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RESEARCH

Conservation genetics of the threatened wetland plant *Eriocaulon nudicuspe*: application of GRAS-Di sequencing technology and implications for restoration

Ikuyo Saeki[1] · Akira S. Hirao[2]

Abstract

Eriocaulon nudicuspe is a threatened plant species distributed at discrete seepage wetlands in Central Honshu, Japan. These wetlands have been present over a million years and functioned as refugia during the glacial periods. The species is an endemic of the wetland ecosystems, but its natural habitats are fragmented by land development. We aimed to identify the genetic structure and diversity of *E. nudicuspe* and to assess the implications for conservation. We performed Genotyping by Random Amplicon Sequencing-Direct (GRAS-Di) and selected 2349 SNPs that were genotyped in 102 individuals from 14 natural and 2 human-established populations. Genetic population structure analyses identified four genetic groups: eastern, northern central, southern central, and western. Demographic modelling suggested that first three groups (eastern, central, and western) simultaneously diverged in prehistoric times, followed by the central group diverging into northern and southern groups. Observed heterozygosity was lower in the populations at the edge of the species' distribution than in the others, probably due to foundation effects associated with the limitation of suitable habitats. The human-established populations had similar genetic diversity and composition to those of the geographically closest natural populations, indicating a low risk of outbreeding depression or genetic bottleneck. We propose four major conservation units, which can be useful for the development of strategies for ex-situ conservation and genetic management of restoration. Although conservation of remnant natural populations should be prioritized, establishing new populations with adequate genetic management seems an effective option to restore connectivity between wetlands isolated by urbanization.

Keywords Conservation unit · Demographic history · Genetic diversity · NGS · Spatial variation

Introduction

Wetlands are among the most important productive ecosystems to humans (Halls 1997). They provide a wide range of ecosystem services, including supply of water and foods, regulation of climate, mitigation of flooding disturbances, and provision of esthetic, educational, and recreational opportunities (Costanza et al. 2014; Mitsch et al. 2015; Xu et al. 2020). Wetlands are often highly biodiverse (Ward et al. 1999; Dudgeon et al. 2006; Balian et al. 2008). Thus, their conservation is crucial for maintenance of local floristic and faunistic diversity as well as promotion of sustainable use of biological resources. In spite of their importance, wetlands are declining rapidly (Reid et al. 2019; Ramsar Convention on Wetlands 2021). Approximately 35% of natural wetlands in the world have been lost since 1970, and the remaining wetlands are facing risks of land development, invasions of exotic species, pollution, overuse, and droughts (Brinson and Malvárez 2002; Albert et al. 2021; Ramsar Convention on Wetlands 2021).

In the Circum-Ise Bay area of Central Honshu, Japan, unique seepage wetlands have formed over a million years (Ueda 1994; Makinouchi 2001; Tomita 2010). They are located in a hilly landscape at low elevation and are characterized by the presence of seeping water, absence of peat deposits, and a low nutrient availability. In recent years,

✉ Ikuyo Saeki
saeki.hus@osaka-u.ac.jp

1 Graduate School of Human Sciences, University of Osaka, 1-2 Yamadaoka, Suita, Osaka 565- 0871, Japan

2 Faculty of Symbiotic Systems Science, Fukushima University, Fukushima, Fukushima 960- 1296, Japan

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many of them have been lost to urbanization, changes to agricultural land use or plantation forests, and natural succession because of underuse (Japan Association for Star Magnolia Conservation 1996; Japanese Red Maple Conservation Group 2003; Saeki 2005). The latest survey has estimated that approximately 1600 such wetlands remain (Study Group of Seepage Marsh 2019). Most of them are smaller than 1 ha (Ueda 1994; Saeki 2005). Nevertheless, these wetlands are considered a biodiversity hotspot; the seepages support many endemic and threatened plant species (Yahara 2002; Study Group of Seepage Marsh 2019; Saeki and Li 2022). The wetlands have functioned as refugia during the glacial periods, which promoted formation of an endemic floristic group, called Tokai Hilly Land Elements (Ueda 1989). The group consists of 15 vascular plant taxa. Many of them are listed in the national red list due to the decline of wetlands (Ministry of Environment Japan 2020).

Eriocaulon nudicuspe Maxim. (Eriocaulaceae) is one of the plant species of Tokai Hilly Land Elements. The species is an annual and dependent on seepage wetlands. It has distinctive white flowers from late August to October, which makes wetlands look like the Milky Way and pleases the eye (Fig. S1). The species seems to be insect-pollinated because insects frequently visit the flowers (Masuda et al. 2017; Saeki, personal observation). The dispersal ability of *E. nudicuspe* seeds is poorly understood but seems to be limited except when seeds become attached to mid- to large-sized mammals. An allozyme study has suggested that the species is diploid (Masuda et al. 2017). Because of wetland disappearance, the natural populations of *E. nudicuspe* have rapidly declined; the species is currently designated as vulnerable (VU) in the national red list (Ministry of Environment Japan 2020).

For conservation of *E. nudicuspe*, it is important to conserve the seepage wetlands where it grows and to monitor its population sizes, reproduction, and genetic diversity. New populations are occasionally established by local conservation groups to restore habitat connectivity in the fragmented landscape (Masuda et al. 2017). Their common approach is to create new wetlands or find appropriate restoration sites (e.g., those where *E. nudicuspe* likely grew in the past), collect seeds from adjacent populations, and sow them. In this process, two considerations must be taken into account from the conservation-genetics perspective. First, seed sources should reflect the spatial genetic variation of *E. nudicuspe*. Introduction of genetically too different populations into native ones may negatively affect the species' fitness due to outbreeding depression through crossing (Hufford and Mazer 2003; Frankham et al. 2011), so introduction of exotic genotypes should be avoided (McKay et al. 2005). Second, when creating new populations by sowing collected seeds, excessive loss of genetic diversity may occur due to

a bottleneck caused by founder effects, which decrease the adaptive potential of the species towards changes in climate and habitat conditions (Hufford and Mazer 2003; Gann et al. 2019). It is essential that restored populations maintain similar levels of genetic variability to those present in the source or reference populations (Ramp et al. 2006). Although establishing new populations of *E. nudicuspe* is commonly practiced, their genetic characteristics are poorly understood.

Genotyping by Random Amplicon Sequencing-Direct (GRAS-Di) is a sequencing technology (Toyota Motor Corporation, Aichi, Japan; Enoki and Takeuchi 2018). GRAS-Di is a non-targeted PCR-based procedure and is characterized by the simplicity of library construction. Although the system was developed relatively recently, its application to genetic analyses of non-model organisms is increasing (e.g., Hosoya et al. 2019; Ikeda et al. 2020; Yoshikawa et al. 2021; Ito et al. 2022; Hirao et al. 2024).

Here, we aimed to investigate the genetic structure and diversity of natural populations of *E. nudicuspe* and to compare them with selected human-established populations. We applied GRAS-Di to (i) identify spatial genetic variation of *E. nudicuspe* as the basis for proposing conservation units (Moritz 1999), (ii) characterize the genetic composition and diversity of human-established populations, and (iii) infer implications for restoration.

Methods

Sample collection

Leaves of 136 individuals of *E. nudicuspe* were collected from 18 populations from August 2022 to November 2022 in the Circum-Ise Bay area, Central Honshu, Japan (Table S1). Leaves were sampled from individuals located at least 10 m away from each other; this distance was shortened to about 3 m at the IW, OKH, and TB sites because of the limited population areas. These populations were located between 34.70°N and 35.50°N and between 136.51°E and 137.78°E. The elevation ranged from 37 m (OKN) to 734 m (KR). Among the 16 natural populations, 14 were proactively managed by local conservation groups, whose activities included mowing, removing non-native plants, fencing to prevent illegal harvesting, vegetation monitoring, and providing environmental education programs. The OBH and OKH populations were newly established by humans for restoration purposes. To establish OBH, seeds were collected from an adjacent natural population and sown about 10 years before leaf collection. OBH is located ca. 1 km away from OBN, but the source population of OBN is unknown (OBH manager, personal communication). At

OKH, a new population was established in 2013 approximately 100 m from the source population, OKN. OKH and OKN populations are located at the same wetland site but are spatially isolated from each other.

DNA extraction and sequencing

The leaf samples were dried with silica gel immediately after collection, brought to the laboratory, and stored at room temperature until the experiment. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The DNA aliquot from each individual was assigned a DNA sample code (Table S1). The DNA concentration was measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The GRAS-Di method—a sequencing approach that relies on a two-step PCR library generation using random primers (Enoki and Takeuchi 2018)—was used for genome-wide sequencing. A GRAS-Di library was prepared and sequenced using a commercial analysis service (GeneBay Inc., Yokohama, Japan); a DNB-SEQ-G400RS (MGI Tech Co., Ltd., Shenzhen, China) was used for sequencing with 150-bp paired-end reads.

SNP genotyping

Pre-processing (adapter trimming and quality filtering) of the raw reads was performed using cutadapt v4.1 (Martin 2011), and only 100-bp reads obtained by trimming were selected. SNPs in these reads were identified using the *de novo* pipeline in the Stack v2.59 software (Rochette et al. 2019). Biallelic SNPs with read depth ≥10 were extracted using vcftools v0.1.16 (Danecek et al. 2011) and were subjected to multi-step filtering for missing data using PLINK v1.90 (Purcell et al. 2007) (see Hirao et al. 2024 for details). For population structure analyses, one SNP with a minor allele frequency (MAF)≥0.05 per contig was retained using vcftools to minimize linkage disequilibrium. To check for cross-contamination and/or clonality, the KING-robust kinship estimator among the genotyped individuals was calculated using PLINK. An alternative allele depth ratio (sequencing depth of an alternative allele to total depth) was assessed to validate diploidy in the species. A single mode of allele depth ratio with a mean of approximately 0.5 is expected for diploidy, while multiple modes are expected for polyploidy.

Genetic diversity and population structure

To investigate the genetic population structure, model-based clustering analyses were implemented using ADMIXTURE v1.3.0 (Alexander et al. 2009), and individual-based principal component analysis (PCA) was conducted using the R package Adegenet (Jombart 2008). A Neighbor-net tree with the P-distance was constructed in SplitsTree (Huson and Bryant 2024). The population-level observed heterozygosity (Ho), expected heterozygosity (He), fixation index ($F$is), and allelic richness (Ar) were calculated for each population to quantify genetic diversity. In addition, we quantified individual-level Ho and compared its average values among the populations. The spatial pattern of individual-level Ho was visualized in ArcGIS PRO (ESRI Inc., Redlands, CA, USA) with interpolation of the inverse distance weighted method. To examine the level of genetic diversity of the two human-established populations, the average individual-level Ho of each population was compared with that of the geographically closest natural population (i.e., OBH vs. OBN; OKH vs. OKN) using two-sample T-tests. On the basis of the results of ADMIXTURE analyses, we classified all populations into four genetic groups (see Results for details) and examined the genetic diversity of each group by comparing the average values of individual-level Ho using the Kruskal–Wallis test. Pairwise differences were examined using the Wilcoxon rank sum exact test with Bonferroni adjustment. To quantify genetic differences among genetic groups, global and pairwise F_{ST} were calculated. The genetic diversity analyses were conducted using the packages dartR (Gruber et al. 2018; Mijangos et al. 2022) and snpR (Hemstrom and Jones 2023) in R v4.4.1. Ar was calculated using the package hierfstat (Goudet 2005) with a standardized sample size of four diploid individuals.

Demographic history

To elucidate the demographic history of *E. nudicuspe*, we performed coalescent demographic modelling based on site frequency spectrum (SFS) in fastsimcoal2 v2.7 (Excoffier et al. 2021). The folded multidimensional observed SFS was calculated using the R script 2D-mSFS (https://github.com/garageit46/2D-msfs-R). The effective length of a sequence (including non-variable sites) was calculated as the average contig size multiplied by the effective number of contigs. Missing data were compensated by bootstrapping alleles within the genetic groups. Ten models were examined that combined five phylogenic topologies with the presence or absence of gene flow (Fig. S2). Models with gene flow were assumed under symmetric migration between the genetic groups. The likelihood of each model was maximized from 100 random starting values in 50 expectation-conditional-maximization optimization cycles and 100,000 coalescent simulations. For model 8, the number of random starting values was reduced to 50 because of the long computational time, but the other values remained unchanged. The best-fitting demographic model was selected using Akaike's information criterion (Akaike 1974). Parameter confidence

intervals (CIs) of the best-fit single-population model were obtained using 100 parametric bootstraps. We scaled the results using an assumed mutation rate of 7×10^{-9} per site per generation inferred for *Arabidopsis thaliana* (Ossowski et al. 2010) and a generation time of one year because *E. nudicuspe* is an annual species.

Results

GRAS-Di sequencing yielded a total of 815 M paired-end reads (122.2 Gbp) from 136 individuals. Raw read files were deposited in DRA/SRA/ERA (PRJDB17330). The effective length of a sequence (including non-variable sites) was 496,496 bp. Of the 136 individuals, 34 were filtered out because of a high proportion of missing SNP data. Their exclusion reduced the number of study populations to 16, including 14 natural and 2 human-established populations (Fig. 1; Table 1) that covered almost the entire natural geographic range of *E. nudicuspe*. Variant calling identified 38,695 SNPs, including singletons, from 102 individuals;

this dataset was used for demographic modelling. After further filtering to one SNP with MAF ≥ 0.05 per contigs, 2349 SNPs were retained and showed a reliable genotyping rate of 86.4% for population structure analyses. The distribution of the alternative allele depth ratio showed a single mode with a median of 0.478 (Fig. S3), indicating diploidy in the species. Using KING-robust kinship estimator, we found no clonal individuals among the 102 individuals genotyped. Average population-level values were 0.17 for Ho and He, 0.09 for Fis, and 1.52 for Ar; population IM ($n=1$) (Table 1) was excluded from these calculations.

ADMIXTURE analyses based on the 2349 SNPs revealed a clear genetic structure (Figs. 1 and 2). Cross-validation error was lowest at $K=4$ and was almost as low at $K=3$ and 5. Analysis at $K=4$ revealed four geographically separated genetic groups; Shizuoka and eastern Aichi Prefectures (group I in Fig. 1), Gifu Prefecture (II), central Aichi Prefecture (III), and Mie Prefecture (IV). Group I corresponded to the eastern region, group II to the northern central region, group III to the southern central region, and group IV to the western region. The pairwise F_{ST} among the groups
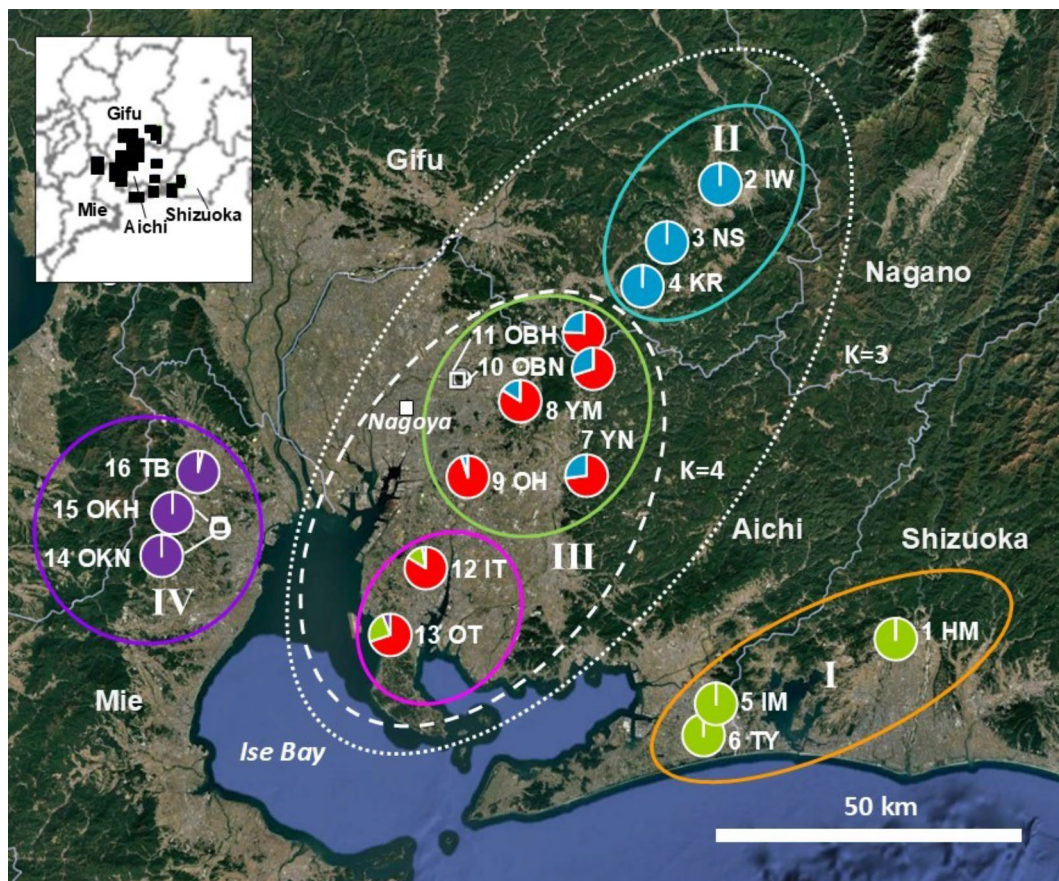

Fig. 1 Localities of sample collection and natural distribution (inset) of *Eriocaulon nudicuspe*. Ellipses indicate three, four, and five genetic groups classified by the ADMIXTURE analyses at $K=3$, 4, and 5, respectively. The pie charts show the results of ADMIXTURE analyses at $K=4$. Roman numerals were arbitrarily given to each group at $K=4$ and correspond to group numbers in Figs. 2, 3 and 4. Dotted, dashed, and solid ellipses indicate the genetic divisions at $K=3$, 4, and 5, respectively. The background in the main map was obtained from Google Earth. The map in the inset was created based on the Ministry of the Environment, Japan (2025)

Table 1 Geographic information, origin, sample size, and population-level genetic diversity indices of 14 natural and 2 human-established populations of *Eriocaulon nudicuspe* in central Honshu, Japan, that were analyzed in this study. See Fig. 1 for the geographic location of each population

ID	Population code	Prefecture	City/town	Origin	Sample size[a]	Ho[c]	He[c]	Fis[c]	Ar[c]
1	HM	Shizuoka	Hamamatsu	Natural	5	0.118	0.112	0.078	1.440
2	IW	Gifu	Nakatsugawa	Natural	6	0.159	0.171	0.175	1.495
3	NS	Gifu	Ena	Natural	9	0.188	0.187	0.066	1.497
4	KR	Gifu	Mizunami	Natural	4	0.171	0.163	0.125	1.646
5	IM	Aichi	Toyohashi	Natural	1	NA	NA	NA	NA
6	TY	Aichi	Toyohashi	Natural	6	0.132	0.135	0.134	1.450
7	YN	Aichi	Toyota	Natural	5	0.178	0.178	0.138	1.600
8	YM	Aichi	Seto	Natural	10	0.178	0.186	0.104	1.493
9	OH	Aichi	Toyoake	Natural	5	0.180	0.164	0.031	1.537
10	OBN	Aichi	Nagoya	Natural	5	0.186	0.177	0.082	1.592
11	OBH	Aichi	Nagoya	Human-established[b]	4	0.198	0.161	-0.049	1.688
12	IT	Aichi	Agui	Natural	6	0.182	0.177	0.081	1.524
13	OT	Aichi	Tokoname	Natural	6	0.173	0.175	0.113	1.508
14	OKN	Mie	Yokkaichi	Natural	10	0.163	0.176	0.138	1.459
15	OKH	Mie	Yokkaichi	Human-established[b]	10	0.186	0.182	0.038	1.463
16	TB	Mie	Komono	Natural	10	0.156	0.172	0.152	1.454
	Subtotal (Natural)				88				
	Subtotal (Human-established)				14				
	Total				102				

[a] Sample size per population passing genotyping quality control

[b] Based on information from local conservation organizations

[c] Ho, population-level observed heterozygosity; He, population-level expected heterozygosity; Fis, population-level fixation index, Ar, allelic richness. These indices were not calculated for the IM population because it had only one sample
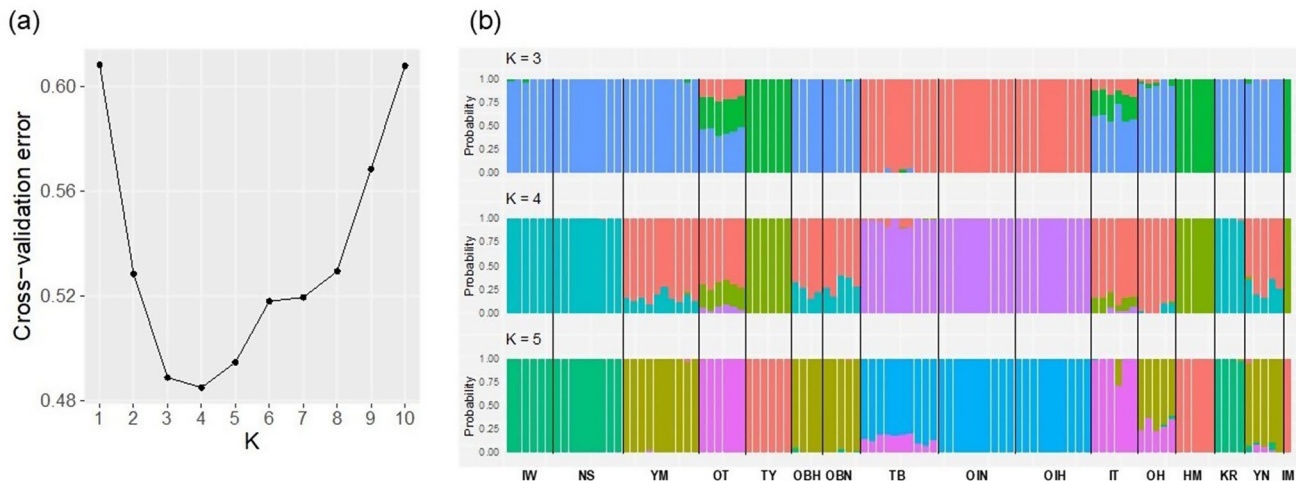
Fig. 2 Results of the ADMIXTURE analyses of *Eriocaulon nudicuspe*. **a** Cross-validation error at different K values. **b** Bar plots for $K=3$, 4, and 5. Population codes are as described in Table 1. See Fig. 1 for the geographic location of each population

Table 2 Pairwise F_{ST} among the four genetic groups identified by the ADMIXTURE analyses for *Eriocaulon nudicuspe*. Values marked with *** are statistically significant at the 0.001 level. See Fig. 1 for geographic locations of the genetic groups and corresponding populations

	II	III	IV
I	0.367***	0.294***	0.361***
II	—	0.103***	0.292***
III	—	—	0.213***

ranged from 0.103 (II and III) to 0.367 (I and II) (Table 2). Clustering by Neighbor-net analysis corresponded to this geographic structure, although the 102 individuals tended to be clustered together by population (Fig. 3). The human-established populations (OBH and OKH) were classified in the same clusters as the geographically closest natural populations (OBN and OKN).

The results of PCA agreed with the clustering by ADMIXTURE analyses, indicating that the populations
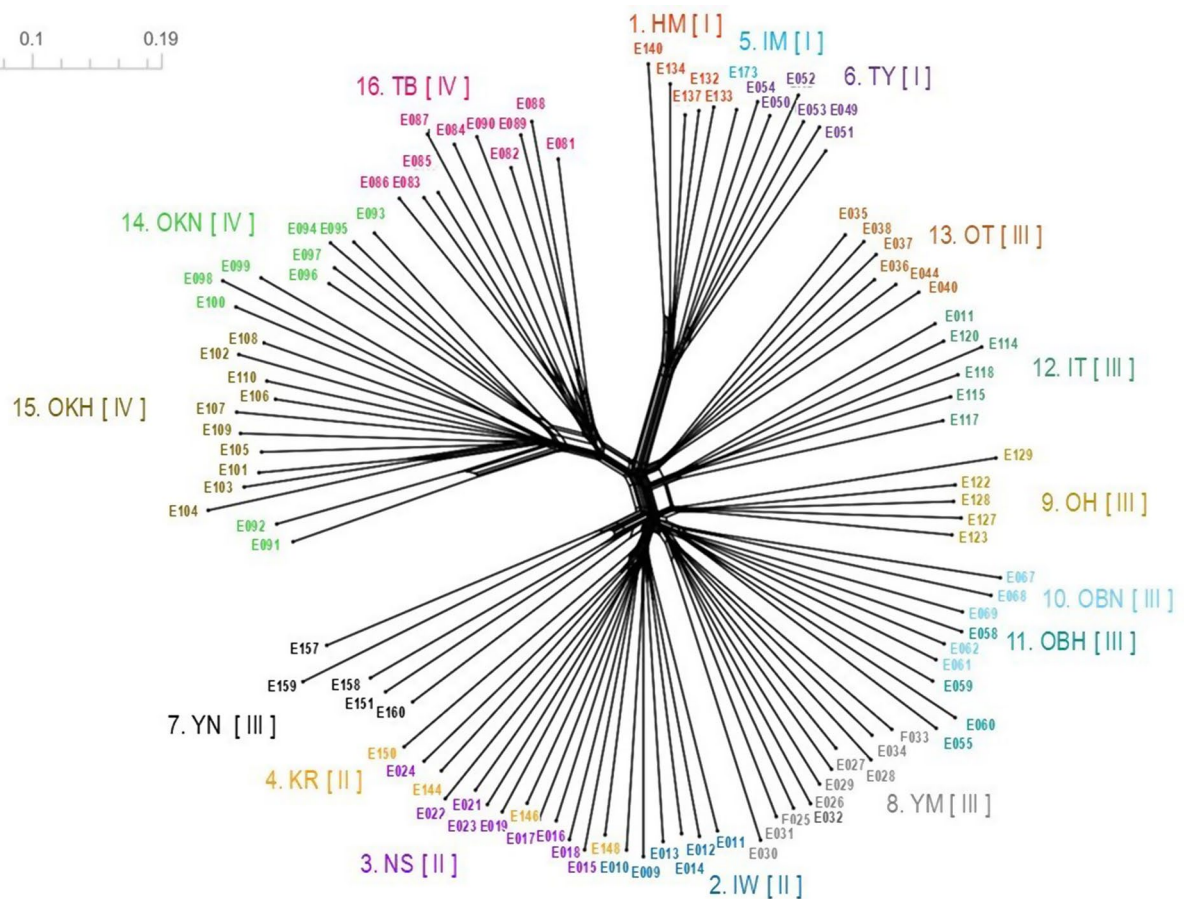
Fig. 3 Neighbor-net network of natural and human-established populations of *Eriocaulon nudicuspe*. Population IDs and codes are as described in Table 1. See Fig. 1 for the geographic location of each population. Roman numerals in brackets correspond to the four genetic groups classified by the ADMIXTURE analysis at $K = 4$. The distance was calculated by UPGMA

that were geographically close to each other have genetically similar compositions (Fig. 4). PC1 explained 13.9% of the total variation, and PC2 and PC3 each explained 9.5%. As above, the two human-established populations showed genetic compositions similar to those of the closest natural populations.

Average Ho of all the individuals was 0.17 (range, 0.08–0.22). At the population level, average Ho was highest in OBH (0.20), followed by NS, OBN, and OKH (0.19) (Fig. 5). Populations at the edge of the species' geographic distribution (i.e., HM, TY, and TB) had lower individual-level Ho than the others (range, 0.12–0.16) (Figs. 5 and 6). No statistically significant differences were detected between the Ho values of the two human-established populations and those of the geographically closest populations (OBH vs. OBN, $p > 0.06$; OKH vs. OKN, $p > 0.26$ in two-sample T-tests). Among the four genetic groups, group I had the lowest average individual-level Ho, followed by group IV (Fig. 7); the differences between group I and the other groups were significant ($p < 0.001$ for all the combinations

in the Kruskal–Wallis test; Table S2). The average Ho was significantly lower in group IV than in group III ($p < 0.01$).

For demographic inference, we used all four groups identified by the population genetic structure analyses. The best supported model was the one with gene flow (Fig. 8; model 10 in Fig. S2; Table S3). The phylogenetic topology of this model indicated that three groups—eastern (I), central (II and III), and western (IV)—simultaneously diverged at the most ancient event time (T_2), and then the central group diverged into II and III at a more recent event time (T_1). The maximum likelihood estimate and 95% CI of the current effective population size were as follows: 344,228 (95% CI: 308,445–379,179) for group I, 347,553 (46,979–391,783) for group II, 1,003,485 (235,021–1,137,862) for group III, and 338,105 (321,054–378,855) for group IV (Table 3). Divergence time was 112.4 Ka (95% CI: 5.9–166.0 Ka) for T_1 and 225.1 Ka (183.7–623.7 Ka) for T_2. Although the divergence-with-gene-flow model was selected as the best fitting one, effective migration rates were estimated to be extremely low (1.22×10^{-5}–3.86×10^{-8}), indicating that migration events were rare.
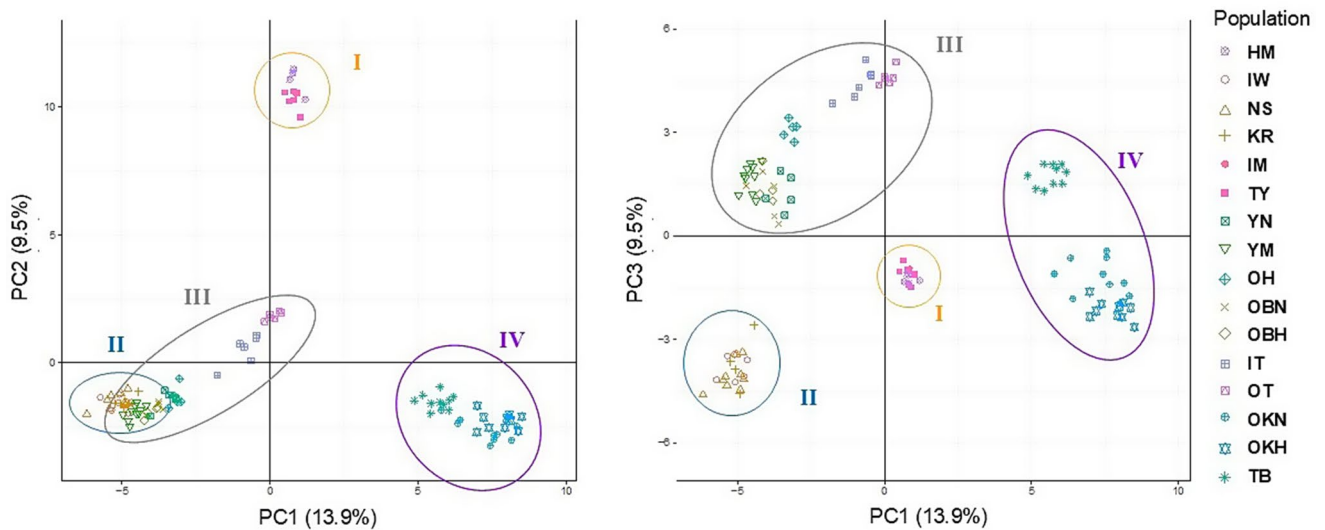
Fig. 4 Principal component analysis based on genotyping information for *Eriocaulon nudicuspe*. Samples collected from 16 populations were divided into four genetic groups (I–IV) by ADMIXTURE analyses at $K=4$. See Fig. 1 for the geographic location of each population
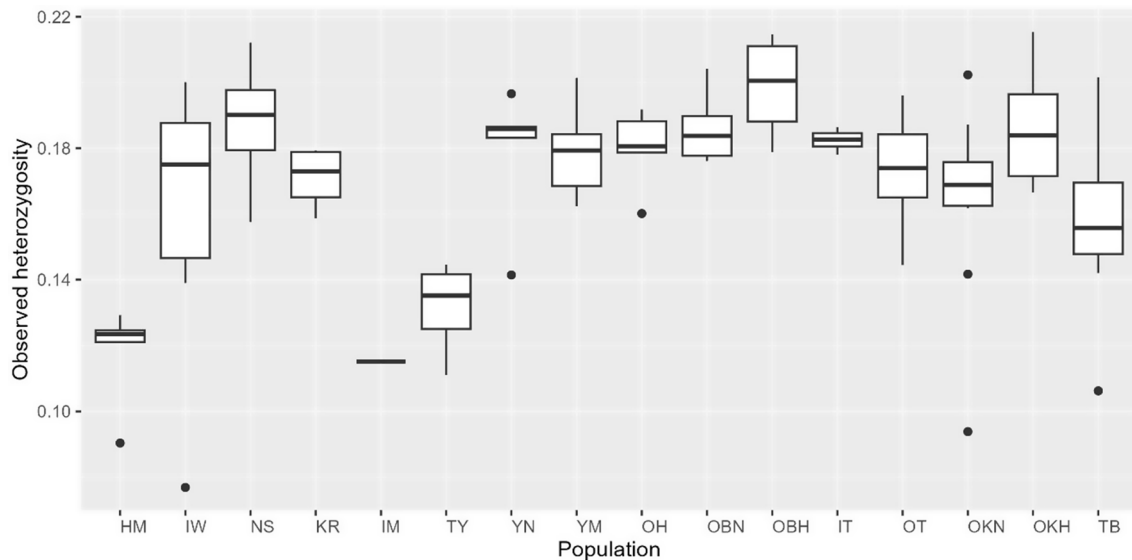

Fig. 5 Individual-level observed heterozygosity in 16 populations of *Eriocaulon nudicuspe*. See Table 1; Fig. 1 for the code and geographic location of each population, respectively. OBH and OKH are human-established populations

Discussion

We found a clear geographic pattern of genetic diversity of the threatened plant species *E. nudicuspe* despite its narrow distribution. The results of ADMIXTURE analyses suggested that its entire population is divided into four major genetic groups (Figs. 1 and 2). The coordinate diagrams of PCA supported this clustering (Fig. 4). Therefore, we propose four conservation units (groups I–IV in Fig. 1); these units can be used for selection of seed sources to establish new populations and development of seed-collection strategies for ex-situ conservation. For the latter purpose, we recommend preserving seeds from at least one population per conservation unit. Since the cross-validation error at $K=5$ was almost as low as the one at $K=4$, we also propose conservation units with five genetic groups, which differentiate populations in southern Aichi Prefecture from the others. This clustering is practically more preferable for preserving genetic diversity of the populations in the central part of its range. Definition of conservation units—whether units reflect only neutral genetic diversity or also ecological traits and variation of adaptive genes—has been debated (e.g., Crandall et al. 2000; Funk et al. 2012). Here, we emphasize that the proposed units were constructed on the basis
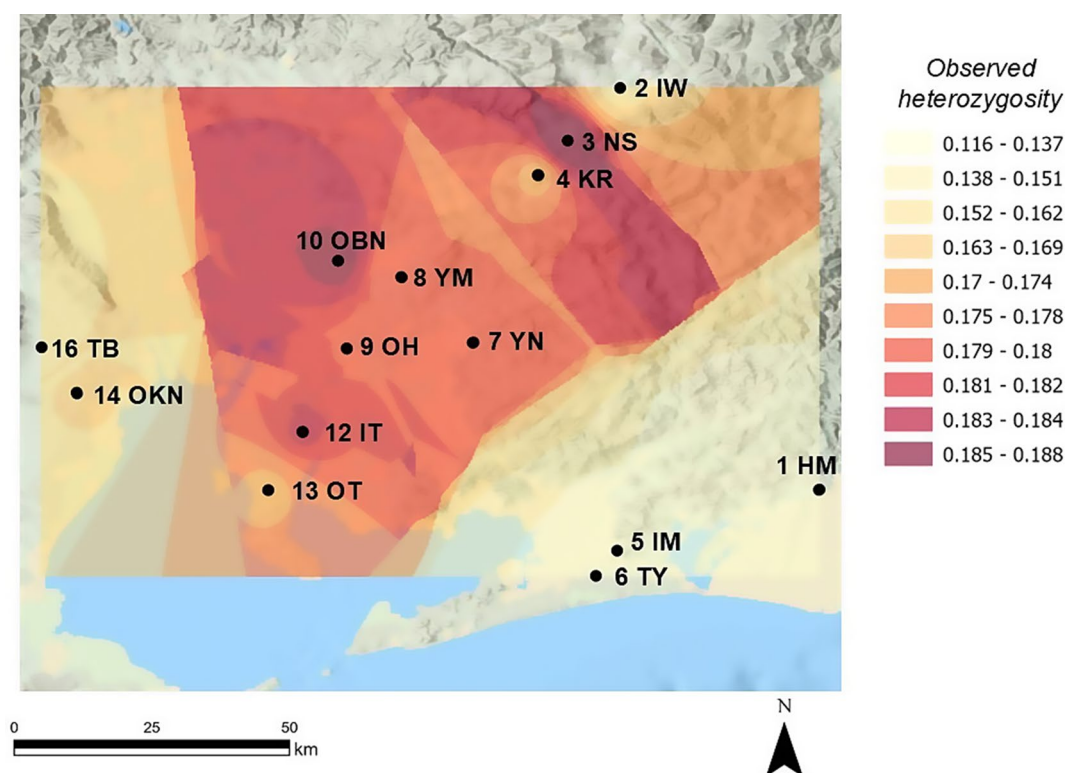
Fig. 6 Spatial patterns of individual-level observed heterozygosity of *Eriocaulon nudicuspe*. Black circles indicate sampling sites for genetic analyses. Values were interpolated by the inverse distance weighted method for 14 natural populations. The base map was provided by ESRI and visualized in ArcGIS PRO
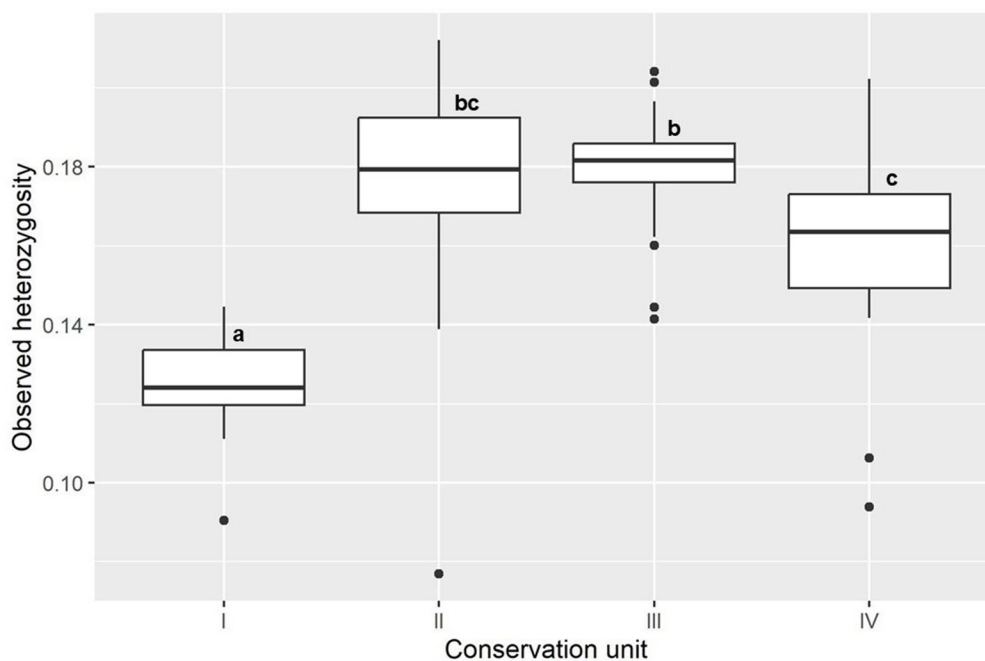

Fig. 7 Individual-level observed heterozygosity in four genetic groups classified by the ADMIXTURE analyses of *Eriocaulon nudicuspe* populations. See Fig. 1 for the geographic location and populations corresponding to each group. Lowercase letters indicate the results of multiple comparisons by the Kruskal–Wallis test. See Table S2 for *p*-values
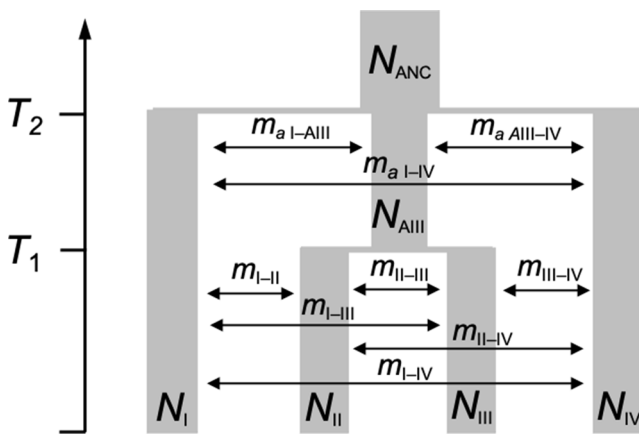
Fig. 8 Schematics of the best demographic model of *Eriocaulon nudi-cuspe*. The four genetic groups (N_I, N_{II}, N_{III}, and N_{IV}) correspond to the ones classified by the ADMIXTURE analyses at $K=4$. The parameters are listed in Table 3

Table 3 Maximum likelihood estimates (MLE) and 95% confidence intervals (CI) of parameters in the best demographic model for *Eriocaulon nudicuspe*. See Fig. 8 and model 10 in Fig. S2 for the schematics and a simplified diagram of this model, respectively

Parameter	MLE (95% CI)
N_I	344,228 (308,445–379,179)
N_{II}	347,553 (46,979–391,783)
N_{III}	1,003,485 (235,021–1,137,862)
N_{IV}	338,105 (321,055–378,855)
N_{AIII}	122,136 (34,458–4,766,167)
N_{ANC}	208,512 (388–285,018)
T_1	112,437 (5911–166,033)
T_2	255,053 (183,654–623,689)
$m_{I–II}$	6.00×10^{-7} (3.34×10^{-7}–2.99×10^{-6})
$m_{I–III}$	8.08×10^{-7} (5.45×10^{-7}–4.12×10^{-6})
$m_{I–IV}$	3.95×10^{-7} (1.39×10^{-7}–5.37×10^{-7})
$m_{II–III}$	2.43×10^{-6} (1.96×10^{-6}–6.78×10^{-6})
$m_{II–IV}$	3.98×10^{-7} (1.96×10^{-7}–9.81×10^{-7})
$m_{III–IV}$	7.26×10^{-7} (5.02×10^{-7}–9.89×10^{-7})
$m_{aI–AIII}$	6.90×10^{-7} (3.72×10^{-8}–2.82×10^{-5})
$m_{aI–IV}$	1.22×10^{-5} (2.73×10^{-7}–6.10×10^{-5})
$m_{aAIII–IV}$	3.86×10^{-8} (3.44×10^{-8}–1.77×10^{-3})

Abbreviations: I, II, III, and IV indicate four genetic groups identified by ADMIXTURE analyses. *N*, effective population size; *T*, event time; $m_{i–j}$, effective migration rate per generation between groups *i* and *j*

of neutral genetic variations for practical use by conservation practitioners for developing genetic strategies in restoration projects.

At a finer scale, the genetic diversity of *E. nudicuspe* tended to be clustered together by population (Fig. 3). Current gene flow to adjacent populations appear to be limited. We were unable to determine whether population-level divergence was developed by a natural demographic and evolutionary process or was caused by recent anthropogenic fragmentation. Without clear evidence of the latter,

we recommend that managers collect seeds for restoration from the closest natural population, as suggested by McKay et al. (2005) ("collect locally if at all possible").

In a phylogeographic study using chloroplast DNA (Saeki et al. 2015), E. *nudicuspe* showed a clear geographic structure, and our GRAS-Di data support it. A clear geographic structure of genetic diversity has also been reported for other plants of Tokai Hilly Land Elements, *Magnolia stellata* (Siebold et Zucc.) Maxim. (Tamaki et al. 2008) and *Acer pycnanthum* K. Koch. (Saeki and Murakami 2009). In contrast, allozyme analyses showed almost no geographic structure in selected populations in Aichi and Shizuoka Prefectures (Masuda et al. 2017). This may be explained by the differences in sampling ranges and resolution of genetic markers.

The populations located at the edge of the species' distribution range had lower *H*o than the others, probably due to foundation effects associated with the limitation of suitable habitats (Figs. 5, 6 and 7). Demographic analyses suggest that these peripheral groups (I and IV) simultaneously diverged at 225.1 Ka (i.e., in prehistoric time) and have remained isolated from the central groups since then (Table 3; Fig. 8). This divergence was estimated to have occurred much earlier than that of groups II and III, and thereby resulted in distinctive genetic compositions (Figs. 2, 3 and 4, and Table 2). Although high levels of neutral genetic diversity do not necessarily increase fitness and long-term survival of a species (Teixeira and Huber 2021), we encourage the managers of edge populations in I and IV to take special care to prevent a decline in population size. For example, the managers can enlarge the area of wetland habitats and remove nonnative species to promote growth and reproduction of *E. nudicuspe*. Nonetheless, we believe that genetic rescue or mixing (Ralls et al. 2018; Hoffmann et al. 2020) is not yet necessary because no obvious deterioration of reproductive ability or survival rates have been reported (managers, personal communications). In our study, the number of samples was smaller for some populations (e.g., IM) than for the others; therefore, we need to interpret the results with caution.

The levels of genetic diversity of re-introduced populations can be lower than those of source populations if only a part of genetic variation is transferred from the source populations (Fraser and Bernatchez 2001). However, the two human-established populations (OBH and OKH) had similar genetic diversity to that of the geographically closest natural ones (Fig. 5). Their genetic compositions were similar to those of the adjacent natural populations (Figs. 3 and 4), confirming that each human-established population has been established without a severe bottleneck or introduction of exotic genotypes,

thereby resulting in a low risk of outbreeding. Seeds of *E. nudicuspe* may be dispersed over long distances by attaching to the bodies of large mammals (e.g., wild boar, deer, and serow). Yet in urbanized areas, such mammals are often absent, which likely promotes isolation of local populations. Our results suggest that collecting seeds from the closest natural populations and sowing them in new wetlands can be an important restoration option to preserve a small population affected by fragmentation as well as maintaining habitat connectivity. Nonetheless, conservation of extant natural populations is crucial for the maintenance of genetic diversity of restored populations because source populations should have high genetic diversity when seeds are collected for restoration. The majority of genetic diversity revealed in this study was assumed to be neutral, and its effects on individual survival and fitness may be much smaller than those of the population size and habitat quality (Yates et al. 2019). In future studies, it will be important to examine the relationships between genetic diversity and individual fitness of *E. nudicuspe*.

Genetic information about threatened species is often critical for the development of effective conservation strategies (Frankham et al. 2011; Rossetto et al. 2021) and is recommended to be applied to genetic monitoring in restoration projects (Aravanopoulos 2011; Flanagan et al. 2018; Van Rossum et al. 2020). However, obtaining such information is challenging for the site managers because it requires time, knowledge, and analytical skills and is expensive (Theissinger et al. 2023). Accordingly, information coming from the conservation genetics field has rarely been used directly for population management and establishment of recovery plans (Mijangos et al. 2015; Willi et al. 2022). Collaboration between conservation geneticists and citizens is necessary for genetic information to be used on-site in grassroot projects (Van Rossum and Hardy 2022; Shaw et al. 2024). We hope that the present study will help to bridge the gap between conservation managers and geneticists, and promote conservation of threatened species in the Circum-Ise Bay Area.

Author contributions Ikuyo Saeki contributed the study conception and design. Material preparation and data collection were performed by Ikuyo Saeki. Genetic analyses and data interpretation were performed by Ikuyo Saeki and Akira S. Hirao. All authors contributed to writing the first draft and approved the final manuscript.

Declarations

Competing interests The authors declare no competing interests.

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