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Disease induced changes in gene flow patterns among Tasmanian devil populations

Anna Brüniche-Olsen [a,*], Christopher P. Burridge [a], Jeremy J. Austin [b,c], Menna E. Jones [a]

[a] School of Zoology, University of Tasmania, Private Bag 5, Hobart 7001, Tasmania, Australia
[b] Australian Centre for Ancient DNA, School of Earth & Environmental Sciences, University of Adelaide, Darling Building, North Terrace Campus, South Australia 5005, Australia
[c] Sciences Department, Museum Victoria, Carlton Gardens, Melbourne, Victoria, Australia

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ABSTRACT

Infectious diseases of wildlife reduce population size and may erode genetic diversity, constituting an extinction threat. The Tasmanian devil (*Sarcophilus harrisii*) is threatened with extinction by an infectious cancer, the devil facial tumour disease (DFTD). In less than two decades, DFTD has caused a more than 85% overall population decline. We used ten polymorphic microsatellite loci to quantify the effects of this decline on genetic diversity, population differentiation, effective population size, and gene flow. Samples from 1999 and 2006 at five locations were analysed, three of which had been affected by DFTD during this time interval. Significant increases in inbreeding coefficient (f) and non-significant reductions in effective population size were observed for both diseased and non-diseased populations, and therefore there was no consistent effect of DFTD. There was significant but stable structuring of genetic variation among locations through time, although a dynamic "source-sink" relationship was evident for gene flow associated with disease-mediated changes in population densities. These changes in gene flow may have contributed to the maintenance of genetic diversity in disease-affected areas. Simulations suggest that the estimated population declines, although severe, have been insufficient to yield significant changes in genetic variation; this may have been exacerbated by disequilibrium between population sizes and genetic diversity at the time of DFTD emergence, owing to elevated devil abundances following the extinction of the previous apex predator—the thylacine—approximately 80 years ago.

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1. Introduction

Emerging infectious diseases of wildlife are recognized as a significant threat to global biodiversity (Smith et al., 2009). They have been shown to reduce population size (N) (Robinson et al., 2010), alter spatial genetic population structure (Lee et al., 2010), population age structure (Lachish et al., 2009), life-history parameters (Jones et al., 2008), and dispersal patterns (Hurtado, 2008), all factors that can compromise a species' long-term survival (Frankham, 2005). Low genetic diversity is common in threatened species (Spielman et al., 2004) and is often caused by population reductions driven by factors such as habitat degradation or fragmentation, over-hunting, or climate change (Chiucchi and Gibbs, 2010; Hansen et al., 2009; MacDonald et al., 2008). The genetic consequences of these population declines may compromise species' resistance to disease (Altizer et al., 2003; Lee et al., 2010), which in turn, can lead to further reductions in population size and genetic diversity.

The world's largest marsupial carnivore, the Tasmanian devil (*Sarcophilus harrisii*), is currently facing extinction threat caused by the transmissible cancer, devil facial tumour disease (DFTD). DFTD is derived from Schwann cells in the peripheral nervous system (Murchison et al., 2010) and is restricted to a single host, the Tasmanian devil. It manifests as facial tumours and is consistently fatal, usually within 6 months of infection (Jones et al., 2008; Lachish et al., 2007). Cancers are not usually infectious—they typically arise and die within a single host (Hanahan and Weinberg, 2000)—and the only other known case in which live tumour cells are infectious is canine transmissible venereal tumour (Murchison, 2009). Intimate, injurious contact is the route for transmission of live tumour cells (McCallum and Jones, 2012; Murchison, 2009). Genetic diversity was low in Tasmanian devils prior to the emergence of DFTD for both the functional MHC genes (Cheng et al., 2012; Morris et al., 2013; Siddle et al., 2007), and for mitochondrial- and nuclear loci (Jones et al., 2004; Miller et al., 2011; Siddle et al., 2010). Reduced MHC diversity preceded the isolation of Tasmania with sea level rise ~13,000 years ago (Morris et al., 2013; Siddle et al., 2013). Although low genetic diversity may have played a role in the evolution of transmissibility, both the devil and the dog cancers have evolved sophisticated mechanisms to evade the immune

* Corresponding author. Tel.: +61 3 6226 2866; fax: +61 3 6226 2631.
E-mail address: annabo@utas.edu.au (A. Brüniche-Olsen).

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system of the host, involving down-regulation of MHC expression in the tumour (Fassati and Mitchison, 2010; Murgia et al., 2006; Siddle et al., 2013).

Currently, the spread of DFTD is reducing devil population size (DPIWE, 2012; Hawkins et al., 2006; McCallum et al., 2007), but with unknown effects on its already low genetic diversity. Since DFTD was first detected in 1996 at Mt. William National Park in north-eastern Tasmania, it has spread to the majority of the species' range, causing more than 85% overall population decline, with local declines in excess of 95%, contributing to "Endangered" listing status (Hawkins et al., 2006; IUCN, 2010; McCallum et al., 2007). DFTD transmission is strongly frequency dependent (McCallum et al., 2009, 2007), creating a risk of disease-driven extinction because transmission is sustained even at very low population densities through the requirement of contact for reproduction (McCallum, 2008; Smith et al., 2009). However, geographic spread of DFTD is likely to be slower than gene flow, as adults (which are the predominant infectious host) are restricted to a home range, while juveniles (which are seldom infected with DFTD) move greater distances away from their natal site to establish their own territory (Jones et al., 2008). Natal dispersal in devils tends to be male biased, perhaps driven by inbreeding avoidance, a pattern common in most carnivores (Gachot-Neveu et al., 2009; Goltsman et al., 2005).

Previous studies of genetic diversity in the Tasmanian devil have either concentrated on spatial variation (Jones et al., 2004; Miller et al., 2011; Siddle et al., 2010), or have tested for temporal changes at diseased sites without comparison to control sites that remained uninfected during the same interval (Lachish et al., 2011). In this paper, we investigate changes in genetic diversity through time at sites that became DFTD infected, relative to changes at sites that did not. We ask the following specific questions: (i) Does genetic diversity change through time and could any changes be attributable to DFTD-induced population declines? (ii) Does genetic population structure, sex-biased dispersal and population connectivity change over time and with respect to DFTD mediated changes in population density? (iii) Has DFTD had a detectable effect on effective population size (N_e)? We interpret our results with respect to the future conservation—and particularly the genetic management—of this species.

2. Materials and methods

2.1. Study area and samples

Ear biopsies from Tasmanian devils were collected in 1999 ($n = 213$) and 2006–2007 ($n = 212$) at five locations (Marrawah, Narawntapu National Park, the Freycinet Peninsula, Little Swanport, and Pawleena) in Tasmania (Fig. 1; total sample sizes in Table 1). Devils were trapped using 30 cm diameter PVC pipe traps baited with meat. Forty traps were set across 25 km^2 study areas at landscape locations that carnivores were likely to encounter during nightly movements, with the exception of Freycinet (60 traps in 100 km^2) and Marrawah (40 traps along a 40 km length of road). Traps were checked daily starting in the early morning. All devils were micro-chipped for individual identification and an ear biopsy taken from each individual. To minimize relatedness, only samples from adults (2 + years old) and independent sub–adults (1 year old) that were beyond natal dispersal age were included in analyses. To analyse the effects of DFTD, we sampled pre-disease and post-disease at Little Swanport, Freycinet, and Pawleena, where DFTD arrived in 1999, 2001, and 2002, respectively (Hawkins et al., 2006; McCallum et al., 2007). For non-diseased locations we surveyed Marrawah and Narawntapu over the same time interval. The first reported DFTD case in Narawntapu was in 2007, which is outside our sampling period, and Marrawah is presently disease-free. We also estimated gene flow using samples taken during 2006 from Dunalley ($n = 29$, DFTD arrival = 2004) and the Forestier Peninsula ($n = 75$, DFTD arrival = 2004). This provided a replicate to examine directionality of gene flow in adjacent populations with different disease effects (Dunalley exhibited substantial declines at the time of sampling, while Forestier exhibited minor declines; Fig. 1; McCallum et al., 2007). We will refer to the period "2006–2007" as "2006" throughout.

2.2. Genotyping

Each individual was genotyped for ten polymorphic microsatellite loci developed for Tasmanian devils following Jones et al. (2003): *Sh2v* (1), *Sh2p* (1), *Sh3o* (1), *Sh6l* (1), *Sh6e* (1), *Sh2i* (1), *Sh2g* (2), *Sh2l* (3), *Sh5c* (6), and *Sh3a* (not analysed), with assignment to each of $n = 7$ chromosomes given in parentheses. All samples from 1999 were genotypes taken from Jones et al. (2004). The 2006 samples from Marrawah and Narawntapu were genotyped during this study, as were subsets of the Freycinet ($n = 28$), Dunalley ($n = 8$) and Forestier ($n = 73$) samples. Remaining 2006 Little Swanport, Pawleena and subset-parts of the Freycinet ($n = 20$), Dunalley ($n = 21$) and Forestier ($n = 2$) samples where genotyped by Lachish et al. (2011). We did not re-genotype any samples.

2.3. Tests for genotyping errors and selection

Data were checked for scoring errors associated with allele stuttering, allele drop-out, and null alleles using MICRO–CHECKER ver. 2.2.3 (Van Oosterhout et al., 2004). Tests for selection was performed using the Bayesian approach in BAYESCAN ver. 2.01 (Foll and Gaggiotti, 2008), which is an extension of Beaumont and Balding's (2004) method. This allows differences in allele frequencies between populations and their ancestral common gene pool, and differences in F_{ST} to be interpreted in light of a demographic model where differences in effective population size and gene flow may contribute in addition to selection (Foll and Gaggiotti, 2008). Posterior odds (PO) are used to evaluate how much more likely the model with selection is compared to the neutral model, and allows the user to control the false-positives rate. BAYESCAN can handle small sample sizes, as it incorporates uncertainty in allele frequencies, without risk of estimate bias. A Reversible Jump Markov chain Monte Carlo (RJ-MCMC) algorithm was used to obtain posterior distributions of the degree of differentiation (F_{ST}) decomposed into a locus-specific component (α), shared by all populations, and a population-specific component (β), shared by all loci. Selection is assumed when α is necessary for explaining the observed pattern of diversity. We tested the two datasets (1999 and 2006) separately using sample size = 5×10^3, thinning interval = 10^2, pilot runs = 10^2, pilot run length = 10^4, and additional burn-in = 5×10^5. These are higher than the default settings, which normally ensure good convergence in most cases (Foll and Gaggiotti, 2008). Convergence of the RJ-MCMC was tested by comparing the sample means of an early segment (first 10% of the RJ-MCMC) and a later segment (last 50% of the RJ-MCMC) for significant deviation (Geweke, 1992), in R ver. 2.12.2 (2011) using the BOA package (Smith, 2007). We used PO of 10 and 100, corresponding to accepting a false-positive rate of 5% and <1%, respectively, to make decisions on whether a locus was under selection (positive or balancing) (Foll and Gaggiotti, 2008). The two PO values are interpreted as "substantial" and "very strong" evidence for selection, respectively (see BAYESCAN program notes for details). The PO values for each locus were calculated in R and outliers identified.

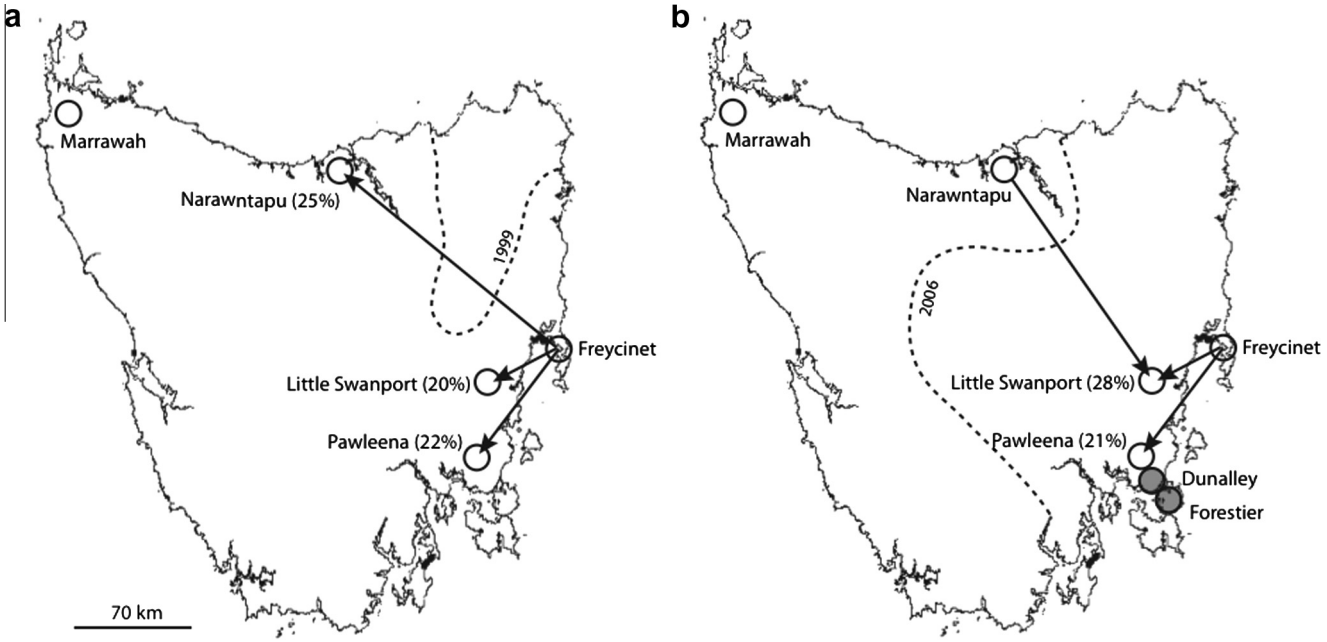

Fig. 1. Sampling locations and gene flow patterns among Tasmanian devil populations in (a) 1999, and (b) 2006. Gene flow directions are indicated with arrows and percentage of gene flow into the population is given in parentheses, and only rates ⩾10% are shown. On the 2006 map gene flow from Dunalley (heavily impacted at time of sampling) and Forestier Peninsula (weakly impacted at time of sampling) are included.

Table 1
Genetic diversity over time in Tasmanian devil populations based on 10 microsatellite loci.

Year	Population	DFTD arrival	N	A_O	H_O	UH_E	f
1999	Marrawah	–	40	2.8	0.404 (0.082)	0.393 (0.068)	−0.030
1999	Narawntapu	–	36	3.4	0.472 (0.046)	0.466 (0.044)	−0.014
1999	Freycinet	–	61	3.2	0.423 (0.069)	0.421 (0.066)	−0.006
1999	Little Swanport	–	37	3.3	0.436 (0.057)	0.427 (0.055)	−0.021
1999	Pawleena	–	39	3.3	0.414 (0.063)	0.423 (0.059)	0.023
2007	Marrawah	–	59	2.7	0.385 (0.066)	0.400 (0.068)	0.038[#]
2006	Narawntapu	2007	54	3.1	0.389 (0.048)	0.434 (0.053)	0.104[*,#]
2006	Freycinet	2001	48	3.1	0.404 (0.071)	0.391 (0.062)	−0.022
2006	Little Swanport	1999	29	3.1	0.428 (0.059)	0.455 (0.049)	0.062[#]
2006	Pawleena	2002	22	3.0	0.405 (0.054)	0.415 (0.055)	0.026

Estimates include: mean number of alleles per locus (A_O), observed heterozygosity (H_O), unbiased expected heterozygosity (UH_E,), and inbreeding coefficient (f). Standard errors are given in parentheses.
[*] Indicates values significantly different from zero ($P < 0.05$; Bonferroni corrected) based on 10^3 randomisations.
[#] Indicates significant difference ($P < 0.05$; Bonferroni corrected) in means among 1999 and 2006 samples.

2.4. Genetic diversity

Observed (H_O) and unbiased expected heterozygosities (UH_E) were estimated using GENALEX ver. 6.4 (Peakall and Smouse, 2006). Mean number of alleles per locus (A_O) was calculated using FSTAT ver. 2.9.3.2 (Goudet, 1995). Significance of deviations from expected genotype frequencies under Hardy Weinberg equilibrium, measured as the "inbreeding coefficient" f (Weir and Cockerham, 1984), were tested using GENODIVE ver.2.0b23 (Meirmans and Van Tienderen, 2004). Deviations from linkage disequilibrium (LD) were tested using GENEPOP ver. 4.0.10 (Raymond and Rousset, 1995). Sequential Bonferroni correction was applied for simultaneous tests (Whitlock and Schluter, 2009).

2.5. Population genetic structure

Population genetic structure was estimated as the fixation index θ (Weir and Cockerham, 1984) between populations and as Φ_{ST} among groups of populations via Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992; Michalakis and Excoffier 1996) in GENODIVE ver. 2.0b17. Significant differentiation was tested using 10^3 permutations of individuals among populations, with sequential Bonferroni correction for simultaneous tests. Population structure was also assessed using Bayesian clustering implemented in STRUCTURE ver. 2.3.3 (Pritchard et al., 2000). We performed analyses five times for each of clusters $K = 1–10$, using a burn-in of 10^4 iterations followed by a MCMC of 10^5 iterations, both with and without incorporating prior information on the source location of each individual. STRUCTURE HARVESTER ver. 0.6 (Earl and vonHoldt, 2011) was used to find the most likely number of clusters in the samples following the ΔK method (Evanno et al., 2005). CLUMPP ver. 1.1.2 (Jakobsson and Rosenberg, 2007) was employed to combine the five STRUCTURE run output files for each K using the "full search" method, and the results were displayed using DISTRUCT ver. 1.1 (Rosenberg, 2004).

2.6. Gene flow

Gene flow (m) was estimated using BAYESASS + ver. 1.3 (Wilson and Rannala, 2003) based on the disequilibrium among genotypes

in each population resulting from the presence of novel multi-locus genotypes introduced by migrants or individuals recently descended from migrants. BAYESASS+ requires fewer assumptions than long-term coalescent-based *m* estimates (i.e. MIGRATE; Beerli and Felsenstein, 2001), and can be applied to non-stationary populations that are far from Hardy–Weinberg Equilibrium. BAYESASS+ allows for arbitrary genotype frequency distributions within populations and is suitable for samples that are taken a few generations apart (Wilson and Rannala, 2003), which we interpret as up to five generations. MCMC commenced with a burn-in of 10^6 iterations followed by 4×10^6 iterations with sampling every 2×10^3 iterations. Delta values were set to 15 for allele frequencies, *m*, and the inbreeding coefficient. The program was run with two different "seed" values (10 and 10^3) to test for convergence in R using the BOA package, as described above. We also estimated gene flow for the 2006 samples from Dunalley and Forestier using the same settings as described above, as an additional replicate with which to examine directionality of gene flow in adjacent populations of different disease status (either disease versus not diseased, or minimal versus severe population decline from disease). We tested for temporal congruence among the estimated gene flow matrices with a Mantel test of 10^4 permutations in R, using the ADE4 package. We also applied the Mantel test to each temporal gene flow matrix and the geographic isolation of populations, measured as straight-line distance (km).

As devils exhibit sex-biased dispersal (Lachish et al., 2011), possible changes in sex biased dispersal patterns over time were investigated using the assignment procedure (Favre et al., 1997; Mossman and Waser, 1999) implemented in GENALEX. The genotype for each individual is compared to the expected genotype frequency—of the population in which it was sampled—based on the observed allele frequency distribution of the population. A negative corrected assignment value (AIc) indicates that the individual is most likely an immigrant. To evaluate whether the estimated dispersal (based on AIc values) differed over time and between sexes we used a linear mixed effects model. In this model, sex and time were fixed effects and population was the random effect, enabling us to account for any potential confounding variation among sites. Mixed effects analyses were undertaken using the package lmer in R, with the package pval.fnc utilized to compute *P*-values using 10^4 MCMC simulations. We also evaluated if sex ratio among our sites changed over time. This might be relevant in situations where sites became diseased at different times (e.g. Narawntapu; Little Swanport and Freycinet) and if patterns of sex-biased migration changed (Lachish et al., 2011). The sex-biased assignment test does not allow for missing data, so individuals that lacked data for one or more loci were omitted from the test. The following populations had individuals lacking data: Freycinet ($n_{1999} = 1$; $n_{2006} = 1$), Little Swanport ($n_{1999} = 3$) and Pawleena ($n_{1999} = 3$). No sex information was available for the majority of individuals from Dunalley, Forestier and Marrawah in 2006; these populations are therefore not included in the sex-biased dispersal test, and due to lack of temporal data from Marrawah this population was excluded from the analysis of temporal changes in AIc values for the sexes.

2.7. Effective population size and bottleneck tests

We used LDNe ver. 1.31 (Waples and Do, 2008) to estimate N_e for each population at each sampling time. LDNe assumes that a linkage disequilibrium signal arises only from genetic drift, and is suitable for samples of 30–50 individuals genotyped for 10–20 unlinked loci (Luikart et al., 2010). We used a model with random mating and excluded alleles with a frequency <0.02, which provides acceptable balance between precision of N_e estimates and possible biases (Waples and Do, 2010). 95% confidence intervals were generated by jack-knife resampling, which performs better than the parametric method (Waples and Do, 2008). Spearman's rank correlation (Whitlock and Schluter, 2009) was used to test for correlation among number of years DFTD presence and N_e.

Past reductions in N_e resulting in genetic bottlenecks were tested using two methods implemented in BOTTLENECK ver. 1.2.02 (Cornuet and Luikart, 1996). First, we used the mode-shift indicator, which is appropriate for detecting bottlenecks that occurred over the last few dozen generations, and is based on expectations of the shape of the allele-frequency distribution. Second, we used the Wilcoxoń signed rank test, which is sensitive to detection of bottlenecks that have occurred during the last 2–4N_e generations, and examines whether the heterozygosity within populations is higher than predicted if they were in mutation-drift equilibrium. We performed 10^4 simulations for the two temporal samples, applying a two-phase mutation (TPM) model comprising 95% single-step mutations and 5% multi-step mutations with a variance of 12, as recommended by Piry et al. (1999).

2.8. Simulation-based expectations of changes through time

Expected changes in genetic diversity and N_e through time were further investigated via simulations. Using BOTTLESIM ver. 2.6 (Kuo and Janzen, 2003) we simulated the genetic changes of a DFTD-affected population (Freycinet) and a DFTD-unaffected population (Narawntapu) from 1999 to 2006. We performed 10^3 simulations of the two populations, assuming Freycinet decreased from 126 to 52 individuals (Lachish et al., 2007), and Narawntapu had a constant population size of ~80 individuals since 1999 (M.J. unpublished data). Marrawah, Little Swanport and Pawleena lacked direct estimates of abundance, with relative changes in density inferred from spotlighting transects, and therefore could not be simulated in absolute numbers. We assumed a dioecious random mating model, 1.1:1.0 ratio of males to females, longevity of 6 years, and onset of reproductive maturity at 2 years. We used a generation overlap of 10^2, which means that all individuals were assigned random age values. Empirical estimates from 2006 of A_O, H_O, *f*, and N_e were compared to the distributions obtained at the end of the simulations. The tests for genetic bottlenecks were also performed on simulated populations using BOTTLENECK, but with 10^3 replicates under the TPM model instead of 10^4 to reduce computational time.

2.9. Testing for temporal differences in parameter estimates

To test for significant differences in parameter estimates (e.g. UH_E, H_O, A_O, *f*, θ, Φ_{ST}, *m*) over time we first employed the *F*-test for differences between variances of temporal population pairs. If the variances were found to be equal, a two-sample *t*-test was used. If the variances were unequal Welch's approximate test was applied. Both tests were followed by sequential Bonferroni correction (Whitlock and Schluter, 2009). *P*-values were calculated using a web-based *t*-distribution calculator (http://stattrek.com/Tables/T.aspx).

3. Results

3.1. Genetic variation

There was no evidence for genotyping errors in the datasets based on the MICROCHECKER output. None of the loci showed evidence of gametic disequilibrium or departures from selective neutrality for the BAYESCAN (PO < 10) selection test (Supplementary Material, Table A1 and A2). All 10 loci were therefore included in analyses. In 1999 ($n = 213$) a total of 39 alleles were observed,

ranging from three (*Sh3a*, *Sh6l*, *Sh2l*, *Sh5c*) to seven (*Sh2v*) alleles per locus (average 3.9). In 2006 ($n = 212$) a total of 42 alleles were observed, with three (*Sh3a*, *Sh2l*, *Sh2l*, *Sh6e*) to 10 (*Sh2v*) alleles per locus (average of 4.2). H_O varied from 0.198 (*Sh2l*) to 0.693 (*Sh2v*) in 1999 (average 0.429), and from 0.192 (*Sh2l*) to 0.639 (*Sh2v*) in 2006 (average 0.399). At the population level, genetic diversity had a tendency to decrease over time (Table 1). All locations experienced a slight decrease in A_O and H_O, but none of these were significant ($P > 0.05$). The inbreeding coefficient f increased significantly at Marrawah ($P = 0.01$), Narawntapu ($P < 0.01$), and Little Swanport ($P = 0.01$), but Narawntapu 2006 was the only sample where f differed significantly from that expected under Hardy–Weinberg equilibrium.

3.2. Population structure and gene flow

All pairwise θ estimates were significantly greater than zero ($P < 0.019$), except for Narawntapu 1999 against Pawleena 2006 ($P = 0.079$) (Table 2). Results from the Mantel test showed that the pairwise θ estimates were significantly correlated across years ($r = 0.97$; $P = 0.02$). Marrawah, on the northwest coast, was the most distinct population during pairwise comparisons ($\theta_{(1999)} > 0.140$; $\theta_{(2006)} > 0.135$), with differentiation among the other populations markedly lower ($\theta_{(1999)} = 0.015–0.049$; $\theta_{(2006)} = 0.010–0.065$). This was also evident from AMOVA (Fig. 2a) and STRUCTURE analyses (Fig. 2b), which suggested two main clusters representing Marrawah and the remaining populations for both temporal samples ($\Delta K_{(1999)} = 72$ and $\Delta K_{(2006)} = 98$), regardless of whether sampling location priors were included. There were no changes in structure over time (Fig. 2).

Gene flow estimates from BAYESASS+ were highest among east coast populations, but most were below 0.10 (Fig. 1), which is assumed to be the upper limit for demographic independence (Hastings, 1993). Gene flow is given as the sum of individual m ($\geqslant 0.10$) into each population (for specific population pair gene flow see Supplementary Material, Table A3). No correlation among m and geographic distance among populations were detected for either 1999 ($r = -0.67$; $P = 0.93$) or 2006 ($r = -0.54$; $P = 0.96$), but the temporal matrices of m among populations were significantly correlated ($r = 0.85$; $P = 0.016$). However, significant increases in gene flow through time were observed from Narawntapu to Little Swanport as the latter became diseased ($m_{(1999)} = 0.01$; $m_{(2006)} = 0.10$; $P < 0.001$). Similarly, analysis of the Dunalley and Forestier 2006 samples showed Forestier as a strong contributor of immigrants to the other east coast populations: Dunalley (22%), Freycinet (23%), Little Swanport (14%) and Pawleena (22%).

The assignment indices showed no evidence for sex-biased dispersal among populations (Table 3). Accounting for among population variation, we found no effect of sex, year or interaction on the estimated number of immigrants (Supplementary Material, Table A4). We did, however, find that the percentage of males increased on average by 14.5% (95% CI 0.02–0.27) across sites from 1999 to 2006 ($P = 0.037$).

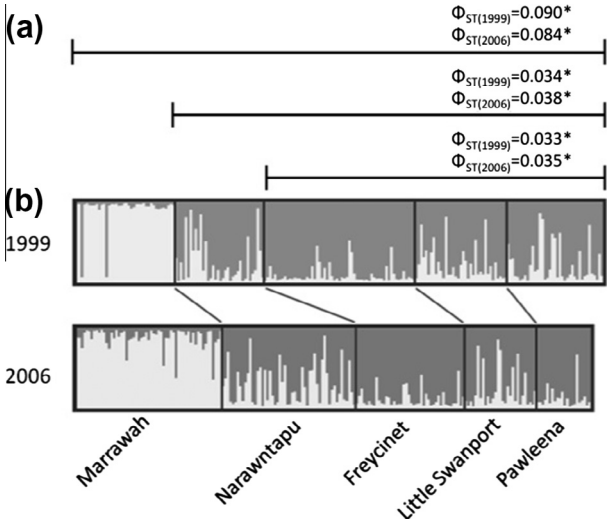

Fig. 2. Temporal population differentiation and structure among Tasmanian devil populations. (a) Analysis of molecular variance (Φ_{ST}) among groups of devil populations partitioned into different geographical groups. (b) Population structure based on Bayesian clustering. Each vertical bar represents an individual and its proportion of ancestry in two clusters. Black vertical lines divide individuals into populations as labelled below the figure. *Indicates significant differentiation ($P < 0.05$; Bonferroni corrected) based on 10^3 permutations.

3.3. Effective population size and bottleneck tests

Although there was no significant change at any site, most sites (both diseased and non-diseased) showed some degree of decline, with the exception of the non-diseased Marrawah, where there was a slight increase (Fig. 3). Years of DFTD presence and N_e were not correlated ($r = -0.64$; $P > 0.05$). None of the populations showed evidence of genetic bottlenecks over time (Supplementary Material, Table A5); estimates from the Wilcoxon test were non-significant for both temporal samples ($P > 0.05$), and the mode-shift test was *L*-shaped for all populations in 1999 and 2006, and therefore not indicative of loss of rare alleles from the populations.

3.4. Simulation-based expectations of changes through time

Declines in A_o and H_o (Fig. 4a and b) were observed during simulations mimicking 1999–2006 population census size histories for Freycinet (declining) and Narawntapu (stable). Empirical values of A_o and H_o were consistent with simulation values with the exception of H_o at Narawntapu in 2006, where simulations returned higher values (Fig. 4a and b). The inbreeding coefficient f had a tendency to decrease during the first 5 years of the simulated population history for both scenarios, but then started to increase, most pronouncedly for the declining population (Fig. 4c). In both scenarios empirical f estimates were lower than simulated values, except

Table 2
Pairwise estimates of genetic differentiation among Tasmanian devil populations.

	Marrawah	Narawntapu	Freycinet	Little Swanport	Pawleena
Marrawah	–	0.141*	0.194*	0.164*	0.178*
Narawntapu	0.144*	–	0.031*	0.049*	0.023*
Freycinet	0.202*	0.065*	–	0.046*	0.020*
Little Swanport	0.136*	0.019*	0.046*	–	0.015*
Pawleena	0.183*	0.010	0.026*	0.022*	–

Genetic differentiation calculated as Weir and Cockerham's (1984) θ. Estimates based on 1999 samples are above the diagonal and estimates based on 2006 samples are below the diagonal.
* Indicates significant differentiation ($P < 0.05$; Bonferroni corrected) based on 10^3 permutations.

Table 3
Mean corrected assignment values (AIc) estimates of sex-biased dispersal for Tasmanian devil populations.

Population	1999			2006		
	N	Males	Females	*N*	Males	Females
Marrawah	17:21	−0.402 (0.302)	0.325 (0.171)	–	–	–
Narawntapu	17:19	0.055 (0.210)	−0.049 (0.292)	29:25	−0.059 (0.224)	0.069 (0.232)
Freycinet	28:32	−0.098 (0.120)	0.086 (0.195)	25:22	−0.224 (0.272)	0.255 (0.216)
Little Swanport	14:20	0.102 (0.452)	−0.072 (0.252)	17:12	0.100 (0.262)	−0.141 (0.389)
Pawleena	18:18	0.116 (0.324)	−0.116 (0.290)	15:7	−0.159 (0.299)	0.341 (0.289)

Sample sizes (*N*) are the number of males to females.
Standard errors are given in parentheses.

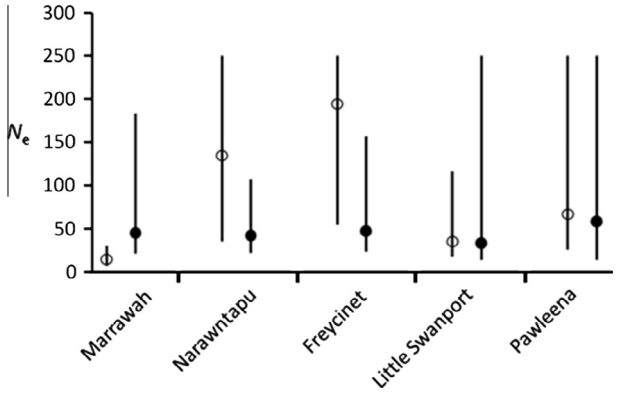

Fig. 3. Temporal changes in effective population size (N_e) for Tasmanian devil populations. Empty symbols represent 1999 samples and filled symbols represent 2006 samples. Bars represent 95% confidence intervals.

for Narawntapu in 2006, which had much greater empirical *f* than simulated (Fig. 4c).

The distributions of simulated N_e estimates were left skewed. Simulations mimicking the stable but initially smaller population (Narawntapu) provided larger N_e estimates than the declining population (Freycinet) (Fig. 5a). Empirical N_e estimates from the 2006 datasets fall within the higher end of the distributions derived from simulations. Less than 3% of simulated datasets returned significant *P*-values for the Wilcoxon bottleneck test (Fig. 5b), and the observed *P*-values from the 2006 empirical datasets fell within the upper 50% of the simulation *P*-value distributions. Recent bottlenecks were revealed by the mode-shift test in 7.9% and 0.4% of simulations mimicking Narawntapu and Freycinet, respectively (Supplementary Material, Table A6).

4. Discussion

Our results indicate that the greatest effect of DFTD appears to be changes in patterns of gene flow, which probably reflect DFTD-induced changes to local population densities and "source-sink" dynamics. While we observed early signatures of changes in genetic variation that might be expected in species undergoing decline, the majority of these changes were not significant and were observed in both DFTD affected and unaffected populations across the same sampling interval.

4.1. Lack of decline in genetic variation

A drastic reduction in *N*, including declines in excess of 95% estimated for some DFTD affected populations, would be expected to lead to loss of genetic diversity, with allelic diversity declining faster than heterozygosity as the former is more sensitive to bottlenecks (Charlesworth, 2009; Hedrick, 2005). Diseases can also impose strong directional selection for individuals with particular genotypes (Hurst, 2009), and as such, can reduce genetic diversity faster than demographic decline alone. The lack of significant

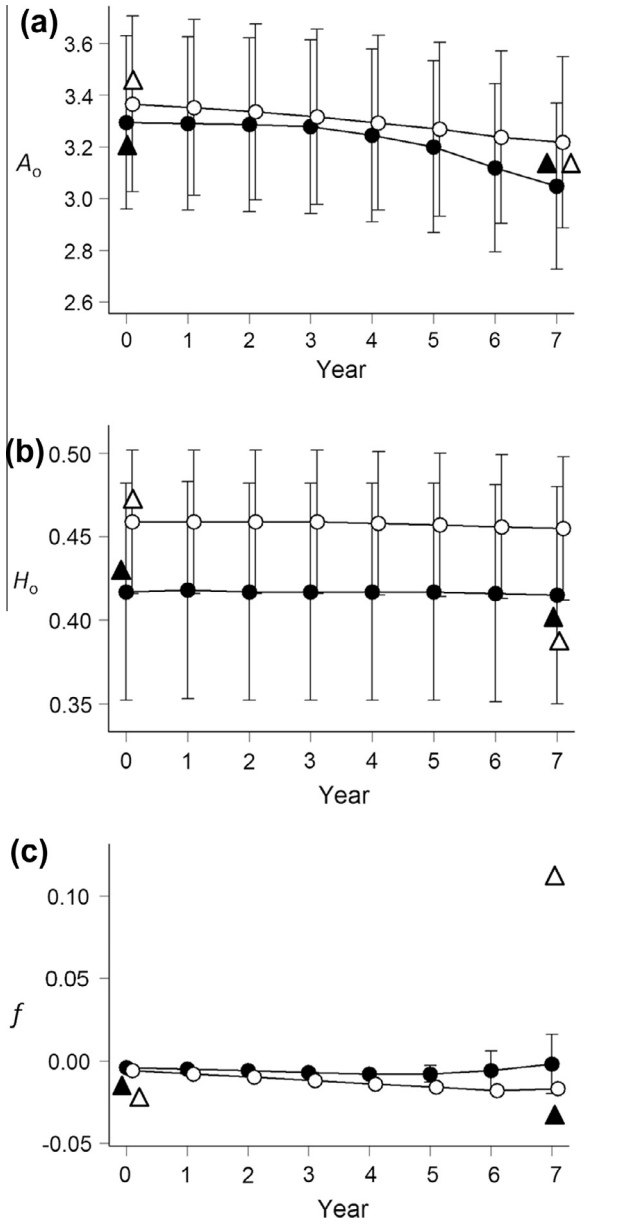

Fig. 4. Simulation results of temporal changes in genetic diversity in Tasmanian devil populations affected and unaffected by DFTD. The simulations are based on 10^3 datasets generated from the 1999 data from Narawntapu (o, DFTD unaffected) and Freycinet (●, DFTD affected). Bars represent standard errors. (a) Mean number of alleles per locus (A_O). (b) Observed heterozygosity (H_O). (c) Inbreeding coefficient (*f*). Empirical estimates from 1999 and 2006 are indicated for Narawntapu (Δ) and Freycinet (▲).

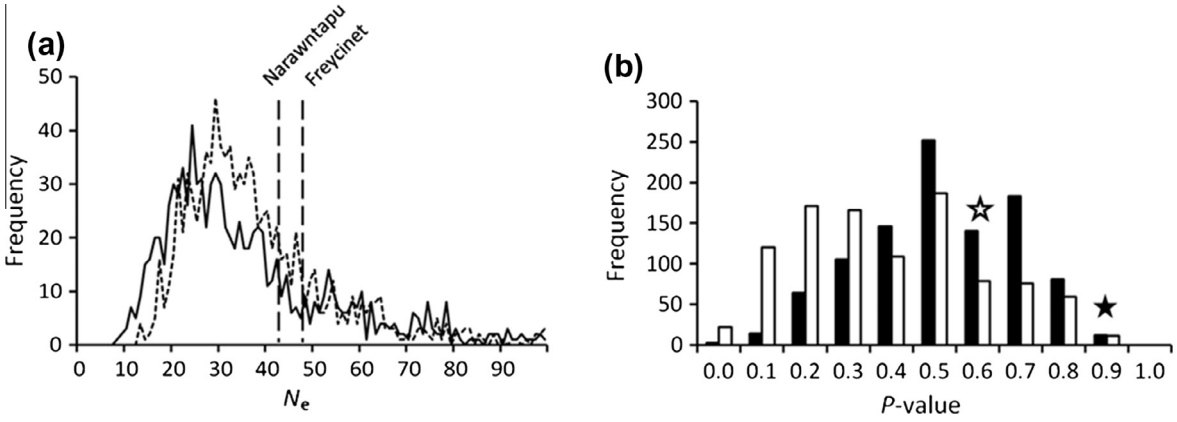

Fig. 5. Effective population size (N_e) estimates and bottleneck test results following simulations of Tasmanian devil populations at Narawntapu (stable population) and Freycinet (declining population) during the period 1999–2006. (a) Estimated N_e-values for Narawntapu (dashed line) and Freycinet (solid line). Estimates from empirical datasets from 2006 are marked with vertical lines. (b) Wilcoxon tests of bottlenecks for Narawntapu (white) and Freycinet (black). *P*-values based on 2006 empirical data are indicated with stars. All simulations are based on 10^3 datasets generated from the 1999 data from Narawntapu and Freycinet.

declines in genetic diversity with population reductions was consistent with simulation results where genetic variation was assumed to be free from the potential influences of selection, as suggested for our loci by BAYESCAN. However, with limited coverage of the genome, it is possible that selection has influenced genetic diversity at parts we have not surveyed. The use of genomic methods that attain a higher loci density and wider genome coverage is necessary to reveal any genetic changes in response to the intense selection pressure from DFTD and its associated high mortality. Selection on devils for any traits that confer a fitness advantage will be intense, such as the observed reduction in age at first reproduction (Jones et al., 2008), although this change may represent phenotypic plasticity in response to reduced intraspecific competition.

Other marsupial species that have experienced smaller reductions in population size show declines in genetic diversity over short periods (<5 generations), such as the northern quoll (*Dasyurus hallucatus*) (Cardoso et al., 2009), or longer periods (>20 generations), as in the case of the Leadbeater's possum (*Gymnobelideus leadbeateri*) (Hansen et al., 2009). Therefore, it may be that the devil decline has been of insufficient duration (2–3.5 generations) to impart a significant reduction in genetic variation, a result that is consistent with our simulations. With low variability in the 10 microsatellite loci, there may also be insufficient statistical power to detect short-term loss of genetic diversity. If rare alleles are lost from populations as *N* declines, which is a likely outcome, this loss will not be mirrored by a similar loss of heterozygosity, which makes changes in genetic diversity difficult to detect. A potential contributing factor is the possibility that levels of genetic variation in devils prior to DFTD emergence were below equilibrium expectations for its population size (Hauser et al., 2002). If the devil population was previously small and then expanded over a short period of time leading up to the emergence of DFTD (Jones et al., 2004), levels of genetic variation would still reflect the historical N_e (Hedrick, 2005). Consequently, the ability of DFTD mediated population declines to affect genetic variation may have been constrained by prior demographic history. While estimates of past abundance are lacking, the devil population may have increased following the early 1900s decline and eventual extinction of Tasmania's previous apex marsupial predator, the thylacine (*Thylacinus cynocephalus*), which may have resulted in greater food availability for devils and reduced intra-guild competition (Jones, 1997). Furthermore, partial land clearance for livestock grazing during the last 200 years may have also increased herbivore abundance and hence food availability for devils, resulting in increasing devil populations prior to DFTD.

4.2. Changes to inbreeding coefficient and population connectivity

Loss of genetic diversity and increase in the inbreeding coefficient over time is expected in populations undergoing decline (Hedrick, 2005), potentially leading to inbreeding depression (Eldridge et al., 1999). In our study, significant increases in *f* were observed in three devil populations, but only one of these (Little Swanport) was DFTD-affected during the sampling time interval. Marrawah was the only population in our study that did not receive any immigrants, the small population size and the lack of gene flow is likely to have resulted in loss of genetic diversity and significant increase in *f* over time. The scenario in Narawntapu is likely to be the result of changed gene flow patterns. From 1999 to 2006 Narawntapu experienced a change from net immigration to net emigration, which could have resulted in loss of genetic diversity, non-random mating and increased *f* as the result of small population size (Frankham, 2002). The changes in immigration observed at Little Swanport from receiving immigrants only from Freycinet (Supplementary Material, Table A3, $m_{1999} = 20\%$) in 1999 to receiving immigrants from both Freycinet and Narawntapu (Supplementary Material, Table A3, $m_{2006} = 18\%$ and $m_{2006} = 10\%$, respectively) in 2006 may have caused a Wahlund effect (Wahlund, 1928), reflecting the mixing of populations with different allele frequencies.

Our simulation results suggested that *f* should behave similarly in both DFTD affected (Freycinet) and unaffected (Narawntapu) populations, and not exhibit significant changes through time (Fig. 4c). However, our observed *f* estimates from Freycinet and in particular Narawntapu differed from simulation expectations, with the DFTD affected population (Freycinet) decreasing slightly in *f* and the non-DFTD population (Narawntapu) increasing in *f* (Fig. 4c). These counter-intuitive trends in *f* most likely reflect changes in gene flow with DFTD effects, with declining populations such as Freycinet potentially receiving more immigrants from surrounding uninfected populations in the years immediately following its decline, lowering *f*, while uninfected populations such as Narawntapu would receive fewer immigrants from neighbouring DFTD-infected populations, thus increasing *f*. These scenarios are consistent with inferences of reduced dispersal from

DFTD-infected populations owing to less competition for resources (Lachish et al., 2011), and in Narawntapu they are also supported by the large changes in gene flow inferred across the two sampling periods (Fig. 1).

4.3. Is population structure and connectivity influenced by DFTD?

As explained above, extrinsic reductions in the density of individuals in a landscape can have implications for gene flow among populations (Haugen et al., 2006). Following severe population decline, competition for food resources is likely to be greatly reduced. This may reduce the net benefits of emigration and increase genetic structuring among populations (Frankham, 2002). In polygynous mammals such as devils (MJ unpublished; Pemberton, 1990) increases in food resources are more likely to reduce dispersal in females, which are driven by the need to secure food resources to raise young, than in males which are driven by inbreeding avoidance irrespective of resources levels (Goltsman et al., 2005; Handley and Perrin, 2007; Lachish et al., 2011). Contrary to this prediction our results failed to show significant sex-biased dispersal or changes in sex-biased dispersal over time in DFTD affected areas (Table 3).

We found significant genetic structuring among all Tasmanian devil populations (Table 2; Fig. 2a), which remained unchanged following DFTD spread. The lack of increase in θ estimates of population structure is likely to be due to compensatory changes in gene flow pattern. Our estimates of gene flow, based on methods that do not assume migration-drift equilibrium, and should be more responsive to short-term fluctuations (Wilson and Rannala, 2003), revealed changes between sites differentially affected by DFTD, but not those more-or-less equally affected. Narawntapu received large numbers of immigrants from Freycinet prior to DFTD affecting east coast populations. Subsequently, Narawntapu increased its contribution of immigrants to Little Swanport (Fig. 1). We further investigated the possibility that changes in relative density influenced gene flow by examining the Dunalley (DFTD arrival 2004, strongly affected by 2006) and Forestier (DFTD arrival 2004, weakly affected by 2006) populations, with the latter exhibiting higher gene flow into Dunalley and other heavily DFTD affected populations than conversely. Therefore, changes in gene flow among populations affected by DFTD may be somewhat compensatory, so that overall structuring among populations remains un-changed, at least during the early stages of DFTD-induced population declines. Therefore, directional estimates of dispersal are recommended when investigating potential density-driven changes in population structure, rather than overall estimates such as F_{ST}. It also appears that it is not changes in *absolute* densities in the landscape that are influential on animal dispersal decisions and thus gene flow, but rather changes in *relative* density across the landscape.

The degree of genetic differentiation among populations has been shown to be important for correct estimates of gene flow (Faubet et al., 2007). Some of our pairwise population θ values are in the range where BAYESASS+ does not have optimal performance ($\leqslant$0.05, Table 2) (Faubet et al., 2007). Therefore, we interpret our results with caution, and acknowledge that the means of m might be overestimated, but are confident that the observed relative changes in gene flow patterns are representative for the individual devil populations (Fig. 1). Likewise, DFTD is primarily spread among adult devils (Jones et al., 2008), and as this segment of the population declines, any dispersal of juveniles from surrounding populations will be proportionally larger relative to the recipient population. Therefore the rates should be seen as indications of changes in gene flow pattern and not as absolute values (Supplementary Material, Table A3).

4.4. Effects of DFTD on effective population size

Declines in N_e over time were only observed for one DFTD affected population, Freycinet, and one unaffected population, Narawntapu, although both were non-significant (Fig. 3). The declines in N_e within these sites were small compared what could be expected from the simulated scenarios (Fig. 5a). There was no evidence of population bottlenecks regardless of DFTD arrival. These bottleneck test results were consistent with the outputs from simulated populations with histories of decline or stability (Fig. 4b). Therefore, despite DFTD-induced declines representing one of the most dramatic observed for a previously healthy wildlife population (McCallum et al., 2009), they do not appear to have been manifested into changes in N_e in a straightforward manner. If, as suggested above, the devil population size might have expanded for a period before the emergence of DFTD, then the sensitivity of genetic tests to identify subsequent population declines will be reduced. Failure to detect genetic bottlenecks can also relate to the number of loci and alleles per locus under investigation; the low number of alleles per locus observed in Tasmanian devils (Jones et al., 2004) can impair sensitivity of the Bottleneck test (Cornuet and Luikart, 1996; Hedrick, 2005).

4.5. Contradictions to previous findings

Our finding of the effect of DFTD on genetic diversity over time contradicts a previous study by Lachish et al. (2011). While DFTD-induced population decline was previously invoked for observed increases in f based on eight of the ten loci used here (Lachish et al., 2011), it was calculated from a pooled sample (Freycinet, Little Swanport, and Pawleena) that also exhibited an increase in θ across the same period. Therefore, the increase in f identified by Lachish et al. (2011) could merely reflect changing genetic structuring among these populations, rather than that within.

The lack of temporal changes in population differentiation we observe also contrasts with previous findings (Lachish et al., 2011), which found no population structuring prior to DFTD at three sites (Freycinet, Little Swanport, Pawleena), but significant differentiation following DFTD ($\theta = 0.005$, becoming $\theta = 0.020$; $P < 0.001$). While we conducted essentially the same analyses (Fig. 2a), Lachish et al. (2011) excluded two of the 10 loci on suspicion of selection and our study differed in terms of the exact individuals used in the analysis (Supplementary Material, Table A1 and Table A2). The high variance of population structuring among neutral loci is well recognised (Lewontin & Krakauer, 1973), and hence the removal of individual loci on the basis of their structuring relative to that of a few others has great potential to influence multilocus estimates of population structuring. Therefore, assessment of the effects of DFTD on population structuring will benefit from analysis of a larger number of loci.

4.6. Implications and future directions

The present study has found evidence for DFTD-mediated changes in gene flow among devil populations following DFTD spread. The observed "source-sink" gene flow dynamics may have compensated for decline in individual populations by contributing genetic diversity. Haag et al. (2010) proposed that species at higher trophic levels may be more susceptible to the effects of disturbance on gene flow, leading to drift–induced differentiation among local populations. However, in our case these interpretations need to be considered relative to the short interval of DFTD effects under investigation (7 years, 3–4 generations and only 2–3 generations since DFTD arrival at a population), and the possibility that devil populations expanded during the ~90 years prior to the emergence of DFTD, with recent genetic diversity still reflecting lower, earlier

abundances (Owen and Pemberton, 2005). Given the possibility of such a scenario, the results from this study may not be directly transferable to genetic effects of population decline in other species, and the pre-DFTD history of genetic variation in the devil requires investigation.

The short timespan under investigation and the low variability of the available microsatellite markers may have limited statistical power to detect signals of population decline. We expect that a larger set of more variable markers, or genome-scale data, could provide a more detailed picture of the effects of DFTD on genetic diversity. Employing more markers—nuclear and mitochondrial—in future studies is needed to gain higher resolution of the genetic effect and ensure contemporary genetic diversity is retained into the future. This includes detailed management of gene flow among populations, both wild and captive.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2013.05.014.

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