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Genetic differentiation among populations of the threatened *Bellevalia webbiana* (Asparagaceae) and its consequence on conservation

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ABSTRACT

The narrow central Italian endemic and threatened Webb's hyacinth (*Bellevalia webbiana*), a perennial herb, is a clear example of a species that has disappeared from several localities due to the development of human settlements. We characterized population genetics of this species to infer possible threats to its viability. We used a dominant DNA fingerprinting approach to infer genetic relationships among the five richest populations known for this species (each with $N > 50$ individuals). We highlighted phenomena of genetic erosion, with values of intrapopulation-gene-diversity quite similar across all populations (mean value 0.113), but a mean F_{st} value only slightly below the mean found in other plant species using similar approaches. Despite an overall genetic similarity among populations, a population from Faenza (Emilia-Romagna) is clearly separated from all the others on genetic grounds, and may be defined as an Evolutionarily Significant Unit, worth of special conservation attention. Interestingly, this latter population is also behaving differently from all the others in terms of both vegetative and reproductive functional strategies. Our results highlight the relevance of evolutionary approaches to conservation biology for preserving a genetic diversity linked to local adaptations.

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Conservation biology; evolutionarily significant units; geophytes; ISSR; monocots; IUCN red lists; Webb's hyacinth

1. Introduction

Italy is one of the main territories that constitute the Mediterranean basin, a well-known biodiversity hotspot (Myers et al. 2000), and it also hosts about one-third of the animal and half of the European plant taxa (Rossi et al. 2013). However, native plant diversity in this hotspot is unfortunately highly threatened by environmental changes, notably human-induced changes (Myers et al. 2000; Marchese 2015). In particular, the endemics are of primary conservation interest, but to pursue a proper conservation of these species, an adequate knowledge of their distribution, systematic relationships and autecology is largely advocated (Orsenigo et al. 2018). Moreover, over the last years, a huge amount of studies underlined the importance of population genetics approaches in understanding the threats on taxa viability (Olivieri et al. 2016). Indeed, the study of the genetic diversity within populations, in particular when related to local adaptations, may supply tools effective in understanding the evolutionary forces that have shaped this diversity (Hansen et al. 2012). This is particularly true for many narrow endemic taxa that face several challenges in the context of climate changes, since they may be associated with small, isolated populations and with highly fragmented habitats.

Italy hosts over 1500 endemic plant species and subspecies (Peruzzi et al. 2014, 2015). However, despite this

richness, some information about genetic diversity is currently available for less than 3% of these taxa (Conte et al. 1998, 2004; Mengoni et al. 2003, 2006; Bellusci et al. 2005, 2008; Bancheva et al. 2006, 2011; Coppi et al. 2008, 2014; Mameli et al. 2008; Crema et al. 2009; De Vita et al. 2009; Bacchetta et al. 2011, 2013; Bedini et al. 2011; Garrido et al. 2012; Nicoletti et al. 2012; Raimondo et al. 2012; De Castro et al. 2013; Vandepitte et al. 2013; Dettori et al. 2014; Maggioni et al. 2014; Gargano et al. 2015; Gentili et al. 2015; Gargiulo et al. 2019).

Amongst Italian narrow endemic plants (Bartolucci et al. 2018), the Webb's hyacinth (*Bellevalia webbiana* Parl., Asparagaceae) is one of the most evolutionarily relevant (Chiarugi 1949; Borzatti von Loewenstern et al. 2013; Astuti et al. 2017a) and threatened species (Orsenigo et al. 2018). According to Gestri et al. (2010), the range of this bulbous perennial herb is restricted to an area of pre-Appennines (100–700 m a.s.l.) in Tuscany and Emilia-Romagna (Central Italy), with two disjunct population groups separated by the mountains of Northern Apennine (about 1100 m a.s.l., in that area). Typical habitats for Webb's hyacinth are open fields and meadows, wood margins, olive groves and vineyards. During the last century, Webb's hyacinth disappeared from several historical localities due to the development of human settlements; for these reasons, this species is currently listed in global IUCN Red List of Threatened Species as Endangered

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(EN A2c) (Peruzzi and Carta 2011). Despite contributions providing important information on the species reproductive and functional traits (Astuti et al. 2018, 2019), no information on population genetics was published so far. Accordingly, the aim of this paper is to contribute to the genetic characterization of this species using an Inter Simple Sequence Repeat (ISSR) DNA fingerprinting approach. The ISSR technique is a simple and quick method based on the use of microsatellite sequences as primers in PCR amplification. Thanks to this molecular approach, we can answer to the following questions: (a) is a genetic differentiation among populations of *B. webbiana* taking place? (b) if yes, which factors are driving this differentiation? (c) is the intrapopulation diversity related to habitat fragmentation and/or other human-induced changes? We may expect: (i) a certain degree of genetic isolation among the two geographically disjunct groups of populations (Tuscany vs. Emilia-Romagna), (ii) a higher genetic diversity values for the largest populations and/or for those living in less disturbed habitats and (iii) genetic drift phenomena, given the scattered distribution of the Webb hyacinth's populations and their heterogeneous habitats (Gestri et al. 2010; Astuti et al. 2019).

2. Material and methods

2.1. Sampling design

We applied ISSR analysis on the five richest populations of our species (each with $N > 50$ individuals): three in Tuscany (Pratolino, PRAT; Uccellatoio, UCC, Tavarnuzze, ROS) and two in Emilia-Romagna (Casola Valsenio, CVAL; Faenza, FAEN; Figure 1). In particular, the largest populations (hundreds of individuals) are ROS and CVAL, followed by PRAT and FAEN (around 100 individuals) and UCC (around 50 individuals). These five populations are the same previously studied by Astuti et al. (2018) concerning reproductive traits and by Astuti et al. (2019) concerning functional traits, and the only populations hosting a sufficient number of individuals to be genetically studied. Indeed, most of the localities where *Bellevalia webbiana* grows are constituted by often less than five individuals. For each population, fragments of foliar tissue were sampled from 15 individuals and dried in silica gel.

2.2. DNA extraction and ISSR amplification

The total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). The primers selected for the ISSR analysis were (Hex)ISSR4, (Tet)ISSR8 (Lazzaro et al. 2018) and CT8 ((CT)₈RT with carboxyl fluorescein as fluorescent staining). The polymerase chain reaction was performed as described in Lazzaro et al. (2018), and the final products were separated by capillary electrophoresis on SeqStudio Genetic Analyzer (Thermo Fisher Scientific). To ensure the reproducibility of the results, replicates were generated for at least three individuals from each population. All the amplified bands were treated as dominant genetic markers and all ISSR profiles were translated into a binary matrix. To this aim, we used the software GeneMarker v. 1.5

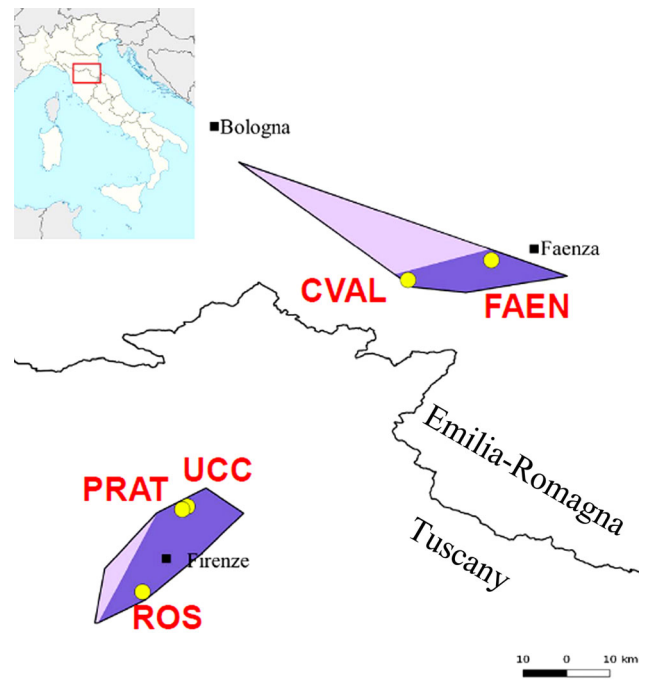


Figure 1. Distribution map of *Bellevalia webbiana* (from Astuti et al. 2019, modified): historical, not confirmed range is in soft violet; presently confirmed range is in deep violet; yellow dots represent the five studied populations, whose acronyms, in red, are superimposed on the figure. The irregular black line indicates the administrative borders between Tuscany and Emilia-Romagna. PRAT: Pratolino, ROS: Tavarnuzze, UCC: Uccellatoio (Tuscany); CVAL: Casola Valsenio, FAEN: Faenza (Emilia-Romagna).

(SoftGenetics LLC, PA, USA). Following the program manual, we adopted a low peak detection threshold by setting a 'stutters peak' filter, to remove stutter peaks within 2.5 bp of each detected fragment peak. After running the data along with the size standard and with the specific panel, the trace comparison report was analysed with the duplicated samples and no mismatch was detected.

2.3. Data implementation and analysis

Within-population genetic diversity was investigated by studying (i) the percentage of polymorphic loci in the dataset, (ii) the estimation of the average gene diversity over loci (Nei 1987), (iii) the observed number of differences (mismatches) between all pairs of individuals in a population (Slatkin and Hudson 1991; Rogers and Harpending 1992) and (iv) by the analysis of the number of private loci (N_{sp}), as described in Lazzaro et al. (2018). Analyses were conducted using the program Arlequin 2.000 (Schneider et al. 2000). The partition of genetic variation within and across populations was estimated by the analysis of molecular variance (AMOVA) (Excoffier et al. 1992), as implemented in the Arlequin 2.000 software (Schneider et al. 2000). The statistical support to different hypothetical groupings of individuals were estimated with 1000 permutations. The binary matrix was subjected to a PCoA multivariate analysis using PAST software (Hammer et al. 2001; Hammer 2020). The genetic structure of the five populations was analyzed by the Bayesian algorithm implemented in STRUCTURE v. 2.3.3 (Pritchard et al. 2000), as explained in detail by Astuti et al.

(2017b). The most likely number of genetic clusters (K) was estimated according to Evanno et al. (2005).

3. Results

The combination of primers used for the ISSR protocol produced a total of 108 loci in the 75 individual profiles, 90 for ISSR4 and 18 for CT8, while ISSR8 was removed from the analysis because of lack of reproducibility of the DNA-fingerprinting products. Shared haplotypes were evidenced for two individuals each from Uccellatoio and Casola Valsenio, whereas private fragments were highlighted only for Faenza. The level of genetic diversity was comparable among populations, with only one population (Uccellatoio) showing a lower number of polymorphic loci and a lower level of genetic diversity (Table 1). The mismatch analysis revealed a multimodal pattern (Figure 2), except for the genetically less variable Uccellatoio, which showed a nearly bimodal pattern associated with a lower frequency of pairwise differences.

AMOVA highlighted that 71.2% of genetic variation was within populations, and 28.8% among populations (p value < .001), with an average $F_{st} = 0.29$. Pairwise F_{st} values of genetic differentiation were reported in Table 2. Despite the low genetic differentiation among populations, the individuals from Faenza were clearly differentiated from the others, showing 11 private loci. Indeed, they group separately in PCoA, despite the first two axes account only for 37.7% of variation (Figure 3). The optimum number of genetic clusters was 2 (Figure 4). The individuals from Pratolino, Uccellatoio, Tavarnuzze and Casola

Valsenio either belong to the first genetic group (33 out of 60 individuals showing a proportion of membership above 80%) or to a genetic admixture among the two genetic groups (27 out of 60 individuals). On the contrary, the proportion of membership to the second genetic group was above 80% in all the 15 individuals from Faenza.

4. Discussion

During the last decades, conservation biologists gathered increasing evidence on the biodiversity crisis, showing species extinction rating up to 1000 times higher than the natural background, whereas the prevision for the future rates seems likely to be 10,000 times higher (De Vos et al. 2015). The main processes that impair biodiversity are habitat loss and fragmentation, as well as its overexploitation or increased pollution (Novacek and Cleland 2001; Pereira et al. 2012). Within this framework, it is well established that human activities catalyze most of the above-described phenomena, identifying the human population as a determinant factor of global biodiversity deterioration. Narrow endemic species could be considered particularly prone to extinction, suffering the consequences of their restricted distribution and, in some cases, low population density (Olivieri et al. 2016; Levin 2019). The Webb's hyacinth is a clear example of a threatened narrow endemic species that has disappeared from several localities due to the development of human settlements (Peruzzi and Carta 2011). Based on the distribution and autecology of this species, and on its habitat loss and fragmentation, it was plausible to foresee genetic erosion processes leading to genetic drift in the most isolated populations. Accordingly, all the examined populations show similar distribution of mismatch profiles, and similar low rates of intrapopulation gene diversity (0.082–0.131, mean value 0.113), suggesting possible genetic bottleneck events. Notably, the smallest among the examined populations (Uccellatoio) shows reduced mismatch values – which tend to a bimodal distribution – and the lowest value of average intrapopulation genetic diversity (0.082). The levels of genetic diversity found in this study are in line with those observed for other threatened Asparagaceae species (*Muscari*

Table 1. Parameters of genetic variability of *Bellevalia webbiana*: percentages of polymorphic loci (PPOLY), the average of gene diversity over loci (AGD) and the number of private loci for each population (Nsp).

ID	PPOLY	AGD	Nsp
CVAL	34	0.131	0
FAEN	35	0.129	11
PRAT	34	0.116	0
ROS	33	0.106	0
UCC	28	0.082	0

PRAT: Pratolino, ROS: Tavarnuzze, UCC: Uccellatoio (Tuscany); CVAL: Casola Valsenio, FAEN: Faenza (Emilia-Romagna).

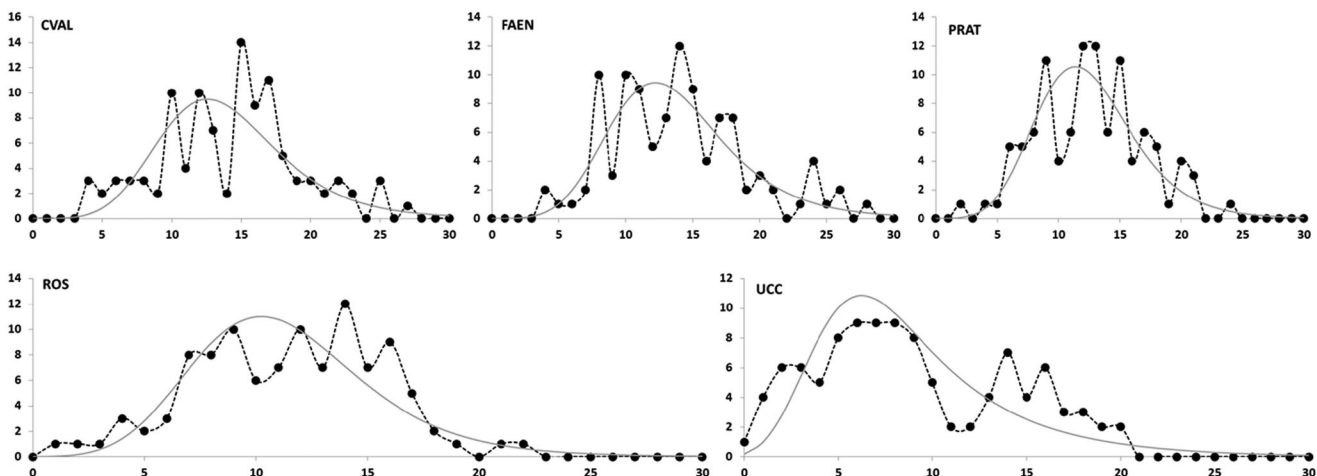


Figure 2. Plots of the distribution of the number of pairwise differences between haplotypes of each population of *Bellevalia webbiana*. X-axis shows the number of pairwise differences, y-axis the number of loci showing the mismatch. PRAT: Pratolino, ROS: Tavarnuzze, UCC: Uccellatoio (Tuscany); CVAL: Casola Valsenio, FAEN: Faenza (Emilia-Romagna).

Table 2. Pairwise genetic differentiation among populations of *Bellevalia webbiana*, based on F_{ST} values.

	CVAL	FAEN	PRAT	ROS	UCC
CVAL	0				
FAEN	0.37	0			
PRAT	0.14	0.37	0		
ROS	0.12	0.41	0.025	0	
UCC	0.18	0.54	0.2	0.14	0

PRAT: Pratolino, ROS: Tavarnuzze, UCC: Uccellatoio (Tuscany); CVAL: Casola Valsenio, FAEN: Faenza (Emilia-Romagna).

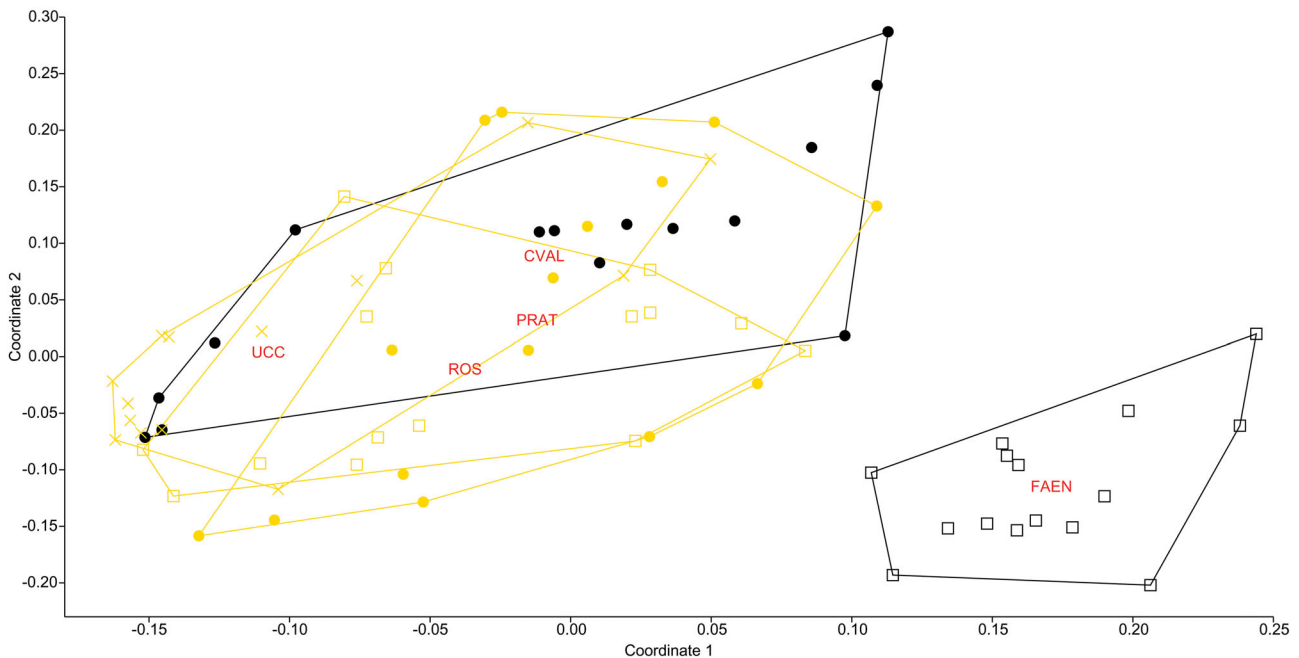


Figure 3. PCoA expressing 37.7% of genetic variation in the first two axes. *Bellevalia webbiana* populations from Tuscany are represented in yellow and populations from Emilia-Romagna in black. PRAT: Pratolino, ROS: Tavarnuzze, UCC: Uccellatoio (Tuscany); CVAL: Casola Valsenio, FAEN: Faenza (Emilia-Romagna).

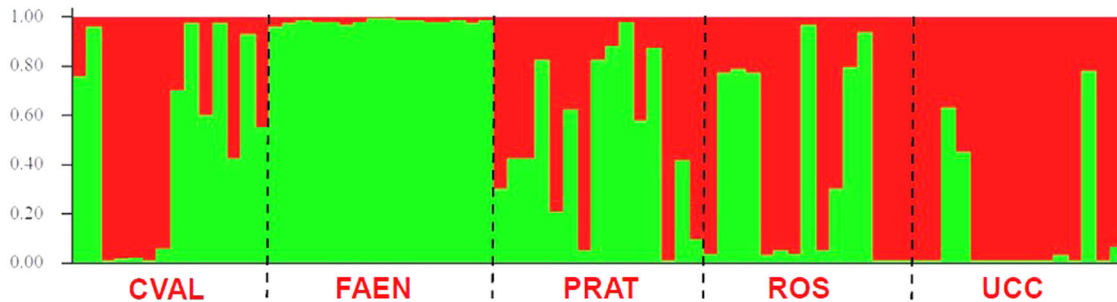


Figure 4. Estimated genetic clustering ($K=2$) obtained by STRUCTURE analysis of 75 individuals of *Bellevalia webbiana*. Each individual is represented by a vertical line partitioned into coloured segments; each colour corresponds to the individual's estimated membership fraction in K clusters. Different populations are separated by vertical black dashed lines. PRAT: Pratolino, ROS: Tavarnuzze, UCC: Uccellatoio (Tuscany); CVAL: Casola Valsenio, FAEN: Faenza (Emilia-Romagna).

gussonei (Parl.) Nyman, see Vandepitte et al. 2013), corroborating the threatened status of Webb's hyacinth. Despite the evidence of genetic erosion, the mean F_{ST} value found in *B. webbiana* is slightly below the overall mean value found using ISSR in plants (see Nybom 2004), indicating only a feeble, probably recent, genetic drift among the populations, that has not compromised the effect of a probable, past amphimictic condition of these populations. Indeed, variable genetic profiles were evidenced in individuals from Pratolino, Uccellatoio, Tavarnuzze and Casola Valsenio, which showed a genetic admixture among the two genetic groups identified by STRUCTURE (Figure 4). Given the higher genetic intrapopulation diversity, Casola Valsenio may,

thus, be considered the populations putatively connecting the germplasm across Tuscany and Emilia-Romagna. The most significant separation among populations was between Faenza and all the others, with the latter being more genetically clustered (Figure 3, Table 2). Unfortunately, no information is available concerning breeding system, pollinators and dispersal strategies of this species, since these traits may have a strong influence on levels of genetic drift and gene flow, and thus, on population genetic structuring and genetic diversity.

Interestingly, however, Faenza is the only population statistically different as far as CSR strategy is concerned (Astuti et al. 2019), being more 'competitor' and less 'stress-tolerant'

compared to the other four populations. As far as reproductive features are concerned (Astuti et al. 2018), the same population shows the tallest inflorescences with the highest number of flowers and the highest fruit set. Consistently, the 'life-history theory' (Bonser 2013) predicts higher reproductive efficiency in plant species exposed to higher levels of competition. This population is also the only conserved one, being actively preserved by the owners of 'Apicoltura Lombardi', by cutting other plant species and fencing the area occupied by the Webb's hyacinth in their property. In this framework, our hypothesis is that Faenza can express its competitive potential by maintaining a preferential outbreeding reproduction and by holding private fragments otherwise lost in the other, disturbed (Astuti et al. 2018, 2019), populations. The peculiar genetically differentiated population from Faenza may be defined as an Evolutionarily Significant Unit (Casacci et al. 2014), deserving special conservation attention, in order to guarantee the survival of this species.

Lastly, Emilia-Romagna may represent the centre of origin for *B. webbia*, by considering that the two populations from this region hold the highest levels of intrapopulation variability, and that Faenza population shows the highest levels of private loci but, at the same time, it shares rare fragments with other populations. A high intrapopulation variability may be also the result of a secondary contact of two formerly well distinct genetic groups, but it seems however at odds with the genetic structure found for Faenza population (Figure 2). However, we cannot exclude that human-induced changes to the distribution range and connectivity among populations may have shaped the current genetic structure. It should be remarked that differences among populations concerning intrapopulation variability can be hardly related to ploidy level, since all the chromosome counts known so far always returned $2n = 4x = 16$ in both Tuscany and Emilia-Romagna for this species (Chiarugi 1949; Borzatti von Loewenstern et al. 2013; Peruzzi et al. 2017).

Our results further highlight the relevance of evolutionary approaches to conservation biology, with the aim of preserving genetic diversity linked to functional diversity. In addition to limitations in land use and habitat exploitation by humans, further useful supplementary conservation actions could rely on germplasm propagation, breeding and reintroductions with genetically well-characterized plant material.

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Disclosure statement

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