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Ecological factors correlate with genome size variation of *Acanthocalyx* (Caprifoliaceae) in the Hengduan-Himalaya Mountains

Nan WANG[1,2], Chih-Chieh YU[1], Yan-Xia JIA[3], Yao-Wu XING[1,*]

1. *CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666303, Yunnan, China.* ***2.*** *University of Chinese Academy of Sciences, Beijing 100049, China.* ***3.*** *Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, China.* [*]*Corresponding author's tel & fax: + 86 691 8715585; email: ywxing@xtbg.org.cn*

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ABSTRACT: While the genome sizes of flowering plants vary c. 2400-fold, it remains little known what factors may have driven the variation. In this study, we investigated the spatial pattern of the genome size of 54 populations of *Acanthocalyx*, which is found in the Hengduan-Himalaya Mountains. Our results showed that the red-flowered lineage of *Acanthocalyx* had significantly larger genomes (ranging from 1.9 to 2.5 Gb) compared to the white-flowered lineage, which had an average genome size of 1.27 Gb. This difference in genome size can be attributed to particular environmental factors. Within the red-flowered lineage, the genome size was positively correlated with soil nitrogen content and mean diurnal range. On the other hand, the genome size of the white-flowered lineage, *Acanthocalyx alba* was negatively correlated with latitude which aligns with the population dynamics of this species during the Pleistocene. Overall, our findings highlight the influence of abiotic factors and geography in regulating the genome size of *Acanthocalyx* species. This study contributes to our understanding of the evolution of alpine plants in the Hengduan-Himalaya Mountains.

KEY WORDS: *Acanthocalyx alba*, *Acanthocalyx delavayi*, *Acanthocalyx nepalensis*, flow cytometry, Pleistocene, speciation.

INTRODUCTION

Genome size (GS) variation is an inherited genetic trait that plays a significant role in the evolution of species at the cellular level (Leong-Skornickova *et al*., 2007). Previous researches have highlighted the importance of chromosome morphology in the ecology and evolution of plants in extreme environments, such as high mountains and subarctic regions (Bennett, 1976; Rieseberg, 2001; Waters *et al*., 2010; Meng *et al*., 2014; Puttick *et al*., 2015; Sedel'nikova, 2016). However, the factors that drive GS variation, both within and between species, in montane regions are still unclear. It has been observed that GS variations are often associated with physiological and climatic niches. Knight *et al*. (2005) proposed the 'large genome constraint' hypothesis, suggesting that organisms with large genomes are typically absent from harsh environments, such as cold and dry regions. In alpine regions, the growth and development of plants are limited by low temperatures (Reeves *et al*., 1998). The GS of these plants is generally lower than the average values within the angiosperms (Dodsworth *et al*., 2015). However, there are significant differences in GS among species and even within species, which may be due to adaptations to the diverse high-altitude environments (Bennett and Smith, 1976; Loureiro *et al*., 2013). For example, Basak *et al*. (2018) found that GS variation in *Brassica rapa* var. *rapa*, which is found at high altitudes, is correlated with climatic variables. Additionally, Terlevic *et al*. (2022) suggested that the presence of refugia during glacial periods may have influenced the geographic patterns of GS in alpine plants of the Caryophyllaceae family in the Balkan Peninsula and the Alps. However, our understanding of GS variation and its underlying factors in biodiversity hotspots, particularly in mountain regions with significant environmental gradients, is still limited.

The Hengduan-Himalaya Mountains (HHM) region consisting of two global biodiversity hotspots is known for its rich plant diversity and high endemism (Wu, 1988; Li *et al*., 2014; Ding *et al*., 2020). Previous researches have focused on the role of polyploid speciation in the evolution of species at the cellular level in this region (Chen *et al*., 2007; Han *et al*., 2020). However, there have been limited studies on the genetic variation within populations. *Acanthocalyx* is a small herbaceous genus that belongs to the Caprifoliaceae. It is endemic but exclusively found in the high-altitude regions in the HHM region, ranging from the Nepalese Himalayas to the Hengduan Mountains (HDM) (Yang and Landrein, 2011). In earlier taxonomic classifications, *Acanthocalyx* was placed under the Section *Acanthocalyx* of *Morina s.l.* (Bunge, 1852). However, recent studies using morphological and molecular analyses have supported the establishment of the genus and its infrageneric classification, recognizing three species: *Acanthocalyx alba* (Hand.-Mazz.) M. J. Cannon, *A. delavayi* (Franch.) M. J. Cannon, and *A. nepalensis* (D. Don.) M. J. Cannon (Cannon and Cannon, 1984; Mu *et al*., 2020) (Fig. 1A). *A. alba*, the white-flowered species, is primarily distributed in the HDM, with a few scattered populations found in the eastern Himalayas (east of the Mekong-Salween divide).

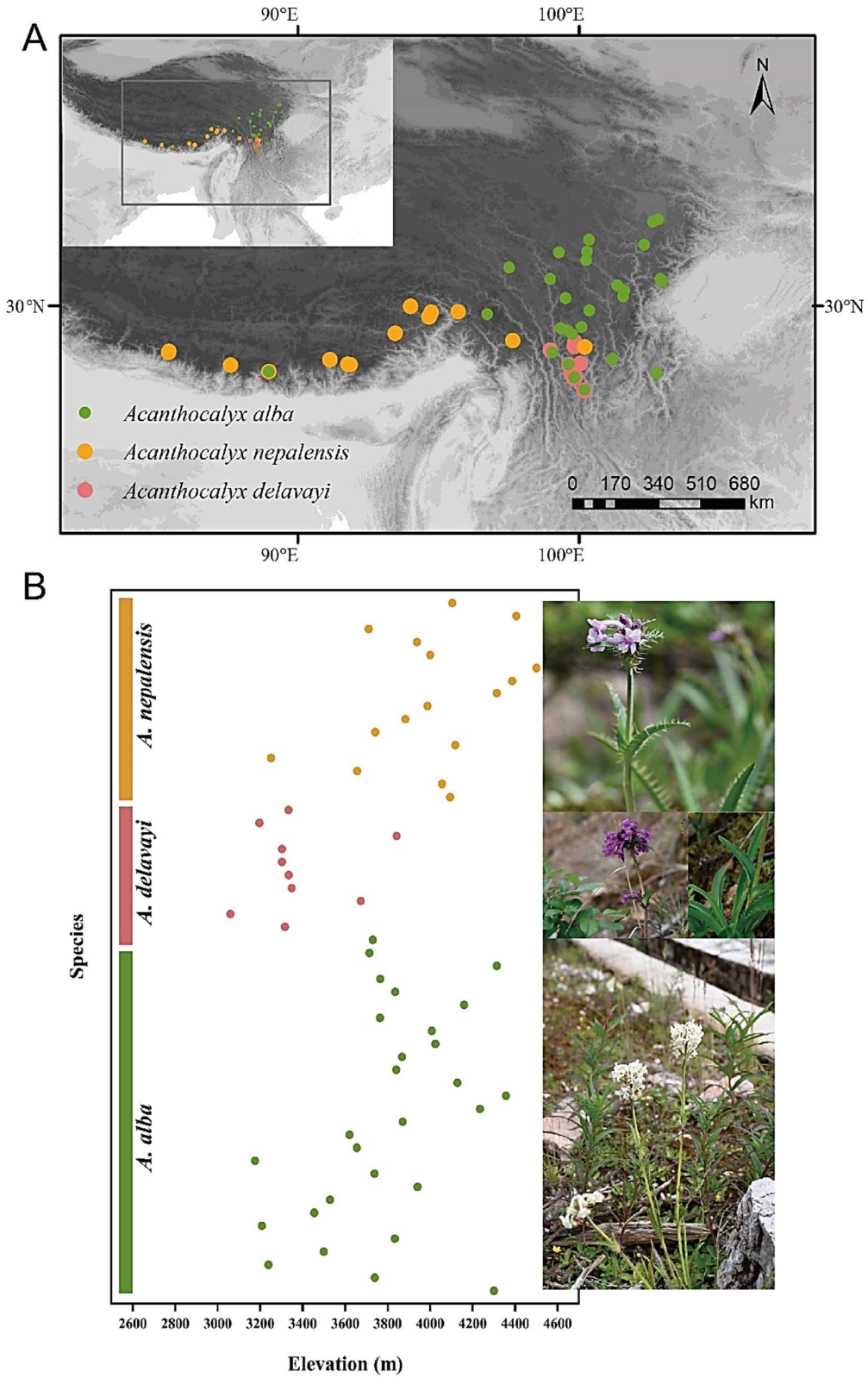

Fig. 1. The basic situation of the research area and research group of this study. **A.** Geographic pattern of GS variation of the red-flowered species (***Acanthocalyx nepalensis*** and ***A. delavayi***) in the HHM, with point size proportional to the GS of the sampled populations. Geographic pattern of GS variation of the white-flowered species of Acanthocalyx in the HHM. **B.** Elevational distribution of species of ***A. nepalensis*** (orange), ***A. delavayi*** (red), A. alba (green). Photos on the right illustrate the habitat and overall habit of each ***Acanthocalyx*** species.

The red-flowered lineage of the genus is represented by *A. nepalensis* and *A. delavayi*. *A. nepalensis* is mainly found at the forest edge and in the forest understory of high mountains in the southern part of the HDM and the Himalayas, while *A. delavayi* is restricted to the southern part of the HDM (Yang and Landrein, 2011) (Fig. 1B). The spatial pattern of GS variation in *Acanthocalyx* is still not well understood.

The objective of this study is to examine the geographic distribution and potential factors influencing the variation in GS of *Acanthocalyx*. Specifically, we will investigate whether there are significant differences in GS between or within species, and identify the abiotic factors that are associated with the variation in GS of *Acanthocalyx*.

MARERIALS AND METHODS

Sample collection

Fifty-four populations of *Acanthocalyx* were sampled from the HHM region covering the natural range of the three species (Fig. 1A and Supplemental Table S1). We collected one to three individuals within each population, where individuals were each isolated at least by 30 m following the protocol of Terlevic *et al*. (2022). In order to ensure the accuracy of the experimental data, one individual from each population was selected randomly for flow cytometry. The GS of each sample was estimated from the basal leaves that were dried by silica gel.

Chromosome count

To determine the chromosome number of *A. alba*, we followed the methods described by Tito *et al*. (1991) and Doležel *et al*. (1992). Healthy seeds were first subjected to water selection and disinfection treatment. They were then placed in a culture box at a temperature of 25 ℃ for soaking. When the root growth reached 0.5–1.0 cm, root tips were collected around 9:30 am. The root tips were pre-treated with 0.002 M 8-hydroxyquinoline in darkness at 5 ℃ for 4 hours. After pre-treatment, the root tips were fixed in a solution of 3:1 absolute alcohol: glacial acetic acid. The fixed root tips were hydrolyzed in 5 M HCl at room temperature and cut to approximately 2 mm in length using a blade. They were then stained with Schiff's reagent for 1 hour. Finally, the stained samples were examined under a microscope and the number of chromosomes was counted. For *A. delavayi*, we collected a young living plant from Lijiang in Yunnan province and counted its chromosomes in the same way when it grew new roots.

Flow cytometry

Flow cytometry was utilized to determine the GS of the sampled *Acanthocalyx* populations. A total of 54 populations were selected for this study, "maize B73 (2.3 Gb)" and "tomato Heinz 1706 (0.9 Gb)" were used as internal standard. The materials were finely chopped using razor blades in 3 ml of ice-cold modified Galbraith's chopping buffer (Galbraith *et al*., 1983) and filtered through a 40 μm pore size mesh to obtain a nuclei suspension. The suspension was collected in a 5 mL tube placed on ice. Prior to measuring the DNA content of the cell nuclei, 500 μL of staining solution (495.5 μL Staining Buffer + 3 μL Propidium Iodide (PI) + 1.5 μL RNaseA) was added to the nuclei suspension, which was then stained on ice in the dark for 0.5–1 hour. The internal reference samples were prepared in the same manner. The stained nuclei suspension samples were analyzed using a BD FACScalibur flow cytometer. The GS was calculated using the following equation: 2C = (sample G1 peak mean/standard G1 peak mean) × standard 2C genome size (Doležel *et al*., 2007). CV was controlled within 5%, and the number of cells collected in each sample was more than 1000.

Environmental data

To assess the environmental differences among three species, we used the RASTER v.2.6-6 package in R (R Core Team, 2018) to extract climatic, soil, and direct normal irradiance (DNI) values for each population. The climate variables were obtained from the WorldClim dataset (Fick and Hijmans, 2017), which includes 19 variables (see Supplemental Table S3 for detailed information). Soil data, including pH, CEC (cation exchange capacity), TN (total nitrogen), and SOM (soil organic matter), were collected from the dataset of Liu *et al*. (2022). DNI data were obtained from https://globalsolaratlas.info. Altitude data were obtained from our field measurements.

Descriptive statistics and niche divergence analysis

We used the Wilcoxon rank-sum test to compare the GS of three species of *Acanthocalyx*: *A. nepalensis* (16 individuals), *A. delaveyi* (11 individuals), and *A. alba* (27 individuals). Based on the results of GS, we grouped *A. delavayi* and *A. nepalensis* with large and similar genomes into the red-flowered lineage, and *A. alba* with small genome into the white-flowered lineage for the following analysis. To test whether there is niche divergence between red-flowered lineage and white-flowered lineage, niche overlap or divergence test were conducted using 19 climatic variables, four soil factors and DNI. We performed Schoener's niche equivalency (identity) and similarity test (D), and Warren's niche background test (I) using the 'humboldt' R package (Brown and Carnaval, 2019). The niche similarity (D) is measured on a scale from 0 to 1, where 0 signifies completely dissimilar niches and 1 denotes completely similar niches. The background test (I) assesses the power to detect the differences between the two lineages. A non-significant equivalency statistic (D) and a significant background statistic(I) support the underlying null hypothesis that species environmental niches are identical (Brown and Carnaval, 2019). It should be noted that since

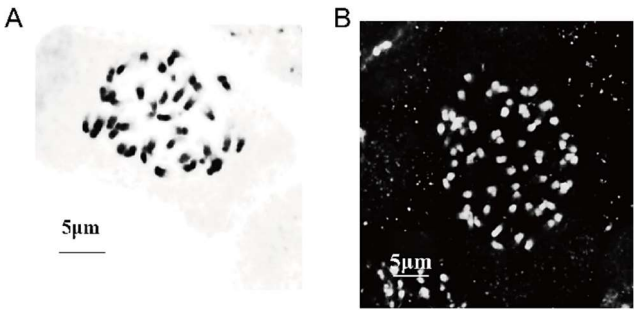

Fig. 2. Chromosome numbers of ***A. alba*** (**A**) and ***A. delavayi*** (**B**).

'humboldt' is suitable for large spaces, the samples used in this paper are far from enough. Therefore, all the samples (126 individuals) collected in the past five years were used to detect the niche divergence of the two lineages, including the samples used in this paper. In addition, we performed Principal Component Analysis (PCA) using environmental factors data (altitude, DNI, 19 climatic variables and 4 soil factors) for 54 individuals to study ecological niche divergence between red-flowered lineage and white-flowered lineage.

Wilcoxon rank-sum tests were performed to compare climatic variables, DNI, latitude, and altitude among the three species. Standardized Pearson correlation analysis was used to investigate the relationship between GS and environmental factors within each species. All results were visualized using the "ggplot2" package in R.

RESULTS

Chromosome count of *Acanthocalyx alba*

The chromosome number of *A. alba* was $2n = 36$, with chromosome lengths ranging from 1-3 μm (Fig. 2A). The chromosome number of *A. delavayi* was $4n = 72$, with chromosome lengths ranging from 1-3 μm (Fig. 2B)

Variation of GS within the *Acanthocalyx*

The GS of *Acanthocalyx* ranged from 1.2 Gb to 2.5 Gb (Fig. 3). Within the white-flowered lineage, *A. alba* had a GS of 1.27 ± 0.044 Gb, which was significantly smaller than the GS of the red-flowered lineage, *A. delavayi* and *A. nepalensis*, with a mean of 2.15 ± 0.141 Gb and ranging from 1.9 Gb to 2.5 Gb (Figs. 3 & S1, Table S2).

Niche divergence between red and white-flowered lineage

In the PCA of environmental data, the first two principal components (PC) accounted for 75% of the variation between the two lineages (43.1% for PC1 and 24.2% for PC2) (Fig. 4A). The variables that contributed the most to the first PC were annual mean temperature, min temperature of coldest month, mean temperature of driest quarter mean temperature of coldest quarter.

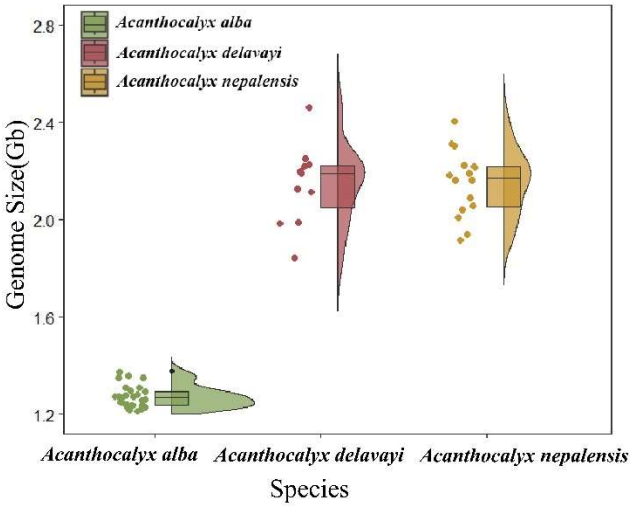

Fig. 3. The variation range of GS among 54 populations of three species of ***A. alba*** (green), ***A. delavayi*** (red), ***A. nepalensis*** (orange), with genome units in Gb. In the violin plot, the central line represents the median, and the edges of the violin show the 25th and 75th percentiles of the data, which represent the interquartile range of data distribution. Whiskers are the lines extending outside 1.5 times the interquartile range in the violin plot, used to depict the overall range of data distribution.

The histograms of niche overlap between red-flowered lineage and white-flowered lineage are displayed in Fig. 4B. The niches of the two lineages overlap greatly. We obtained a non-significant niche equivalency test statistic ($D=0.12$, $p=0.41584$, Fig. S2D), and significant background test statistic ($p=0.05051$, Fig. S2E). Niches of the two lineages in two dimensional E-space in Fig. S3. These results indicate that there is no significant niche divergence between red-flowered lineage and white-flowered lineage.

However, in individual ecological factors, we found differences between red-flowered lineage and white-flowered lineage. For climatic variables, the annual mean temperature of red-flowered lineage was significantly higher than that of the white-flowered lineage ($p < 0.05$, Fig. 5E) while precipitation seasonality ($p < 0.01$, Fig .5F), temperature seasonality ($p < 0.01$, Fig. 5D), DNI ($p < 0.01$, Fig. 5B), mean diurnal range ($p < 0.01$, Fig. 5G), and temperature annual range ($p < 0.0001$, Fig. 5H) of the red-flowered lineage were smaller than the white-flowered lineage. For soil variables, the soil pH of the red-flowered lineage had higher values ($p < 0.05$, Fig. 5C), while the white-flowered lineage was shown to grow in the soil with higher nitrogen content ($p < 0.01$, Fig. 5A). Finally, the red-flowered lineage was distributed in lower latitudes compared to the white-flowered lineage ($p < 0.0001$, Fig. 5I).

Correlation of GS variation and abiotic variables within two lineages of *Acanthocalyx*

The variation in GS within the white-flowered lineage was not influenced by climatic factors (Table 1), but it

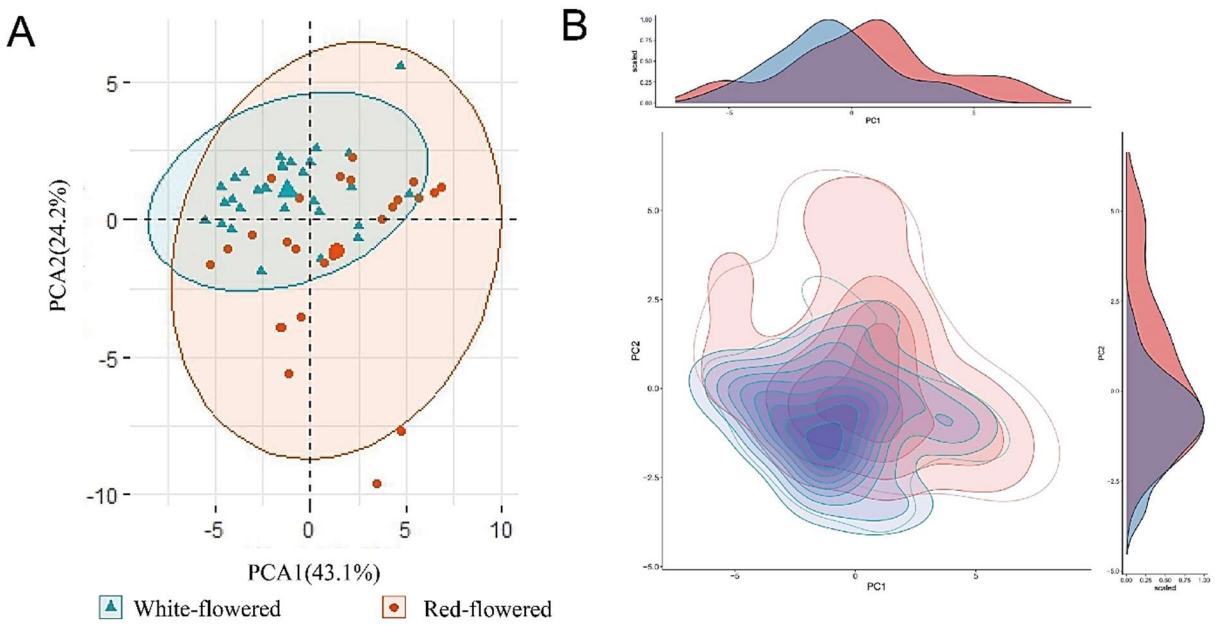

Fig. 4. Niche divergence between red and white-flowered lineage. Scatter plot of PC1 vs PC2 (**A**), each data set was used to present the potential niche differences between the white-flowered (blue triangle) and red-flowered lineages (red circle). Histogram density plots for the red-flowered lineage vs. white-flowered lineage (**B**). For each PC, the density of each species E-space is displayed. Lines representing the kernel density isopleths from 1-100% kernel densities, (red=red-flowered lineage and blue=white-flowered lineage).

Table 1. Linear regression analysis of GS variation within the red and white-flowered lineages in relation to environmental variables.

Environmental	Red-flowered		White-flowered	
variable	R^2/Pearson	P/Pearson	R^2/Pearson	P/Pearson
Bio1	0.0484	0.33	0.0625	0.21
Bio2	0.1936	*	0.09	0.13
Bio3	0.1369	0.064	0.0049	0.73
Bio4	0.0225	0.49	0.000256	0.93
Bio5	0.0121	0.59	0.0289	0.38
Bio6	0.000081	0.96	0.0484	0.28
Bio7	0.0676	0.2	0.1296	0.067
Bio8	0.01	0.61	0.003136	0.75
Bio9	0.001681	0.85	0.1369	0.066
Bio10	0.001225	0.86	0.006084	0.7
Bio11	0.006561	0.69	0.1296	0.068
Bio12	0.1369	0.072	0.0144	0.56
Bio13	0.0841	0.15	0.005929	0.71
Bio14	0.007225	0.68	0.0256	0.42
Bio15	0.0529	0.26	0.0361	0.35
Bio16	0.001764	0.83	0.0576	0.22
Bio17	0.000529	0.91	0.0289	0.4
Bio18	0.0841	0.15	0.002025	0.82
Bio19	0.000441	0.92	0.0324	0.38
PH	0.1156	0.062	0.1521	0.062
TN	0.1521	*	0.1024	0.11
SOM	0.001369	0.86	0.0196	0.49
CEC	0.001156	0.87	0.1024	0.1
Altitude	0.0121	0.58	-0.000961	0.88
Latitude	0.0027	0.25	0.1521	*

The number of asterisks (*) indicates the significance level of the differences: *, p <= 0.05, **, p <= 0.01, ***, p <= 0.001, ****, p <= 0.0001

showed a negative correlation with latitudes ($p = 0.046$, $R^2 = 0.1521$; Fig. 6C, Table 1). On the other hand, the variation in GS within the red-flowered lineage was strongly correlated with the mean diurnal range (Bio2, $p = 0.026$, $R^2 = 0.1936$; Fig. 6A, Table 1), as well as the soil TN content ($p = 0.047$, $R^2 = 0.1521$; Fig. 6B, Table 1).

DISCUSSION

Chromosome number of *Acanthocalyx alba* and its taxonomic significance

The chromosome number of *Acanthocalyx* in the Caprifoliaceae has not been previously reported. Our study reveals that *A. alba* has a chromosome number of $2n = 36$, which is the first report for this genus. Another species within the Caprifoliaceae, *Morina longifolia*, has a chromosome number of $2n = 34$ (WFO, 2023). Previous research has suggested that *Acanthocalyx* belongs to the *Morina* genus (Bunge, 1852). However, our findings, supported by morphological and palynological evidence (Blackmore and Cannon, 1983; Cannon and Cannon, 1984), indicate that *Acanthocalyx* and *Morina* should be considered as separate genera due to their distinct chromosome numbers. Additionally, we have discovered that the chromosome number of *A. delavayi* is $4n = 72$. Considering that the GS of *A. delavayi* is nearly double that of *A. alba* ($2n=36$), we hypothesize that *A. delavayi* might be a tetraploid. This hypothesis is based on the widespread phenomenon of polyploidization among closely related species in the HHM (Chen *et al*., 2007). Although we were unable to obtain the chromosome

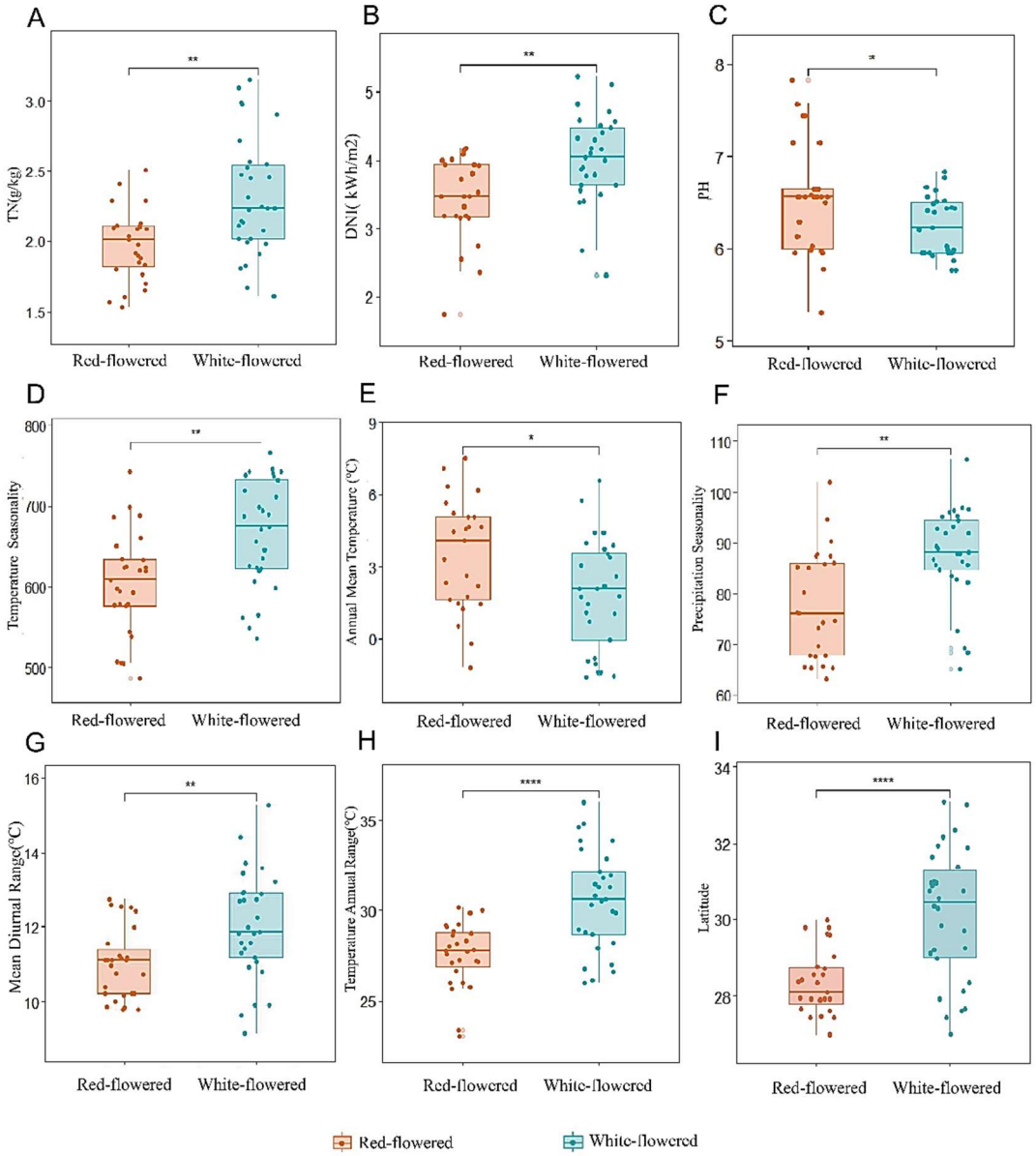

Fig. 5. Box plots illustrating the ecological niche divergence between red-flowered lineage and white-flowered lineage in ***Acanthocalyx*** (A-I). The boxes represent the 25th and 75th percentiles, the horizontal line represents the median, the whiskers represent the 5th to 95th percentiles, and the circles represent outliers. The number of asterisks (*) indicates the significance level of the differences: *, p <= 0.05, **, p <= 0.01, ***, p <= 0.001, ****, p <= 0.0001.

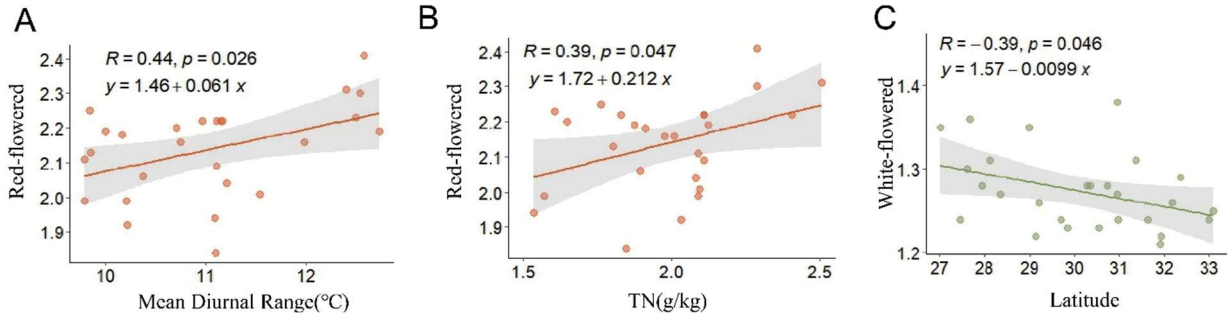

Fig. 6. Linear regression analysis of GS variation within the red and white-flowered lineages in relation to (**A**), mean diurnal range (**B**), TN and (**C**), latitude. The red dots represent the rede-flowered lineage and the green dots represent the white-flowered lineage.

count for *A. nepalensis* due to material limitations, we speculate that *A. nepalensis* could also be a polyploid, given its similar GS to *A. delavayi.* Polyploidization may have facilitated speciation within *Acanthocalyx*.

GS variation and preference between red-flowered lineage and white-flowered lineage

In the study of angiosperm GS evolution, Leitch *et al*. (1998) defined a small genome as GS ≤ 3.5 pg. *Acanthocalyx* falls within this category, both among angiosperms and within the Caprifoliaceae, which has a range of 0.20 to 10.45 pg (Pellicer and Leitch, 2020). The similar GS of the red-flowered species *A. nepalensis* and *A. delavayi* suggest a close phylogenetic relationship, supporting recent phylogenetic analysis of this genus (Mu *et al*., 2021).

Although our results showed that there was no major niche differentiation between red-flowered lineage and white-flowered lineage, it may be due to very recent divergence of these species (Mu *et al*., 2021). In fact, species of *Acanthocalyx* generally occur in the high-altitude regions across the Himalaya and Hengduan Mts, showing similar habitat preferences. However, while analyzing particular environmental factors separately, we did find significant differences between the red and white flowered lineages, and which may indicate further microhabitat differentiation within the dominant alpine habitat. Specifically, the red-flowered lineage, which has a larger GS, tends to grow in regions with milder climates characterized by lower temperature seasonality, precipitation seasonality, mean diurnal range, and temperature annual range (Fig. 5D, F, G, H). This is consistent with previous research indicating that species in regions with smaller climate variations have longer growing seasons, leading to faster evolutionary rates and larger genomes (Qiu *et al*., 2019). Furthermore, the red-flowered lineage with larger genomes is predominantly found in low latitude regions (Fig. 5I), while the white-flowered lineage with smaller genomes is more common in high latitude regions (Fig. 5I). This pattern aligns with the findings of Laurie and Bennett (1985), who proposed a positive correlation between GS and cell cycle duration, suggesting that plants in low latitude regions with longer reproductive cycles tend to evolve larger genomes (Bennett, 1972). The white-flowered lineage receives more direct normal irradiance (DNI) than the red-flowered lineage (Fig. 5B), which aligns with the ecological preference of the white-flowered species for high mountain meadows and the distribution of red-flowered species in forest understories. Previous studies by Guignard *et al*. (2016) and Pellicer *et al*. (2018) indicate that species with large genomes have higher energy requirements for body construction and maintenance, especially in nutrient-limited environments. Consequently, these species are expected to have lower competitiveness. Therefore, red-flowered lineage with large genomes may thrive in environments with lower nutrient availability and less competition, such as the understory, to minimize their reproductive costs.

Drivers of GS variation within species of *Acanthocalyx*

Previous studies have shown that environmental factors can influence the geographic distribution of various plant groups (Bures *et al*., 2004; Grotkopp *et al*., 2004; Du *et al*., 2017). However, in our study, we did not find a correlation between the GS and environmental variables in the white-flowered lineage. Instead, we observed a negative correlation between GS and latitude (Fig. 6C). This may be related to the evolutionary history of *Acanthocalyx alba*, which originated in the southern part of the HDM and expanded to the northern part during the ice age (Mu *et al*., 2021). The genetic mechanism underlying genome reduction in *A. alba* remains unclear. Similar findings have been reported in studies on the Caryophyllaceae in the Balkan Peninsula and the Alps, where GS was correlated with the history of population differentiation (Terlevic *et al*., 2022).

In the red-flowered lineage, we found a positive correlation between intraspecific GS and total nitrogen in soil (Fig. 6B). This suggests that nutrient availability plays a role in determining GS, as organisms require nutrients for growth and reproduction. Previous research has also shown that GS is associated with adaptation strategies to changes in nutrient availability (Giovannoni *et al*., 2014). Studies on bacteria have demonstrated that low-nutrient concentrations select for genomic features that reduce the cost of reproduction, such as smaller GS (Chuckran *et al*., 2023). Our results support the idea that total nitrogen can serve as an indicator of nutrient concentration, particularly within the red-flowered lineage. Furthermore, we found a positive association between GS and mean diurnal range (Fig. 6C) in the red-flowered lineage. This suggests that larger genomes may be more adaptable to pronounced climatic fluctuations, as demonstrated by Hutang *et al*. (2023).

CONCLUSION

Our study investigates the variation of GS in *Acanthocalyx* at both interspecific and intraspecific levels. We found that the distribution of GS in this genus is correlated with ecological factors, although the genus exhibits similar major niche type among species. Notably, the evolution of GS in *A. alba* appears to be linked to the historical differentiation of populations. Consequently, the GS variation in *Acanthocalyx* may reflect distinct adaptive strategies among different lineages in the Hengduan-Himalaya Mountains. Our findings highlight the potential significance of GS evolution in driving the diversification of plant groups in these two biodiversity hotspots.

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