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Genetic Diversity and Effective Population Size of the Endangered Crozet Killer Whales

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Genetic diversity and effective population size (N_e) are two key parameters involved in the long-term persistence of a population and, as such, are important metrics to assess the conservation status of a population (Frankham et al. 2010; Hoban et al. 2020; Lande and Shannon 1996). The population standing genetic variation (i.e., pre-existing genetic variation) is a basis for evolution and enables organisms to adapt to changing environmental conditions (Barrett and Schluter 2008; Frankham et al. 2010; Matuszewski et al. 2015; Reed and Frankham 2003). Furthermore, the risk of extinction of a population is greater for small effective population sizes, as it results in the loss of genetic diversity due to genetic drift and increases the risk of inbreeding depression (Gilpin and Soulé 1986; Hedrick and Kalinowski 2000; Kardos et al. 2023; Keller and Waller 2002). Events in the population demographic history, such as bottlenecks or founder events, can result in reductions of genetic diversity and in small effective population sizes (Allendorf et al. 2013; Garner et al. 2005). It has been estimated that an effective population size of at least 1000 effective breeders is required to ensure long-term persistence and sufficient adaptive potential for a given population, while an effective population size > 100 individuals should prevent negative effects due to inbreeding (Frankham 1995; Frankham et al. 2014).

The killer whale (*Orcinus orca*) is a cosmopolitan apex predator whose populations rarely include more than a few 100 individuals and, therefore, can be subject to erosion of genetic diversity and risks of extinction (Foote et al. 2023; Ford et al. 2018; Hoelzel et al. 2002; Kardos et al. 2023; Parsons et al. 2013). These populations can show large variation in their habitat, level of specialization in their feeding preferences and behavior,

but also in their demographic trajectories and the types and levels of threats to which they are exposed (De Bruyn et al. 2013; Foote et al. 2016; Ford et al. 1998; Hoelzel et al. 2007; Jourdain et al. 2024; Morin et al. 2010; Riesch et al. 2012). In particular, over the past decades, some populations have greatly declined due to decreases in prey availability and/or negative interactions with humans (Beck et al. 2014; Ford et al. 2011, 2018; Guinet et al. 2015; Tixier et al. 2017), while others have shown significantly positive trends (Matkin et al. 2014; Towers et al. 2015).

Two morphologically, ecologically, and genetically distinct forms of killer whales, the so-called “Crozet killer whales” (Figure 1) and “Type D killer whales,” are encountered around the Crozet archipelago (French subantarctic islands) (Amelot et al. 2022; Tixier et al. 2016). While type D killer whales have only been recognized as a distinct ecotype in the last ~15 years (Pitman et al. 2010, 2019) and their population trend is unknown, 50 years of photo-identification monitoring data have shown a 60% decline of the Crozet killer whales over the last 30 years (Amelot et al. 2022; Guinet et al. 2015; Poncelet et al. 2009; Tixier et al. 2017, 2021). This decline is likely due to both prey limitations and negative interactions with fishers operating illegally in the area and using lethal means (firearms or explosives) to repel the whales depredating on their catch (Guinet et al. 2015; Tixier et al. 2017). While this raises concern about potential risks of extinction of the population, which is estimated to number between 89 and 94 individuals in 2020 (Tixier et al. 2021), the genetic delineation of the population is still unclear. For instance, movements of individuals and social groups have been documented between the Crozet islands and the adjacent Prince Edward and Marion islands, where killer whales of

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FIGURE 1 | Crozet killer whales surfacing (Tixier).

the same form are monitored (hereafter referred to as “Marion killer whales”), suggesting potential genetic connectivity and a larger population encompassing both areas (Jordaan et al. 2019, 2023; Jourdain et al. 2024; Tixier et al. 2021).

The present study aimed at assessing the genetic diversity, inbreeding level, and effective population size of the Crozet killer whale population to evaluate its risk of extinction using multi-locus genotypes obtained from 31 individuals at 19 microsatellite loci.

Killer whale skin samples were obtained through skin biopsy collection from free-ranging individuals from fishing vessels operating legally around the Crozet islands and the shores of Île de la Possession between 2011 and 2024. Individuals > 2 years old were biopsied remotely in the medio-lateral region of the body below the dorsal fin using a crossbow and sterilized stainless-steel darts when surfacing within a 15-m range of the vessel or the shore (Reisinger et al. 2014). Individuals were identified and then sampled based on an existing photo-identification catalogue (Tixier et al. 2021). The samples were stored in 70% ethanol until used for genetic analyses.

Genomic DNA was extracted from approximately 25 mg of skin tissue from 31 individuals using the extraction protocol of genomic DNA from the Macherey-Nagel NucleoSpin Tissue kit. Three samples that were difficult to genotype due to lower DNA quality were re-extracted using the New England BioLabs Monarch Genomic DNA Purification kit. DNA concentrations were determined by absorbance using the Nanodrop8000 Spectrophotometer and normalized to 20 ng/μL for samples with higher concentrations. Sex was determined following Rosel (2003) by amplifying a 382 base-pair fragment on the ZFX region of the X chromosome in both sexes and a 339 base-pair fragment on the SRY region of the Y chromosome in males only. Nineteen microsatellite loci were amplified (Tables 1 and S1) for each sample in 15 μL of reaction volume containing 1× Promega GoTaq reaction buffer (containing 1.5 mM MgCl₂), 200 μM of dNTP, 0.8 U of Promega GoTaq DNA Polymerase, 0.2 μM of each primer, and 1 μL (≈20 ng) of sample DNA, the rest of the volume consisting of ultra-pure water. The PCR profile used was as follows: 30 s at 94°C; followed by 33 cycles of 20 s at 94°C, 30 s at T_a°C (Table S1) and 30 s at 72°C; then a last extension of 10 min at 72°C. PCR products were then analyzed using a Thermo Fisher

Scientific SeqStudio Genetic Analyzer and Applied Biosystems ABI LIZ500 as an internal size standard. The PeakScanner software (Applied Biosystems) was used on the Thermo Fisher Connect web interface for genotyping. All genotypes were determined independently by two different readers.

Replicates of four of the 31 samples (DNA obtained from individuals biopsied multiple times) were included in the dataset and were genotyped in order to check the genotyping consistency. Moreover, the standard probability of identity (PI) and the probability of identity for siblings (PI_{sib}) (Waits et al. 2001) were estimated using GenAEx version 6.5 (Peakall and Smouse 2012). These values, respectively, illustrate the probability that two randomly selected individuals in the population and that two related individuals in the population have identical genotypes. Genetic diversity was estimated after the removal of the replicates. Number of alleles per locus (Na), allelic richness (AR), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) were obtained using FSTAT version 2.9.4 (Goudet 1995) and GenAEx. A probability test of a departure from the Hardy–Weinberg equilibrium (HWE) was performed using GENEPOP version 4.7 with the Markov Chain Monte Carlo method (10,000 iterations) for each locus and for the whole set of markers (Raymond and Rousset 1995). The effective population size was calculated using the linkage disequilibrium method with NeESTIMATOR version 2.0 (Do et al. 2014) in order to get a short-term estimate of the effective population size, that is, on the scale of the last few generations.

Molecular sexing led to the identification of 20 females (13 reproductive, 5 post-reproductive, and 2 juveniles) and 11 males (3 adults, 4 subadults, and 4 juveniles). The age and the reproductive status were either determined or estimated from the long-term monitoring of individuals by photo-identification based on the methodology and life history parameters used for killer whales in the north-eastern Pacific (Tixier et al. 2021; Towers et al. 2019, 2020). All individuals were unambiguously identified from their multi-locus genotypes, even when they were siblings, given the low standard probability of identity and probability of identity for siblings (1.10⁻¹⁶ and 4.10⁻⁷, respectively).

Crozet killer whales showed high levels of genetic diversity when compared to killer whales from other populations using similar microsatellite markers such as North Pacific resident and transient (or “Bigg’s”) killer whales (Parsons et al. 2013) as well as Icelandic killer whales (Tavares et al. 2018). Indeed, they showed the highest levels of expected and observed heterozygosity and among the highest allelic richness alongside the Pacific transients (Table 2). None of the loci showed a significant departure from HWE ($p > 0.05$), and little difference between expected (0.649) and observed heterozygosity (0.677) led to negative values of the F_{IS} for most loci and an overall value of -0.043, reflecting a non-significant excess of heterozygosity among Crozet killer whales for this set of microsatellite markers (Table 1). The estimated contemporary effective population size (N_e) of the Crozet killer whales was 53 (95% CI [40–74]) breeders (Table 3), which is consistent with the number of reproductive individuals estimated at 48 using observation data (Tixier et al. 2021). The estimated N_e represents about 0.59 of the census size of around 90 individuals frequently observed in Crozet waters as estimated through photo-identification monitoring in Tixier et al. (2021).

TABLE 1 | Summary of genetic diversity measures for 19 microsatellite markers, based on the 31 genotypes obtained from biopsied Crozet killer whales.

Locus	Citation	Number of alleles	Allelic richness	Expected heterozygosity	Observed heterozygosity	Inbreeding coefficient	HWE departure probability test (p-val)
<i>Dde66</i>	Coughlan et al. 2006	5	4.968	0.641	0.677	-0.057	0.581
<i>Dde70</i>	Coughlan et al. 2006	7	6.967	0.436	0.484	-0.110	0.923
<i>Dde72</i>	Coughlan et al. 2006	7	6.968	0.760	0.774	-0.019	0.166
<i>EV37</i>	Valsecchi and Amos 1996	11	10.935	0.748	0.774	-0.035	0.866
<i>FCB17</i>	Buchanan et al. 1996	8	7.967	0.806	0.871	-0.081	0.936
<i>FCB5</i>	Buchanan et al. 1996	8	7.967	0.779	0.806	-0.035	0.219
<i>GATA53</i>	Palsbøll et al. 1997	4	4.000	0.579	0.733	-0.267	0.383
<i>KMW12a</i>	Hoelzel et al. 1998	9	8.967	0.092	0.097	-0.051	0.627
<i>KW199</i>	Ford et al. 2011	2	2.000	0.530	0.581	-0.096	1.000
<i>KW4</i>	Ford et al. 2011	6	5.967	0.858	0.774	0.098	0.724
<i>MK5</i>	Krützen et al. 2001	6	6.000	0.659	0.867	-0.314	0.212
<i>MK9</i>	Krützen et al. 2001	4	4.000	0.564	0.433	0.231	0.197
<i>Ttr04</i>	Rosel et al. 2005	4	4.000	0.560	0.549	0.020	0.210
<i>Ttr11</i>	Rosel et al. 2005	6	5.999	0.689	0.774	-0.124	0.868
<i>Ttr34</i>	Rosel et al. 2005	4	4.000	0.570	0.613	-0.076	0.859
<i>Ttr48</i>	Rosel et al. 2005	7	6.935	0.775	0.774	0.001	0.140
<i>TtruAAT44</i>	Caldwell et al. 2002	7	6.935	0.720	0.613	0.149	0.067
<i>TtruGT39</i>	Caldwell et al. 2002	7	6.999	0.794	0.839	-0.056	0.817
<i>TtruGT48</i>	Caldwell et al. 2002	7	7.000	0.771	0.833	-0.081	0.827
Overall	—	6.263	6.241	0.649	0.677	-0.043	0.744

Note: Number of alleles refers to the alleles found in the present study.

This ratio is greater than those estimated for southern resident and transient killer whale populations in the North Pacific, with effective size/census size (N_e/N) ratios of approximately 0.36 and less than 0.25 respectively, and well above the N_e/N ratio of less than 0.05 observed in Alaskan resident killer whales (Kardos et al. 2023) (Table 3). Such high levels of allelic richness, heterozygosity, and N_e/N ratio observed for a small and declining cetacean population (Vachon et al. 2018) could corroborate the hypothesis that gene flow occurs between Crozet and neighboring killer whales such as Marion killer whales, which have been

observed in Crozet's waters and vice-versa (Jordaan et al. 2019; Tixier et al. 2021). Crozet and Marion killer whales have similar ecologies. However, the individuals monitored in coastal waters of Marion Island show higher survival and population growth rates than the Crozet individuals, and among other potential factors, this could be explained by a lower exposure of Marion killer whales to the negative impacts of interactions with Patagonian toothfish fisheries than that of Crozet killer whales (Amelot et al. 2022; Guinet et al. 2015; Jordaan et al. 2020, 2023; Jordaan et al. 2019; Jourdain et al. 2024; Tixier et al. 2017). Furthermore,

TABLE 2 | Comparison of the overall genetic diversity measures of the Crozet killer whales with other killer whale populations.

Microsatellites	Crozet killer whales	Resident killer whales	Transient (Bigg's) killer whales	Icelandic killer whales (average of the three genetic units)
	Present study	Based on Parsons et al. (2013)		Based on Tavares et al. (2018)
<i>n</i>	31	263	143	61
<i>NA</i>	6.263	4.000	6.769	3.387
<i>AR</i>	6.241	3.647	6.701	3.253
<i>H_o</i>	0.677	0.441	0.597	0.590
<i>H_e</i>	0.649	0.479	0.647	0.480
<i>F_{IS}</i>	-0.043	0.113	0.075	-0.233

Abbreviations: AR: allelic richness, FIS: inbreeding coefficient, He: expected heterozygosity, Ho: observed heterozygosity, *n*: Sample size, Na: number of alleles.

TABLE 3 | Comparison of the estimated contemporary effective population size (N_e) with a 95% confidence interval ([95% CI]) of the Crozet killer whales with other killer whale populations.

	Crozet killer whales	Southern resident killer whales	Alaskan resident killer whales	Transient (Bigg's) killer whales
	Present study	Based on Kardos et al. (2023)		
<i>N</i>	89–94	70–80	> 700	> 350
N_e [95% CI]	53 [40–74]	27 [25–35]	39 [25–115]	86 [60–185]

Note: Census population sizes (*N*) were based on Tixier et al. (2021) for Crozet killer whales, Carretta et al. (2023) for southern resident killer whales, Matkin et al. (2014) for Alaskan resident killer whales, and Towers et al. (2019) for transient (Bigg's) killer whales.

despite a high level of photo-identification observation effort in both areas, there does not appear to be an increase in the number of Crozet killer whales observed around the Prince Edward and Marion islands or Marion killer whales observed around the Crozet archipelago over time (Jordaan et al. 2019; Tixier et al. 2021). Together, these observations suggest demographic independence between Crozet and Marion killer whales, without excluding potential gene flow between killer whales of the two adjacent areas. If occasional gene flow is occurring, the estimated N_e of 53 breeders could refer to a larger population than the killer whales frequently observed in Crozet, implying that the corresponding genetic population would not only include frequently observed individuals based on the Crozet photo-identification catalogue (Tixier et al. 2021). Gene flow could be mediated by adult males mating with females belonging to different social units, which is common in large odontocete species (Barrett-Lennard 2000; Cassens et al. 2005; Engelhaupt et al. 2009; Frère et al. 2010; Hoelzel et al. 2007; Möller and Beheregaray 2004; Pilot et al. 2010). Connectivity and dispersal data among killer whales observed around subantarctic islands are needed to better understand the geographical extent of our estimate of N_e (i.e., whether this estimate corresponds to the local population or rather a larger one including Marion killer whales) and to appropriately interpret the N_e/N ratio obtained here for the Crozet killer whales (Hare et al. 2011; Hoelzel et al. 2007; Kalinowski and Waples 2002; Wang and Whitlock 2003; Waples 2005; Whitlock and Barton 1997).

Levels of genetic diversity observed for the Crozet killer whales suggest that the population has maintained adaptive potential. High levels of genetic diversity were also observed

in Australasian killer whales, especially in the Southwestern Australia population, likely due to admixture and Antarctic-like ancestry that helped maintain high N_e and levels of genetic diversity, as well as population fitness and adaptive potential (Reeves et al. 2025). Whole genome analyses indicate that Crozet and Western Australia killer whales display highly similar evolutionary trajectories with stable long-term N_e , which could explain such levels of genetic diversity (Foote et al. 2021). Moreover, the N_e/N ratio of 0.59 is well above the average ratio recorded for wild populations of any taxon studied, between 0.1 and 0.2 (Frankham 1995; Palstra and Ruzzante 2008), and is greater than ratios generally observed in mammal populations (Clarke et al. 2024). Demographic stochasticity and a population decline as observed for the Crozet killer whales can increase N_e/N ratios; however, consequences from these demographic events are difficult to predict as they will depend on their effects on reproductive variance (Palstra and Ruzzante 2008), especially since our estimate of N_e corresponds to the parental generation, and not to the generation actually sampled (Hare et al. 2011; Waples 2005). Thus, it will be important to obtain a new estimate of N_e in 20–30 years to evaluate whether a further decline in the number of breeders is observed. However, the current estimate of contemporary N_e is nearly half the minimum of 100 effective breeders assumed to be needed to avoid negative effects of inbreeding in future generations and to ensure long-term population viability (Frankham 1995; Frankham et al. 2014). It would also be necessary to complement the present results obtained from neutral markers by a genome-wide estimation of genetic diversity, which would inform on the amount of functional genetic diversity that natural selection could act on, to bring a better understanding of how

well the Crozet killer whales could potentially adapt to changing environmental conditions.

These results highlight the need for conservation actions to prevent any additional human-caused mortality of killer whales of the Crozet archipelago, whose number of individuals remains low. Indeed, the combination of the decline in the number of individuals and the erosion of the genetic diversity due to a low N_e could lead to the extinction of the population (Gilpin and Soulé 1986).

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Ethics Statement

This project operated under animal ethics approval from the French Government (APAFIS permit #33165) and from the Scientific Committee of the French National Nature Reserve of the French Southern Lands.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the [Supporting Information](#) of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1.**