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RESEARCH ARTICLE

A first genetic assessment of the newly introduced Isle Royale gray wolves (*Canis lupus*)

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Abstract
The gray wolf (*Canis lupus*) population of Isle Royale National Park suffered an extreme population decline where by 2017 only two wolves that were both half-siblings and a father-daughter pair remained with low probability of producing viable young. This precipitous decline was in part due to the negative fitness consequences associated with inbreeding. To restore the Isle Royale ecosystem 19 gray wolves were translocated in 2018 and 2019. The founders were translocated from Grand Portage, MN (n = 4), western Upper Peninsula, MI (n = 4), Jostle Lake, ON (n = 3), and Michipicoten Island, ON (n = 8), and genotyped using 18 microsatellite loci. Allelic richness and heterozygosity of translocated Isle Royale founders was similar to reference populations. Population structure assigned the Isle Royale founders to gray wolves with little evidence of admixture from eastern wolves (*Canis lycaon cf*). In addition, we confirmed wolves translocated from Michipicoten Island were a single family-group. Through simulation and empirical analysis of the new Isle Royale founders we projected a loss in genetic variation over the next 50 years and an increase in inbreeding. However, varying levels of immigration may allow the retention of some genetic variation. Our findings indicate Isle Royale founders are genetically diverse and representative of the Great Lakes region, but the numerical dominance of a single family group may have negative implications for retaining genetic diversity and success of establishment for specific wolves, reinforcing the importance of continued monitoring of genetic fitness.

Keywords Conservation · Translocation · Restoration · *Canis lupus* · Inbreeding · Pedigree

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Introduction

Wildlife restricted to islands are often more vulnerable to extinction than their mainland counterparts due to isolation and small population sizes (e.g. Frankham 1998). Vulnerabilities facing island wildlife have prompted researchers to study and identify key components that maintain viable island populations (Diamond 1975; Reed et al. 2003; Traill et al. 2007). One important component for maintaining a viable island population is the retention of heterozygosity (Frankham 1997; Frankham et al. 2014), which is critical due to its impact on survival (Coltman et al. 1998), reproductive output (Hedrick and Fredrickson 2008), disease susceptibility (Niskanen et al. 2013), and evolutionary potential (Harrisson et al. 2014), an especially important aspect of genetic variation in an era of rapidly shifting climate. Island populations also tend to be more susceptible than mainland populations to losing genetic variation and heterozygosity from genetic drift and inbreeding (Frankham et al. 2014).

Inbreeding, where consanguineous mating events tends to increase in genome-wide homozygosity, is particularly impactful, given well-documented deleterious effects on fitness (e.g. Sams and Boyko 2019; Robinson et al. 2019; Hooper et al. 2020). As such, monitoring of island species should include assessments of genetic health such as, the potential negative fitness consequences associated with reduced genetic variation and inbreeding.

Nowhere are these concerns truer than Isle Royale National Park (ISRO), an island in Lake Superior, Michigan USA, where reduced heterozygosity due to inbreeding has adversely impacted population viability of gray wolves (*Canis lupus*) (Räikkönen et al. 2009; Hedrick et al. 2014). It is believed that one female and two males first colonized the island in either 1949 or 1950 (Wayne et al. 1991; Adams et al. 2011). The establishment and subsequent fluctuations in the ISRO wolf population through time have generated an especially well-documented case for the consequences of inbreeding. At their peak in 1980, the ISRO wolves numbered 50 individuals but suffered a precipitous population decline to just 14 individuals in 1982 due to the combined negative influence of high density and introduction of novel canine parvovirus to the island (Peterson et al. 1998). Following this severe bottleneck event, the inbreeding coefficient (F) was estimated as high as 0.81, using estimates of effective population size and generation time over time during the mid-1990s (Adams et al. 2011). Furthermore, throughout the history of this population, the incidence of congenital bone deformities increased, demonstrating a potential connection between reduced heterozygosity and reduced fitness (Räikkönen et al. 2009), with reduced fitness of ISRO wolves as a consequence of fixed deleterious alleles in a small population recently confirmed (Robinson et al. 2019).

As of 2017 only two highly related wolves remained on ISRO, a male (M183) and female (F193) who never produced viable offspring (Hedrick et al. 2016). The functional extirpation of ISRO wolves, and the subsequent unmitigated population growth of moose (*Alces alces*), the primary prey of wolves on ISRO, prompted efforts to translocate wolves from the surrounding mainland to restore wolf predation (NPS 2018). Specifically, wolves were trapped and relocated to ISRO from Grand Portage, MN, western Upper Peninsula, MI, Jostle Lake, ON and Michipicoten Island, ON. All wolves translocated to ISRO, hereafter ISRO founders, were native to the Great Lakes region. The Great Lakes region possesses a mosaic of wolf ancestry including gray wolf, eastern wolf (*Canis lycaon cf*), and coyote (*Canis latrans*) (Leonard and Wayne 2007; Wheeldon and White 2009; Rutledge et al. 2010; Fain et al. 2010). The history of Great Lakes wolves suggests that eastern/gray wolf hybrids from Manitoba, Ontario, and Minnesota recolonized into Wisconsin and the Upper Peninsula, MI (Wheeldon and White 2009), encompassing the region where ISRO founders were captured. Also, Algonquin Park, ON is the epicenter of eastern wolves' current geographic range (Rutledge et al. 2010; Heppenheirmer et al. 2018), making it plausible for eastern wolves or their ancestry to be translocated to ISRO through a founding individual from Ontario.

In addition to the implications for potential eastern wolf ancestry, the ISRO founders translocated from Michipicoten Island were a suspected family group (B.R. Patterson et al., unpublished data). Michipicoten Island was originally colonized by wolves in 2014 via an ice bridge, after which there was minimal movement on and off the island. Thus, understanding their pedigree is critical for monitoring relatedness and genetic fitness of the new ISRO founders.

The potential admixture of eastern wolves and the presence of related ISRO founders necessitates genetic monitoring of this population to help ensure long-term goals for this introduction are successful. Mixed ancestry between two species of canids can complicate management decisions. For example, in Ontario, gray wolves are classified as not-at-risk and eastern wolves are considered threatened provincially and nationally (COSSARO 2016; COSEWIC 2015). Further, to prevent the same fate as the contemporary ISRO population, it is necessary to track genetic variation and inbreeding to identify fitness consequences associated with reduced genetic variation during and after the establishment of the ISRO founders. Here, we present the first genetic assessment of the ISRO founders and compare genetic variation of the founders relative to reference populations of the Great Lakes region. We utilized autosomal microsatellite markers to: (1) summarize genetic diversity and structure of ISRO founders relative to surrounding Great Lakes wolf populations, (2) identify relationships within the ISRO founders translocated from similar regions and (3) simulate the trajectory of heterozygosity and inbreeding over the next 200 years.

Materials and methods

Translocation details, status of wolves, and reproduction

Nineteen wolves (ISRO founders) were moved to ISRO from September 2018 through September 2019. ISRO founders were captured with foot-hold traps or net-gunned via helicopter and translocated from Grand Portage, MN (n = 4), Michipicoten Island, ON (n = 8), Jostle Lake, ON (n = 3), and western Upper Peninsula, MI (n = 4). A twentieth wolf from Grand Portage, MN was trapped, but died in holding prior to release to the island (ISRO-002).

Of the 19 ISRO founders released on the island, seven wolves have died, and one wolf left the island during the winter of 2019 on an ice bridge (Table 1). Intraspecific

Table 1 Summary of translocated wolves on Isle Royale

ID	Source Location	Estimated Birth Year	Sex	Status	Date of Release	Date of Death	Cause of Death	Potential Breeder
ISRO-001	Grand Portage, MN	2014	F	Alive	27 Sep. 2018	–	–	Yes
ISRO-002*	Grand Portage, MN	Unknown	F	Dead	NA	Died in Captivity	Adverse Reaction to Anesthetics	No
ISRO-003*	Grand Portage, MN	2014	F	Alive	3 Oct. 2018	–	–	No
ISRO-004	Grand Portage, MN	2017	F	Dead	4 Oct. 2018	29 Sep. 2019	Intraspecific Aggression	No
ISRO-005*	Jostle Lake, ON	2017	F	Dead	26 Feb. 2019	6 Jan. 2020	Unknown	No
ISRO-006	Jostle Lake, ON	2017	M	Dead	28 Feb. 2019	31 Mar. 2019	Unknown	Yes
ISRO-007	Michipicoten, ON	2016–2017	M	Alive	28 Feb. 2019	–	–	Yes
ISRO-008	Grand Portage, MN	2013	M	Dead	27 Sep. 2018	17 Oct. 2018	Pneumonia	No
ISRO-009	Michipicoten, ON	<2014	M	Alive	1 Mar. 2019	–	–	Yes
ISRO-010	Michipicoten, ON	2015	M	Dead	23 Mar. 2019	9 Jan. 2020	Intraspecific Aggression	Yes
ISRO-011	Michipicoten, ON	2016	F	Alive	23 Mar. 2019	–	–	Yes
ISRO-012	Michipicoten, ON	2017	M	Alive	23 Mar. 2019	–	–	Yes
ISRO-013	Michipicoten, ON	2017	M	Alive	23 Mar. 2019	–	–	Yes
ISRO-014	Michipicoten, ON	<2014	F	Alive	23 Mar. 2019	–	–	Yes
ISRO-015	Michipicoten, ON	2016	F	Alive	23 Mar. 2019	–	–	Yes
ISRO-016	Jostle Lake, ON	Unknown	M	Alive	23 Mar. 2019	–	–	Yes
ISRO-017	Baraga County, MI	2015–2017	M	Alive	6 Sep. 2019	–	–	Yes
ISRO-018	Baraga County, MI	2015–2017	F	Dead	8 Sep. 2019	6 Jan. 2020	Intraspecific Aggression	No
ISRO-019	Baraga County, MI	2015–2017	M	Alive	11 Sep. 2019	–	–	Yes
ISRO-020	Baraga County, MI	2015–2017	F	Dead	13 Sep. 2019	14 Sep. 2019	Cellulitis and Suspected Septicemia	No
M183	Isle Royale, MI	2008	M	Dead	NA	15 Oct. 2019	Intraspecific Aggression	No
F193	Isle Royale, MI	2010	F	Alive	NA	–	–	No

*ISRO-002 died in captivity before reaching ISRO, not included in analysis, ISRO-003 left the island via ice bridge in 2019, ISRO-005 was not genotyped for this study

aggression was the predominant cause of death in the population, while disease, trapping injury, and unknown causes have also led to deaths (Romanski et al. 2020).

Genetic diversity estimates such as, private alleles, allelic richness, and heterozygosity, were calculated for ISRO founders that are believed to have contributed or have the potential to contribute to reproduction. Based on visual and GPS-based observations, it is likely ISRO-014 was the only female to reproduce in 2019 where she copulated on Michipicoten Island, ON and gave birth on ISRO (Romanski et al. 2020). In 2020, females ISRO-001 and ISRO-015 both showed signs of denning activity suggesting they may have reproduced as well (Romanski et al. 2020). Potential breeders should also include wolves that were alive on the island for a span of time where they could potentially have bred. We explicitly define potential breeders as: (1) ISRO founders currently alive on the island, (2) male ISRO founders who were alive for at least a period that overlapped with the months ranging from January to April representing the typical window of copulation (Mech 1974), or (3) female ISRO founders that were alive for at least one full calendar year starting on January 1st. These criteria are set so males

only need to be present for copulation, but females must have been present for copulation through post-birth rearing up to one year to be considered a potential breeder. All ISRO founders that meet the criteria to be a potential breeder (n = 13) are alive except for males ISRO-006 and ISRO-010 (Table 1).

Female F193 and male M183 were the two native wolves of ISRO when translocation events began. M183 was found dead from intraspecific aggression in the Fall of 2019 making it plausible for him to classified as a potential breeder. Further, F193 is classified as alive at this time and could also be a potential breeder. However, because of the high levels of inbreeding within the population prior to translocation events, it is unlikely these two individuals would reproduce with ISRO founders or each other.

Microsatellite genotyping and regional reference samples

Whole blood was obtained from each ISRO founder following capture and was used for genomic DNA extraction (100 μL) using Qiagen DNeasy Blood and Tissue Kit (Qiagen,

Ltd.) following the manufacturer's guidelines. We analyzed 18 microsatellite loci of the ISRO founders: cxx147, cxx225, cxx250, cxx253, cxx377, cxx410, cxx442 (Ostrander et al. 1993, 1995), Pez5, Pez8, Pez11, Pez16 (Neff et al. 1999), FH2004, FH2611, FH2658, FH2914, FH3047, FH3399 (Guyon et al. 2003), FH2324 (Mellersh et al. 1997) and Amelogenin sex determination primers DS1 (Yan et al. 2013). Amplification was done in two multiplex reactions with fluorescently labelled forward primers: Multiplex A included Pez8, FH2324, FH3853, FH3965, cxx410, Pez11, cxx253, FH3399, cxx147, and DS1; Multiplex B included FH2914, FH3047, FH2004, Pez16, Pez5, cxx377, cxx225, cxx442, cxx250, FH2611, and FH2658. We dropped FH3853 and FH3965 from Multiplex A due to irregular binning patterns that created inconsistent scores across runs. A 10×2 μM primer mix of 1 mL was made for each multiplex reaction and frozen at -20 °C. Total volume for each reaction was 12 μL with 2 μL of DNA (standardized to 1.25 ng/μL), 1x primer mix, 1x Qiagen Multiplex PCR Master Mix (Qiagen, Toronto ON; Cat. No. 206143) and 2.8 μL of DNAase-free molecular grade water. Cycling conditions were as follows: initial denaturation and polymerase activation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 58 °C (A) or 59 °C (B) for 90 s, and 72 °C for 60 s followed by a 30 min final extension at 72 °C. We combined 6 μL of GenScan 500 LIZ size standard (Applied Biosystems) with 1 mL of HiDi Formamide and then visualized amplified DNA on an ABI3730 (Applied Biosystems) by combining 1 μL of PCR product with 9 μL of HiDi Formamide-500 LIZ mixture. Samples were genotyped with standardized bins in Genemarker v 7.1 (SoftGenetics).

We used gray wolves, eastern wolves, and eastern coyotes as reference populations to identify if ISRO founders contained greater assignments to gray wolf, eastern wolf, or eastern coyote clusters as determined using population structure analyses. Specifically, we used the following reference populations: eastern wolves from Ontario (n = 24), northeastern, Ontario gray wolves (n = 30), and northwestern, Ontario gray wolves (n = 22). We also included coyotes from Ontario (n = 55) as a secondary outgroup to understand if ISRO founders contained admixture from coyote (see supplementary Fig. 1 for the inclusion of coyote in population structure analyses). To remove potential misidentified or admixed reference samples, we ran an initial analysis of the reference samples mentioned above (excluding ISRO founders) in STRUCTURE v2.3 (Pritchard et al. 2000) using the admixture model (assuming correlated allele frequencies) with 20,000 MCMC replications after a burnin period of 20,000. Assuming the number of clusters was three (representing coyotes, eastern wolves, and gray wolves) individuals with Q-values less than 0.9 (threshold previously used by Heppenheimer et al. 2018) to the cluster representing their species assignment were removed from future analyses (24

samples removed, which did not meet criteria). The final reference population contained 15 eastern wolves from Ontario, 22 northeastern, Ontario gray wolves, 22 northwestern, Ontario gray wolves, and 48 coyotes from Ontario.

Statistical Analyses

Genetic Diversity

We measured standard genetic diversity estimates of ISRO founders classified as potential breeders (Table 1) and compared estimates against reference populations using R package, ADEGENET (Jombart 2008). Diversity estimates included allelic richness, private alleles, frequency of private alleles per locus, observed heterozygosity (H_O) and expected heterozygosity (H_E). We estimated the number of alleles per locus using rarefaction, in R package, HIERFSTAT (Goudet 2005), while the number of private alleles per population were identified using R package, POPPR (Kamvar et al. 2014). F_{is} was calculated following Nei (1987) for three reference population and ISRO founders.

Population Structure

Our initial analysis of population structure after the removing admixed/misidentified reference samples, included coyotes from Ontario (n = 48), eastern wolves (n = 15), northeastern, Ontario gray wolves (n = 22), northwestern, Ontario gray wolves (n = 22), and 18 ISRO founders, which excludes ISRO-002 who left the island via an ice bridge shortly after arrival, ISRO-005, and two native gray wolves of Isle Royale (F193 and M183) which we were unable to genotype for this study (Table 1). We determined population structure by running the admixture model (assuming correlated allele frequencies) using the software, STRUCTURE v2.3 (Pritchard et al. 2000) from K = 1 to 15 (10 iterations per K) with 20,000 MCMC replications after a burnin period of 20,000. Coyotes were included in the initial model to assess potential admixture among coyote and wolf species but then dropped given no evidence of substantial admixture (Supplementary Fig. 1). Also, during the initial analysis, we identified ISRO founders translocated from Michipicoten Island had their own independent cluster with high assignment (see Supplementary Fig. 1, K = 4). Because population structure-based analyses assume random sampling within each group, any family-group present will bias that estimate (Falush et al. 2003). To avoid this bias for our second analysis excluding coyotes, we excluded ISRO founders from Michipicoten Island except for ISRO-009 and ISRO-014, which are the inferred parents of the other Michipicoten wolves (see Relatedness and Relationships section for description of relationships). After the removal of the family group, we identified

loci deviating from Hardy–Weinberg equilibrium (HWE) using a χ^2 test with R package, PEGAS (Paradis 2010).

To explicitly test if ISRO founders contained ancestry from eastern wolves, we ran a second population structure model. As mentioned above, coyotes were excluded from the reference population as well as all ISRO founders from Michipicoten Island, Ontario except for the inferred parent ISRO-009 and ISRO-014, leaving 12 ISRO founders for the analysis. We selected the most supported number of clusters (K) using Structure Harvester (Dent and vonHoldt 2012), which implements the criteria outlined by both Pritchard et al. (2000) and Evanno et al. (2005). The same parameters were retained from the initial model using software STRUCTURE v2.3 except allele frequencies were not correlated.

We also used multivariate analyses to explore population structure using principal components analysis (PCA) and discriminant analysis of principal components (DAPC) using R package, ADEGENET (Jombart 2008; Jombart and Collins 2015). For both PCA and DAPC we used the same reference samples and ISRO founders from the second model ran in software, STRUCTURE mentioned above. We used PCA to visualize the general structure of the three reference populations (eastern wolves, northeastern, ON gray wolves, and northwestern, ON gray wolves) relative to the 12 ISRO founders included in the analysis. DAPC allowed us to estimate the number of clusters to summarize a dataset and the probability of assignment for each individual to a cluster. We used DAPC to identify the number of clusters by selecting the cluster set with the lowest Bayesian information criterion. Also, DAPC differs from PCA in that it can describe data using a reduced set of principal components, while describing most of the variance. DAPC uses an approach where variance in samples are partitioned into a between-group and within-group components to maximize discrimination between groups. We utilized DAPC to explicitly define ISRO founder's assignment to clusters associated with either gray or eastern wolves. For the probability of assignment, we retained 60 principal components (Supplementary Fig. 2A) given little information was gained by retaining the remaining principal components.

For pairwise F_{st} estimates, we used the same reference samples and ISRO founders from the second model ran in software, STRUCTURE mentioned above. We calculated cases of significant pairwise differentiation (F_{st}) between two populations by calculating bootstrap confidence intervals for variance components (number of bootstraps = 5,000), in R package, HIERFSTAT (Goudet 2005).

Relatedness and Relationships

Related individuals translocated together can impact the success of the translocation (Reading et al. 2013) and accelerate inbreeding depression if the individuals mate. Therefore, we identified the average pairwise relatedness within each geographic region and each translocated wolf group to understand if fine-scale social structure was present. We calculated pairwise relatedness using ML RELATE, a maximum likelihood approach, where a pairwise value of 0.0 is unrelated, 0.5 parent/offspring, and 1.0 is identical (Kalinowski et al. 2006). We also assessed the significance in regional differences in relatedness using a Kruskal–Wallis test. Because founders translocated from Jostle Lake contained only a single pairwise relatedness comparison (as they consist of only two individuals), they are not included in the Kruskal–Wallis test. Results of the pairwise relatedness was graphed in R using the GGPLOT2 package (Wickham 2016).

To explicitly define the relationships among ISRO founders, we constructed a pedigree using program COLONY, which implements a maximum likelihood approach to estimate discrete relationship assignments (Wang 2004). ISRO founders translocated from Michipicoten Island had a known birth year, which we used to inform the analysis. Further, a pedigree of all Michipicoten wolves that once occupied Michipicoten Island was used to reconfirm our assignments and overall accuracy (T.J. Wheeldon et al., Ontario Ministry of Natural Resources and Forestry, unpublished data).

Projected Inbreeding and Heterozygosity

To project the level of inbreeding and retention of heterozygosity overtime, we designed an agent-based model in NETLOGO (Wilensky 1999). The agents within the model represent the wolves of Isle Royale and we will refer to agents as wolves when describing the model. The rule sets applied to the wolves reflect the choices wolves make when selecting mates, reproducing, and migrating. For variables associated with the genetic makeup of each wolf, we informed the model using empirical data from this study. The full list of parameters and variables associated with wolves and the structure of the overall model is available in the supplementary file (Overview, Design Concepts, and Detail (ODD); Grimm et al. 2006, 2010, 2020). Here, we present a summarized overview of the model.

The model assumed up to three packs could form each year (Peterson and Page 1988; Vucetich et al. 1997). Within each pack, the mating system was assumed monogamous where the same male and female (designated as alphas) bred each year. If the male alpha died, the female alpha selected a new mate (at random but meeting the criteria mentioned below), but if the female alpha died, a new female alpha was designated for the respective pack and she selected a new mate. Both male and female wolves must be at least two years of age to become sexually mature or available to become alphas and female alphas selected their mate at random to support the idea that gray wolves rely more on

dispersal than kin recognition to avoid inbreeding (Geffen et al. 2011).

Once mates were selected, female litter size was drawn from a random uniform distribution ranging from four to eight pups. ISRO-014 previously had a litter size of eight pups (B.R. Patterson et al., unpublished data) informing our maximum litter size, and while litter sizes as small as a single pup have been reported for wolves on Isle Royale (Peterson and Page 1988), these observations were rare and led us to set a more biologically relevant minimum litter size of four pups (Peterson and Page 1988; Sidorovich et al. 2007). In addition, we implemented a steady state population where the maximum population size could not exceed 45 wolves, which allowed a random subset of offspring to be recruited into the population for the following year up to the maximum population size.

For offspring to be recruited into the population, mortality was informed using age-specific survival of a wolf population from Yellowstone National Park (Cubaynes et al. 2014) where the age-specific survival determined the probability of mortality. The only exceptions were at age 0 where all offspring had a 100% chance of survival, but only a subset were recruited into the population and age 11 was set to a 0% chance of survival making it the oldest possible age for a wolf.

To calculate the H_o for each wolf, 18 microsatellite loci were assigned to each wolf, which we used empirical data of the ISRO founders to set the initial ISRO founders within the simulation. When offspring were produced, one of the two alleles from each parent was assigned to the offspring. To calculate inbreeding, we used R package PEDIGREE (Coster 2013) which calculates the inbreeding coefficient (F) from a reconstructed pedigree by tracking each wolf's parents. ISRO founders were assumed to have no known parents and shared no relationship to other founders except for offspring of founders ISRO-009 and ISRO-014 translocated from Michipicoten Island (see Results: Relationships).

Migration to ISRO had two components; first an ice bridge must form to allow the possibility of migration and second the probability that a wolf would migrate across said ice bridge. We explicitly tested the probability of an immigrant wolf entering the population in a given year by allowing the probability of migration to be 0.0 (no migration), 0.5, or 1.0, while each year the probability of an ice bridge forming was set to 0.1 (Licht et al. 2015). Immigrants entering the population had alleles assigned using a probability proportional to the average allele frequencies calculated from the three reference populations of this study including the ISRO founders themselves. Each migration treatment was run with 100 replications. Further, immigrant wolves were assumed to have no known parents or relation to the wolves currently occupying the island.

The starting conditions for all treatments in the model were identical where only the 13 ISRO founders designated as potential breeders (see Table 1) occupied the first time step. To explicitly test the differences in outcomes when varying probabilities of migration were imposed, we used the final time step (year = 199), but to measure significant differences for both F and H_o within a realistic management timeframe we also measured significant differences at time step 49. Significant differences for both F and H_o were determined using a Kruskal–Wallis and one-way ANOVA respectively between probability of migration treatments (0.0, 0.5, and 1.0). Significant differences between treatments were explored using a pairwise Wilcox test for F, and Tukey multiple pairwise comparison for H_o.

Results

Genetic Diversity

Estimated allelic richness of ISRO founders was 4.78 ± 1.39, which is within the range of the allelic richness of reference populations sampled (Table 2). We observed the greatest number of private alleles in the eastern wolf reference population, while ISRO founders contained no private alleles (Table 2, Supplementary Table 1).

Averaged F_{is} values across loci were 0.05 ± 0.04, 0.01 ± 0.02, 0.01 ± 0.02, and -0.08 ± 0.03 for eastern wolves, northeastern, ON gray wolves, northwestern, ON gray wolves, and ISRO founders respectively (Supplementary Table 2).

Table 2 Summary statistics of 18 microsatellites by population. Allelic richness (A) was calculated using rarefaction, private alleles (PA) are alleles unique to their respective populations, observed (H_o) and expected (H_E) heterozygosity are presented across loci with standard deviations. Isle Royale, MI population used for calculations presented in this table are a subset of individuals classified as potential breeders within the population (see methods section 2.1)

Location	Species	N	A	PA	H_o	H_E
Ontario	*Canis lycaon cf*	15	4.88 ± 0.89	16	0.68 ± 0.14	0.70 ± 0.08
Northeastern, ON	*Canis lupus*	22	5.44 ± 1.54	4	0.76 ± 0.10	0.75 ± 0.09
Northwestern, ON	*Canis lupus*	22	5.09 ± 1.49	4	0.73 ± 0.11	0.72 ± 0.11
Isle Royale, MI	*Canis lupus*	13	4.78 ± 1.39	0	0.80 ± 0.18	0.71 ± 0.10

Population Structure

We did not remove the four loci deviating from HWE (FH2611, FH2914, cxx377, and FH2324) because the removal of these four loci did not change population structure (Supplementary Fig. 3). Pairwise F_{st} values were all significantly different between populations, except for ISRO founders and Northwestern, ON (Table 3). PCA demonstrated substantial genetic separation between gray wolves and eastern wolves with principal component one explaining 6.5% of the variation and principal component two explaining 4.1% (Fig. 1). There was overlap among gray wolf populations from northeastern ON, northwestern ON,

Table 3 Pairwise F_{st} values according to Nei 1987 of Eastern wolves of Ontario, gray wolves of northeastern, Ontario, gray wolves of northwestern, Ontario, and gray wolves of Isle Royale

	Eastern Wolf	Northeastern, ON	Northwestern, ON
Northeastern, ON	0.0966**	–	–
Northwestern, ON	0.1052**	0.0093*	–
Isle Royale, MI	0.0919**	0.0132*	0.0008

Pairwise F_{st} values with significant differentiation between samples indicated by asterisk ($p < 0.05$*; $p < 0.01$**)

and ISRO founders. From the DAPC, the best fitted K was K = 2 representing the eastern and gray wolf clusters, where we observed high assignments of individuals to their respective species (Supplementary Fig. 2).

Population structure inferred from STRUCTURE coincided with results from the PCA and DAPC. We estimated the most supported model in STRUCTURE to be K = 2 again representing eastern and gray wolf, where individuals showed high probability of assignment to their respective clusters (Fig. 2, Supplementary Fig. 4). Before excluding all but two of the ISRO founders translocated from Michipicoten Island from analysis, they were assigned to their own cluster with a high probability of assignment (Supplementary Fig. 1, K = 4).

Relatedness and Relationships

Average pairwise relatedness significantly differed between regions of wolves ($\chi^2 = 153$, df = 5, p-value < 0.001). We observed an average pairwise relatedness value of 0.46 within the ISRO founders translocated from Michipicoten Island demonstrating a highly related family group relative to other ISRO founders, which ranged from 0.0 to 0.16 (Fig. 3). High pairwise relatedness among ISRO founders from Michipicoten was confirmed by finding that ISRO-009 (male) and ISRO-014 (female) were the inferred parents of all other ISRO
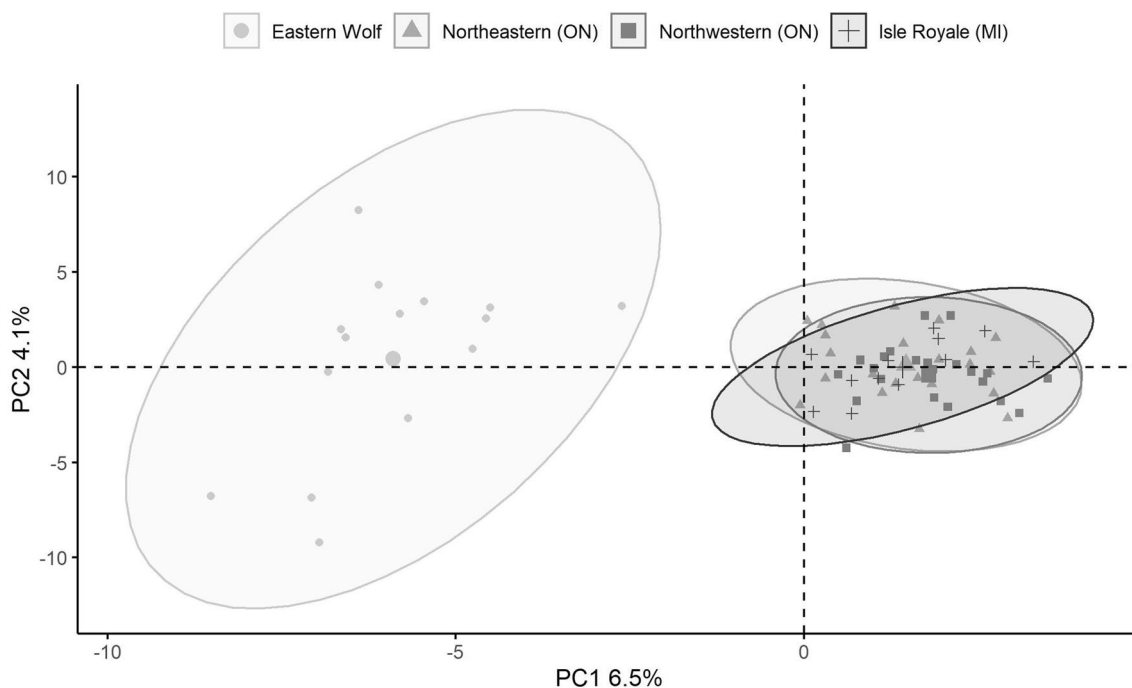
Fig. 1 Principal Component Analysis of gray and eastern wolves sampled across the Great Lakes region including wolves translocated to Isle Royale. Ellipses group 95% of individuals into their respective population assignments. Principal components one and two are represented in this figure with their percent variance explained listed on their respective axes
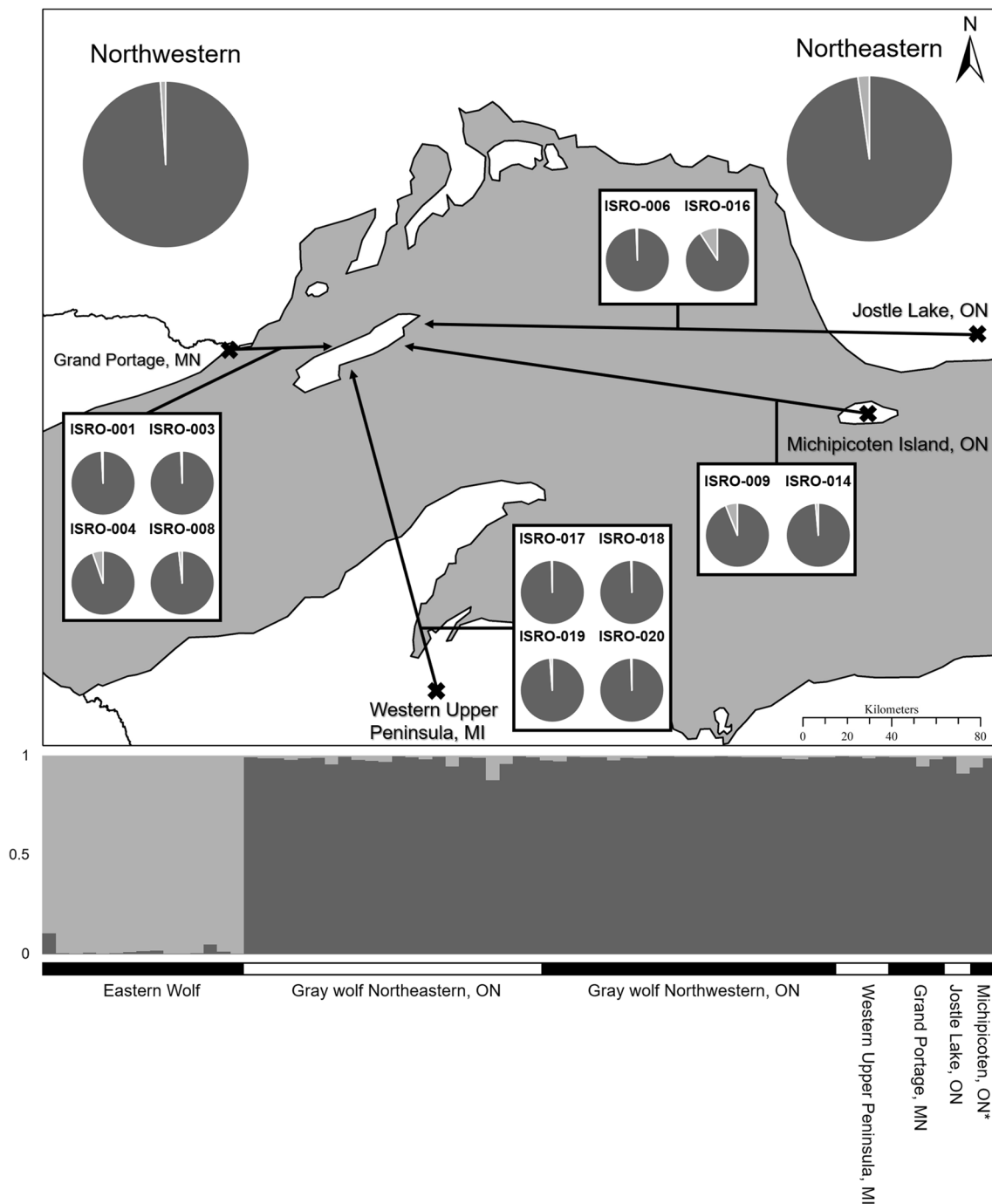
Fig. 2 Geographic distribution for the probabilities of assignment (K = 2) using software STRUCTURE v2.3 for Isle Royale founders and gray wolf reference populations. Individuals in STRUCTURE plot below the spatial figure are organized relative to their taxonomic grouping with reference wolf populations listed with horizontal labels and Isle Royale founders located on the far right with their respective location of origin listed vertically. *Indicates two Michipicoten wolves used as the representatives for the Michipicoten group translocated to Isle Royale (see methods for details)

founders translocated from Michipicoten Island (Table 4). Although average pairwise relatedness was 0.46 for founders translocated from Michipicoten Island, ON, a single pairwise relatedness value of 0.0 can be observed

in Fig. 3 for this group, which is the estimated pairwise relatedness between the two inferred parents (ISRO-009 and ISRO-014). All other ISRO founders had no shared ancestry when defining discrete relationships.
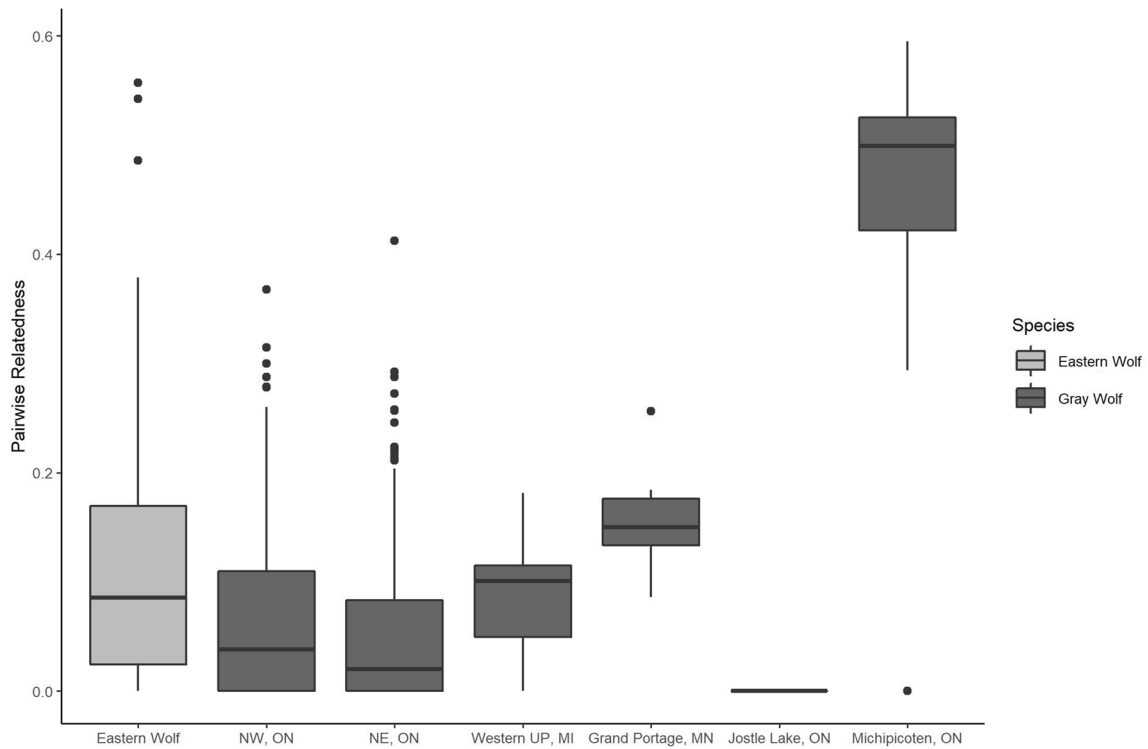
Fig. 3 Pairwise relatedness calculated using MLRELATE within reference populations of eastern wolf, northwestern gray wolf (NW,ON), and northeastern gray wolf (NE,ON) as well as pairwise relatedness within Isle Royale founders grouped by location of origin before translocation. Note Jostle Lake, ON only contained two individuals resulting in a single pairwise relatedness observation

Table 4 Parental assignments to Isle Royale founders using a maximum likelihood approach in program COLONY

ID	Source Location	Father	Mother	Probability
ISRO-001	Grand Portage, MN			
ISRO-003	Grand Portage, MN			
ISRO-004	Grand Portage, MN			
ISRO-006	Jostle Lake, ON			
ISRO-007	Michipicoten, ON	ISRO-009	ISRO-014	1.00
ISRO-008	Grand Portage, MN			
ISRO-009	Michipicoten, ON			
ISRO-010	Michipicoten, ON	ISRO-009	ISRO-014	1.00
ISRO-011	Michipicoten, ON	ISRO-009	ISRO-014	1.00
ISRO-012	Michipicoten, ON	ISRO-009	ISRO-014	1.00
ISRO-013	Michipicoten, ON	ISRO-009	ISRO-014	0.99
ISRO-014	Michipicoten, ON			
ISRO-015	Michipicoten, ON	ISRO-009	ISRO-014	1.00
ISRO-016	Jostle Lake, ON			
ISRO-017	Western Upper Pennisula, MI			
ISRO-018	Western Upper Pennisula, MI			
ISRO-019	Western Upper Pennisula, MI			
ISRO-020	Western Upper Pennisula, MI			

Each Isle Royale founder is listed followed by their inferred mother and father, and the probability of the parental pair assignment to the Isle Royale founder. Blank cells represent no inferred parent to the Isle Royale founder
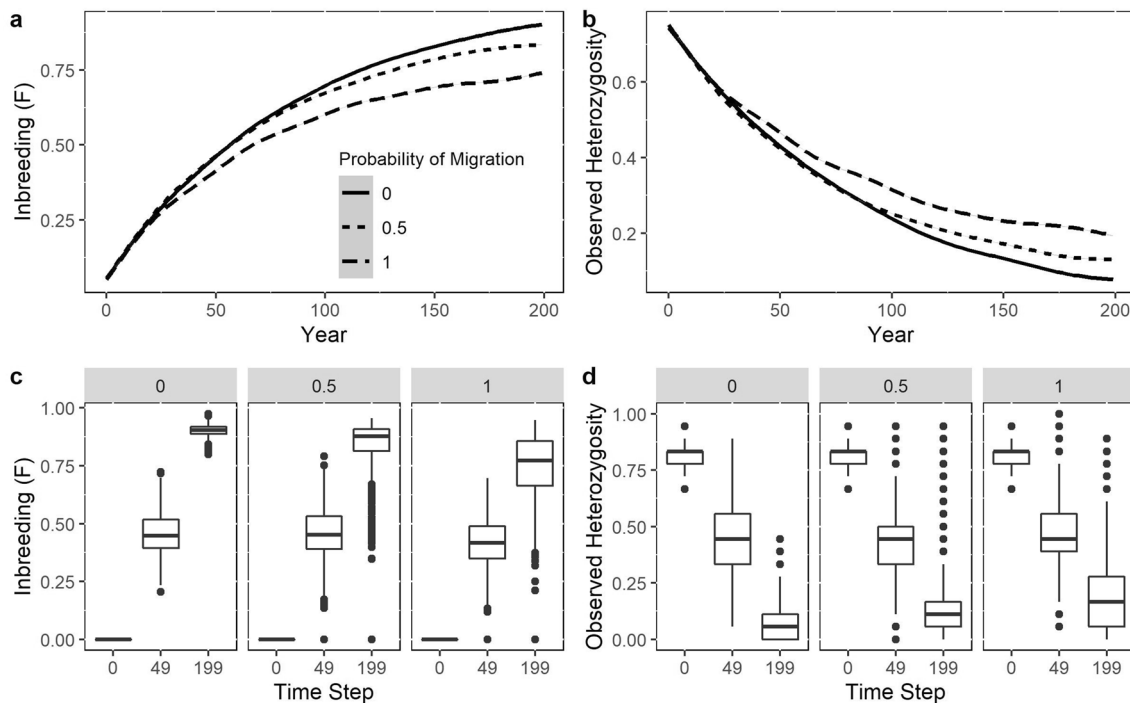
Projected Inbreeding and Heterozygosity

The projection of F over 200 years increased, while H_o decreased over the same period regardless of the probability of migration (Fig. 4a, b). The 50-year projection of average F for different probabilities of migration was significant ($\chi^2 = 431.68$, df = 2, p-value < 0.001), where treatment groups 0.5 and 0.0 (p-value = 0.3) did not differ, but all others did (both p-values < 0.001). The average difference of F at the 50-year time step between probability of migration treatment groups 0.5–0.0, 1.0–0.0, and 1.0–0.5 were 0.002, 0.047, 0.045, respectively. Average H_o was also significant for the probability of migration treatment groups ($F_{(2, 13472)} = 106.30$, p-value < 0.001), where again the treatment group with a probability of 1.0 significantly differed from other treatments (both p-values < 0.001), but treatment 0.5 did not significantly differ from 0.0 (p-value = 0.42, Fig. 4d). Average differences of H_o between treatment groups 0.5–0.0, 1.0–0.0, and 1.0–0.5, were 0.003, 0.036, 0.039, respectively.

The 200-year projection of average F was significantly different across different treatment groups for probability of migration ($\chi^2 = 4621$, df = 2, p-value < 0.001), and a pairwise Wilcox test confirmed all treatment groups significantly differed from each other (all pairwise comparisons had p-values < 0.001). The average difference of F between treatment groups 0.5–0.0, 1.0–0.0, and 1.0–0.5, were 0.068, 0.163, 0.095, respectively. The average 200-year projection of H_o by probability of migration group was also significant ($F_{(2, 13487)} = 1155$, p-value < 0.001) and a Tukey multiple pairwise comparison confirmed all treatment groups differed significantly (all p-values < 0.001). The average difference of H_o between treatment groups 0.5–0.0, 1.0–0.0, and 1.0–0.5, were 0.057, 0.122, 0.065, respectively.

Discussion

The previous ISRO population of gray wolves provided an extensive understanding of the negative effects associated with inbreeding and loss of genetic diversity (e.g. Räikkönen et al. 2009; Robinson et al. 2019). As the ISRO wolf population faced extirpation, the management decision was made to translocate wolves back to the island with the goal of restoring predation to the ISRO ecosystem (NPS 2018). Previous restoration efforts of inbred populations in the wild have experienced success, including the Florida panther (*Felis concolor coryi*) (Land et al. 1999), adders (*Vipera berus*)

Fig. 4 Projected inbreeding and observed heterozygosity for Isle Royale wolves with 100 replications for each migration treatment (see supplementary file for full description of simulation parameterization). Projected levels of inbreeding **a** and observed heterozygosity **b** over 200 years assuming the annual probability of migration of one animal to the island was 0.0, 0.5, and 1.0 plotted using a smooth-ing function. Box and whisker plots for inbreeding **c** and observed heterozygosity **d** for the starting population of Isle Royale founders (year = 0), 50 years (year = 49), and 200 years (year = 199) under varying treatments for the probability of migration to the island (0.0, 0.5, and 1.0)

of southern Sweden (Madsen et al. 1999), and the greater prairie chicken (*Tympanuchus cupido pinnatus*; Bouzat et al. 2009).

Although relocation success is possible, wildlife populations still face increased habitat fragmentation, making the ability to restore and maintain genetic diversity within mainland populations more difficult, but also making ISRO's isolated setting more applicable to these mainland systems when establishing methods to monitor and mitigate the effects of inbreeding depression. To that end, rarely do researchers have the opportunity to study the founding individuals of a population. To capitalize on this unique opportunity, we identified and reported on the genetic diversity and population structure of ISRO founders. By understanding each wolf's genetic composition, we can establish a baseline for assessing genetic diversity through time.

We sought to characterize the genetic diversity of the new ISRO founders compared to other canid populations of the Great Lakes region and lay the groundwork for long-term genetic monitoring. Importantly, the ISRO founders have comparable estimates of heterozygosity and allelic richness to all three reference populations. Interestingly, we observed an excess of H_o relative to H_E in the ISRO founders, which is surprising given the pooling of slightly differentiated populations should lead to an excess of H_E not H_o. This observation could be an artifact of small population size ($n = 13$) and/or potentially a subset of loci not amplifying certain alleles.

No private alleles were identified for the ISRO founders and eastern wolves contained many private alleles relative to other populations studied here. However, three populations of gray wolves should contain greater overlap in unique alleles while the eastern wolves we examined came from a single population. When relocating wildlife, especially large carnivores, it is difficult or sometimes impossible to strategically select individuals that will promote genetic variation in the new founding population, unless drawing from a captive population (Hedrick and Fredrickson 2008). However, here we demonstrated that a variety of metrics to assess genetic variation of ISRO founders suggests they were similar to reference populations. To ensure these metrics of genetic variation hold true, future studies could assess genetic load or recessive deleterious traits in the ISRO founders, which could be high despite similar measures of genetic diversity in reference populations. These are important parameters that will need to be evaluated with genome level data.

Previous studies have suggested that Great Lakes wolves, the source of all of the ISRO founders, are gray wolf/eastern wolf hybrids (Wheeldon et al. 2010; Wheeldon and White 2009), thus we wanted to explicitly compare an eastern wolf reference population against all ISRO founders to note any admixture. However, after accounting for the family group within the ISRO founders translocated from Michipicoten Island, the population structure of the founder's clustered with reference populations assigned as gray wolf with no observed admixture from eastern wolves or eastern coyotes.

ISRO founders originating from Jostle Lake, ON and western Upper Peninsula, MI had little shared relatedness within their groups. Founders translocated from Grand Portage had a higher than average pairwise relatedness when compared to gray wolf reference populations and other translocated wolf groups (excluding Michipicoten wolves). However, their average pairwise relatedness was 0.16 (less than half siblings).

ISRO founders from Michipicoten Island contained high pairwise relatedness values indicating that these eight wolves were part of a single family-group. Specifically, the family-group translocated from Michipicoten Island, ON contained a mother, a father, and their six offspring of varying ages. It is not surprising that wolves from Michipicoten Island share close relation to one another given they were from a small, closed population that descended from a single founding event. If family groups are maintained during translocations, their survivorship may be higher (Fritts et al. 1985; Bradley et al. 2005; Reading et al. 2013), increasing the chance of survival for ISRO founders translocated from Michipicoten Island. Including family groups is considered a viable strategy where inbreeding is not a concern, but the isolated population of ISRO presents a unique paradox where two contradicting goals exist: maximize relocation success and maximize genetic variation retained through time. Other wolf reintroduction projects have strategically relocated family groups with high success, while increasing heterozygosity and maintaining low inbreeding depression, but these were not island systems (vonHoldt et al. 2008). The current population of ISRO founders appear genetically diverse, but if ISRO founders of the same family group mate, the loss in heterozygosity and allelic richness will be reduced at a greater rate than if nonrelated wolves mate.

We confirmed this observation by explicitly simulating how the level of F and H_o will change over the next 50 and 200 years assuming varying levels of migration to the island. Interestingly, at the 50-year mark, we observed very little separation between values of F and H_o for varying probabilities of migration, which is likely due to values of F and H_o not yet reaching their asymptote (see supplementary ODD file for additional migration treatment groups). However, at the 200-year mark, in the absence of migration, the rate of F increases, and H_o decreases at a substantially greater rate relative to when migration is present (Fig. 4a, b). We chose not to incorporate kin avoidance during female mate selection, but if it were present, we may see larger and earlier differences of H_o and F at different migration treatment groups given migrant male wolves would have a higher probability of being selected as a mate relative to the other males highly related to female alphas. The purpose of our projected

results is not to designate when we should expect to intervene with this population, but to demonstrate that the loss of genetic variation and increase in inbreeding is inevitable if immigration does not occur. Also, our projection is likely optimistic, given we did not incorporate inbreeding depression into our simulation (but see supplementary ODD file). Documented immigration events have occurred in the ISRO population over monitored years (Hedrick et al. 2014). However, ice bridges forming between ISRO and the mainland are projected to decrease into the future (Licht et al. 2015).

Island species are more susceptible to extirpation where inbreeding is thought to play a role (Frankham 1998) and history has demonstrated ISRO wolves are no exception (Hedrick et al. 2019). Conservation efforts for isolated populations should ensure genetic monitoring is considered to inform when management action is required. In the era of high throughput sequencing, the opportunity to study functional genomic health is increasing and future studies should incorporate monitoring that goes beyond baseline estimates of genetic variation (e.g. Kleinman-Ruiz et al. 2017). The current ISRO wolf population acts as a unique experimental opportunity to study the persistence or demise of genomic-scale health and will provide a framework of restoration for inbred populations. Success of previous restoration efforts in other wildlife populations and increased use of novel genomic-scale methods (e.g. Campbell et al. 2015) bode well for informing management, and if needed, future restoration of the ISRO wolf population. Monitoring the genetic fitness of ISRO wolves will help inform the timing of potential relocation efforts, if warranted for ensuring wolf persistence, and thus helping maintain the health of the ISRO ecosystem as a whole.

Conclusions

The original wolf population inhabiting Isle Royale National Park through 2017 succumbed to stochastic environmental events and severe inbreeding (Hedrick et al. 2019). We presented the first assessment of genetic diversity, population structure, and parentage analyses to inform management decisions relating to the sustainability of genetic diversity through time. The ISRO founders now inhabiting the island are genetically diverse and represent the gray wolves of the Great Lakes region. However, it is vital to monitor this population's genetic diversity through time to ensure its persistence given the likelihood of a precipitous increase in F and decrease in H_o without future immigration events and with the presence of a family group within one of the translocation groups. Future, studies should incorporate genome-wide data to understand diversity and monitor functional genes, assessing their impacts on phenotypes to inform if additional management actions will be necessary in the future.

Data availability Microsatellite data needed to replicate the analysis were deposited into Zenodo, https://doi.org/10.5281/zenodo.4677512

Code availability The Netlogo file containing the agent-based model, R code for reformatting data from the Netlogo simulation output and R code calculating inbreeding coefficients were deposited into Zenodo, https://doi.org/10.5281/zenodo.4677512

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval Ontario Ministry of Natural Resources & Forestry AUC permit no. 19-441.

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