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Genetic diversity and conservation of *Carex kobomugi* in Taiwan

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ABSTRACT: *Carex kobomugi* is native to the coastal sand dunes and sandy beaches of East Asia, including China, Japan, Korea, Taiwan, and the Russian Far East. In Taiwan, *C. kobomugi*, which is categorized as threatened species (category of critically endangered, CR), is narrowly distributed in northern Taiwan and offshore islands. First recorded in 1924, the species disappeared for a time but was rediscovered in 2020. However, it is currently threatened by increasing human activities and faces the risk of local extinction. In this study, we employed molecular markers (*rpL16* spacer of cpDNA, ITS region of nrDNA and eleven microsatellite loci) to evaluate the genetic diversity and population structure. The results showed that the low genetic diversity of *C. kobomugi* in Taiwan may be attributed to small population size, and geographic isolation based on cpDNA and nrDNA. In contrast to the identical nrDNA sequences across different regions, a unique cpDNA haplotype identified exclusively in Taiwan underscores the need for focused conservation efforts. A moderate level of genetic diversity was found for microsatellite loci, and STRUCTURE and principal coordinates analysis revealed that the two genetic groups corresponded to their geographical distributions. Based on these findings, two distinct management units are recommended for further conservation. Actions such as manually conducted beach cleanups, species inventories, and both *in-situ* and *ex-situ* conservation have been implemented to mitigate human disturbance.

KEY WORDS: *Carex kobomugi,* conservation, ITS, microsatellite, population genetics, *rpL16*.

INTRODUCTION

Gene flow is an important force in maintaining species boundary by introducing genetic variation into populations and preventing population differentiation by local adaptation and genetic drift (García-Ramos and Kirkpatrick, 1997; Morjan and Rieseberg, 2004; Takayama *et al*., 2008; Sexton *et al*., 2011; M'Baya *et al*., 2013). Species with a high dispersal ability are expected to maintain species boundary through gene flow. In contrast, species with a low dispersal ability are more likely to diverge due to random mutations and genetic drift (Suárez *et al*., 2022). For coastal species, ocean currents play a crucial role in shaping their distribution (Schönswetter *et al*., 2008; Geng *et al*., 2021; Han *et al*., 2022). Specific morphological and ecophysiological adaptations can enable species to achieve long-distance dispersal via ocean currents (Takayama *et al*., 2006, 2008; Geng *et al*., 2021). In Taiwan, certain species, such as *Kandelia candel* (Chiang *et al*., 2001a) and *Ipomoea pes-caprae* (Miryeganeh *et al*., 2014), have demonstrated that ocean currents play a crucial role in the long-distance dispersal of mangroves or seaside plants. Conversely, ocean currents can act as geographic barriers, hindering gene flow between populations (Takayama *et al*., 2006; Yamamoto *et al*., 2019). Ochoa-Zavala *et al*. (2020) showed that the genetic discontinuities of *Avicennia germinans* along the coasts of Mexico were generated by ocean currents and a physical barrier. Ocean currents between the Andaman Sea and the Malacca Strait have been attributed to the distinct genetic discontinuity of *Rhizophora mucronata* (Wee *et al*., 2014).

Taiwan, a continental island located approximately 150 km off the Southeast Asian coast, harbors significant species diversity (Chiang and Schaal, 2006). More than 4,000 taxa of vascular plants have been recorded in Taiwan (Hsieh *et al*., 1994). The island's coastal ecosystems are shaped by complex ocean currents, which play a crucial role in species distribution. The coastal community consists primarily of taxa with a Pacific Basin distribution, such as *Kandelia obovata*, *I. pes-caprae*, *Artemisia capillaris*, and *Wollastonia biflora* (Hsieh *et al*., 1994). However, long-term human activities have led to the fragmentation of coastal habitats, which has made it difficult to study the population genetics of seaside species. Chiang *et al*. (2001a) suggested that two differentiated lineages of *K. candel* corresponding to geographical regions. Low levels of genetic differentiation

between and within mainland and island populations indicated conspicuous long-distance seedling dispersal across oceans. Ruan *et al*. (2013) proposed that the population structure of *K. obovata* is affected by both different sources of colonizers and the geographic barrier. Long-term isolation by the Taiwan Strait differentiated the Taiwanese and Chinese populations. While mangroves have received more research attention (Chiang *et al*., 2001a; Ruan *et al*., 2013; Huang *et al*., 2015), studies on other seaside plants remain limited (Huang and Hsu, 2023). Among these, *Carex kobomugi* Ohwi, a sand sedge found along Taiwan's coasts, was overlooked until 2020.

Carex L. (Cyperaceae), with about 2000 species, is one of the most species-rich genera among angiosperms (Ford *et al*., 2006). *Carex kobomugi* Ohwi is naturally distributed in the coastal sand dunes and sandy beaches of East Asia, including China, Japan, Korea, Taiwan, and Russian Far East (WFO, 2024). There are several studies on the phylogeny (Ford *et al*., 2006; King *et al*., 2009; King and Roalson, 2009), reproductive system (Ohsako, 2010), invasion (Charbonneau *et al*., 2020; Riffe and Zinnert, 2024). *Carex kobomugi* is closely related to *C. macrocephala*, which is naturally distributed in the coastal sand dunes and sandy beaches of North American, Russian and Japan (Ford *et al*., 2006; King and Roalson, 2008, 2009). King and Roalson (2009) adopted molecular data (chloroplast *rpL16* and microsatellite DNA) to clarify the relationships of the *C. macrocephala* species complex, which includes the Asian *C. macrocephala*, North American *C. macrocephala*, and Asian *C. kobomugi.* The divergence among these lineages is estimated to have occurred during the Late Pleistocene epoch. Incomplete lineage sorting is considered the cause of the non-reciprocal monophyly observed among these lineages. Additionally, *C. kobomugi* was accidentally introduced to North America in 1929 and has become widespread along the Northeast coast of the USA. As an invasive species in the USA, efforts have been made to eradicate it (Wootton *et al*., 2005; Riffe and Zinnert, 2024).

In Taiwan, *C. kobomugi*, which is categorized as a threatened species (category CR: critically endangered) (Editorial Committee of the Red List of Taiwan Plants, 2017), is restricted to northern Taiwan and offshore islands. It was first recorded in 1924 and the specimen was deposited in NTU Herbarium (TAI no. 22663). However, few accurate records were reported until 2020. The known populations in Taiwan are only restricted to Fulon (FL, 福隆) and Mazu (MZ, 馬祖). The FL population, which has larger population size compared to MZ, was threatened by increasing human activities and was under the pressure of possible local extinction. A large amount of construction waste was dumped from 2011 to 2013 and was latter covered by sands, creating a habitat where most of *C. kobomugi* grows. In response to the impacts of habitat disturbance and human activities, the conservation project of *C. kobomugi* has been ongoing since 2020. The construction waste is being manually removed to prevent machinery from affecting the population. However, limited knowledge about *C. kobomugi* in Taiwan complicates efforts to preserve the native populations. Specimen misidentification and incorrect information of sampling locations make it difficult to confirm the distributions of *C. kobomugi* in Taiwan. For known populations, urgent conservation measures, such as *in situ* preservation, germplasm collection and minimizing human disturbance, have been implemented to maintain the wild population size. The next critical question is whether these measures will be sufficient to preserve genetic diversity and structure.

Chloroplast DNA, internal transcribed spacer of nuclear ribosomal (nrDNA) and microsatellite DNA (simple sequence repeat, SSR) are widely used genetic markers for investigating phylogenetic relationships and population genetics (Ge *et al*., 2012; Feng *et al*., 2014; Liu *et al*., 2024; Wang *et al*., 2024). Different DNA markers have specific mutation rates, inheritance patterns, and dispersal mechanisms (Schlotterer, 2000; Kay *et al*., 2006; Huang *et al*., 2012). Chloroplast DNA evolves at a relatively slow rate and is maternally inherited, primarily reflecting the influence of seed dispersal on genetic diversity. In contrast, nrDNA, which evolves at a medium rate, and SSR, which evolves more rapidly, exhibit biparental inheritance, making them useful for assessing the impact of pollen flow on genetic diversity patterns (Feng *et al*., 2014; Liu *et al*., 2024). Furthermore, SSR serves as a valuable tool for analyzing the population structure of endangered species and providing essential information for conservation strategies (Wahlsteen, 2021; Hu *et al*., 2024; Ko *et al*., 2024). Species delimitation and identification of genetically distinct populations provide vital information for further effective conservation measures (Wang *et al*., 2024). In this study, we employed *rpL16* (cpDNA), nrDNA and SSR markers to assess genetic diversity, population differentiation, and genetic structure, with the aim of informing conservation strategies.

MATERIALS AND METHODS

Plant materials

Thirty-three individuals in Taiwan were sampled (Fig. 1, Table 1). Despite several field surveys conducted in the coastal region of northern Taiwan from 2020 to 2023, only two extent populations (FL and MZ) were confirmed. The habitat in FL has been partly covered by construction waste since 2011, leading to changes in the landscape. Although later buried by sand, the waste remains to this day. Based on personal observations, most *C. kobomugi* individuals in FL are found on the sand dunes above the waste, with an estimated population size of approximately 600 individuals. In contrast, the population size in MZ is estimated to be fewer than 100

Table 1. Sample locations, code, coordination, sample size, nucleotide diversity (π), haplotype (h) and haplotype diversity (Hd) of ***Carex kobomugi*** in Taiwan.

Species	Location	Code	Longitude	Latitude	Sample size	nrDNA			cpDNA		
						π	h	Hd	π	h	Hd
Carex kobomugi					33	0	1	0	0	1	0
	Fulong	FL	121.937	25.029	18	0	1	0	0	1	0
	Mazu	MZ	119.981	25.966	15	0	1	0	0	1	0

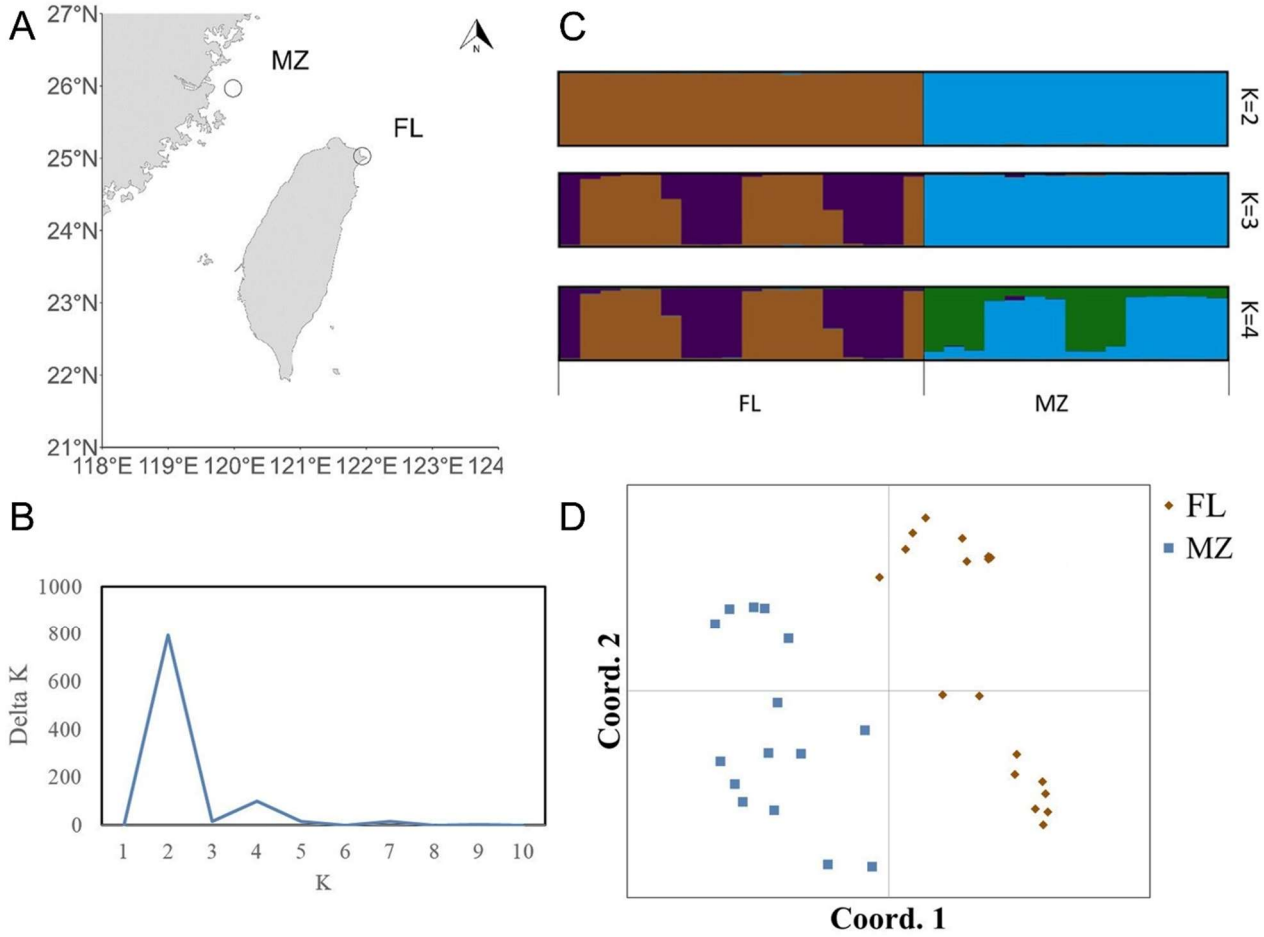

Fig. 1. (**A**) The sample locations of ***Carex kobomugi*** in Taiwan, (**B**) graphical depiction of the relationship between K and ΔK. (**C**) STRUCTURE histogram of genetic cluster membership generated from analysis of microsatellite loci for K =2–4. (**D**) Principal coordinates analysis (PCoA) of ***C. kobomugi*** based on microsatellite loci. FL: Fulon, MZ: Mazu.

individuals. Eighteen and fifteen individuals were collected form FL and MZ, respectively. Yong leaves were collected and dried using silica gels for genomic DNA extraction. The collected specimens were stored in TESRI herbarium.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from powdered tissues following the CTAB procedure (Murray andThompson, 1980). PCR amplification of the *rpL16* spacer (cpDNA; rpL16F71: GCTATGCTTAGTGTGTGACTCGTTG; rpL16R1516: CCCTTCATTCTTCCTCTATGTTG) (Shaw *et al*., 2005) and ITS (nrDNA; ITS4: TCCTCCGCTTATTGATATGC; ITS5: GGAAGTAAAAGTCGTAACAAGG) (Chiang *et al*., 2001b) was performed in a 50 μ L reaction using 10 ng template DNA, 25 μ L GoTaq ® Green Master Mix (Promega, Madison, WI, USA), and 5 pmol of each primer. PCRs was performed in a PCR cycler using the following profile: an initial 10 min denaturation at 94℃, 30 cycles of 45 s denaturation at 94 °C, 1 min 15 s annealing at 55 °C for two markers, and 1 min 30 s extension at 72 °C, followed by a 10 min final extension at 72 °C. All PCR products were purified and then sequenced directly in both directions on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA). Direct sequencing of PCR products generates heterozygous base-calling fluorescence (double peak)

chromatograms, which show paralogous genes within individuals (Chang *et al*., 2012). In this study, the ITS sequences obtained from all individuals did not exhibit any double peaks for any of the sites in the chromatogram obtained by direct sequencing.

Eleven microsatellite loci (Ohsako and Yamane, 2007) were performed in a 25 μ L reaction using 5 ng template DNA, 12.5 μ L GoTaq ® Green Master Mix (Promega, Madison, WI, USA), and 2.5 pmol of each primer. PCRs was performed in a PCR cycler using the following profile: an initial 10 min denaturation at 94℃, 30 cycles of 45 s denaturation at 94 °C, 1 min of specific annealing temperature of each microsatellite locus, and 1 min extension at 72 °C, followed by a 5 min final extension at 72 °C. The PCR products were then analyzed using capillary electrophoresis on the 5200 Fragment Analyzer System (Agilent Technologies, USA), and allelic sizes were determined with the ProSize Data Analysis Software (Agilent Technologies, USA).

Data analysis

Sequences from National Center for Biotechnology Information (NCBI, https:// www. ncbi. nlm. nih. gov/; Supplementary table 1) were used as outgroups to infer the population genetics of *Carex* in Taiwan. For cpDNA and nrDNA, nucleotide sequences were aligned using MAFFT version 7 (Katoh *et al*., 2019) and later adjusted visually. The best-fit models of nucleotide substitution for the alignments were estimated with jModeltest 2.1.10 (Darriba *et al*., 2012). Maximum-likelihood (ML) analyses and Bayesian inference (BI) algorithms were performed to infer the relationships among the studied *Carex* haplotypes. ML analyses were performed using PhyML v. 3.67 (Guindon *et al*., 2010). The bootstrap consensus values were calculated using 1,000 replicates. BI tree was generated with the program MrBayes 3.2.7 (Huelsenbeck and Ronquist, 2001). Two independent Markov chain Monte Carlo (MCMC) runs with 5 X 10^6 generations were performed for the analysis. Trees were sampled every 1,000 generations. The first 25% of the sampled trees were discarded as burn-in, and the remaining trees were used to build a 50% majority-rule consensus tree. FigTree version 1.4.4 (Rambaut, 2018) and the "ggtree" package (Yu, 2020) were applied to depict the ML and BI trees. Levels of genetic diversity within species were quantified with measures of nucleotide diversity (π) (Nei, 1987), haplotype number (*h*), haplotype diversity (*Hd*) using DnaSP 6 (Rozas *et al*., 2017). To depict relationship among *Carex* population belonging to different geographic regions, TCS network of cpDNA haplotype was conducted by PopART V.1.7 (Leigh and Bryant, 2015). CpDNA was used to estimate the divergence times. A mean mutation rate, $\mu = 1 \times 10^{-8}$ /site/year, with an interval set from 1×10^{-7} /site/year to 1×10^{-9} /site/ year, was used for the cpDNA data (Wolfe *et al*., 1987; Willyard *et al*., 2007). The divergence time between Asian *C. macrocephala* and *C. kobomugi* (160,000 years with a 95% confidence interval of 75,000 to 296,000 years) was used as a calibration point (King and Roalson, 2009). We used BEAST version 2.6 to estimate the time of divergence with 10^7 iterations and a burn-in of 10^6 under the HKY model and a strict molecular clock (Bouckaert *et al*., 2019). TRACER v1.7.1 (Rambaut *et al*., 2018) was used to examine the convergence of chains to stationary distribution. TREEANNOTATOR v2.6.3 (Bouckaert *et al*., 2019) and FigTree version 1.4.4 (Rambaut, 2018) were used to summarize and display the sampled tree, respectively.

For microsatellite DNA, to ensure the unique genotype, the multilocus genotypes (MLGs) were identified using R package "poppr" version 2.9.6 (Kamvar *et al*., 2014). The genetic diversity parameters, including the number of individuals (*N*), number of alleles (*Na*), the number of effective alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), Shannon's information index (*I*), fixation index (*F*) were calculated using GenAlEx 6.51 (Peakall and Smouse, 2012). Deviations from Hardy-weinberg equilibrium in each population across all loci were tested using a chi-square test (X^2) in GenAlEx 6.51 (Peakall and Smouse, 2012). Polymorphic information content (PIC) was determined using Cervus 3.0.7 (Marshall *et al*., 1998). The results of analysis of molecular variance (AMOVA), and pairwise F_{ST} analysis were performed to assess genetic differentiation within and among populations by ARLEQUIN 3.5.2.2 (Excoffier and Lischer, 2010). Significance tests were conducted using 10,000 permutations. Principal coordinates analysis (PCoA) was computed by GenAlEx 6.51 (Peakall and Smouse, 2012). Genetic structure was analyzed using a Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard *et al*., 2000). Independent simulations were run of each K (K = 1–10) with 500,000 burn-in steps followed by 5,000,000 MCMC steps. Each analysis was repeat 5 times for each consecutive value of K. StructureSelector was utilized to visualized results from Structure (Li and Liu, 2018). BAYESASS software (Wilson and Rannala, 2003) that use a Bayesian approach and a Markov chain Monte Carlo (MCMC) algorithm was applied to estimate the recent migration rates between populations. We used 50,000,000 iterations, with a burn-in of 5,000,000 generations, and a sampling interval of 2000. The mixing parameters for migration rates (m), allele frequencies (a) and inbreeding coefficients (f) were optimized to achieve posterior acceptance rates of 20–60% for each parameter. Convergence of the MCMCs was checked by comparing the traces of each run using TRACER v. 1.7 (Rambaut *et al*., 2018). We conducted five independent runs with different random seed numbers to ensure consistent results.

Temporal changes in population size were estimated by the VarEff model, that use the coalescent method and

Table 2. Characteristics of the 11 polymorphic SSR markers used in this study.

Locus	N	*Na*	*Ne*	*I*	*Ho*	*He*	PIC	*F*
	32.909	6.545	3.890	1.414	0.586	0.669	0.633	0.122
Cko_1_9	33	11	7.358	2.118	0.545	0.864***	0.849	0.369
Cko_1_12	33	8	4.694	1.748	0.697	0.787***	0.759	0.114
Cko_1_47	33	12	7.003	2.168	0.545	0.857***	0.843	0.364
Cko_1_68	33	4	2.814	1.205	0.939	0.645***	0.599	-0.457
Cko_1_80	33	4	2.672	1.079	1.000	0.626***	0.554	-0.598
Cko_2_56	33	6	2.593	1.237	0.576	0.614***	0.577	0.063
Cko_2_112	33	6	4.605	1.636	0.939	0.783***	0.751	-0.200
Cko_2_113	33	3	2.562	1.005	0.000	0.610***	0.531	1.000
Cko_2_118	33	8	3.582	1.563	0.545	0.721***	0.688	0.243
Cko_2_135	33	3	1.130	0.263	0.091	0.115***	0.111	0.211
Cko_2_139	32	7	3.779	1.536	0.563	0.735***	0.697	0.235

N: number of individuals; *Na*: number of alleles; *Ne*: number of effective alleles; *I*: Shannon's information index; *Ho*: observed heterozygosity; *He*: expected heterozygosity; PIC: polymorphic information content; *F*: fixation index.

approximate likelihoods in a Monte Carlo Markov Chain approach (Nikolic and Chevalet, 2014). The VarEff method was implemented in the R package "VarEff"

Temporal changes in population size were estimated by the VarEff model, that use the coalescent method and approximate likelihoods in a Monte Carlo Markov Chain approach (Nikolic and Chevalet, 2014). The VarEff method was implemented in the R package "VarEff" (https://qgsp.jouy.inra.fr). This analysis was performed assuming a single step model, S, with a microsatellite mutation rate, $\mu = 2 \times 10^{-4}$ (Escudero *et al*., 2013), and burn-in of 10,000 over the past 10,000 generations. The parameter values included setting the assumed prior values for effective size to 10,000, number batch to 50,000, the length and space batch to 10, an acceptance rate of 0.25 and diagonale of 0.5, following recommendations from Nikolic and Chevalet (2014). The median of the posterior distribution was used to visualize demographic inference. Generation time was set to one, based on the fact that *C. kobomugi* is a perennial herb with annual reproduction (Ohsako, 2010).

To predict the suitable habitats for *C. kobomugi*, the maximum-entropy (MaxEnt) model was utilized (Phillips *et al*., 2006). The geographic distribution information of *C. kobomugi* was obtained from the data of Global Biodiversity Information Facility (GBIF, https://www.gbif.org/zh-tw/). 110 occurrence points from Japan, Korea, and Taiwan, recorded between 1970 and 2000, were used to compute species distribution models in MaxEnt. Nineteen bioclimatic factors and elevation data at 30 arc-second (~1km) resolution, for both current (1970–2000) and future (2081–2100, ACCESS-CM2, SSP 5-8.5) scenarios, were obtained from the WorldClim v2.1 database (https://www.worldclim.org/data/worldclim21.html). Bioclimatic factors corresponding to the species distribution were extracted and imported into "dismo" (Hijmans *et al*., 2024) package in R. Strong correlations among environmental variables can reduce model accuracy due to multicollinearity. To address this, Pearson's correlation coefficients (r) were calculated for all pairs of variables and excluded one variable from each pair with $|r| > 0.8$ (Supplementary Fig. S1) (Rana *et al*., 2017). Variables contributing more than 1% to model prediction results were initially retained (Dai *et al*., 2022). Among these, the most influential environmental variables, based on contribution rates in the initial model were selected for further analysis.

Species distribution models were constructed using the MaxEnt function from the "dismo" package (Hijmans *et al*., 2024). The model was run with 30 replicates under a cross-validation scheme, with 75% of the occurrence data randomly selected for training and the remaining 25% for testing in each replicate. The regularization multiplier was set to 1. The average value was used to calculate the potential geographic distribution. The accuracy of the operation results was evaluated using the ROC curve and the area under the curve (AUC) value. Values of AUC range from zero to one, with those closer to one indicating a more accurate model.

RESULTS

Genetic diversity of cpDNA, nrDNA and microsatellite loci

The levels of cpDNA (*rpl16*) and nrDNA genetic diversity of *Carex kobomugi* in Taiwan were examined, as showed in Table 1. The consensus sequences for cpDNA and nrDNA were 756 and 646 bp in length, respectively. No polymorphic sites were detected in either cpDNA (GenBank accession numbers: PQ351396) or nrDNA (GenBank accession numbers: PQ345421), indicating that individuals in both regions were fixed in the single haplotype. Nucleotide (π) and haplotype (*Hd*) diversity were both found to be zero in cpDNA and nrDNA.

Genetic diversity was assessed in 33 individuals from two *C. kobomugi* populations using 11 microsatellite markers (Table 2). Allele size ranged from 157 bp (Cko_1_47) to 293 bp (Cko_2_118), with a total of 72 alleles across all loci. The number of alleles per locus (*Na*) ranged from 3.000 (Cko_2_135) to 12.000 (Cko_1_47), with a mean value of 6.545. The number of effective alleles (*Ne*) varied from 1.130 (Cko_2_135) to 7.358 (Cko_1_9), with an average of 3.890. Observed (*Ho*) and expected (*He*) heterozygosity ranged from 0 to 1.000 and 0.115 to 0.864, with a mean values of 0.586 and 0.669, respectively. The polymorphic information content (PIC) ranged from 0.111 to 0.849, with a mean value of 0.633. A total of 33 multilocus genotypes (MLGs) were identified from 33 samples using the "poppr" package in R. Significantly deviation from Hardy-Weinberg equilibrium (HWE) were detected in all of the loci.

The levels of genetic diversity in the two populations

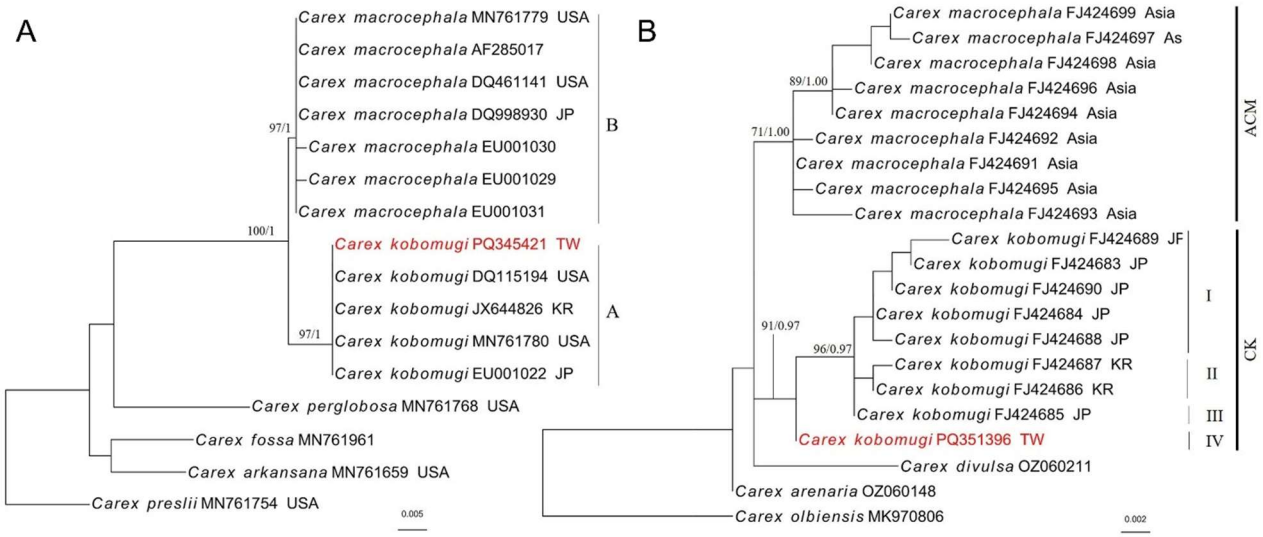

Fig. 2. Phylogenetic tree of nrDNA (**A**) and cpDNA (**B**) based on a maximum-likelihood analysis. The numbers on the branches are bootstrap values (>60)/posterior probabilities (>0.6). Sequence obtained in this study is shown in red, and those retrieved from NCBI are presented in regular black text. ACM: Asian ***Carex macrocephala***, CK: ***Carex kobomugi***. Sequence obtained in this study is shown in red, and those retrieved from NCBI are presented in regular black text.

Table 3. Genetic diversity of ***Carex kobomugi*** in Taiwan.

Species	Location	N	*Na*	*Ne*	*I*	*Ho*	*He*	*F*
Carex kobomugi		32.909	6.545	3.890	1.414	0.586	0.669	0.122
	FL	18	5.273	3.713	1.327	0.833	0.668	-0.220
	MZ	15	3.273	2.316	0.831	0.285	0.461	0.457

N: number of individuals; *Na*: number of alleles; *Ne*: number of effective alleles; *I*: Shannon's information index; *Ho*: observed heterozygosity; *He*: expected heterozygosity; *F*: fixation index. FL: Fulon; MZ: Mazu.

Table 4. Analysis of Molecular Variance (AMOVA) based on SSR loci for *Carex kobomugi.*

Source of variation	df	Sum of squares	Variance components	Percentage variation
Between populations	1	33.3417	0.92089	21.93
Within populations	64	209.856	3.27899	78.07
Total	65	234.273	4.19988	100

are shown in Table 3. *Na* ranged from 3.273 to 5.273, while *Ne* varied from 2.316 to 3.713. *Ho* and *He* ranged from 0.285 to 0.833 and 0.461 to 0.668, respectively. *F* varied from -0.220 to 0.457, and number of private alleles ranged from 1.273 to 3.273, respectively. Higher genetic diversity was detected in FL population compared to MZ.

Genetic differentiation

A single nrDNA haplotype (PQ345421) was found in Taiwan, identical to NCBI samples from Japan (EU001022), South Korea (JX644826), and the USA (MN761780) (Fig. 2A). In contrast, a higher level of genetic differentiation in cpDNA was detected between Taiwan (PQ351396) and others countries (Taiwan vs. Japan (FJ424683–FJ424685, FJ424688–FJ424690), F_{ST} = 0.746; Taiwan vs. South Korea (FJ424686–FJ424687) F_{ST} = 0.889) (Fig. 2B).

For microsatellite loci, pairwise F_{ST} values and AMOVA were conducted to evaluate genetic variation between two *C. kobomugi* populations in Taiwan (Table 4). The genetic differentiation between FL and MZ was moderate (F_{ST} = 0.200, $p < 0.05$), indicating a moderate genetic differentiation between two populations. The AMOVA results indicated that 79.97 % of the total genetic variation occurred within populations and 21.93% was attributed to differences between populations.

The BAYESASS was applied to estimate the recent migration rates between populations based on microsatellite DNA. The rate of gene flow from FL population to MZ population was 0.017 (95% CI = 0.0004–0.0586), while the rate in the opposite direction was 0.019 (95% CI = 0.0005–0.0683). In contrast, the rates of gene flow within FL and MZ populations were 0.983 95% (CI =0.9414–0.9996) and 0.981 (95% CI = 0.9317–0.9995), respectively.

Phylogenetic reconstruction and Genetic structure

The nrDNA and cpDNA phylogenetic trees, reconstructed using both ML and BI methods, revealed consistent topologies. Therefore, only the ML trees are presented, with Bayesian posterior probabilities indicated at the corresponding nodes. (Fig. 2). The HKY+G model, identified as the most suitable model by jModeltest, was used to construct the phylogenetic trees for nrDNA and cpDNA. *Carex preslii* was set as outgroup for nrDNA, while *C. olbiensis* was used as outgroup for cpDNA.

For nrDNA (Fig. 2A), Clade A contains haplotypes of *C. kobomugi* (PQ345421 from Taiwan is identical to NCBI sequences from Japan (EU001022), the USA (DQ115194, MN761780), and South Korea (JX644826)).

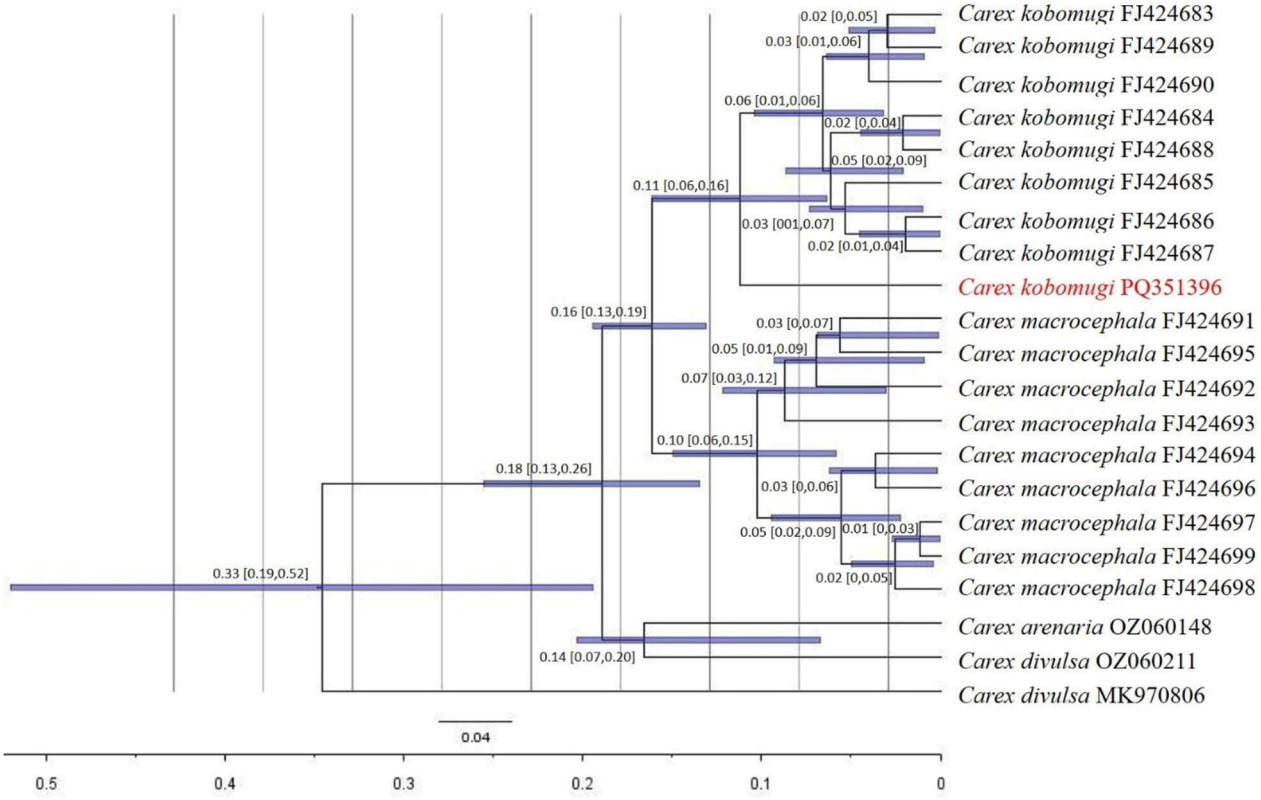

Fig. 3. Results of coalescence time estimations performed using BEAST, v 2.6. The numbers at each nodes and in parentheses represent the estimated divergence times (in Mya) and their corresponding 95% highest posterior density (HPD) intervals. Sequence obtained in this study is shown in red, and those retrieved from NCBI are presented in regular black text.

Clade A is closely related to clade B, which consisted of all *C. macrocephala* (AF285017, EU001029, EU001030, and MN761779).

For cpDNA (Fig. 2B), *C. kobomugi* formed a monophyletic clade (Clade CK), which comprised subclade I (Japan: FJ424683–FJ424684, FJ424688–FJ424690), II (South Korea: FJ424686–FJ424687), III (Japan: FJ424685) and IV (Taiwan: PQ351396). Subclade IV was distantly related to others. Clade CK was closely related to clade ACM (Asian *C. macrocephala*: FJ424691–FJ424699). *Carex divulsa* (OZ060211), *C. arenaria* (OZ060148) and *C. obliensis* (MK970806) were used as outgroups.

Bayesian estimates of the divergence time of *C. kobomugi* based on cpDNA were obtained using the BEAST program (Fig. 3). The Taiwan lineage (PQ351396) was the first to diverge from the other lineages, with an estimated divergence time of approximately 0.11 may (95% HPD: 0.06–0.16).

The TCS network of cpDNA haplotypes revealed thatthe clustering corresponded with geographical distribution, with samples from different regions forming distinct clades. The haplotype from Taiwan was unique and distinct from those in Japan and South Korea (Fig. 4).

The STRUCUTRE results revealed that ΔK reached a maximum value when $K = 2$ (Fig. 1), indicating that the 33 samples can be divided into two subgroups. The subgroup membership probabilities of 33 individuals from the two populations are shown in Fig. 1. These two subgroups corresponded to their geographical distribution. Also, the PCoA results (Fig. 1) were consistent with the STRUCTURE results.

VarEff was used to estimate the current and past effective population sizes and the changing times in *C. kobomugi* (Figs. 5 & S2). The results showed that the effective population size remained stable until approximately10,000 years ago, after which it began to decline, assuming a generation time of one year and a fixed mutation rate of 2×10^{-4} (Ohsako, 2010; Escudero *et al*., 2013). Different populations (FL and MZ) experienced different demographic histories. FL underwent recent population expansion following a dramatic decline, maintaining a larger population size, while MZ experienced population decline and remained smaller.

Distribution of suitable habitats for *C. kobomugi* in Taiwan

The MaxEnt model for *C. kobomugi* had high predictive power (mean AUC value = 0.994) for inferring the suitable habitats in Taiwan (Fig. 6). Based on MaxEnt modeling results, Precipitation of Warmest Quarter (bio18; 27.6%), Elevation (26.1%) Precipitation of Driest

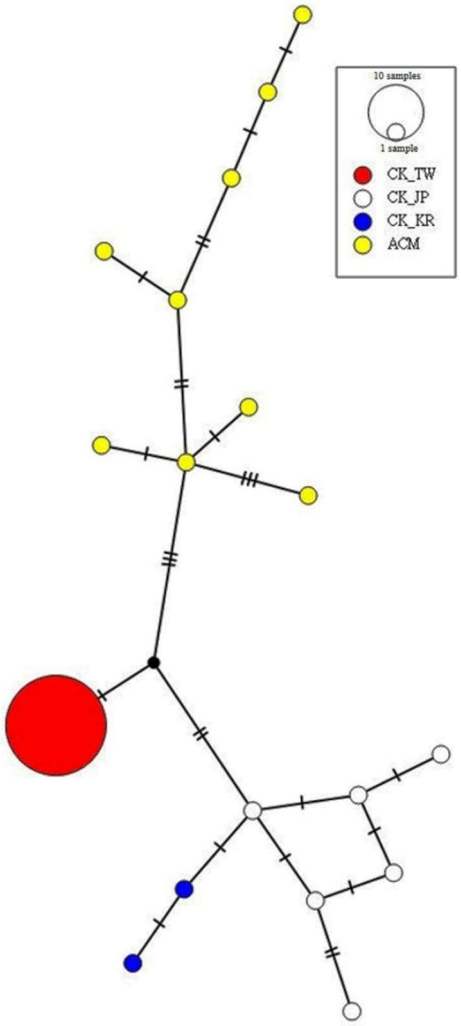

Fig. 4. The TCS network of ***Carex macrocephala*** and ***C. kobomugi*** based on cpDNA haplotype. Each circle represents a haplotype, and the circle size is proportional to the haplotype frequency. ACM: Asian ***C. macrocephala***, CK: ***C. kobomugi*** (KR: South Korea, JP: Japan, TW: Taiwan).

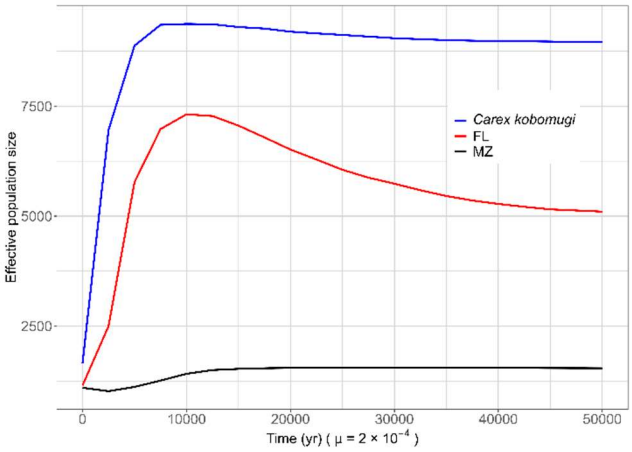

Fig. 5. Population size fluctuation of ***Carex kobomugi***. μ, mutation rate per locus per generation. FL: Fulon, MZ: Mazu.

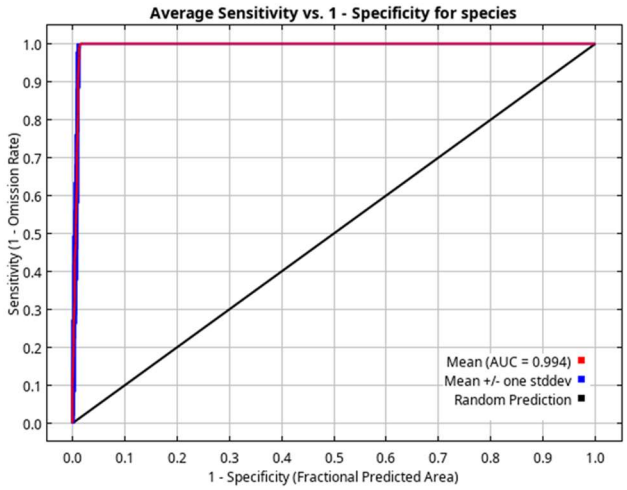

Fig. 6. ROC curves in the developing ***Carex kobomugi*** distribution model. The red (training) shows the fit of the model to the training data. The blue (testing) line indicates the fit of the model to the testing data.

Month (bio14; 21.0%), and Temperature Seasonality (bio4; 18.9%) accounted for over 93.6% of the contribution (Supplementary table S2). These results indicates that precipitation, temperature variation and elevation are critical factors influencing the suitability of *C. kobomugi* (Fig. 7). We selected four variables to predict the current and future distributions for *C. kobomugi* (Figs. 8 & S3). The current potential distribution areas of *C. kobomugi* are located in the coastal regions of northern Taiwan and match the observed distributions. The future potential areas of *C. kobomugi* in Taiwan are contracted to several suitable habits scattered in the current distribution range. Environmental variables differed between FL and MZ, including Precipitation of Warmest Quarter (708 mm vs. 372 mm), Precipitation of Driest Month (179 mm vs. 41 mm), Temperature Seasonality (466.439 vs. 648.710) and Elevation (18 m vs. 30 m).

DISCUSSION

Genetic diversity

CpDNA and nrDNA, which have been widely used for phylogenetic analyses of *Carex* (Roalson and Friar, 2004; King and Roalson, 2008, 2009; Yano *et al*., 2010), were collaboratively applied to study the genetic diversity of *Carex* in Taiwan. For *C. hakkodensis* Franch, *C. scita* Maxim and *C. stenatha* Franch. Et Sav., only one to four cpDNA haplotypes were identified in Japan (Senni *et al*., 2005), while a higher number of cpDNA haplotypes were detected in *C. conica* Boott (Yano *et al*., 2010), *C. macrocephala* and *C. kobomugi* (Japan and South Korea) (King *et al*., 2009). In this study, a single haplotype was found for both cpDNA and nrDNA, revealing that *C. kobomugi* in Taiwan possessed low genetic diversity (Table 1). *Carex kobomugi,* which is primarily distributed

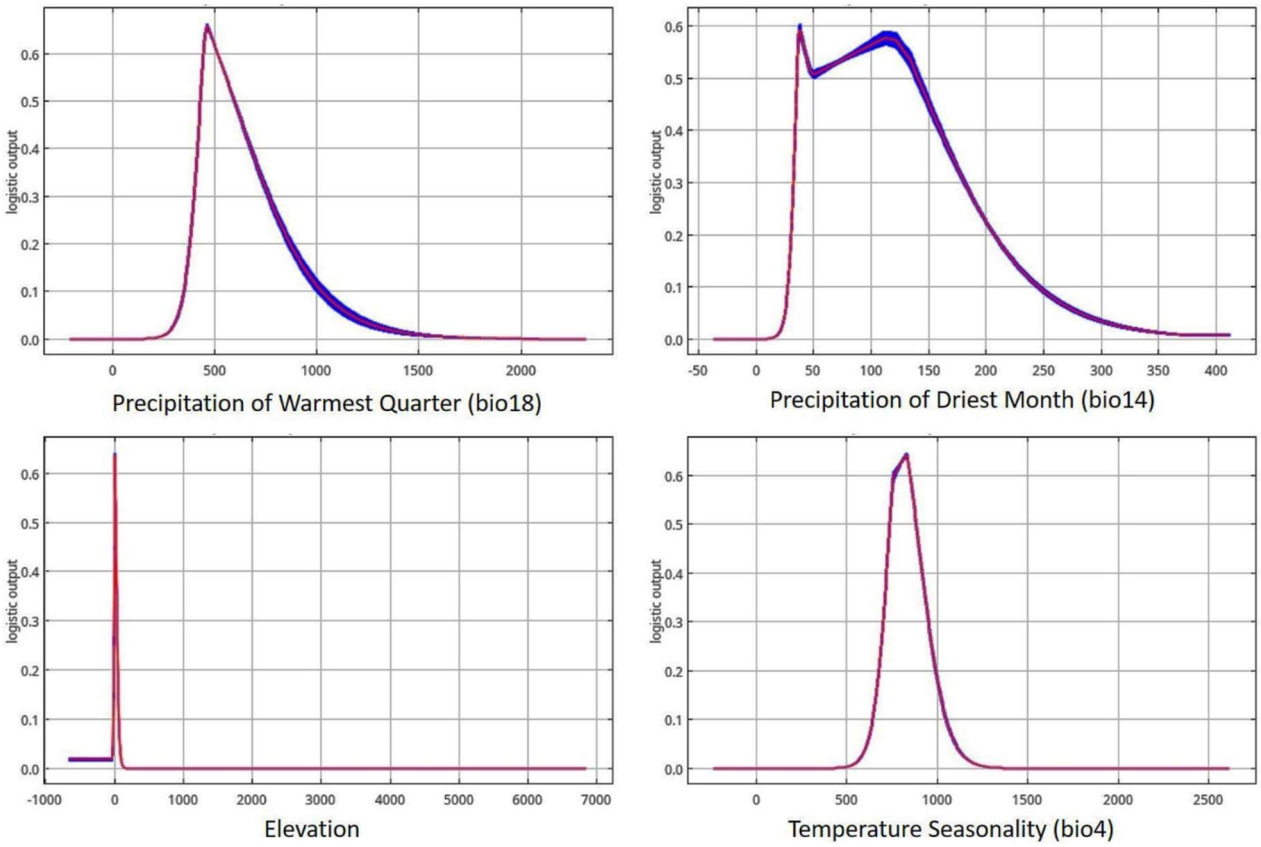

Fig. 7. Response curves (blue indicates standard deviation) for important environmental predictors in MaxEnt model for ***Carex kobomugi***:

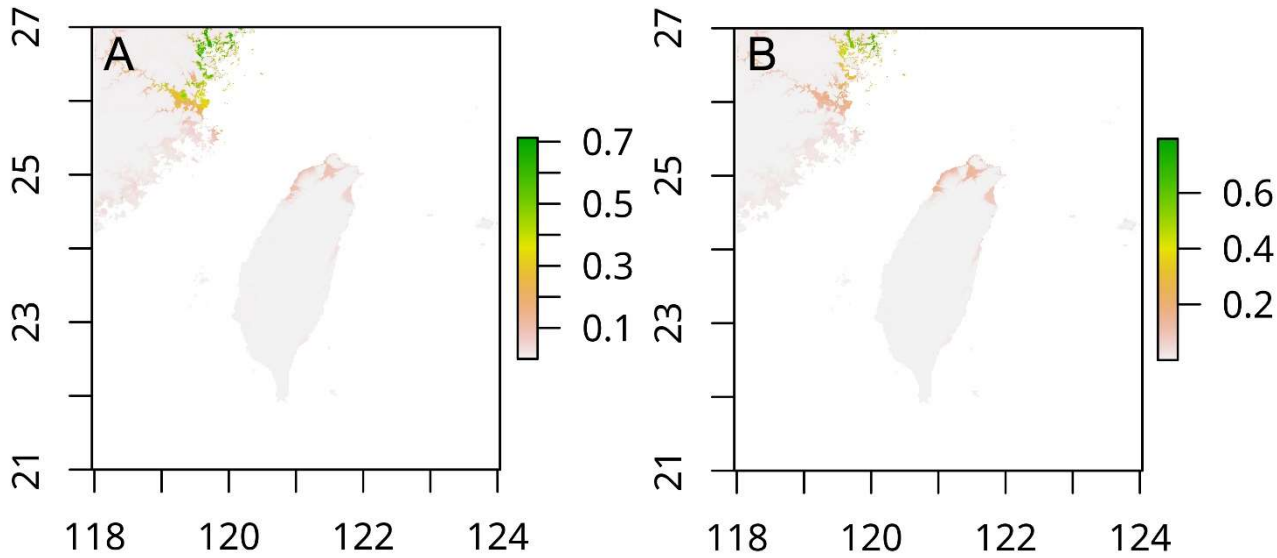

Fig. 8. Potential distributions of ***Carex kobomugi***. (**A**) Present (1970–2000) and (**B**) future potential distribution (2081–2100) were predicted using MaxEnt based on four bioclimatic variables.

in temperate Asia, has a peripheral distribution in Taiwan. Peripheral populations are often located in less optimal environments and are isolated from central andcontinuous populations. The smaller population size of peripheral populations make them more vulnerable to stochastic or catastrophic demographic events (Lammi *et al*., 1999; Lesica and Allendorf, 1995). Peripheral populations are expected to exhibit low genetic diversity (Channell, 2004). The lack of genetic diversity in *C. kobomugi* in Taiwan may be attributed to suboptimal habitats, small population sizes and prolonged isolation from other populations (Madsen *et al*., 1999; Furlan *et al*., 2012).

Different molecular markers are used to assess genetic diversity, population structure, and phylogeographical patterns. Microsatellite loci provide more detailed information on genetic structure, while cpDNA and nrDNA sequences are more effective for detecting phylogeographical patterns (Zhang *et al*., 2020, 2021; Liu *et al*., 2024). Microsatellite loci have been widely applied to evaluate the genetic diversity of *Carex* species, such as *C. breviculmsi* (*Ho* = 0.057), *C. hebes* (*Ho* = 0.105) (M'Baya *et al*., 2013), *C. macrocephala* (*Ho* = 0.041) (King *et al*., 2009) and *C. kobomugi* (*Ho* = 0.575–0.658) (Ohsako, 2010).

A total of 33 multilocus genotypes (MLGs) were identified among 33 samples in Taiwan. Our analysis of *C. kobomugi* from two populations revealed significant deviations from HWE due to heterozygote deficiency (table 2). King *et al*. (2009) proposed that high levels of selfing (*F* = 0.979) contributed to departures from HWE in *C. macrocephala*. Similarly, the positive *F* value (*F* = 0.122) observed in *C. kobomugi* suggests nonrandom mating led to deviations from HWE (table 3).

A lower level of heterozygosity (*Ho* = 0.586) was observed compared to that in Japan (*Ho* = 0.575–0.658) at the same loci (Ohsako, 2010). The low levels of genetic diversity in cpDNA, nrDNA and SSR suggested that *C. kobomugi* in Taiwan corresponds to peripheral populations, which tend to occur in less suitable habitats and are often smaller, making them more prone to extinction due to stochastic or catastrophic demographic events.

However, different populations have experienced different population dynamics. Peripheral populations often exhibit genetic and morphological divergence from central populations (Lesica and Allendorf, 1995). In the FL population, higher observed heterozygosity (*Ho* = 0.833) and a negative inbreeding coefficient (*F* = -0.220) were detected, whereas the MZ population showed a lower observed heterozygosity (*Ho* = 0.285) and a positive inbreeding coefficient (*F* = 0.457). Geographic isolation, decreased gene exchange, and small population sizes increase the risk of genetic drift and inbreeding (Ellstrand, 1993; Madsen *et al*., 1999; Ko *et al*., 2024). The VarEff results (Fig. 5) showed that the FL population underwent recent population expansion following a dramatic decline, maintaining a larger population size, while the MZ population experienced population decline and remained smaller. The FL population, characterized by a larger population size and a tendency for outcrossing, displayed greater genetic diversity. Ohsako (2010) proposed that the prolonged lifespan of individuals and mating system of dioecy could contribute to the maintenance of genetic diversity. In contrast, the MZ population, with its smaller size and inclination toward inbreeding, exhibited lower genetic diversity.

Phylogeographic pattern, genetic differentiation and genetic structure

King and Roalson (2009) proposed that the divergence among Asian *C. macrocephala*, North American *C. macrocephala*, and *C. kobomugi* occurred during the Late Pleistocene epoch based on *rpL16* spacer of cpDNA and microsatellite loci. The estimated divergence time between Asian *C. macrocephala* and *C. kobomugi* is approximately 0.16 mya. Most of Taiwan' flora is thought to have originated during the Pleistocene glacial cycles (Chen *et al*., 2023). The single nrDNA haplotype from Taiwan was identical to NCBI sequences from Japan, the USA, and South Korea, which is consistent with King and Roalson's finding that nuclear markers show little to no variation within *Carex* (King and Roalson, 2008). In contrast, the single cpDNA haplotype from Taiwan was distantly related to those from other regions. In this study, the phylogeographic pattern of *C. kobomugi* was assessed using the cpDNA. Based on the BEAST results, all *C. kobomugi* haplotypes coalesced approximately 0.11 mya, and PQ351396 (the Taiwan-inclusive haplotype) diverged from the rest, suggesting that *C. kobomugi* migrated into Taiwan during the Late Pleistocene. Furthermore, based on microsatellite loci, the VarEff results showed that *C. kobomugi* maintained a stable population size but experienced a recent population decline after the Last Glacial Maximum. During the Pleistocene glacial period, the retreat of coastlines on both sides of the Taiwan Strait led to the disappearance of mangrove habitats in the region (Ruan *et al*., 2013). Consequently, habitat degradation following the Last Glacial Maximum may have contributed to the population decline of *C. kobomugi.* The persistently small effective population size observed in MZ suggests that MZ has long experienced suboptimal environmental conditions, possibly due to the loss of suitable coastal habitats during the Last Glacial Maximum. In contrast, the population decline in FL appears to have occurred more recently, likely as a result of habitat loss caused by post-glacial sea-level rise.

For both nrDNA and cpDNA, a single haplotype was detected in Taiwan populations, indicating the lack of genetic differentiation between populations. However, the genetic structure of *C. kobomugi* was found to consist

of two distinct groups: FL and MZ, as revealed by the STRUCTURE and PCoA of microsatellite loci. The F_{ST} (0.200) also suggested moderate genetic differentiation between the FL and MZ populations. The study revealed genetic difference in population structures between FL and MZ populations. Two possible explanations could account for this pattern.

First, the higher contemporary gene flow within populations (m = 0.980–0.983) were detected than that between populations (m = 0.017–0.020). These results correspond with the central-marginal hypothesis that peripheral populations have lower genetic diversity and higher genetic similarity because of genetic drift and higher local gene flow (Eckstein *et al*., 2006). Takayama *et al*. (2008) proposed that the high differentiation observed in African populations of *Hibiscus tiliaceus* was due to the geographical barrier of the African continent, whereas the differentiation in *H. pernambucensis* was attributed to the bifurcating South Equatorial Current. Yamamoto *et al*. (2019) suggested that ocean currents could act as cryptic barriers, corresponding to the population differentiation observed in *Vigna marina*. The buoyant seeds of *C. kobomugi* are capable of sea-water dispersal (Ishikawa *et al*., 1993; Yang *et al*., 2012). Ishikawa *et al*. (Ishikawa *et al*., 1993) proposed that the moist-chilling condition in winter help *C. kobomugi* seeds germinate in the following spring. The southward current, the Zhejiang-Fujian Coastal Current, the northward Taiwan Warm Currents, and the incursion of the Kuroshio Current branch, control the hydrological conditions in the Taiwan Strait (Liao *et al*., 2018). The combined effects of these currents may act as a geographic barrier, hindering gene flow between populations. Therefore, differentiation between the *Carex* populations might have been caused by successful colonization by sea-drifted seeds followed by an interruption of gene flow.

Second, local adaptation may shape the population structure (Savolainen *et al*., 2013). Selective factors, such as climate, edaphic factors and parasites can contribute to local adaption, and evolved traits can respond to selection pressures (Savolainen *et al*., 2007). Compared to genetic drift, which leads to random population structure changes (Yan *et al*., 2022), local adaption may drive the structure towards a specific composition based on selective pressures. Fournier-Level *et al*. (2011) proposed that the fitness-associated loci of *Arabidopsis thaliana* exhibited local adaptation with geographic and climatic signatures. Fan *et al*. (Fan *et al*., 2023) proposed that the correlation between genetic variation in microsatellite markers and climatic variables is stronger than that with geographic variables, suggesting the climatic factors are the primary drivers of local adaptation of *Toona ciliata* var. *pubescens*. Based on the worldclim2 data, environmental variables (Precipitation of Warmest Quarter and Driest Month and Temperature Seasonality) differed between FL and MZ. These differences in climate and environmental factors could drive the FL and MZ populations into distinct genetic structure, leading to local adaption. Further studies using correlation analysis between morphological traits and genomic data are recommended for future research (Zimmerman *et al*., 2020).

SDM analysis indicated that Precipitation of Warmest Quarter, Precipitation of Driest Month, Temperature Seasonality and Elevation contributed the most to model development. Based on the current potential distribution areas (Fig. 8), Taiwan represents a less suitable habitat, which is consistent with hypothesis of peripheral populations. The contracted habitats in the future potential areas implies that *C. kobomugi* in Taiwan is in critical situation and requires conservation efforts.

Conservation strategies of *Carex kobomugi* in Taiwan

Maintaining higher genetic diversity, adaptive potential and avoiding inbreeding depression are crucial for conservation (Kardos *et al*., 2021). The cpDNA results suggest that the haplotype in Taiwan is distinct from those in Japan and South Korea. Two possible explanations could account for this: First, populations in Taiwan, located at the southern boundary of the species' range, may have a different evolutionary history compared to northern populations. One lineage may have split from the northern populations, migrated to Taiwan, and gradually undergone lineage sorting. Second, only limited genetic studies have focused on *C. kobomugi* (Ford *et al*., 2006; King *et al*., 2009; King and Roalson, 2009; Ohsako, 2010). The haplotype found exclusively in Taiwan could potentially be present elsewhere but has yet to be discovered due to insufficient sampling. Currently, the eight cpDNA haplotypes available from the NCBI database constitute the only genetic evidence used for this study.

The identification of Management Units (MUs) provide essential information for conservation. Preserving MUs, which are populations with significant genetic divergence, helps maintain the species diversity (Palsbøll *et al*., 2007; Coates *et al*., 2018). *Carex kobomugi*, with only two populations and small population size in Taiwan, has been categorized as Critically Endangered (CR) (Editorial Committee of the Red List of Taiwan Plants, 2017). Recent increases in human activities may lead to habitat loss. Microsatellite loci results suggest that FL and MZ exhibit different genetic structures and high percentage of variation (78.07 %) within populations, indicating that the two populations should be considered independent units. Furthermore, FL shows higher genetic diversity and a larger population size, making it a priority for conservation efforts.

Conservation efforts for *C. kobomugi* in Taiwan were initiated after its rediscovery in 2020. Actions such as manually conducted beach cleanups (replacing machinery), species inventories, and both *in-situ* and *ex-*

situ conservation have been implemented to mitigate human disturbance. Clugston *et al*. (2024) employed RADSeq data to confirm the uniqueness of *Sabal antillensis* and *S. lougheediana,* proposing further conservation implications and recommendations to protect these species. This study confirmed the presence of a unique cpDNA haplotype in Taiwan, and both populations exhibited distinct genetic components, suggesting that each should be considered in further conservation strategies. The genetic variation of *C. kobomugi* exists mainly within populations, and the conservation efforts to maintain the genetic diversity within populations are suggested.

First, to protect the existing populations, meetings with authority in charge were held and management strategies were modified to reduce human disturbances. For example, construction waste is being manually removed to prevent machinery from impacting the population. Second, germplasm collections from two populations are essential for preserving genetic diversity. During our field observations of the FL population in 2023–2024, approximately 600 individuals were recorded and seed production was found to be low, restricting the potential for seed-based reproduction in *ex-situ* conservation. Therefore, asexual reproduction via cutting propagation is required to maintain the population size. Identifying the MLGs of *C. kobomugi* for asexual reproduction helps sustain higher genetic diversity. Currently, genetic materials from both populations have been collected and *ex-situ* preserved at TBRI (Taiwan Biodiversity Research Institute). *Carex kobomugi* is a perennial herb that can propagate sexually via wind pollination and asexually via elongating rhizomes (Ohsako, 2010). The identification of different MLGs using SSR markers, along with continued germplasm collection, is recommended to enhance *ex-situ* conservation efforts.

Global climate change, which causes the rise in sea level, poses a significant threat to coastal plants (Garner *et al*., 2015). Inundation, flooding, and erosion, which are caused by sea level rise, could lead to habitat loss. This study provides insights into the genetic conservation and population dynamics of *C. kobomugi.* We identified distinct management units and collected genetic materials for *ex-situ* preservation. These actions not only help preserve genetic diversity but also contribute to the conservation of *C. kobomugi* in Taiwan. Our comprehensive approach aims to ensure the survival of *C. kobomugi* through appropriate management strategies.

CONCLUSION

In summary, this study used molecular markers to assess the genetic diversity, population differentiation and genetic structure of *C. kobomugi*. As peripheral populations in Taiwan, *C. kobomugi* exhibits a unique haplotype compared to those from Japan and Korea. A distinct evolutionary history and geographic isolation have led to the genetic differentiation between the FL and MZ populations. Based on these findings, two distinct management units are recommended for further conservation efforts. Actions such as manually conducted beach cleanups, species inventories, and both *in-situ* and *ex-situ* conservation have been implemented to mitigate human disturbance.

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