0.24	0.07
			SE	0.22	0.00	0.07	0.07	0.04
	Total Number		**250**	-	-	-	-	-
Captive / reintroduced	Magrath, Alberta	MA	18	2.56	0.11	0.49	0.46	-0.04
			SE	0.25	0.07	0.08	0.07	0.07
	Upper Little Bitterroot River, Montana	ULBR	25	3.40	0.36	0.57	0.57	0.03
			SE	0.39	0.28	0.08	0.08	0.07
	Vancouver Aquarium	VA	40	1.81	0.00	0.27	0.26	-0.02
			SE	0.23	0.00	0.08	0.07	0.07
	Total Number		**83**	-	-	-	-	-
Historical	Fort Steele	FS	10	2.24	1.78	0.38	0.36	-0.01
			SE	0.34	0.39	0.09	0.09	0.07

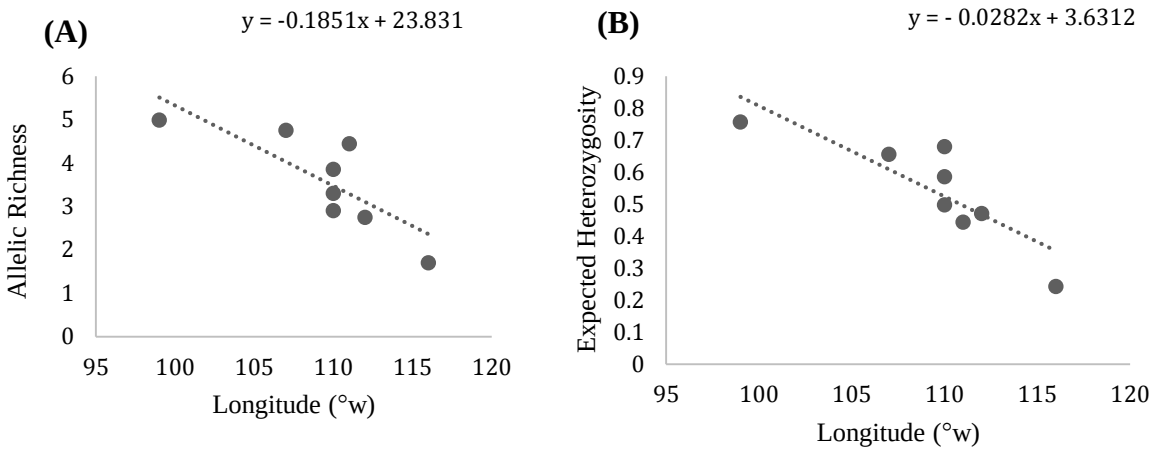

Figure 3. Linear regression between genetic diversity and longitude of wild northern leopard frog populations in Canada. Diversity is measured by (A) allelic richness (r^2 = 0.6439, p = 0.017), and (B) expected heterozygosity (r^2 = 0.7193, p = 0.007).

Table 2. The effective population size (N_e) of sampling sites based on linkage disequilibrium after omitting alleles with frequencies lower than 5%. Codes for population identifications are given in Table 1. Sampling sites in each population type are ordered from east to west.

Population Type	**ID**	**n**	**Ne (0.050)**	**95% CI**
Wild Populations	YQP	19	14.3	10.3-20.9
	VM	9	305.1	16.9-Infinite
	EM	5	61.2	1.9-Infinite
	CH	31	18.2	12.5-27.9
	PS	32	29.9	14.8-95.2
	SS	10	6.8	2.2-40.9
	DK	36	20.6	11.7-41.1
	CV	119	2.0	1.2-2.9
Captive and reintroduced populations	MA	18	14.9	5.1-118
	ULBR	25	18.1	10.7-36.1
	VA	40	3.5	1.9-10.4

Populations from Alberta were mostly different from each other, with the reintroduced population of Magrath showing the highest F_{st} with Drain K in the province (0.27, Table 3). The South Slave population (Northwest Territories) was very greatly differentiated from other populations except the Cypress Hill and Yellow Quill Property populations located in Alberta and Manitoba, respectively, to which it was still greatly differentiated. The Val-Marie population (Saskatchewan) had a moderate genetic differentiation from the only

sampled population in Manitoba (Yellow Quill Property) and the Cypress Hills population in Alberta.

Table 3. F_{st} distance (above diagonal) and linear geographical distance (Km; below diagonal) between non-captive northern leopard frog populations in Canada.* represents a reintroduction site, and ^{H} represents the historical population. Acronyms for the population are given in Table 1.

	CV	**MA***	**DK**	**SS**	**PS**	**CH**	**EM**	**VM**	**YQP**	**FS^{H}**
CV	-	0.625	0.560	0.573	0.523	0.500	0.579	0.585	0.507	0.698
MA*	272.5	-	0.275	0.339	0.255	0.201	0.224	0.275	0.213	0.514
DK	339.4	109.1	-	0.286	0.206	0.221	0.155	0.290	0.175	0.523
SS	1237.3	1181.8	1082.0	-	0.309	0.241	0.272	0.308	0.199	0.525
PS	483.3	239.4	144.6	1027.3	-	0.231	0.188	0.236	0.167	0.494
CH	469.6	196.9	160.2	1155.0	127.4	-	0.172	0.128	0.101	0.383
EM	510.8	267.7	172.5	1013.8	28.5	143.6	-	0.187	0.101	0.489
VM	649.4	375.9	344.6	1228.6	258.3	186.2	253.1	-	0.097	0.415
YQP	1253.8	980.5	929.2	1425.1	801.8	784.4	780.4	607.8	-	0.347
FS^{H}	88.3	200.2	259.6	1182.8	400.1	392.6	427.4	575.4	1176.6	-

There did not appear to be a relationship between the geographic and genetic distances across all wild populations (P-value = 0.42; Figure 4A) even after the Creston Valley population was removed from the analysis (P-value = 0.14; Figure 4B).

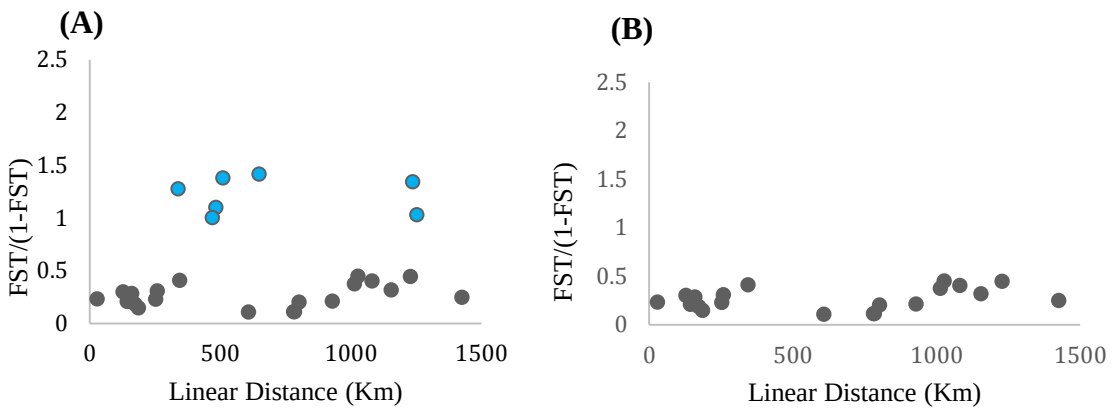

Figure 4. Correlation of genetic (FST/ (1- FST) and geographical distances (Km) between wild northern leopard frog populations. A is with all Canadian wild populations (P = 0.42), blue dots are Creston Valley, while B is without Creston Valley.

The Structure analysis revealed a strong signal at K=2 and K= 10 (Figure 5), showing the existence of 2 main genetic clusters and 10 populations among the data. The Vancouver Aquarium and Creston Valley populations constituted one cluster, further indicating that they are genetically related.

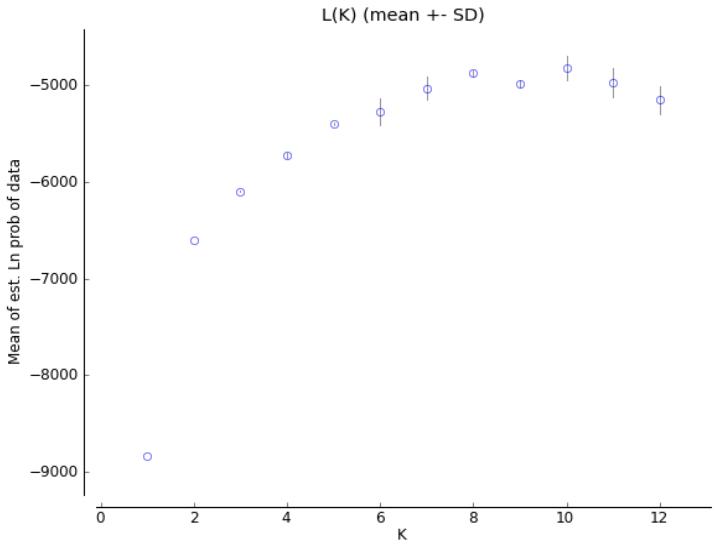

Figure 5. Plot of the log probability of the data [LnP (D)] as a function of the numbers of clusters (K) calculated by Structure 2.3.1.

The historical Fort Steele population and the other 8 Canadian populations sampled in this study were grouped together in a second genetic cluster. However, the historical population of Fort Steele had evidence of admixture with the remnant Creston Valley population (Figure 6). Also, there was some similarity between the Cypress Hills and Magrath sites when K=10.

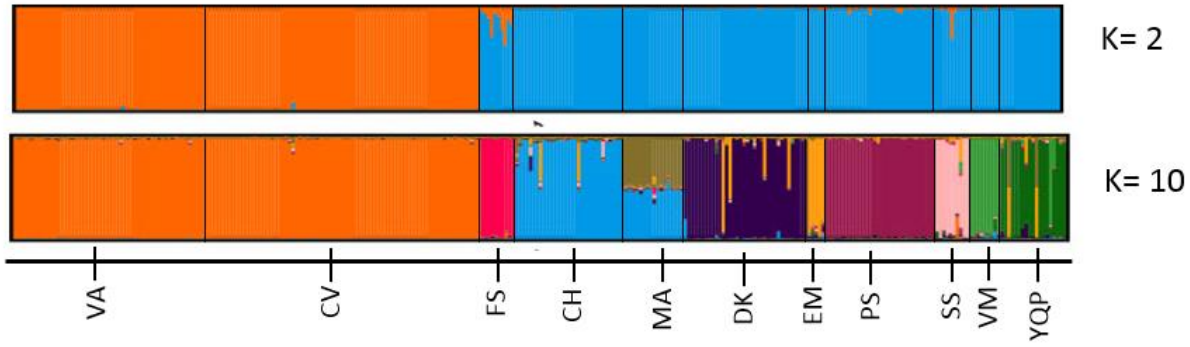

Figure 6. STRUCTURE result of admixture plots for northern leopard frog sampled in Canada. Admixture plots showing individual membership to K clusters for K values of 2 (above) and 10 (below) based on genotypes. Unique clusters are represented by color. Each vertical line represents an individual. Codes for population identifications are given in Table 1.

Change in Population Genetic Metrics Over time

Genetic diversity of the extinct population of Fort Steele population in British Columbia was higher than the current Creston Valley population as measured by the rarefied allelic richness, rarefied private allelic richness, H_o, and H_e (Table 1). However, these standard errors of these indicators of genetic diversity overlapped, as did the inbreeding coefficient of both populations (Table 1). In general, the extinct Rocky Mountain population was highly differentiated from current NLF populations, including the Creston Valley population, and was more genetically similar to populations of the species east of the Rocky Mountains as compared to the current Creston Valley population (Table 3).

The current genetic data for Cypress Hill, Prince's Spring, Drain K, and Creston Valley were also compared to 2004 (Wilson et al., 2008; Table 4). Although the sample size was smaller in 2004 than in 2019, there was not a considerable change in rarefied allelic richness, rarefied private allelic richness, H_o, and H_e (Table 4). In contrast, rarefied allelic richness and expected heterozygosity of the Creston Valley population decreased by 26% and 44%, respectively, between 2000 (Hoffman and Blouin 2004b) and 2019 (Figure 7). The rarefied allelic richness of the population in 2000 was 2.32 ± 0.28 and the expected heterozygosity was 0.43 ± (0.08) (Hoffman and Blouin 2004b) while rarefied allelic richness of it in 2019 was 2.07 ± 0.21 and expected heterozygosity was 0.39 ± 0.07 (Table 1).

Table 4. Rarefaction allelic richness (AR), Rarefaction private allele (PA), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient from Wilson et al. (2008) study. The populations are ordered from east to west. Acronyms for the population are given in Table 1.

ID	n	AR	SE	PA	SE	Ho	SE	He	SE	Fis	SE
CH	4	2.79	0.29	1.13	0.28	0.58	0.08	0.503	0.070	- 0.02	0.09
PS	20	2.35	0.29	0.43	0.15	0.52	0.11	0.456	0.081	- 0.08	0.08
DK	3	2.56	0.38	0.89	0.29	0.44	0.10	0.420	0.089	0.14	0.11
CV	17	2.07	0.22	0.62	0.20	0.37	0.08	0.396	0.076	- 0.10	0.06

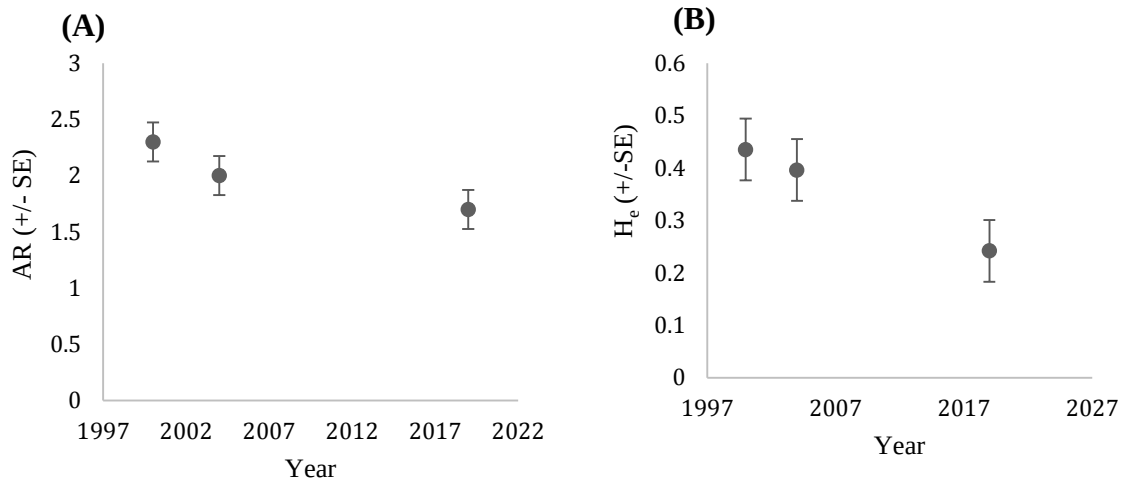

Figure 7: changes in rarefaction allelic richness (AR) and expected heterozygosity (H_e) of the Creston Valley population at three time points in 2000 (Hoffman et al. 2004), 2004 (Wilson et al. 2008) and 2019.

Discussion

Population Genetic Structure of Current Northern Leopard Frogs in Western Canada

Modern genetics play a key role in understanding the genetic health and population structure of at-risk species by evaluating the trends of their genetic diversity (Frankham et al. 2010, Frankham 2019). The northern leopard frog populations in Canada are highly genetically differentiated, and their genetic diversity declines from east to west. The Creston Valley population, the most western sampled population and belonging to the Rocky Mountain DU, had the lowest genetic diversity. In northern landscapes, amphibians are at the periphery of their distribution and typically show reduced genetic variability (García-Ramos and Kirkpatrick 1997, Lesbarrères et al. 2014, D'Aoust-Messier and Lesbarrères 2015). Additionally, this trend might be due to historical bottlenecks or founder effects associated

with either human activities or natural events such as glacial and post-glacial range expansion (Wilson et al. 2008).

South Slave (Northwest Territories), Empress (Alberta), and Creston Valley populations showed evidence of inbreeding. The South Slave population is the most northern population sampled. Populations inhabiting the northern limits of the species' range, must adapt to the harsh weather conditions, and they usually have a small population size due to founder effects. Therefore, these limitations may explain the evidence of inbreeding in these populations (Lesica and Allendorf 1995, Arnaud-Haond et al. 2006). Additionally, the sample size was low for both Empress and South Slave populations which can reduce accuracy in estimating the true inbreeding coefficient by affecting genotype distribution (Pruett and Winker 2008). The estimation of relatedness is often inaccurate whenever a substantial proportion of close relatives are included. In certain species for instance, family members (especially juveniles) tend to cluster spatially, so sampling without being aware of and accounting for this structure may result in only a few large families being represented in the sample (Wang 2014).

Effective population size estimates the genetic drift and inbreeding of a population (Frankham 2005, Allendorf 2009, Sládkovičová et al. 2022). To minimize inbreeding depression in wild populations to 10% over 5 generations, an effective population size equal to or larger than 100 is required (Frankham et al. 2014). Furthermore, to maintain evolutionary potential, the effective population size needs to be equal or larger than 1000 (Frankham et al. 2014), a figure never observed in all the populations sampled in this study. Small sample size (e.g., Empress, South Slave, Val Marie and Yellow Quill Property) can bias estimates of true effective population size by not yielding properly weighted allele

frequencies (Waples and Yokota 2007). In the case of the South Slave population, Creston Valley, and Vancouver Aquarium, founder effects may have resulted in the small effective population size of these populations (Arnaud-Haond et al. 2006, Waples and Yokota 2007, Ogden et al. 2020). The effective population size is smallest in the Creston Valley, which is also consistent with research that found that the effective population of northern leopard frogs declined from east to west (Phillipsen et al. 2011). Small effective population size results in both inbreeding and low allelic richness due to genetic drift, in turn increasing the likelihood of population extinction (Newman and Pilson 1997). Therefore, inbreeding in the population can be associated with small effective population size, both of which can increase the probability of extinction of the population (Ogden et al. 2020).

The genetic distance observed between NLF populations varied from moderate to very greatly differentiated. Such results have also been observed in previous genetic research conducted on the species (Kimberling et al. 1996, Wilson et al. 2008), potentially reflecting a long period since these populations have diverged. This genetic differentiation may also result from the large geographical distance between populations and presence of physical barriers, such as the Rocky Mountains between the Rocky Mountain DU and Prairie/Boreal DUs, as well as discontinuous habitats and the shift between the main eco-regions (Wilson et al. 2008). In most cases, the linear distance between populations was greater than 150 km, with only 6 pairs of populations geographically closer than that. There was no relationship between genetic and geographic distance. These results were consistent with those found by Wilson (2008), suggesting that genetic drift is a major driver of genetic differences. It is also possible that the low genetic diversity and effective population size of the Creston Valley population resulted from a population bottleneck (Frankham et al. 2010, Frankham 2019).

The Creston Valley and Vancouver Aquarium populations appear as one genetic cluster, which is unsurprising since the former serves as the source population for the latter (Randall et al. 2021). Additionally, the Creston Valley population shows the highest genetic distance from other extant populations of the species. Interestingly, the Creston Valley population shows even very great genetic distance from the historical Rocky Mountain population of Fort Steele in British Columbia. This was contrary to the results of a mitochondrial DNA study by Hoffman and Blouin (2004b), that showed that these two populations share the same haplotypes and are clustered together. Moreover, the extinct historical population clusters with the Boreal/Prairie populations in Alberta, Saskatchewan, and Manitoba, although there is some evidence of genetic admixture with the Creston population. The current admixture analysis thus refines the results from Wilson et al. (2008) which only distinguished populations from British Columbia and Alberta. When more complicated genetic structures were considered, there was considerable similarity between the Cypress Hills and Magrath populations which is not surprising given that two of the source populations for the Magrath reintroduction were from Medicine Hat which is only about 50 km from the Cypress Hills (Romanchuk and Quinlan 2006).

Changes in Population Genetic Structure Over Time

My results show a lack of difference in genetic diversity, inbreeding, and effective population size between the extinct population of Fort Steele and the Creston Valley population but they were very greatly differentiated from each other when it comes to genetic distance. However, private alleles present in the former suggest the loss of genetic diversity

in Creston Valley was due to a population bottleneck (Sonsthagen et al. 2017). Although the sample size is small, the low genetic diversity of the extinct population of Fort Steele shows that the historical populations of the Rocky Mountains, inhabiting the northern limits of the species range, already had low genetic diversity, consistent with Hoffman and Blouin (2004b). One possible reason for the Fort Steele population's extinction and other populations within the Rocky Mountain DU might be due to loss or reduced level of diversity of major histocompatibility complex (MHC) genes which are important for immunity and the spread of diseases such as Bd (*Batrachochytrium dendrobatidis)* (Trujillo et al. 202l).

Genetic monitoring of the Cypress Hill, Drain K, Prince Spring, and Creston Valley populations between 2004 (Wilson et al. 2008) and 2019 shows little change in their population genetic parameters. However, the comparison of the Creston Valley allelic richness and expected heterozygosity between 2000 (Hoffman and Blouin 2004a), 2004 (Wilson et al. 2008) and 2019 show that the population's genetic diversity has declined over 19 years, suggesting that it may not be possible to detect changes in genetic diversity over shorter time periods. Hoffman and Blouin (2004b) concluded that genetic diversity was always low in these peripheral populations and therefore artificial gene flow was unnecessary, however the results of this study reveal that genetic diversity has since declined, so perhaps this idea should be revisited. This reveals the need for government and conservation managers to revise recovery plans in order to improve the genetic health of these populations, particularly the Creston Valley population which has a high probability of extinction.

Conclusion

For the most part, the northern leopard frog populations in western Canada are highly differentiated from each other, and their genetic diversity declines from east to west. At the edge of the western range and as a peripheral population, the Creston Valley population presents signatures of a severe bottleneck as evidenced by its low genetic diversity and effective population size. The Vancouver Aquarium captive population also shows a low level of genetic diversity as a result of Creston Valley being its only source population. These results suggest that genetically augmenting the Creston Valley population might help its long-term survival. Additionally, continued genetic monitoring of the northern leopard frog populations in western Canada may be helpful in tracking changes in the populations' genetic structure over time and will allow for a better evaluation of conservation actions (Schwartz et al. 2007). Moreover, a comprehensive genetic study using modern genomic techniques across the eastern and western clades, particularly in the province of Manitoba, as well as the western United States will benefit conservation managers to better understand the recent genetic changes of the species' population and its designatable units, as well as inform the poorly understood boundary between the eastern and western clades in Canada.

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General Conclusion

Only about half of the DUs that were assessed by COSEWIC incorporated genetic data, and very few had genetic monitoring. Furthermore, significant taxonomic bias in the availability of genetic information exists, with mammals and fishes showing the highest number of genetic while there was little available genetic information for lichen. Therefore, the government, the scientific community, and conservation managers should include a comprehensive plan for integrating genetic monitoring as genetics and genomic approaches can help better address conservation concerns associated with anthropogenic activity on at-risk species and their respective DUs.

In western Canada, northern leopard frog populations are highly genetically differentiated. In particular, the most western population of the species (Creston Valley) had the lowest genetic diversity and effective population size. The existence of private alleles in the extinct Rocky Mountain population of Fort Steele may also provide evidence of a severe bottleneck in the Creston Valley population. Genetic monitoring of four populations between 2008 (Wilson et al. 2008) and 2019 did not reveal drastic changes in genetic diversity, suggesting stable conditions in the monitored populations of the species over this time period. However, a comparison of allelic richness, and heterozygosity showed that these had declined in the population since 2000. Considering the results of both chapter 1 and chapter 2 of my thesis, including genetic information in management plans and recovery strategies of at-risk species is essential. Practically, the results of this study will help the northern leopard frog recovery team to manage the population of the Creston Valley and can help inform reintroduction efforts. Yet, a comprehensive genetic study using modern genomic techniques across the eastern and western clades, particularly in the province of Manitoba as well as the

western United States, is encouraged. Moreover, the boundary between the eastern and western clades is poorly understood in Canada, so this study would help identify where these two DUs meet.

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Appendix

Table S1. Information of samples used in this study.

Sampling site	Population Type	Number of Individuals	Life status			Sampling Method	Season	Year
			Number of Tadpoles	Number of Juveniles	Number of Adults			
Yellow Quill Property, Manitoba	Wild	19	0	Unknown	Unknown	Buccal Swab	Summer	2020
Val-Marie, Saskatchewan	Wild	9	0	5	4	Buccal Swab	Spring/ Summer	2020
Empress, Alberta	Wild	5	0	3	2	Buccal Swab	Summer	2019
Cypress Hill, Alberta	Wild	31	0	18	13	Buccal Swab	Spring	2019
Prince's Spring, Alberta	Wild	32	0	26	6	Buccal Swab	Summer	2019
South Slave, Northwest Territories	Wild	10	0	Unknown	Unknown	Toe Clips	Spring/ Summer	2009
Drain K, Alberta	Wild	36	0	28	8	Buccal Swab	Spring	2019
Creston Valley, British Columbia	Wild	120	30	52	38	Tissue/ Buccal swab	Spring/ Summer/ Fall	2019
Magrath, Alberta	Reintroduced	18	0	16	2	Buccal Swab	Summer	2019
Upper Little Bitterroot River, Montana	Reintroduced	25	0	Unknown	Unknown	Buccal Swab	Summer	2020
Vancouver Aquarium	Captive	40	1	Unknown	Unknown	Tissue/ Buccal swab	Summer	2019
Fort Steele, British Colombia	Historical	10	0	Unknown	Unknown	Skin	-	1973