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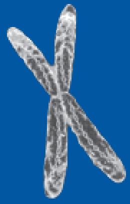

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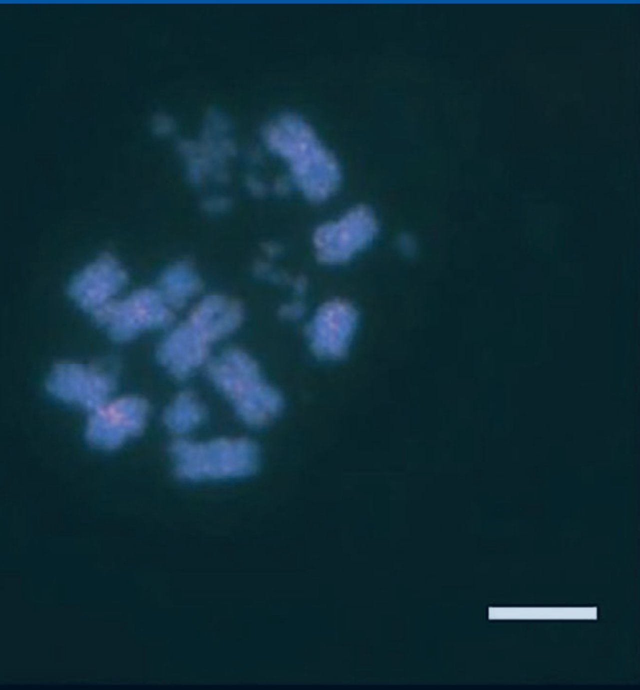

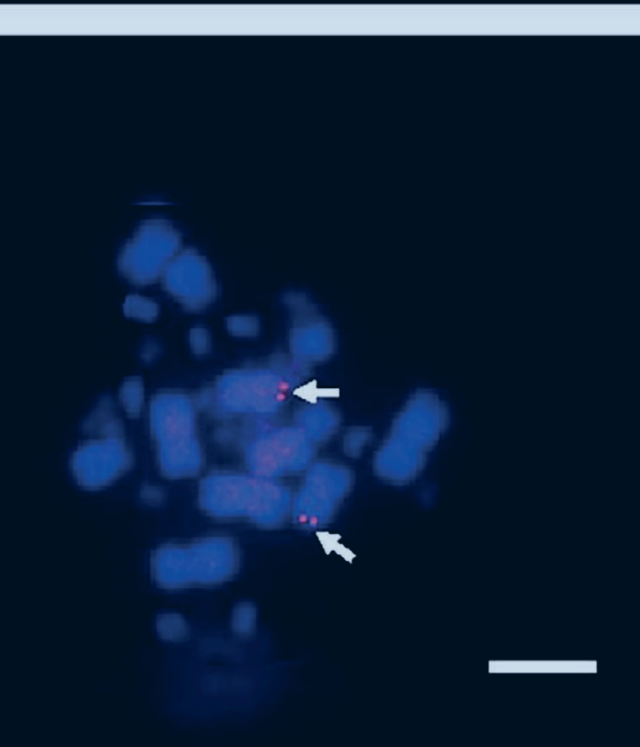

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COVER: figure from the article inside by Praween Supanuam et al. First chromosome characterization and repetitive DNA of Barred Gliding Lizard, *Draco taeniopterus* Günther, 1861 (Draconinae: Agamidae: Squamata).

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The role of chromosomal rearrangements, polyploidy, and genome size variation in the diversity and ecological distribution of *Asparagus* L. species: a landscape cytogenetics meta-analysis approach

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Abstract. The *Asparagus* genus includes a group of plants with economic and medicinal importance. Although numerous cytogenetic and genetic studies have been conducted on *Asparagus* species, there are no reports on landscape genetics, landscape cytogenetics, or *Asparagus* cultivation in response to climate change. Therefore, we designed this study to answer the above-mentioned objectives. We performed a meta-analysis involving landscape genetic studies based on available cytogenetic data and reported DNA C-values for several *Asparagus* species from different countries. Additionally, species distribution modeling (SDM) was performed on some selected *Asparagus* species. This combined study not only identifies the genetic fragmentation and genetic clines within plant species but also predicts their growth and distribution across different regions and in response to climate change. We used discriminant analysis of principal components (DAPC) to group *Asparagus* taxa based on karyotype and chromosome pairing data. We also performed random forest (RF) analysis to determine the contribution of cytogenetic traits to *Asparagus* speciation based on the Gini index. An association study was performed using redundancy analysis (RDA) of cytogenetic data with geographic variables (longitude and latitude). We used spatial principal component analysis (sPCA) to analyze the contribution of spatial variables to the cytogenetic structure of the studied *Asparagus* species. We used Bioclim and Maxent species distribution models (SDMs) to predict and identify areas suitable for selected *Asparagus* species in response to climate change by 2050. Results indicated that ploidy and chromosome size, the occurrence of heterozygote translocation, frequency and distribution of chiasmata, and genome size play role in *Asparagus* species diversification and adaptation. These cytogenetic characters are significantly associated with spatial variables and *Asparagus* species formed cytogenetic clines in response to local environmental conditions. SDM analyses showed that a combination of temperature and precipitation factors affect *Asparagus* species distribution and that in the coming future, some of these species may have a reduced cultivation area due to climate change which must be tackled by planning a proper conservation program worldwide.

Keywords: *Asparagus*, cytogenetic clines, genome size, Maxent, RDA.

INTRODUCTION

The genus *Asparagus* contains about 240 species, with *Asparagus officinalis* L., commonly known as the garden *Asparagus* as the most famous and economically important species. This edible vegetable crop is cultivated, grown, and used all over the world for its edible spear (Kanno et al. 2011). However, along with garden *Asparagus*, other species are also cultivated such as *Asparagus maritimus* L., or have been proposed as a genetic source for breeding programs like *Asparagus acutifolius* L.

Moreover, the young shoots of some wild *Asparagus* spp. namely, *A. acutifolius* L., *A. aphyllus* L., *A. acerosus* Thunb. ex Schult. & Schult. f., and *A. laricinus* Burch., are consumed fresh. Similarly, *A. verticillatus* L. is used for medicinal purposes and *A. densiflorus* (Kunth) Jessop as well as *A. plumosus* Baker have been grown as ornamental plants (Kubitzki et al. 1998).

Polyploidy has been reported in both the garden *Asparagus* (4x, and 8X), as well as in several species related to garden *Asparagus*, for example in *Asparagus falcatus* (2x, and 4x), *Asparagus racemosus* var. *javinica* (2x, and 4x), *Asparagus maritimus* (4x, and 6x) (see for example, Sheidai and Inamdar 2017; Mousavizadeh et al. 2021; Sala et al. 2023).

Although numerous cytogenetic and genetic studies have been conducted on *Asparagus* species, there are no reports on landscape genetics, landscape cytogenetics, or *Asparagus* cultivation in response to climate change. Therefore, we designed this study to answer the abovementioned objectives. We used our own cytogenetic data (Sheidai and Inadar 1997) and publicly available cytogenetic and nuclear DNA quantity data reports (C-value data) (Pires et al. 2006; Bouberta et al. 2017; Suma et al. 2017; Mousavizadeh et al.2021; Plath et al.2022), and performed a meta-analysis in a landscape genetic context. Additionally, species distribution modeling (SDM) was performed on some selected *Asparagus* species. This combined study not only identifies the genetic fragmentation and genetic clines within plant species but also predicts their growth and distribution across different regions and in response to climate change.

Cytogenetic studies, including chromosome morphology and chromosome pairing analysis, are essential for plant breeding, QTL hybridization and molecular mapping, and genetic transfer of beneficial genes between different species and cultivars. Moreover, the frequency and location of recombination through chiasmata formation can promote adaptation in a species by creating new genetic combinations (Rice 2002). Recombination can vary within chromosomes, between chromosomes, and between individuals, sexes, populations and species and its proportions can be influenced by environmental and demographic factors (Charlesworth 1976; Rice 2002), but are inherited, maintained, and selected for at specific genetic loci (Chinnici 1971). Moreover, studies across different taxonomic scales have shown that recombination frequency and occupying a particular landscape may be controlled by different mechanisms in different taxa (Ortiz-Barrientos et al. 2015; Johnston et al. 2016).

Landscape genetics considers the genetic basis of diversity in response to spatial variables such as geographic distance, altitude, and latitude (Provost et al. 2022). The frequency and distribution of chiasmata (cross-over) which are genetically controlled, as well as the occurrence of heterozygote translocations, result in genetic variation through chromosome genetic rearrangement. It is also known that polyploidy is one of the main genetic mechanisms in *Asparagus* genus speciation (Sheidai and Inamdar 2017). Therefore, landscape genetics and species distributions allow for determining the role of global and regional spatial variables as well as climatic variables in the genetic makeup of *Asparagus* species and potentially suitable growing regions. Species distribution models (SDMs) are methods that study the current geographic distribution of plant species and predict future events in the face of climate change. Through this, suitable habitats for the cultivation of the plant species of interest can be identified, and conservation measures can be proposed if there is a possibility that the cultivation area of the target plant species will decrease (Elite and Litwick 2009; Lee-Yaw et al. 2021).

MATERIAL AND METHODS

For landscape cytogenetic studies we used both karyotype and chromosome pairing data of 18 *Asparagus* taxon (Table 1). For genome size analysis we used the freely available published data of Plath et al (2022). For the species distribution modeling (SDM), we used the occurrence data points for the selected *Asparagus* species from GBIF (the Global Biodiversity Information Facility), as well as the published materials.

DATA ANALYSES

Cytogenetic grouping

We used discriminant analysis of principal components (DAPC), for grouping of *Asparagus* taxa for both karyotype and chromosome pairing data. An analysis of

Table 1. Karyotype data of *Asparagus* species used in present study.

Species	Country	Locality	Longitude	Latitude	2n	Ploidy	Total chromatin length	Mean chromatin length	Shortest chromosome	Longest chromosome	Ratio
A. racemosus var. *Javanica*-1	India	Orissa	84.27	20.23	40	4	66.5	3.325	1.00	2.60	2.60
A. racemosus var. *Javanica*-2	India	Pune-University	73.82	18.55	20	2	38.52	3.852	1.13	2.93	2.59
A. densiflorus cv. Myers	India	private nursery Pune	73.88	18.51	40	4	78.58	3.929	1.30	2.82	2.16
A. laevissimus	India	J.N.H-Pune	73.87	18.53	40	4	66.12	3.30	0.91	2.91	3.20
A. myriocladus	India	Pune-University botanical garden	73.05	18.03	40	4	92.2	4.61	1.00	3.66	3.66
A. racemusus subacerosa	India	Pune-law college hills	73.82	18.51	40	4	65.2	3.26	1.06	2.26	2.13
A. sprengeri	India	Agharkar Research Institute	73.50	18.31	40	4	58.82	2.94	1.06	2.47	0.78
A. virgatus	India	Fergussen college	73.50	18.31	40	4	67.26	3.363	1.12	2.49	2.22
A. gonoclados	India	Pune	73.05	18.03	60	6	87.38	4.369	1.96	5.30	2.70
A. adsendens	India	J.N.H-Pune	73.87	18.53	20	2	73.3	7.33	0.99	2.00	2.02
A. falcatus	India	private nursery Pune	73.88	18.51	20	2	28.48	2.85	1.00	1.99	1.99
A. Officinalis-1	India	Fergussen college	73.50	18.31	20	2	85.68	8.57	2.58	5.85	2.27
A. racemosus	Bangladesh	University of Dhaka	90.39	23.77	20	2	18.18	1.81	0.53	1.23	2.32
A. setaceus	Bangladesh	Dakha	90.39	23.77	20	2	18.50	1.85	0.48	1.47	3.06
A. albus	Algeria	Tipaza	2.27	36.25	20	2	44.47	4.44	3.19	6.25	1.95
A. acutifolius	Algeria	Senalba	3.10	34.38	20	2	52.07	5.2	4.21	6.61	1.57
A. horridus	Algeria	Emir Khaled	2.12	36.08	20	2	36.04	3.6	2.91	4.46	1.53
A. Officinalis-2	Algeria	Tessala El Merdja	2.54	36.37	20	2	51.18	5.11	3.15	6.52	2.06

variance (ANOVA) test was performed on the cytogenetic data to reveal a significant difference between *Asparagus* species in different countries. We also performed random forest (RF) analysis to reveal the contribution of cytogenetic characters in *Asparagus* species differentiation based on the Gini index. These were performed in the adegenet package of R. 4. 2.

Redundancy analysis (RDA)

Association studies were performed using redundancy analysis (RDA) of cytogenetic data with geographic variables (longitude and latitude). RDA which is a constrained ordination method models the linear relationships between environmental predictors and genetic variation (Capblancq and Forester, 2021). This analysis was performed through 999 permutations in PAST ver. 4.

Spatial principal components analysis (sPCA)

We used the Spatial principal components analysis (sPCA), to analyse the spatial variables' contribution to the studied *Asparagus* species cytogenetic structuring. The sPCA is a multivariate method that is independent of Hardy Weinberg expectations and produces estimates summarizing both genetic variation and spatial structure between individuals (or populations) (Jombart et al.2008). Global structures (patches, wedges, and intermediate junctions) are statistically comparable to local structures (strong genetic differences between neighbors) and random noise. The sPCA also performs Moran's I test and IBD (isolation by distance). The sPCA analyses were performed with the adegenet package version R. 4. 2.

Species Distribution Modeling (SDM)

We used species distribution modeling (SDM) to predict and identify suitable regions for selected *Asparagus* species in response to climate change by 2050. In species distribution modeling, we used layers of forecast climate data for the current period (~1950-2000) and 2050 (2050-2061 average) based on 19 bioclimatic variables at a 5-minute resolution. Data was loaded from the WorldClim database. To represent the impact of climate change, future climate variables in 2050 were projected

according to the "representative concentration trajectory" (RCP, 2. 6).

We used bioclimatic variables derived from the monthly temperature and rainfall values. These bioclimatic variables are coded as follows:
BIO1 = Annual Mean Temperature
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3 = Isothermality (BIO2/BIO7) (×100)
BIO4 = Temperature Seasonality (standard deviation ×100)
BIO5 = Max Temperature of Warmest Month
BIO6 = Min Temperature of Coldest Month
BIO7 = Temperature Annual Range (BIO5-BIO6)
BIO8 = Mean Temperature of Wettest Quarter
BIO9 = Mean Temperature of Driest Quarter
BIO10 = Mean Temperature of Warmest Quarter
BIO11 = Mean Temperature of Coldest Quarter
BIO12 = Annual Precipitation
BIO13 = Precipitation of Wettest Month
BIO14 = Precipitation of Driest Month
BIO15 = Precipitation Seasonality (Coefficient of Variation)
BIO16 = Precipitation of Wettest Quarter
BIO17 = Precipitation of Driest Quarter
BIO18 = Precipitation of Warmest Quarter
BIO19 = Precipitation of Coldest Quarter

To build the SDM model, we used the Bioclim and maximum entropy methods implemented in the Dismo package in R 4. 2 and the Maxent program. SDMs require the occurrence data points on which the pseudo-absences (PAs) points are estimated, followed by model prediction. All the models were constructed with 80% training and 20% testing of occurrence data. The model evaluation was performed by both the threshold method and AUC determination (ROC curve).

Bioclim models the species distributions in relation to climatic variables and thus assumes that species occurrence is influenced by climate at the scale of climate variables and that these variables are normally distributed. Similarly, Maxent (Maximum Entropy Modeling) predicts the occurrence of a species by finding the one closest to the most common or uniform distribution, taking into account the limits of environmental variables in a known location (Phillips et al.2004). I n this method, the fit is measured as gain, which is basically a likelihood statistic that maximizes the probability of being present for background data adjusted for the case where all pixels have an equal (uniform) probability. The final probability distribution becomes the basis for fitted predictor variable coefficients. (Phillips et al.2004). The importance of bioclimatic variables influencing the distribution of *Asparagus* species was assessed by the Jackknife incremental method and the AUC value.

RESULTS

Cytogenetic data based on the country of origin of the *Asparagus* species used in this study are shown in Tables 2 and 3. An analysis of variance (ANOVA) test performed on the cytogenetic data revealed a significant difference between these countries in karyotype data ($p<0.01$, Fig. 1). These results demonstrate that genetic variation accompanies *Asparagus* species diversity in different regions of the world. A similar analysis for chiasma frequency and chromosome pairing could be performed on the species studied and not among the countries, but a significant result ($p<0.01$), indicated the species cytogenetic differences even within a particular country i. e. India.

Cytogenetic grouping of the studied *Asparagus* species based on karyotype data is presented in the DAPC plot (Fig. 2). These species are scattered in three distinct groups based on their country of origin.

Association analyses performed by CCA and RDA for karyotype data produced significant results ($p<0.01$, Fig.

Table 2. The mean value for chiasma frequency and distribution, and chromosome pairing in *Asparagus* species studied.

Asparagus species	Terminal chiasmata	Intercalary chiasmata	Total chiasmata	Ring bivalents	Rod bivalents
A. racemosus javanica	14.2	1.3	16	7.3	1.37
A. densiflorus cv. Myers	34.3	2.6	37	17.2	2.4
A. laevissimus	15.8	4	20	8.5	1.48
A. racemusus subacerosa	35.62	0.47	36.1	16.78	2.81
A. sprengeri	39	0.347	39.347	19.26	0.74
A. virgatus	36.1	1.3	37.4	18	1.7
A. gonoclados	55.28	5	60.23	26.84	2.46
A. adsendens	16.8	4.425	21.225	9.8	0.325
A. officinalis	13.8	1.36	14.54	5.54	3.36

Table 3. The genome size (1C-value) of *Asparagus* species used in the landscape cytogenetic analyses (data obtained from freely available published paper (Plath et al., 2022)).

Species	Country	Country-code	Longitude	Latitude	Ploidy (X)	C-value
A. acutifolius	Italy, Vittoria	1	14.53	36.95	2	1.35
A. aethiopicus	Spain, Malaga	2	4.42	36.71	6	0.86
A. albus	Portugal	3	8.22	39.39	2	1.23
A. amarus	Italy	1	12.56	41.87	6	1.37
A. arborescens	Canary	4	16.62	28.29	2	1.32
A. maritimus 1	Italy	1	12.56	41.87	6	1.33
A. maritimus 2	Italy	1	12.56	41.87	6	1.30
A. maritimus 3	Italy	1	12.56	41.87	6	1.28
A. maritimus 4	Italy, Vign	1	13.04	43.37	6	1.29
A. officinalis 'Darlise'	France	6	2.21	46.22	2	1.47
A. officinalis 'Ravel'	Germany	7	10.45	51.16	2	1.53
A. officinalis 'Steiners Violetta'	Germany	7	10.45	51.16	4	1.59
A. pastorianus	Macaronesia	8	16.84	28.23	4	1.40
A. plumosus	Cuba	9	82.36	23.11	2	0.42
A. plocamoides	Canary	4	16.62	28.29	2	0.71
A. prostratus 1	France, Ploemever	6	3.25	47.44	4	1.48
A. prostratus 2	France, Gavres	6	3.35	47.69	4	1.53
A. prostratus 3	France, Damgan	6	2.57	47.51	4	1.52
A. prostratus 4	France, Houat Is.	6	2.95	47.39	4	1.54
A. pseudoscaber	Italy	1	12.56	41.87	6	1.28
A. ramosissimus	Angola	10	17.87	11.20	2	1.15
A. scoparius	Africa, Cape Verde	11	23.04	16.53	2	0.73
A. stipularis 1	Ibiza	2	1.42	38.90	2	0.81
A. stipularis 2	Ibiza	2	1.42	38.90	2	1.13
A. stipularis 3	Ibiza	2	1.42	38.90	2	1.21
A. stipularis 4	Cyprus	12	33.42	35.12	2	1.19
A. albus	Portugal	2	8.22	39.39	2	1.23
A. ramosissimus	Angola	2	17.87	11.20	2	1.35
A. stipularis 4	Cyprus	2	33.42	35.12	2	0.86
A. scoparius	Africa, Cape Verde	12	23.04	16.53	2	1.23

3, A). These results showed an association between ploidy level, the ratio of the longest to the shortest chromosome the somatic chromosome number (2n). Moreover, the random forest result (Fig. 3, B) identified the ploidy level and somatic chromosome number as the main karyotype characters that differentiate the studied taxa.

A similar analysis performed on chiasma frequency and chromosome pairing of *Asparagus* species did not produce significant association (p >0.1). This may be due to the fact that we obtained and used only the meiotic data of *Asparagus* species from India.

Spatial principal components analysis (sPCA)

The results of sPCA are presented in Fig. 4, A-F. The preliminary analysis of sPCA Eigenvalues showed the presence of strong positive and global spatial constraints over the cytogenetic characteristics of the studied *Asparagus* species (Fig. 4, A). This was supported by a significant global test obtained (p= 0.01, Fig. 4, B).

Similarly, the isolation by distance test (IBD), produced a significant result (p= 0.01, Fig. 4, D), indicating that cytogenetic differences among *Asparagus* species increased with increasing geographic distance.

The connection network (Fig. 4, E), showed a closer relationship (common shared cytogenetic features) between species from India and Bangladesh species, and the cytogenetic clines plot (Fig. 4, F), showed that the species studied in all three countries formed cytogenetic clines probably due to their spatial adaptation. Moreover, Moran's I test was not significant (p> 0.1), indicating that the similar spatial and geographical regions have similar effects on the studied cytogenetic features.

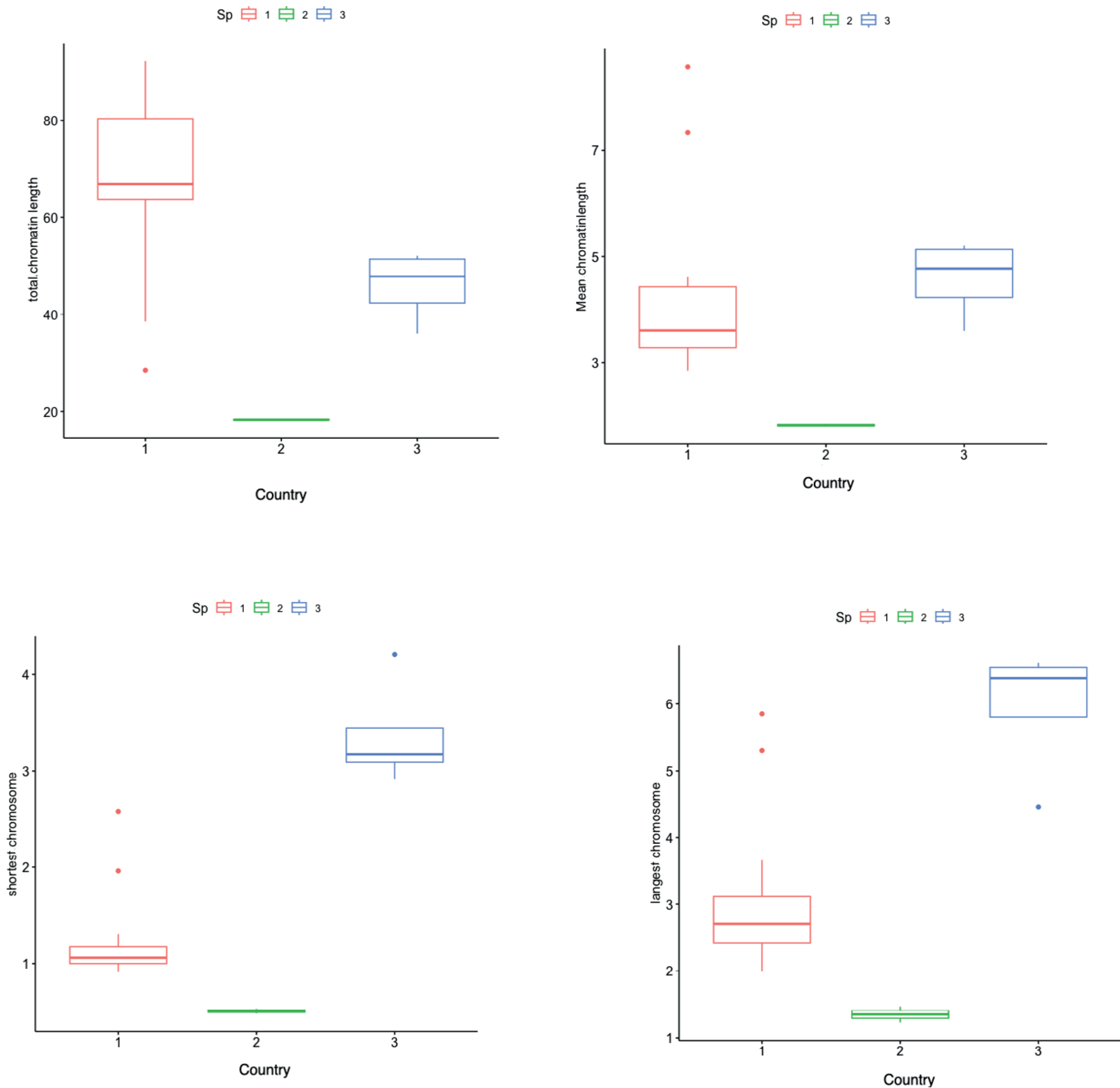


Figure 1. Representative box plots of Karyotypic data ANOVA between *Asparagus* species based on the countries. (Abbreviations for the country are 1= India, 2=Bangladesh, and 3 = Algeria).

Heterozygote translocations

Some of the studied *Asparagus* species showed the occurrence of heterozygote translocations in the pachytene stage of meiosis (Fig. 5, A). These translocations would result in multivalent formation in the metaphase stage (Fig. 5, B and C), (Sheidai 1985).

Genome size (C-value) analysis

The 1C value of the *Asparagus* species obtained from the published freely available work (Plath et al.2022) and their country of origin with spatial variables (longitude and latitude) are presented in Table 3. ANOVA performed on the amount of C-value produced significant results among the *Asparagus* species studied ($p< 0.01$, Fig. 6). The RDA analysis (Fig. 7) also showed a signifi-

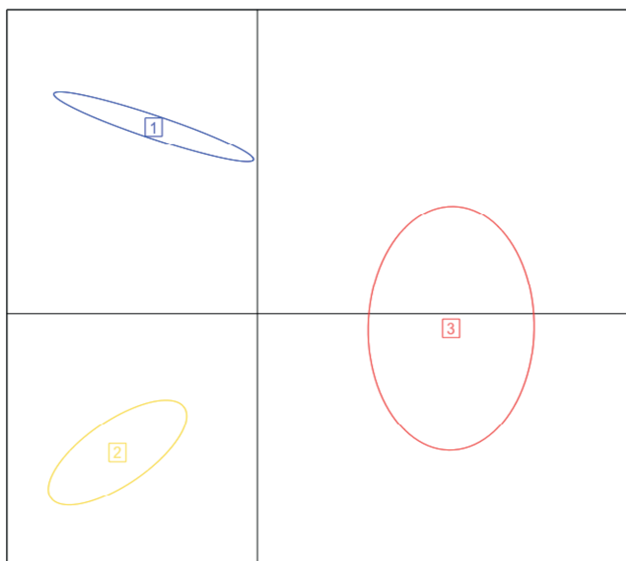


Figure 2. DAPC grouping of *Asparagus* species based on karyotype data. Abbreviations: 1= India, 2= Bangladesh, and 3 = Algeria.

cant association (p= 0.01), and spatial variables. Therefore, the longitudinal as well as latitudinal distribution of *Asparagus* species analyzed affect the ploidy level and their 1C-value genomic content. The longitude and latitude data are in degrees, and minutes, respectively.

Spatial principal components analysis (sPCA) of 1C-value data

The results of sPCA are presented in Fig. 8, A-D. The preliminary analysis of sPCA Eigenvalues showed the presence of strong positive and global spatial variables over the ploidy level, and the genomic 1C-value content of the studied *Asparagus* species (Fig. 8, A). This was supported by a significant global test obtained (p =0.10). Similarly, the isolation by distance test (IBD), did not produce a significant result (p= 0.01), indicating that the 1C-value content difference among *Asparagus* species is not increased with increasing geographic distance.

The connection network (Fig. 8, B), showed similarities (common shared cytogenetic features) between the species studied in different countries, and the genome size clines plot (Fig. 8, C), showed that the species studied in all these countries formed ploidy and 1C-value content clines due to their spatial adaptation. Moreover, Moran's I test was not significant (p> 0.1), indicating that the similar spatial and geographical regions have similar effects on the studied cytogenetic features.

The contribution plot (Fig. 8, D), revealed that the ploidy level plays a more pronounced role compared to that of 1C-value content in the analyzed *Asparagus* species in response to spatial variables.

Species distribution modeling (SDM) results

SDM analysis of selected *Asparagus* species provides insight into the climatic variables affecting the growth and occurrence of these important species worldwide and can predict their response to climate change in the future. These findings help conservation programs.

The results of present-time predicted distribution versus *Asparagus* species distribution by the year 2050 are presented in Figs. 9 and 10. The probable distribution of these species under the influence of climate change obtained from both BIOCLIM and Maxent models was almost the same. These results revealed that the area under cultivation for the studied species would be much reduced in extent by the year 2050. This statement holds true, particularly for *Asparagus verticillatus*.

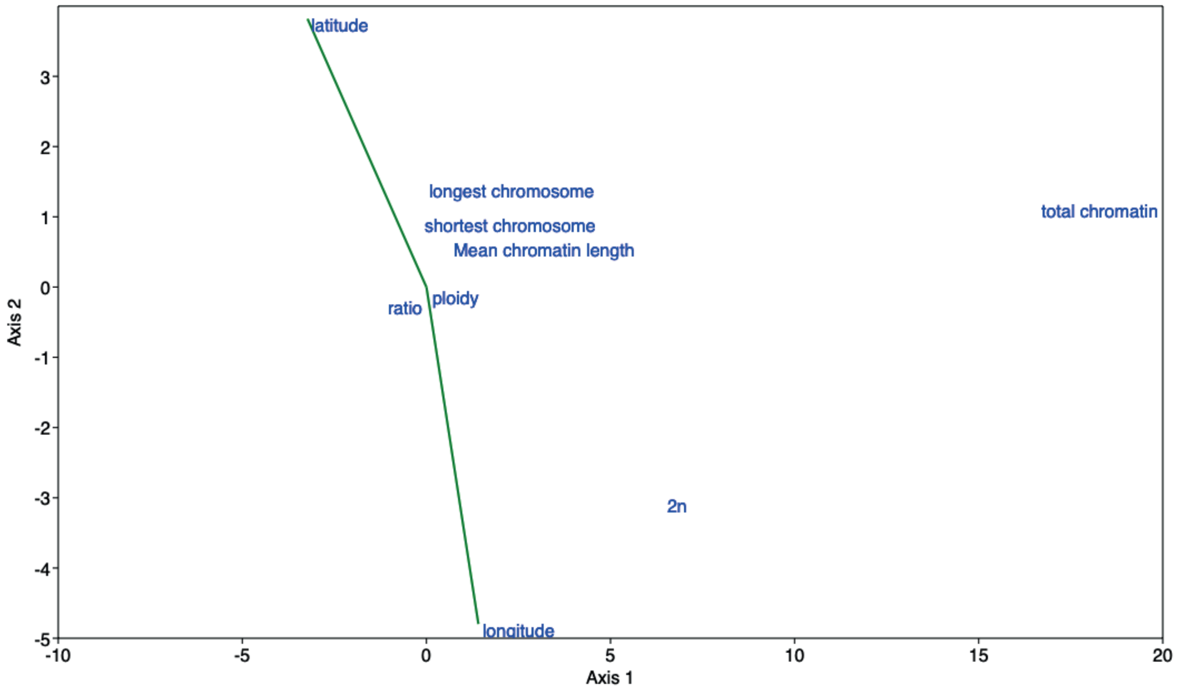
The importance of climatic variables based on the Jackknife method and ROC curve (AUC value) are presented in Fig. 10. The AUC values obtained for both present time prediction and by the year 2050, were all above 0.90 which supports the modeling results.

Important and influential bioclimate variables identified by the Jackknife method revealed that BIO3= Isothermality, BIO5= Max Temperature of Warmest Month, BIO13= Precipitation of Wettest Month, BIO14= Precipitation of Driest Month, BIO15= Precipitation Seasonality, BIO17= Precipitation of Driest Quarter and BIO18= Precipitation of Warmest Quarter, are among the most important bioclimate variables affecting the distribution of *Asparagus* species.

DISCUSSION

This study showed that global and local spatial patterns influence the genetic structure of *Asparagus* species through cytogenetic changes like polyploidy, structural changes of chromosomes (heterozygote translocations), chromosome size, and the plant genome size. Moreover, bioclimatic variables determine the geographical distribution of these plants worldwide.

Chromosomal evolution has played an important role in plant diversification and speciation, especially in the genus *Asparagus* (Plath et al. 2022). In the *Asparagus* genus, Plants with different ploidy levels within the same population are very common. For example, triploid, pentaploid, hexaploid, and octoploid plants were found in



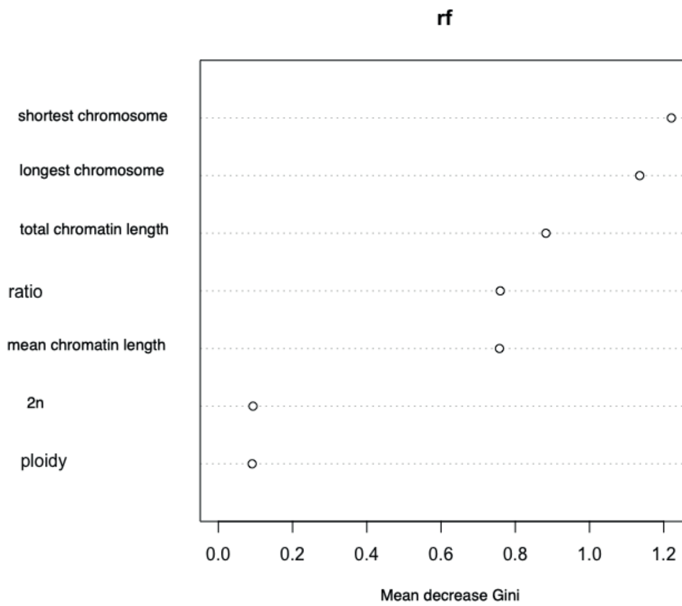


Figure 3. A= RDA plot of karyotype data in the studied *Asparagus* species shows a significant association between ploidy level, longest to shortest chromosome ratio, and somatic chromosome number. B = Random Forest plot of the same data showing the importance of karyotype characters in differentiating the studied taxa.

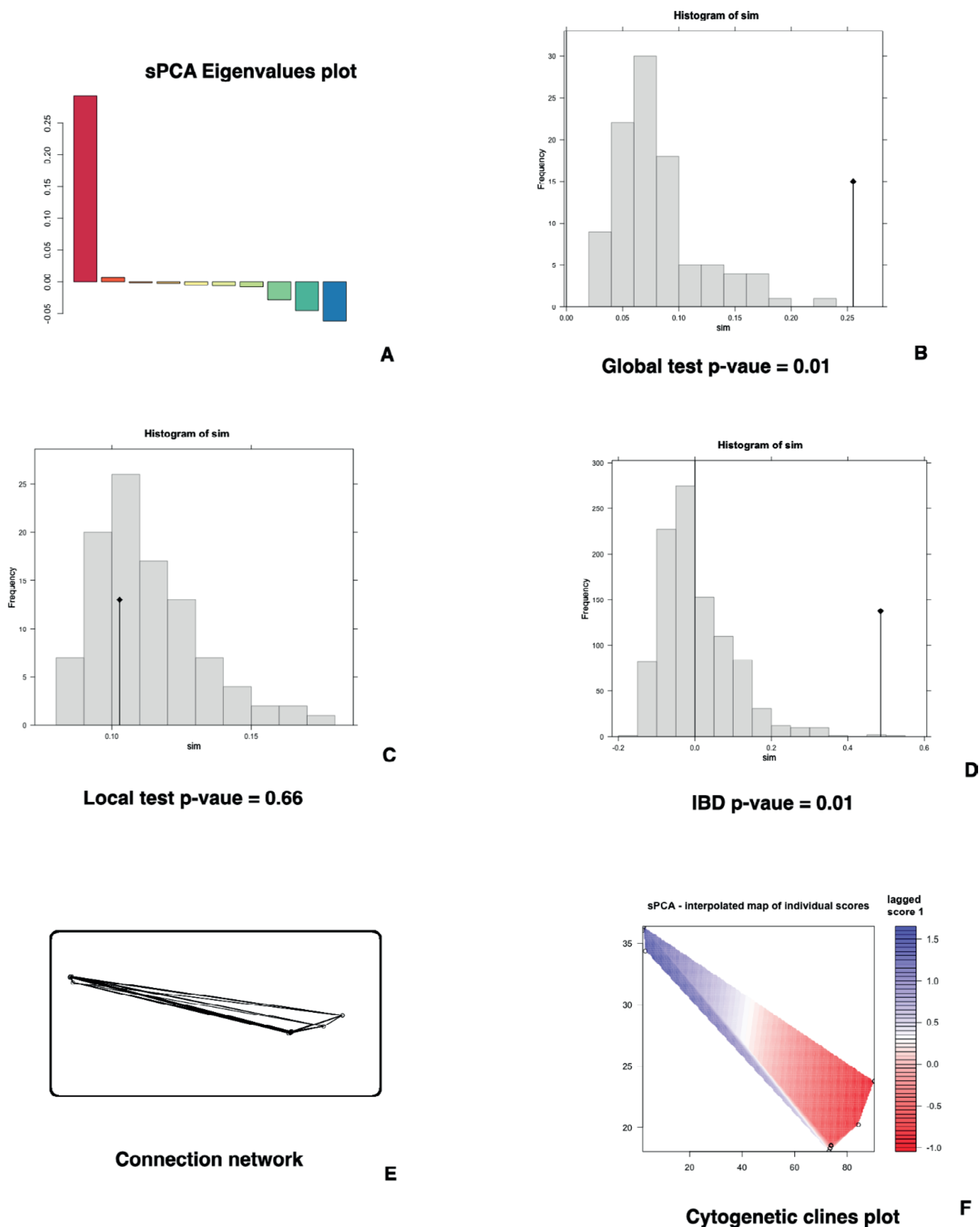
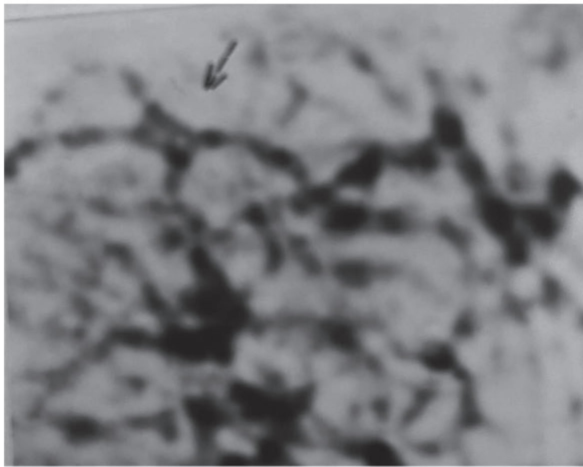


Figure 4. Representative sPCA plots of cytogenetic data in the studied *Asparagus* species. A = Plot of Eigenvalues revealed a strong effect of positive (global) spatial features for the studied taxa (the part shown in red), B-D = Global, local, and IBD test results showed significant p-values in the first two tests. E, and F = The connection and cytogenetic clines plots showed closer relationships between species from India and Bangladesh, with cytogenetic clines formed respectively in all three countries.



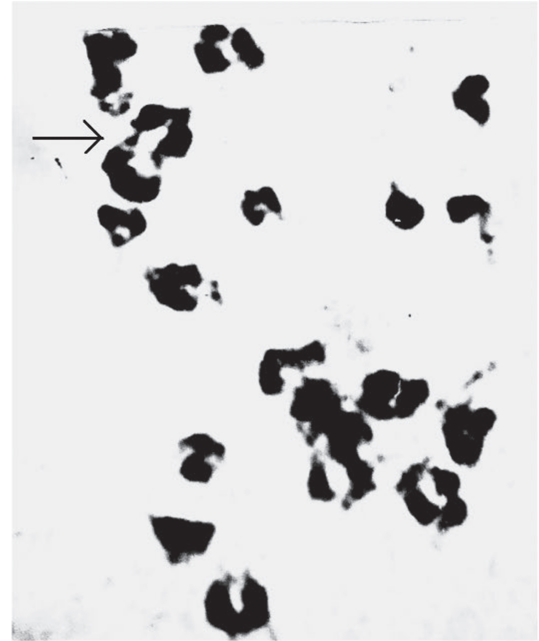


Figure 5. A = A heterozygote translocation (arrow) in *Asparagus racemosus* sub *acesora,* B, and C = Multivalent formation (arrows) in Asparagus gonoclados, and *A. officinalis*, respectively. (Figures are from one of the coauthors i.e. Sheidai 1985, Ph.D thesis)

the Spanish landrace 'Morado de Huetor' (Moreno et al. 2006). Additionally, Ozaki et al. (2014) discovered spontaneous triploid *Asparagus* plants from crosses with diploid parents.

Mousavizadeh et al. (2021), studied the influence of climate on the geographical distribution of diploid and polyploid *Asparagus* plants of *A. officinalis*, *A. persicus*, *A. verticillatus*, and *A. breslerianus* growing in Iran and reported changes in the ploidy levels of vegetation across different zones. These changes are related to humidity, average minimum and maximum temperatures, and soil salinity. They observed that the species with 8X and 10X species live at higher altitudes and are able to adapt to drier and more salinity lands than 2X and 4X plants.

The number and shape of plant chromosomes, the amount and composition of nuclear DNA, the frequen-

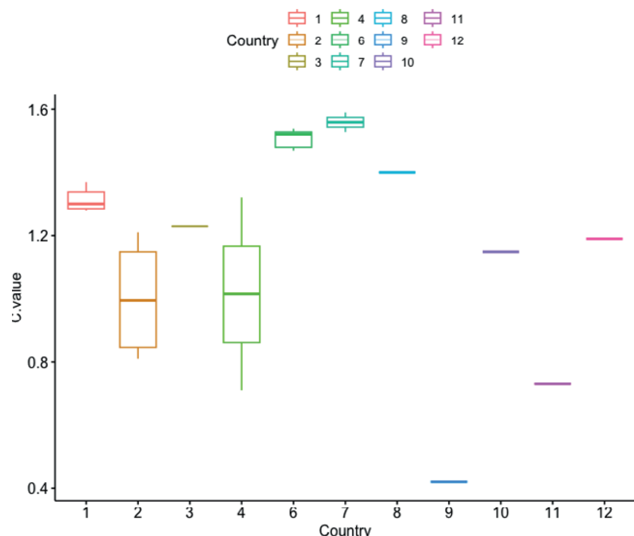


Figure 6. Box-plot of 1C-value quantity among the countries of origin of *Asparagus* species (The country code 1-12, are as in Table 3).

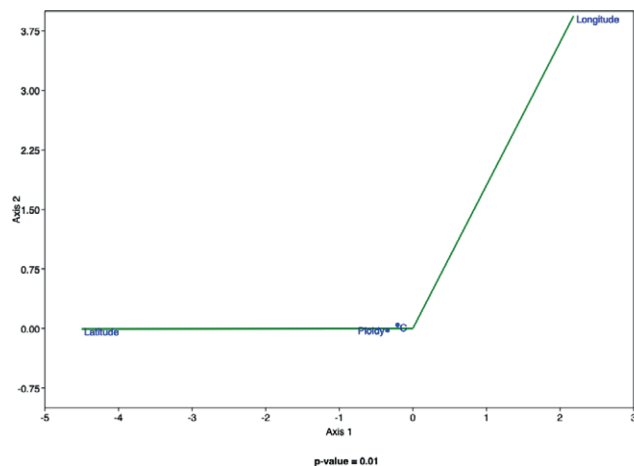


Figure 7. RDA plot shows a significant association (p=0.01), between the polyploidy level and 1C-value with spatial variables.

cy of chiasmata, and the chromosomal meiotic behavior of chromosome pairs vary greatly among plant species. In particular, meiotic behavior is genetically regulated, and changes in the frequency and location of crossovers within chromosome arms affect the genetic diversity of the offspring (Rees and Jones 1977). Therefore, the existence of significant differences in the chiasmata frequency and distribution, and ring and rod bivalents, among *Asparagus* species growing in different parts of the world may indicate their genomic differences (Sheidai et al. 2002) and may act as the genomic adaptation to environmental variables that have been reported in other plant crops (see for example, Sheidai et al. 2012).

Genetic variation can be exploited through local environmental and climatic selection to achieve ecological diversity even in the absence of physical barriers. Because new beneficial mutations or chromosomal rearrangements are unlikely to accumulate rapidly, it has been suggested that rapid adaptation may involve selection based on persistent genetic variation i. e. the genetic variation present in ancestral populations before divergence occurred (Ortiz-Barrientos et al. 2016).

Van Belleghem et al. (2018) presented a scenario for the emergence of persistent genetic variation that describes the demographic history of a population or species. When alleles involved in adaptation arise from independent mutations or chromosomal changes, they occur either at different loci or randomly in lineages from the same locus. Therefore, lineages are not identical because new adaptive mutations may occur in different haplotypes in different regions. On the other hand, if ecological differentiation is based on alleles or genetic loci that exist as persistent genetic variation in an ancestral population, derived alleles have the same origin but differ greatly in their evolutionary history.

Plath et al. (2022) reported that 2C DNA content can vary not only across accessions within a species but also across Asparagus species growing in different geographical regions of the world. The causes of these changes are thought to be polyploidization and differences in chromosome size.

However, other cytogenetic abnormalities and mechanisms, such as aneuploidy (Sheidai and Inamdar 1992; Ozaki et al. 2004), the presence of B-chromosomes (Sheidai and Inamdar 1993), cytomixis (Sheidai et al. 1993), or desynapsis (Sheidai 1992), are the other potentially effective cytogenetic changes found in the genus *Asparagus*.

Landscape genetics and population-level cytogenetic studies can reveal habitat fragmentation and identify the genetic clines within the geographic range of a plant species (Anderson et al. 2011). Studying global climate change also tests the ecological and evolutionary responses of species to predicted conditions. Knowledge and understanding of how habitat fragmentation affects adaptive evolution under projected climate change is very limited (Anderson et al. 2011). The present study found that *Asparagus* species could see their geographic distribution significantly reduced in the future due to climate change.

It has been suggested that environmental stresses (e. g., climate change) may result in inbreeding depression. As a result, inbred, fragmented populations may have a lower ability to adapt to contemporary and changing conditions compared to large, unfragmented

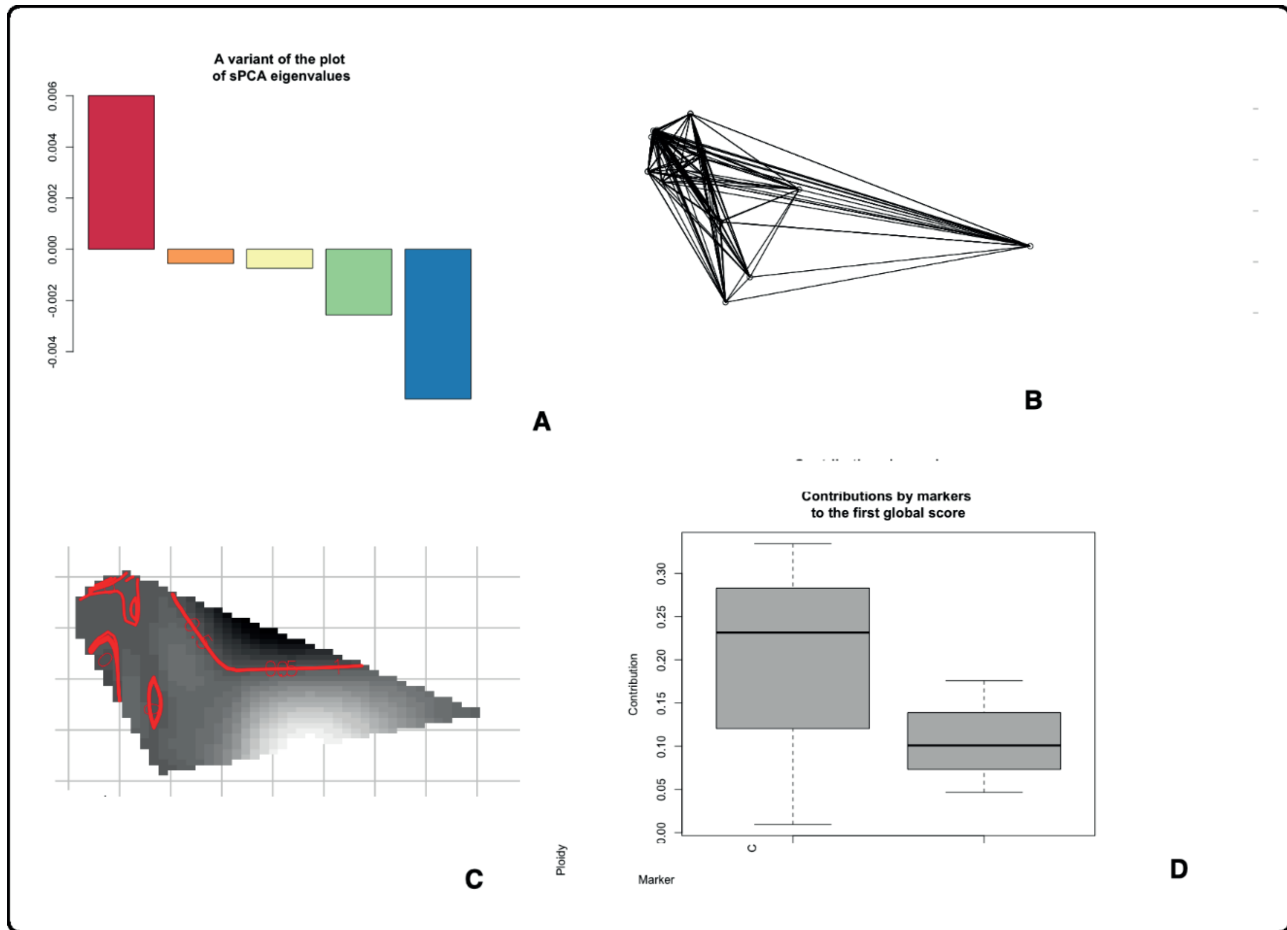


Figure 8. The sPCA analysis plots of the ploidy level and 1C-value content of *Asparagus* species. A the Eigenvalues plot, B the connection plot, C = Genetic clines formed due to both ploidy level and 1C-value data, and D = The contribution role of the studied variables viz. Ploidy and 1C-value.

populations. Fragmented populations with reduced genetic diversity may lack variation in key ecological traits such as drought tolerance. In such situations, assisted migration to suitable habitats along with the conservation of habitat corridors, may be necessary to prevent dramatic declines in species and genetic diversity (Anderson et al. 2011).

Polyploidy may play a role in adaptation to new habitats and environmental conditions. This appears to be positively related to latitude, altitude, and recent sea ice (Stebbins, 1984; Brochmann et al. 2004). Higher ploidy rates are generally observed at higher latitudes or altitudes than related diploids, especially in herbaceous perennial grasses (Zhang et al. 2019). Likewise, genome size is correlated with the environment and geographic distribution of species (Bottini et al. 2000; Bennett and Leitch 2011), and changes in DNA C values are correlated with many phenotypic traits of cells and organisms. This can affect important ecological traits of plant species in natural habitats, such as spring growth timing, cell size and leaf expansion rate early in the growing season, frost tolerance, and dry conditions (Zhang et al. 2019). Therefore, in conclusion, we report that both spatial and bioclimatic variables influence the genetic structure and geographical distribution of *Asparagus* species worldwide and that general conservation programs against climate change are needed.

AUTHOR CONTRIBUTION STATEMENT

Masood Sheidai and Fahimeh Koohdar: conceptualization of the project; Parisa Fouroutan; data collection and lab work

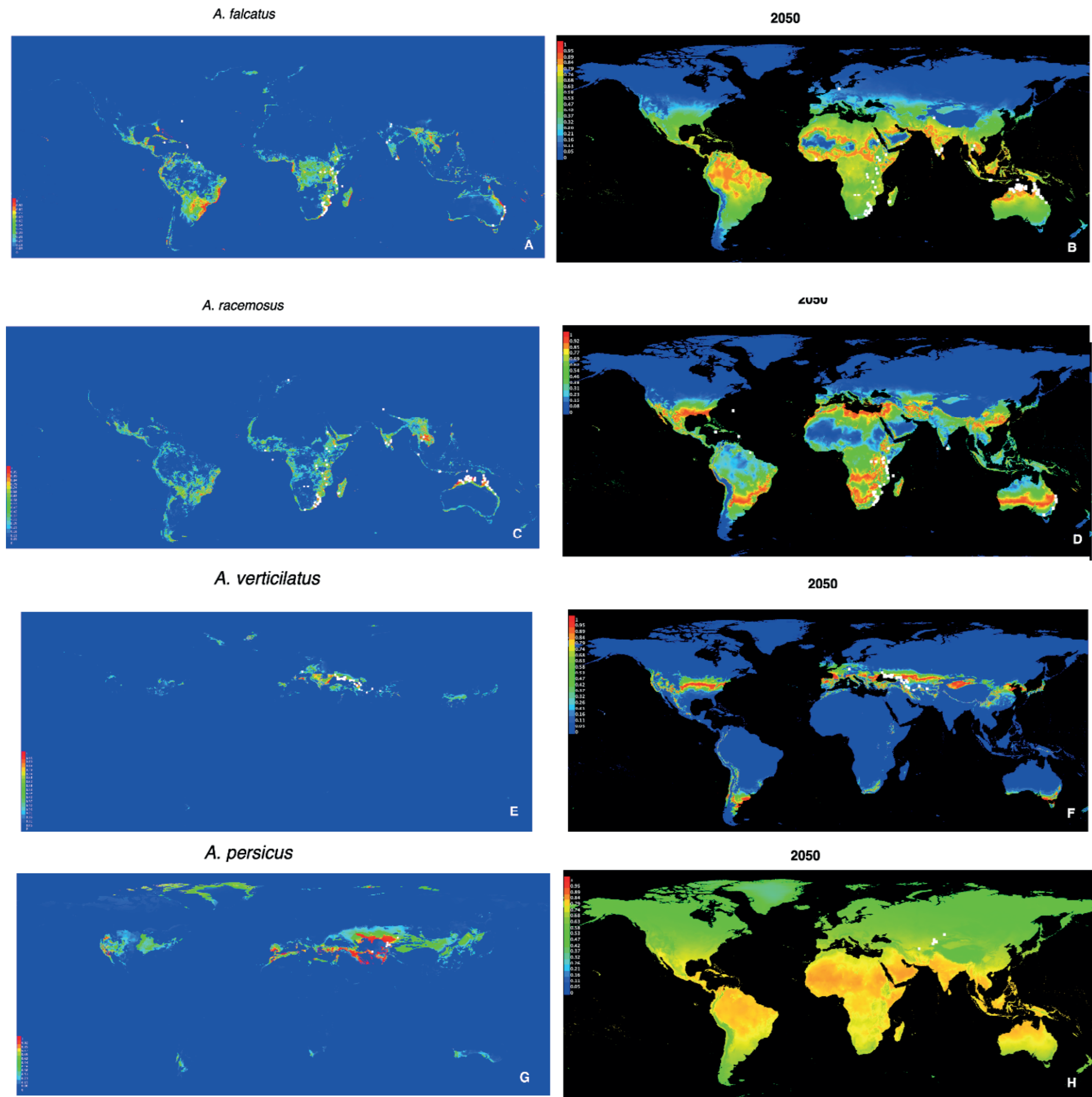


Figure 9. SDM results of the Maxent model show the predicted occurrence of *Asparagus* species at the present time versus the year 2050.

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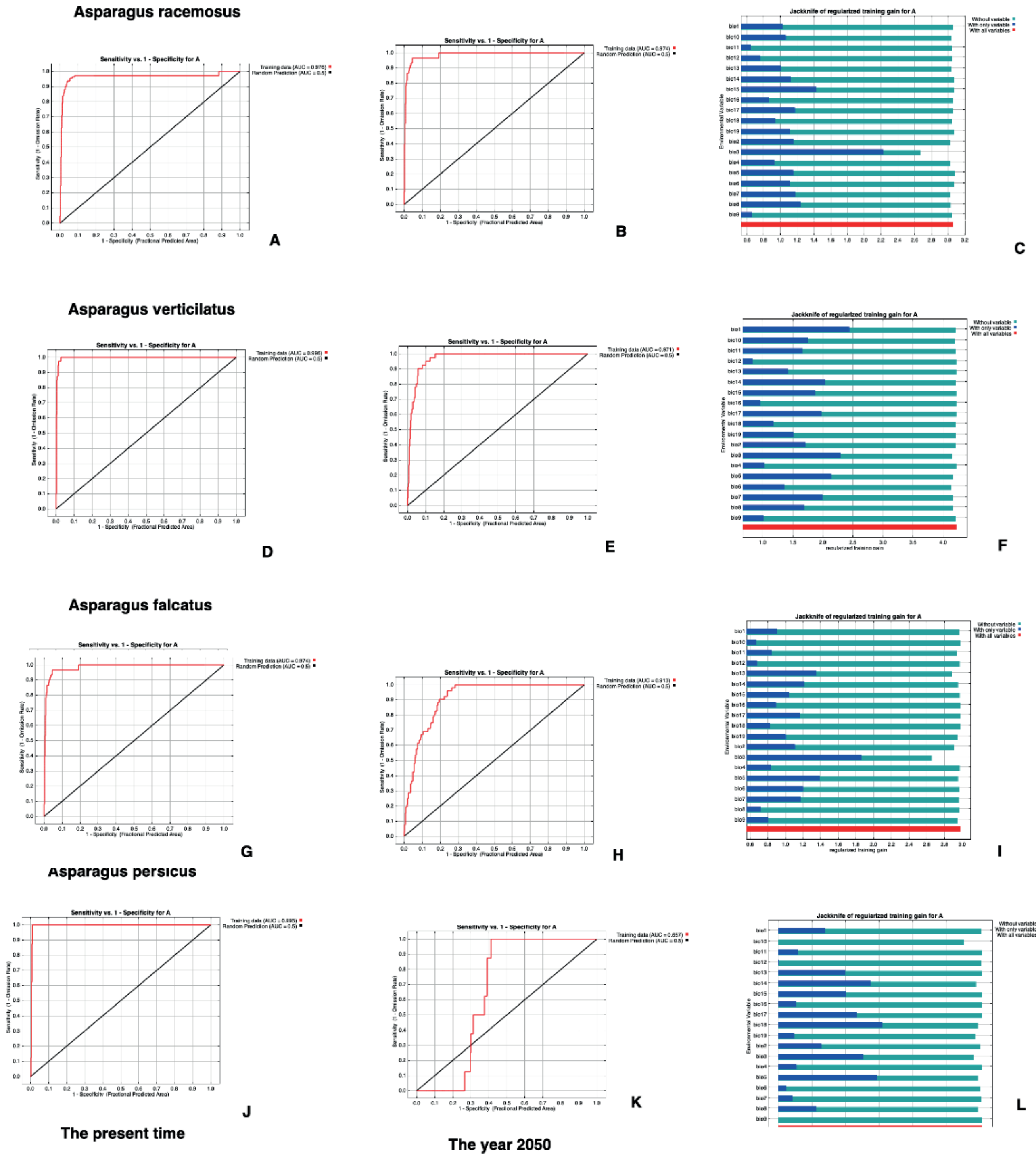

Figure 10. The representative plots of AUC values (ROC curve), and the importance of bioclimate variables affecting *Asparagus* species geographical distribution.

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First chromosome characterization and repetitive DNA of Barred Gliding Lizard, *Draco taeniopterus* Günther, 1861 (Draconinae: Agamidae: Squamata)

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Abstract. This research was the first report on karyological analysis and distribution patterns of repetitive DNA using the fluorescence in situ hybridization (FISH) technique on the barred gliding lizard, *Draco taeniopterus* Günther, 1861. The 10 male and 10 female specimens were collected from Than To district, Yala province, Thailand. Chromosome preparation was performed by direct method using bone marrow and testis. The chromosomes were stained using conventional staining, NOR-banded, and FISH technique with $d(GC)_{15}$, $d(TA)_{15}$, $d(CAG)_{10}$, and $d(CAA)_{10}$ microsatellite probes. The karyotype of the barred gliding lizard reveals a diploid chromosome number of 34 and a fundamental chromosome number of 46, comprising of 8 pairs of large metacentric chromosomes, 2 pairs of small metacentric chromosomes, 2 pairs of large submetacentric chromosomes, and 22 pairs of microchromosomes, no sex chromosome detection between male and female karyotype. The metaphase I showed 17 bivalents and metaphase II showed haploid, n=17. The NOR is observed on the telomeric region of the last microchromosome pair 17th. Microsatellite repeat patterns indicate the presence of $d(GC)_{15}$ and $d(CAG)_{10}$ show specific regions, 2qter and 3qter respectively. While $d(TA)_{15}$ and $d(CAA)_{10}$, show cumulative signals dispersed throughout the chromosomes. This research can provide additional fundamental information for future genetic studies. The barred gliding lizard has the following karyotype formula: $2n=34=L^{m}_{8}+L^{sm}_{2}+S^{m}_{2}+22mi$.

Keywords: *Draco taeniopterus*, Chromosome, Cytogenetics, Repetitive DNA.

INTRODUCTION

Flying lizard genus *Draco* are classified in family Agamidae, subfamily Draconinae which consists of 34 genera and 272 species, the important genera such as *Acanthosaura, Calotes, Diploderma, Draco, Gonocephalus, Japalura* and *Sitana*. In the genus *Draco*, 40 species are found, which is distributed from Southwest India through Southeast Asia, including the Malay Peninsula, the Philippines, and Thailand (Honda et al. 2000; McGuire & Heang 2001; Hoser 2014; Denzer et al. 2015; Nampochai et al. 2021).

Barred gliding lizard or spotted flying dragon (*Draco taeniopterus* Günther, 1861), It is a species that indicates the abundance of tropical rainforest ecosystems. The typical characteristics of this lizard are its small body size (66-78 mm from the tip of the mouth to the anus and the tail 136-153 mm long), flat body, small head, the extended dulap (chin flap) is yellow-orange. Patagium has 4-5 distinct dark transverse bands alternative to light transverse bands and presence of light spots in the middle of dark bands. These colors are useful to help camouflage there to match the bark. Tympanum uncovered with scales. Snout without a series of scales forming a Y-shaped figure. Nostril directed upward (Figure 1). The *Draco taeniopterus* is found in Myanmar, Thailand, Cambodia, and Malaysia. The conservation status of this species is least concern (Honda et al. 1999; 2000; Srichairat et al. 2014; 2015; 2017; Visoot et al. 2023)

Cytogenetic review of the genus *Draco* has only 2 reports in 3-4 species with conventional technique. The Draco karyotype is 2n=34 with 16 macrochromosomes and 18 microchromosomes, without sex-chromosomes.

Figure 1. General characteristics (A.) and its patagium (B.) of barred gliding lizard, *Draco taeniopterus*, Draconinae, Agamidae) from Ban Wang Sai, Mae Wat subdistrict, Than To District, in Yala Province, Thailand.

The karyotype report of Draconinae also not prevailing. The diploid number of this subfamily has appeared in several genera, *Gonocephalus* (2n=36), *Calotes* (2n=32-34), *Japalura* (2n=34,46), *Sitana* (2n=34, 36, 46) (Ota and Hikida 1989; Sharma and Nakhasi 1980; Li et al. 1981; Ota 1988; Solleder and Schmid 1988; Ota et al. 1992; Kritpetcharat et al. 1999; Diong et al. 2000; Ota et al. 2002; Singh and Banerjee 2004; Zongyun et al. 2004; Patawang et al. 2015a).

This research is first report on molecular cytogenetics of the genus *Draco*. Conventional, meiotic configuration, Ag-NOR banding and molecular cytogenetic techniques using microsatellite DNA probes including, $d(TA)_{15}$, $d(CG)_{15}$, $d(CAA)_{10}$ and $d(CAG)_{10}$ were applied to detect. This study is useful for taxonomy, conservation and basic and in-depth cytogenetic information of this species.

MATERIALS AND METHODS

The 10 male and 10 female specimens, barred gliding lizard (*Draco taeniopterus*) were collected from Ban Wang Sai, Mae Wat subdistrict, Than To District, in Yala Province, Thailand. The flying lizard were transferred to the laboratory and identified according to the morphological criteria of KEY (Chan-Ard et al. 2015; Das 2015). Experiments were performed in accordance with ethical protocols, as approved by the Ethics Committee of Prince of Songkla University (Ref No.AI003/2022).

Chromosomes were directly prepared *in vivo* (Patawang et al., 2018a) as follows. The animals were injected on their abdominal cavity with colchicine. Then leaved for 24 hours. Chromosome preparation containing bone marrow for mitosis and testis for meiosis were conducted by the colchicine-hypotonic-fixation-air drying technique. The chromosomes were stained with 20% Giemsa's for 30 minutes and identified for NORs by Ag-NOR staining according to Howell and Black (1980) and Verma and Babu (1995). Chromosomal checks were performed on mitotic metaphase cells under light microscope.

FISH experiments were performed under high stringency conditions (Yano et al. 2017) to classify microsatellite sequences, specifically $(TA)_{15}$, $(GC)_{15}$, $(CAA)_{10}$, and $(CAG)_{10}$. These sequences were directly labeled by Cy3 at the 5'end during synthesis (Sigma, St. Louis, MO, USA) . FISH was performed under stringent conditions and hybridization occurred overnight in a moist chamber at 37 °C. Chromosomes were counterstained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1.2 μg/ml) mounted in antifade solu-

tion (Vector, Burlingame, CA, USA,) (Aiumsumang et al. 2022; Patawang et al. 2022; Prasopsin et al. 2022; Thongnetr et al. 2022).

At least 20 metaphase spreads per individual were analyzed to confirm the diploid number, karyotype structure, NORs and FISH data. Images were captured using an Axioplan II microscope (Carl Zeiss Jena GmbH, Germany) with CoolSNAP and processed using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified according to centromere position as metacentric (m), submetacentric (sm) and acrocentric (a) (Tanomtong et al. 2019). For the chromosomal arm number (NF; fundamental number), m, sm, a were scored as bi-armed while t as mono-armed. The microchromosomes are chromosomes that are 5 times less long than the largest pair of chromosomes (Patawang et al. 2016; 2017; 2018b).

RESULTS AND DISCUSSION

Barred gliding lizard (*Draco taeniopterus*) had a diploid number of 34. The karyotype comprised eight large metacentric, two large submetacentric, two small metacentric and 22 microchromosomes. The fundamental number was 46 in both sexes and no sex chromosome heteromorphisms were evident (Table 1 and Figure 2). The karyotype formula of *Draco taeniopterus* is $2n=34=L^{m}_{8}+L^{sm}_{2}+S^{m}_{2}+22mi$. The diploid chromosome number is following previous studies of 4 species of genus *Draco* (Ota and Hikida 1989, Kritpetcharat et al. 1999). The diploid numbers of subfamily Draconinae are 2n=32-46 in 7 genera 21 species (Ota and Hikida 1989; Sharma and Nakhasi 1980; Li et al. 1981; Ota 1988; Solleder and Schmid 1988; Ota et al. 1992; Kritpetcharat et al. 1999; Diong et al. 2000; Ota et al. 2002; Singh and Banerjee 2004; Zongyun et al. 2004; Patawang et al. 2015a). Some species of Draconinae has polymorphism, the *Calotes versicolor* from India has 2n=32 and 34, the *Japarula swinhonis swinhonis* from Taiwan has 2n=36, 40, and 46. This species exhibits no sex differences in karyotypes between males and females, no cytologically distinguishable sex chromosome was observed to be similar to the *Draco cornutus, D. haematopogon, D. quinquefasciatus and D. belliana*. The karyotypes of this genus are quite similar. All species have 12-16 macrometacentric or submetacentric chromosomes, and 18-22 microchromosomes. The mechanism of chromosomes rearrangement maybe fission, fusion and/or pericentric inversion. Comparative chromosome studies of subfamily Draconinae is show on Table 2 (Ota and Hikida 1989; Sharma and Nakhasi 1980; Li et al. 1981; Ota 1988; Solleder and Schmid 1988; Ota et al. 1992; Kritpetcharat et al. 1999; Diong et al. 2000; Ota et al. 2002;

Table 1. Mean length of short arm chromosome (Ls), length of long arm chromosome (Ll), length of total chromosomes (LT), relative length (RL), centromeric index (CI) and standart deviation (SD) from 10 metaphases of male and female of barred gliding lizard (*Draco taeniopterus*), 2*n* (diploid)=34.

Chromosome pairs	Ls (µm)	Ll (µm)	LT (µm)	CI±SD	RL±SD	Chromosome size	Chromosome type
1	7.69	8.61	16.30	0.528±0.000	0.184±0.000	Large	metacentric
2	5.97	8.45	14.41	0.586±0.000	0.163±0.000	Large	metacentric
3	5.37	6.09	11.46	0.532±0.000	0.129±0.000	Large	metacentric
4	5.25	5.59	10.84	0.515±0.000	0.122±0.000	Large	metacentric
5	2.87	6.15	9.02	0.683±0.000	0.102±0.000	Large	submetacentric
6	2.83	3.44	6.27	0.550±0.000	0.070±0.000	Small	metacentric
7	0.00	2.22	2.22	1.000±0.000	0.025±0.000	microchromosome	
8	0.00	2.13	2.13	1.000±0.000	0.024±0.000	microchromosome	
9	0.00	2.06	2.06	1.000±0.000	0.023±0.000	microchromosome	
10	0.00	2.04	2.04	1.000±0.000	0.023±0.000	microchromosome	
11	0.00	1.96	1.96	1.000±0.000	0.022±0.000	microchromosome	
12	0.00	1.89	1.89	1.000±0.000	0.021±0.000	microchromosome	
13	0.00	1.67	1.67	1.000±0.000	0.019±0.000	microchromosome	
14	0.00	1.66	1.66	1.000±0.000	0.019±0.000	microchromosome	
15	0.00	1.71	1.71	1.000±0.000	0.019±0.000	microchromosome	
16	0.00	1.58	1.58	1.000±0.000	0.018±0.000	microchromosome	
17*	0.00	1.45	1.45	1.000±0.000	0.016±0.000	microchromosome	

* = NORs bearing chromosomes.

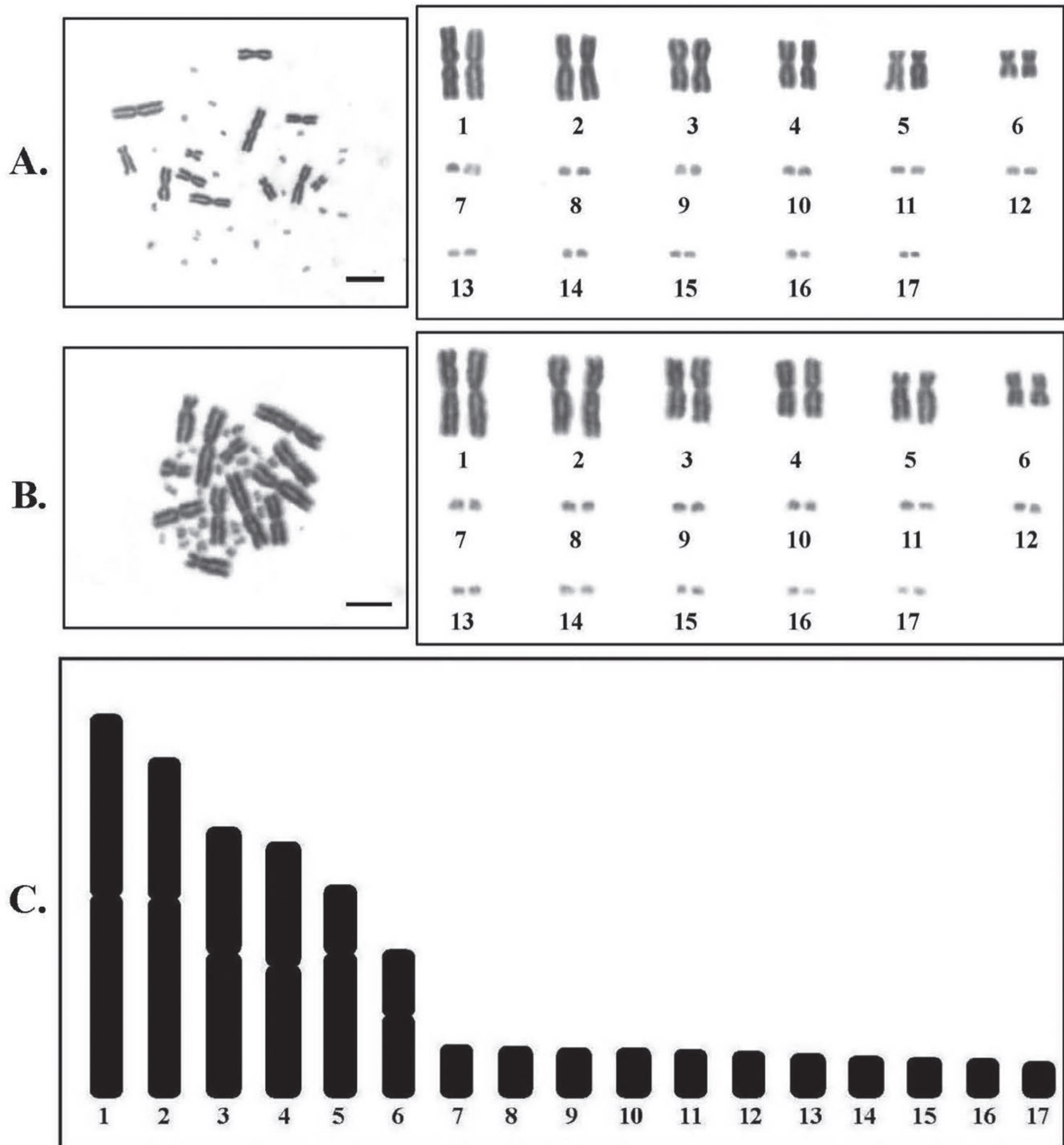

Figure 2. Metaphase plates and standardized karyotypes of male (A.), female (B.) and Idiogram (C.) of barred gliding lizard, *Draco taeniopterus*, 2n=34 by conventional staining (Scale bars = 10 µm).

Singh and Banerjee 2004; Zongyun et al. 2004; Patawang et al. 2015a).

The first cytogenetic study of *Draco taeniopterus* carried out by Ag-NOR banding technique was obtained from this research. We found NORs observed in the region adjacent the last smallest microchromosomes (pair 17th) (Figure 3). The report of NOR position in Draconinae was located on telomeric region of q-arm of pair 2nd in 4 species of *Calotes* including *C. cristatellus, C. emma, C. mystaceus,* and *C. versicolor* (Solleder and Schmid

Table 2. Comparative chromosome studies of subfamily Draconinae.

Species	2n	Karyotype	NOR	Locality	References
Acanthosaura armata	32	12m+20mi	-	Malaysia	Ota et al. (2002)
Bronchocela cristatella	34	14m+20mi	-	Singapore	Ota et al. (2002)
	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
Calotes cristatellus	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
C. emma					
	34	12m/sm+22mi	-	Thailand	Kritpetcharat et al. (1999)
	34	12m+22mi	-	Malaysia	Ota et al. (2002)
	34	-	-	India	Singh and Banerjee (2004)
C. jerdoni	34	12m/sm+22mi	-	India	Sharma and Nakhasi (1980)
	34	-	-	India	Singh and Banerjee (2004)
C. mystaceus	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
	34	12m/sm+22mi	-	Thailand	Kritpetcharat et al. (1999)
	34	-	-	India	Singh and Banerjee (2004)
	34	10m+2m+22mi	2qter	Thailand	Patawang et al. (2015a)
C. versicolor	34	12m/sm+22mi	-	India	Sharma and Nakhasi (1980)
	34	12m/sm+22mi	2qter	Asia	Solleder and Schmid (1988)
	34	12m/sm+22mi	-	Thailand	Kritpetcharat et al. (1999)
	34	12m+22mi		Singapore	Ota et al. (2002)
	32, 34	-	-	India	Singh and Banerjee (2004)
	34	12m/sm+22mi	2qter	Thailand	Patawang et al. (2015a)
Draco cornutus	34	16m+18mi	-	Malaysia	Ota and Hikida (1989)
D. haematopogon	34	16m+18mi	-	Malaysia	Ota and Hikida (1989)
D. quinquefasciatus	34	16m+18mi	-	Malaysia	Ota and Hikida (1989)
D. belliana	34	12m/sm+22mi	-	Thailand	Kritpetcharat et al. (1999)
D. taeniopterus	34	10m+2sm+22mi	17	Thailand	This study
Diploderma splendidum (as *Japarula splendida*)	34	12m+22mi	-	China	Zongyun et al. (2004)
	36	10bi+26a	-	Central Taiwan	
Di. swinhonis (as *Japarula swinhonis swinhonis*)	40	6bi+34a	-	Central Taiwan	Ota (1988)
	46	46a	-	Northern Taiwan	
Gonocephalus chamaeleontinus	42	22m+20mi	-	Malaysia	Diong et al. (2000)
G. liogaster	42	22m+20mi	-	Malaysia	Diong et al. (2000)
G. bellii	42	22m+20mi	-	Malaysia	Diong et al. (2000)
G. grandis	42	30m/sm+12t	-	Boeneo	Ota et al. (1992)
	42	22m+20mi	-	Malaysia	Diong et al. (2000)
G. myotympanum	42	30m/sm+12t	-	Boeneo	Ota et al. (1992)
G. robinsonii	32	12m+20mi	-	Malaysia	Diong et al. (2000)
Japalura variegata	34	-	-	India	Singh and Banerjee (2004)
J. varcoae	34	12m+22mi	-	China	Li et al. (1981)
Ptyctolaemus gularis	34	12m/sm+22mi	-	India	Sharma and Nakhasi (1980)

Remark: 2n=diploid number, m=metacentric, sm=submetacentric, a=acrocentric, t=telocentric, mi=microchromosome, qter=terminal region of long arm.

1988; Patawang et al. 2015a). The NOR position of *Draco taeniopterus* was more conserved than the genus *Calotes*.

Chromosomes of barred gliding lizard testis for meiosis was observed. The metaphase I has 17 bivalents comprising 6 ring bivalents of macrochromosomes and 11 small rod bivalents of microchromosomes. The metaphase II has n=17 haploid comprising 5 metacentric, 1 submetacentric macrochromosomes and 11 microchromosomes (Figure 4). The meiosis karyotypes of other species in Agamidae are showed in lndo-Chinese water dragon, *Physignathus cocincinus* which has 2n =36 with 6 ring bivalents of metacentric or submetacentric macrochromosomes and 12 rod bivalent of microchromosomes (Patawang et al. 2015b). In addition, the meiotic

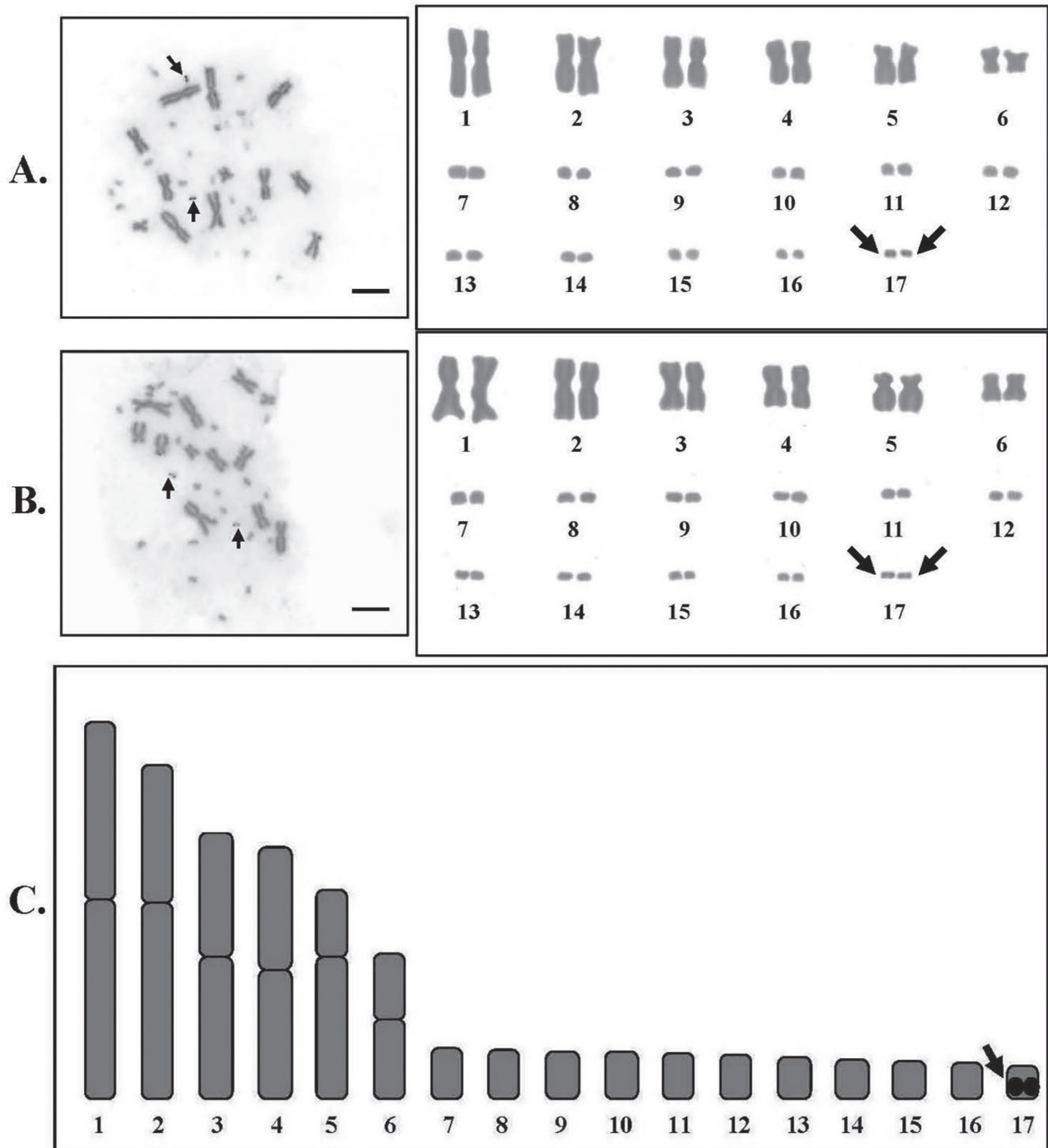

Figure 3. Metaphase plates and standardized karyotypes of male (A.), female (B.) and Idiogram (C.) of barred gliding lizard, *Draco taeniopterus*, 2n=34 by Ag-NOR banding, arrows indicate NORs (Scale bars = 10 μm).

configurations of another lizard were revealed in butterfly lizard, *Leiolepis reevesii rubritaeniata* (Agamidae) and long-tailed grass lizard, *Takydromus sexlineatus* (Lacertidae) (Phimphan et al. 2013; Patawang et al. 2018b).

Microsatellite repeat patterns of *Draco taeniopterus* indicated the presence of $d(GC)_{15}$ and $d(CAG)_{10}$ showed specific regions, 2qter and 3qter respectively. While $d(TA)_{15}$ and $d(CAA)_{10}$, showed cumulative signals dis-

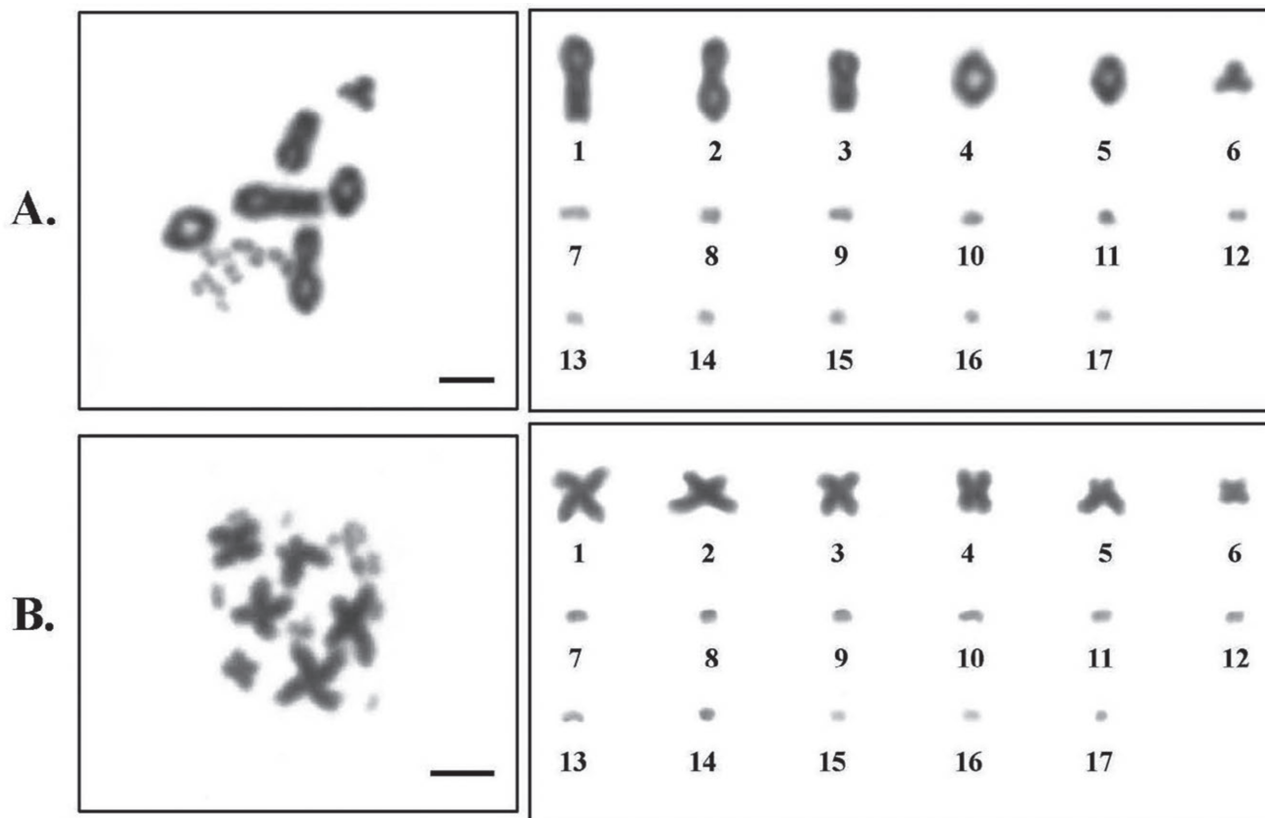

Figure 4. Meiotic cell divisions and karyotypes of metaphase I (A.) and metaphase II (B.) of barred gliding lizard, *Draco taeniopterus*, 2n=34 by conventional staining (Scale bars = 10 μm).

persed throughout the chromosomes (Figure 5 and 6). The microsatellite loci were highly evolved loci. Therefore, in many species there may be different forms of repetitive sequences. Most of them were found distributed throughout the genome. But in some species it was found on the telomere position. However, in some species it may be found in a specific location. This study, the short tandem repeats of $d(GC)_{15}$ was found on q-arm telomeric of pair 2nd and $d(CAG)_{10}$ was found on q-arm telomeric of pair 3rd. The molecular cytogenetics applying microsatellite probe of family Agamidae in previous study has *Leiolepis reevesii rubritaeniata*, 2n=36 (12bi+24mi) using $(TTAGGG)_n$ probes presented on telomeric and interstitial some chromosomes and *Tympanocryptis lineata* and *Rankinia diemensis*, 2n=32 (12bi+20mi) using (TTAGGG)7 presented on centromeric and telomeric region in some chromosomes (Jantarat et al. 2018; Srikulnath et al. 2009; Alam et al. 2021). We suggest employing GC and CAG probes in different Drago to have a deeper understanding of the relationship.

The barred gliding lizard, *Draco taeniopterus* from Than To district, Yala province, Thailand has 2n=34, NF=46. The karyotype comprises four pairs of large metacentric chromosomes, one pairs of small metacentric chromosomes, one pairs of large submetacentric chromosomes, and 11 pairs of microchromosomes. The metaphase I showed 17 bivalents and metaphase II showed haploid, n=17. The NOR was located on the last microchromosome pair 17th. Microsatellite repeat patterns indicated the presence of $d(GC)_{15}$ and $d(CAG)_{10}$ showed specific regions, 2qter and 3qter respectively. While $d(TA)_{15}$ and $d(CAA)_{10}$, showed cumulative signals dispersed throughout the chromosomes. This study is useful in supporting our understanding of the evolution of flying lizards and promote conservation of wildlife resources in tropical rainforests.

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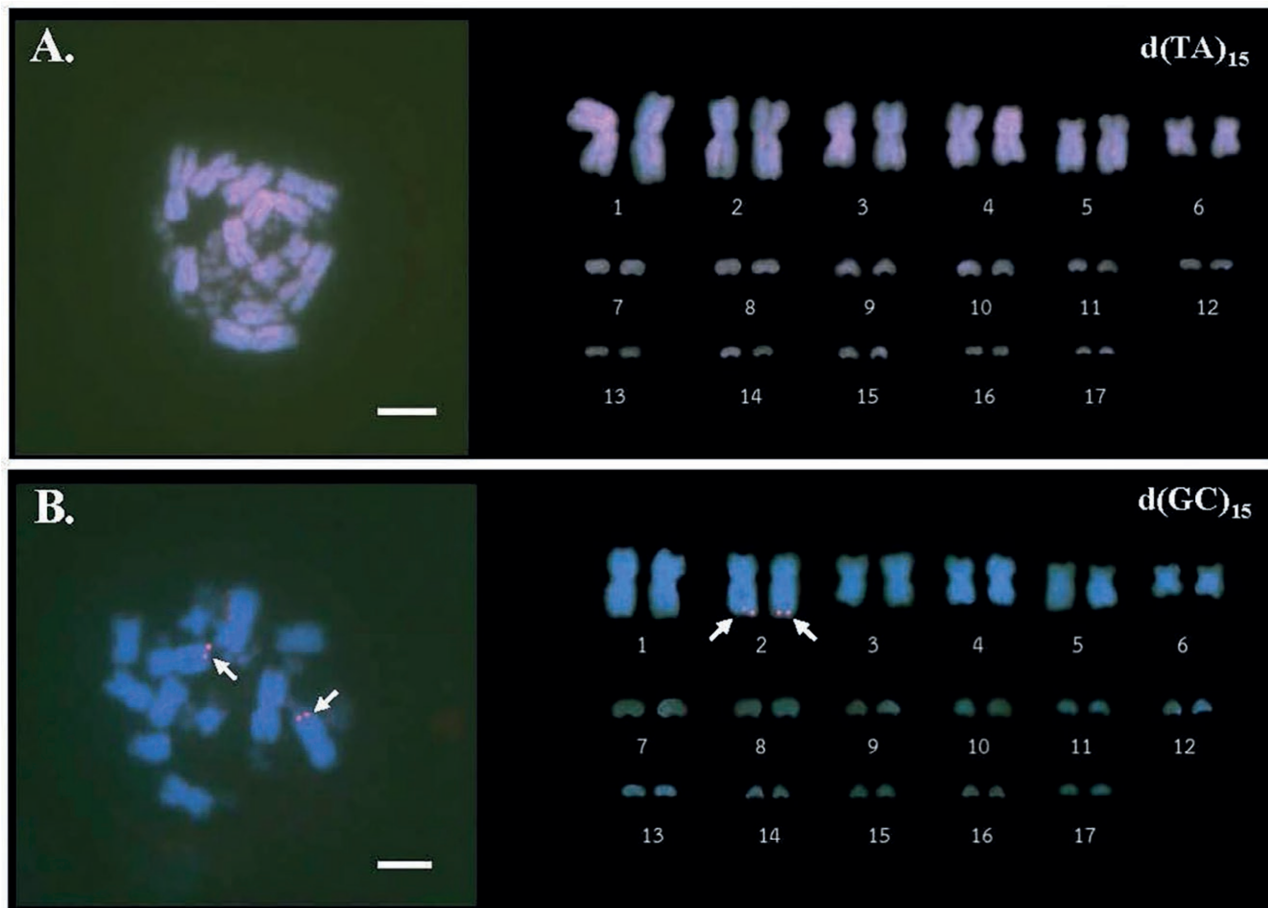

Figure 5. Metaphase plates and hybridization patterns with microsatellite probes $d(TA)_{15}$ (A.) and $d(CG)_{15}$ (B.) (red signals) on metaphase plates of barred gliding lizard, *Draco taeniopterus*, 2n=34, chromosomes were counterstained with DAPI (blue) (Scale bar = 10 μm).

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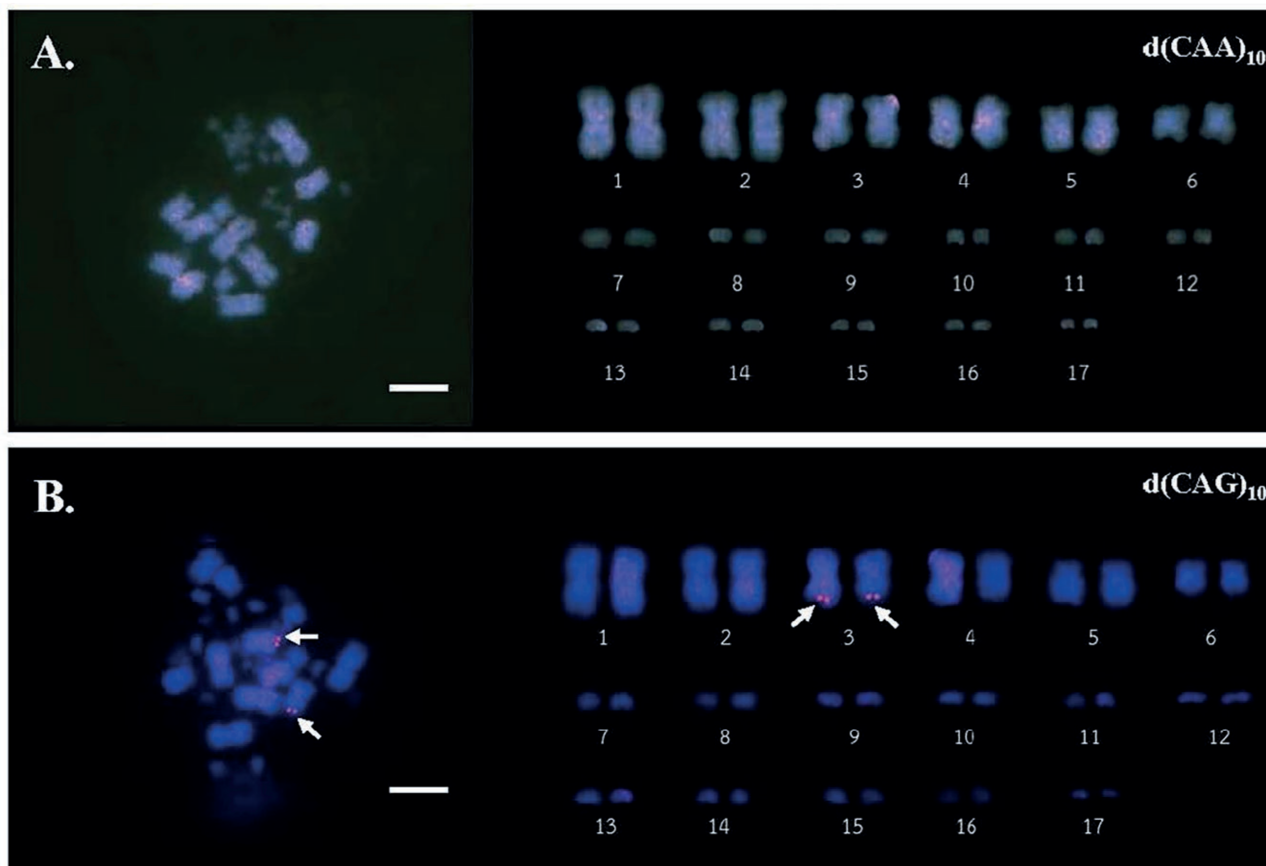

Figure 6. Metaphase plates and hybridization patterns with microsatellite probes d$(CAA)_{10}$ (A.) and d$(CAG)_{10}$ (B.) (red signals) on metaphase plates of barred gliding lizard, *Draco taeniopterus*, 2n=34, chromosomes were counterstained with DAPI (blue) (Scale bar = 10 μm).

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Cytotoxic assessment of aqueous extracts of *Heliotropium keralense* Sivar. & Manilal on *Allium cepa* root tip cells

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Abstract. *Heliotropium keralense* Sivar. & Manilal is an endangered medicinal plant native to the Indian state of Kerala. Cytotoxic effects of aqueous extracts of leaves, stems, and roots of *H. keralense* were evaluated using *Allium cepa* L. root tip method. *Allium cepa* bulbs were exposed to extracts of different parts of the plant for 24 hours. Compared to the negative control, a significant decrease in the length, root number and mitotic index of *Allium cepa* was observed with 5 to 25% aqueous extracts of *H. keralense.* Chromosomal abnormalities such as single, double, and multiple lesions in interphase, single and double lesions in prophase, diagonal metaphase, diagonal anaphase, bridged anaphase, strap-shaped nuclei, giant cells and chromosome loops are identified in positive control and treatments. The highest percentage of chromosomal aberration was observed in the (95.18±2.07%) positive control and 25% (71.76±7.46%) leaf extract. The analysis showed that the aqueous plant parts of *H. keralense* had anti-mitotic and cytotoxic effects. This study shows that *Heliotropium keralense* contains strong cytotoxic substances that can cause chromosomal aberrations.

Keywords: *Heliotropium keralense, Allium cepa*, Cytotoxicity, Chromosomal aberrations, Anti-mitotic.

INTRODUCTION

Phytomedicines play a crucial role in the treatment of human and animal diseases. They are safer than the synthetic drugs. These plant derived metabolites can be isolated, identified, tested and used against new diseases. Plant extracts are extensively used in medicine and the food industry; therefore, evaluating the cytotoxicity of plants against other cell lines and organisms appears crucial to determine non-toxic concentrations at which they can be safely used (Gazala et al., 2018). Many people use these plants for food and therapeutic purposes without having sufficient knowledge about the safe use of these medicinal plants and its products. Herbal products and herbal medicines must be properly evaluated and researched for the phytochemicals and their potential risk of adverse side effects due to overdose and toxicity.

It becomes necessary to investigate the toxicity of phytochemicals because of the adverse effects associated with their use in conventional medicine.

Heliotropium is a large genus of the Boraginaceae family with a wide distribution in tropical and temperate areas. *Heliotropium keralense* Sivar. & Manilal is endemic to Kerala, India. The plant is used as remedy against worms, skin diseases, scorpion and snake poisoning, asthma, cough, anemia, insanity, and epilepsy (Sivarajan, 1994). Flavonoids and terpenoids are abound throughout the plant. The plant contains several pharmacologically active compounds with therapeutic effects of antibacterial, antiviral, anti-inflammatory, and anticancer activities (Nayar, 1996). *Heliotropium keralense* Sivar. & Manilal (Thelkatta) is a tribal medicinal plant used by Mullukuruma tribes in Wayanad district of Kerala. A paste made from the leaves of the plant is applied on the bitten area in the treatment of sting bite (Silja et al., 2007). Due to the lack of knowledge about the genotoxic and cytotoxic potential of the plant, it is important to evaluate the effect of the plant extract on the cell and genetic material. The present study deals with the anti-mitotic and cytotoxic effects of aqueous extracts of different parts of *H. keralense*. The cytotoxicity was tested on *Allium cepa* root tip cells. The simplest and most ideal method for examining the impact of mitosis in plant cells was to examine the root tip of *Allium cepa*. It is a fast and inexpensive system for measuring the cytotoxic effects of pollutants, chemical substances, and plant extracts (Barman et al., 2021; Das et al., 2021).

MATERIALS AND METHODS

Plant collection and preparation of extract

Heliotropim keralense was collected from a paddy field in Pathanamthitta District, Kerala, India (latitude 9°15’26.4”N; longitude 76°49’35.6”E). Plant parts are separated and cleaned. Shade-dried plant parts are powdered. 5g, 10g, 15g, 20g, and 25g of plant parts (leaf, stem, and root) were weighed and boiled in 100ml of distilled water for 10 min. The extracts were filtered using Whatsman No.1 filter paper and used for the treatment.

Effect of plant extracts on root tip cells of Allium cepa

The *Allium cepa* test was used to investigate the cytotoxic activity of the aqueous extracts from the leaf, stem, and root of *H. keralense*. Commercially available *A. cepa* bulbs of the same size (4-5g) were used, carefully de-scaled and placed on top of test tubes filled with distilled water for germination for 48h. The germinated bulbs were then transferred to various concentrations (5, 10, 15, 20 & 25 g/100ml) of aqueous extract of *H. keralense* plant parts for 24h. *Allium cepa* germinated in distilled water was used as negative control. Onions germinated in distilled water followed by treatment with hydrogen peroxide (7%) for 1h were considered as positive control. After treatment, the roots were counted and the length of the roots in each bulb was measured. The roots were fixed in Carnoy’s fluid (ethanol: acetic acid, 3:1). After fixation, the roots were hydrolyzed in 1M/L Hydrochloric acid for 1 min at 60°C. The roots were placed on microscopic slides, crushed using 2% acetocarmine and observed under a microscope. Mitotic index was expressed as number of dividing cells/total number of cells counted (Ozmen and Summer, 2004). Chromosomal aberrations were determined by randomly selecting five zones per slide. Mitotic index was determined using the equation

Mitotic Index = (Number of dividing cells) ÷ (Total number of cells) × 100

The chromosome aberration frequency was expressed as a percentage. This was calculated by using the equation

Chromosome aberration frequency = (Number of cells with chromosome aberration) ÷ (Total number of cells) × 100

Statistical analysis

Ten random samples were taken to analyze the root growth of *Allium cepa* grown in different concentrations of aqueous extracts. Mitotic index was counted under the oil emersion (100x) microscopy. Mitotic index and chromosomal aberrations were determined by randomly picking five zones per slide. Photo documentation was taken using microscope Olympus CX 41 attached with camera Cmos Cam (3.0m pixels). Data on root number, root length, mitotic index and chromosomal aberration percentage in *Allium cepa* were subjected to statistical analysis. One way ANOVA was performed to determine the significance of tests using SPSS free trial Software.

RESULTS

Effect of various treatments on root growth of Allium cepa

The effects of different concentrations (5, 10, 15, 20, and 25%) of aqueous extracts of *H. keralense* plant parts (leaf, stem, and root) on root number and root length were significant (P<0.001). The mean numbers of root in the negative and positive controls (Table 1) were 56.4±12.54 and 7.4±1.81 respectively. The average number of roots of *Allium cepa* bulbs is higher in 5% extracts (leaf, stem, and root) of *H. keralense* is 39.8±2.77, 53.8±9.03 and 35.8±2.38. The mean root number in 25% of extracts of leaf, stem, and root are 8±2.44, 11.2±2.16, and 13.4±2.96 per individual bulb of *Allium cepa*. The number of roots decreases with the increasing concentration of plant extracts.

The mean root length was found to be4.32±0.80 cm in negative controls and 0.06±0.56cm in the positive control (Table 1). A continuous decrease in root growth was observed from lower concentration of the treatments to its higher concentrations. Root growth in 5% aqueous extracts of leaf, stem, and root of *H. keralense* is 5.66±0.86, 4.52±0.32, and 3.6±0.43cm, respectively. *Allium cepa* root growth is reduced in 25% of leaf, stem, and root extracts (0.6±0.38, 0.28±0.08, and 0.86±0.43cm, respectively). *Allium cepa* root lengths decreased with increasing the concentration of extracts.

Average root length and root number of treatments are (5, 10, 15, 20, and 25%) lower than the negative control. Root number and root length of *Allium cepa* were reduced from a lower concentration of extracts to a higher concentration of extracts of different plant parts. The highest concentrations of plant extract showed maximum inhibitory effects on root growth. The mean values of treatments are significantly (P<0.001) lower than the negative control. Treatments of *H. keralense* in *A. cepa* root apical meristem cells showed a concentration-dependent inhibitory effect on root growth.

Effect of various treatments on mitotic index of Allium cepa root cells

Significantly high mitotic index was observed in negative control (Table 2). A significantly (P<0.001) low mitotic index was observed in the aqueous extracts of leaves, stems, and roots of *H. keralense* compared to the negative control (Table 2). Aqueous extract of the stem, leaf, and root of *H. keralense* actively inhibits cell division in *Allium cepa* roots. In different concentrations of extracts, the number of dividing cells decreases with increasing concentration of the extracts. Among the different treatments, the lowest mitotic index was observed in the leaf, stem, and root (25%) extracts (13.54±5.27, 18.37±3.84, and 14.53±4.49). In leaf extracts, the mitotic index of *Allium cepa* cells is reduced from 5% (62.62±8.56) to 25% (13.54±5.27) of the extracts. In stem extracts, the mitotic index was 51.88±10.38 in 5% and 18.37±3.84 in 25% extracts. In cells treated with root extract, 52.6±5.65 mitotic index in 5% and 14.53±4.49 mitotic index in 25% extracts. Mitotic index decreases with the increasing concentration of plant extracts (Table 2).

Cytological effect of various treatments on Allium cepa root tip cells.

Compared to the negative control (Fig. 1c,d,e), chromosomal aberrations were found to be very high in the treatments. The positive control shows the maximum percentage of aberration. All cells treated with the plant

Table 1. Effect of aqueous extracts of *Heliotropium keralense* on *Allium cepa* root growth.

Treatment	Leaf extract		Stem extract		Root extract	
	Root number	Root length (cm)	Root number	Root length (cm)	Root number	Root length (cm)
Negative control	56.4±12.54[a]	4.32±0.80[a]	56.4±12.54[a]	4.32±0.80[a]	56.4±12.54[a]	4.32±0.80[a]
Positive control	7.4±1.81[d]	0.06±0.56[d]	7.4±1.81[c]	0.06±0.56[c]	7.4±1.81[c]	0.06±0.56[c]
EX 5%	39.8±2.77[b]	5.66±0.86[a]	53.8±9.03[a]	4.52±0.32[a]	35.8±2.38[b]	3.6±0.43[a]
EX 10%	34.6±1.81[b]	3.86±0.39[b]	34±4.06[a,b]	3.14±0.19[a]	33±4.84[b]	3.08±0.65[a]
EX 15%	19.2±2.38[c]	3.02±0.70[b]	20.8±3.27[b]	1.84±0.43[b]	22.8±2.58[b,c]	2±0.72[b]
EX 20%	16.4±4.61[c]	1.52±0.34[c]	13.2±3.27[c]	1.26±0.11[b]	21.2±4.43[b,c]	1.42±0.35[b]
EX 25%	8±2.44[d]	0.6±0.38[d]	11.2±2.16[c]	0.28±0.08[c]	13.4±2.96[c]	0.86±0.43[c]
Main effect F df (n-1) = 6	99.14***	59.09***	62.59***	191.43***	32.43***	22.06***

EX: means the different concentrations of plant extract. *** Significant at P<0.001 level. Means within column followed by the same letters are not significantly (P<0.05) different as determined by DNMRT.

Table 2. Mitotic index of *Allium cepa* cells treated with *Heliotropium keralense* extract.

Treatment	Leaf extract	Stem extract	Root extract
Negative control	62.62±8.56[a]	51.88±10.38[a]	52.6±5.65[a]
Positive control	33.11± 3.55[b]	33.37±6.69[b]	39.36±9.49[b]
EX 5%	28.9±11.79[b,c]	32.62±2.02[b]	34.61±1.03[b]
EX 10%	23.05±8.26[b,c]	25.8±3.58[c]	23.99±6.8[c]
EX 15%	13.54±5.27[c]	18.37±3.84[d]	14.53±4.49[c]
EX 20%	68.58±5.8[a]	68.58±5.8[a]	68.58±5.8[a]
EX 25%	12.12±2.06[c]	12.12±2.06[d]	12.12±2.06[c]
Main effect F df (n-1) = 6	26.645***	21.041***	27.978***

EX: means the different concentrations of plant extract. ***Significant at P<0.001 level. Means within column followed by the same letters are not significantly (P<0.05) different as determined by DNMRT.

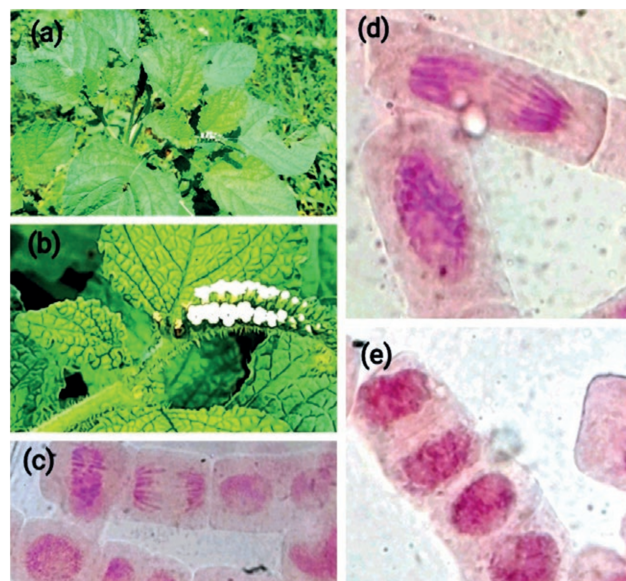

Figure 1. (a) *Heliotropium keralense*. (b) *H. keralense* infloresecence. (c) Normal mitotic phases (interphase,metaphase, and anaphase)in *Allium cepa* root tip .(d) Prophase and anaphase. (e) Telophase.

extract show aberrations. The percentage of aberrations increased with increasing concentration of extracts of individual parts of the *H. Keralense* plant. The leaf extract of *H. keralense* showed a higher percentage of aberration compared to other plant parts. The percentage of aberration increases with the increase with the concentration of aqueous extract of leaves, stem, and root of *H. keralense*.

The mean percentage of aberrations for all treatments is significantly (P<0.05) low compared to positive control. Among the treatments, lowest percentage of aberration was observed in 5% leaf extract was 21.88±3.78. The percentage of aberration of 25% of leaf, stem, and root extracts (71.76±7.46, 67.17±7.22, and 53.43±1.97%) was significantly (P<0.05) lower than that of positive control (95.18±2.07%). Lesions were the common abnormality seen in the interphase and prophase. From the analysis, it was found that plant extracts produce more aberrations in interphase and prophase.

In 25% leaf extract, 34.6±2.6% aberrations are observed in interphase, 17.4±1.14% aberrations in prophase, 11.8±2.49% in metaphase, 9.7±2.58% in anaphase (Table 3). The percentage of aberrations of the 25% leaf extract (71.76±7.46) was significantly (P<0.05) lower than that of the positive control (95.18±2.07%).

In the stem, a significantly low chromosomal aberration was recorded in the 5% stem extract (21.85±2.10) compared to higher concentrations (Table 4). Among

Table 3. Cellular abnormalities observed in *Allium cepa* exposed to the leaf extract of *Heliotropium keralense*

Conc.	Total no. of cells	Percentage of aberrant cells	Percentage of abnormality			
			Interphase	Prophase	Metaphase	Anaphase
5%	118.8±29.82[a]	21.88±3.78[b]	11.2±2.28[c]	8.2±1.3[c]	4.6±1.81[b,c]	2.2±0.83[b,c]
10%	112.6±24.37[a]	28.35±2.66[b]	13.4±2.07[c]	10.6±3.64[b,c]	4.4±1.14[b,c]	3.6±1.14[b,c]
15%	104.8±12.21[a]	33.73±8.30[b]	15.4±2.4[c]	10.8±3.03[b,c]	5±2.34[b]	4.4±0.83[b]
20%	91.6±3.5[b]	56.13±10.67[b]	24.4±2.3[b]	14.6±3.5[b]	6.8±1.3[b]	5.8±0.83[b]
25%	102.4±6.65[a]	71.76±7.46[a]	34.6±2.6[b]	17.4±1.14[b]	11.8±2.49[a]	9.7±2.58[a]
Negative Control	90.2±7.32[b]	9.9±1.65[c]	5±2[d]	2±0.7[d]	1±0[c]	1±0[c]
Positive Control	116.2±6.22[a]	95.18±2.07[a]	53.8±10.94[a]	23.8±2.77[a]	19.4±4.39[a]	13.7±2.86[a]
Main effect F df(n-1)=6	1.587[NS]	53.962***	85.419***	8.804**	13.35***	21.069**

[NS] non significant, **P<0.005,***P<0.001. Means within column followed by the same letters are not significantly (P<0.05) different as determined by DNMRT.

Table 4. Cellular abnormalities observed in *Allium cepa* exposed to the stem extract of *Heliotropium keralense.*

Conc.	Total no. of cells counted	Percentage of aberrant cells	Percentage of abnormality			
			Interphase	Prophase	Metaphase	Anaphase
5%	121.2±13.8[a]	21.85±2.10[b]	11±1.58[c]	7.6±2.88[c]	6.4±1.51[c]	1.8±0.83[b]
10%	93.6±20.18[b]	26.68±2.69[b]	11.8±2.68[c]	4.6±1.14[c]	6.4±2.07[c]	2.2±1.3[b]
15%	99.2±20.51[b]	30.72±6.11[b]	13±3.8[c]	5.8±1.78[c]	8.2±1.78[c]	3.8±2.04[b]
20%	109.8±23.95[a]	44.98±12.06[b]	17.4±4.03[c]	12.2±1.92[b]	10.4±2.51[b]	9.6±1.81[a]
25%	94.8±15.35[b]	67.17±7.22[b]	25.2±3.76[b]	14.4±3.5[b]	12.8±2.58[b]	11.6±6.34[a]
Negative Control	90.2±23.74[b]	9.9±1.65[c]	5±2.23[d]	2±0.7[c]	1±0[c]	1±0 [b]
Positive Control	116.2±33.15[a]	95.18±2.07[a]	53.8±12.51[a]	23.8±6.9[a]	19.4±6.8[a]	13.8±2.86[a]
Main effect F df(n-1)=6	1.863[NS]	41.541***	15.819*	15.392**	8.342*	10.089***

[NS] non significant, *P<0.01, **P<0.005, ***P<0.001, ***P<0.001. Means within column followed by the same letters are not significantly (p<0.05) different as determined by DNMRT.

Table 5. Cellular abnormalities observed in *Allium cepa* exposed to root extract of *Heliotropium keralense.*

Conc.	Total no. of cells	Percentage of aberrant cells	Percentage of abnormality			
			Interphase	Prophase	Metaphase	Anaphase
5%	118.2±18.64[a]	19.31±3.89[b]	9.6±2.07[c]	6.4±2.3[c]	5.8±2.16[b,c]	1.6±0.54[c]
10%	88.6±10.59[b]	25.29±5.91[b]	9.8±1.64[c]	6.6±2.96[c]	4.4±1.51[b,c]	1.8±0.44[c]
15%	114.6±17.91[a]	30.02±8.30[b]	15.6±6.58[b,c]	8.8±2.86[c]	7.4±2.88[b]	2.8±0.83[c]
20%	98.4±3.91[a]	41.8±19.27[b]	17.8±5.49[b,c]	9.8±3.42[c]	8.8±1.92[b]	5±1.58[b]
25%	104.8±11.25[a]	53.43±1.97[b]	27.6±6.46[b]	12.4±2.96[b]	9.8±2.38[b]	6.2±2.68[b]
Negative Control	90.2±2.86[b]	9.9±1.65[c]	5±2.82[d]	2±0.89[d]	1±0[c]	1±0[c]
Positive Control	116.2±18.15[a]	95.18±2.07[a]	53.8±14.46[a]	24±7.68[a]	19.4±7.63[a]	13.8±4.96[a]
Main effect F df (n-1)=6	3.921*	13.630***	11.106***	3.586***	4.84**	9.47**

*P<0.01, **P<0.005, ***P<0.001, ***P<0.001. Means within column followed by the same letters are not significantly (p<0.05) different as determined by DNMRT.

the treatments, highest aberrations were noticed in 25% extract. In 25% stem extract, treated cells showed 25.2±3.76% aberrations in interphase, 14.4±3.5% in prophase, 12.8±2.58% in metaphase, and 11.6±6.34% in the anaphase (Table 4).

In root, a significantly low chromosomal aberration was noticed in 5% (19.31±3.89%) extract compared to higher concentrations. In root tip cells treated with 25% root extract, 27.6±6.46% of aberrations in interphase, 12.4±2.96% aberration in prophase, 9.8±2.38% in metaphase, and 6.2±2.68% in the anaphase (Table 5). All parts of this plant show an undifferentiated range of aberration on the cells of the *Allium cepa* root tip. Lesions are seen in interphase and prophase. More chromosomal aberrations are seen in metaphase and anaphase.

Chromosomal lesions and chromatid bridges are high in 20 and 25% of the plant extracts and in the positive control. All treatments showed varying levels of chromosomal aberrations, but this was less compared to the positive control. Major abnormalities (Fig. 2) such as giant cells, strap-shaped nuclei, single, double, and multiple lesions in interphase, single and double lesions in prophase, metaphase clumping, diagonal metaphase, diagonal anaphase(Fig. 2g), bridged anaphase (Fig. 2e), lagging chromosome, metaphase clumping (Fig. 2f) and chromosome loops were observed in treatment and positive control. Sticky chromosomes and chromosome loops are high in higher concentrations (20 & 25%) of the leaf extract and on the positive control.

Most of the aberrations are noticed in metaphase and anaphase and are in the higher concentrations (20 & 25%) of the plant extracts. Prophase and interphase lesions also increase in higher concentrations of the plant extracts. These aberrations inhibit the cell division and also cause the cell death.

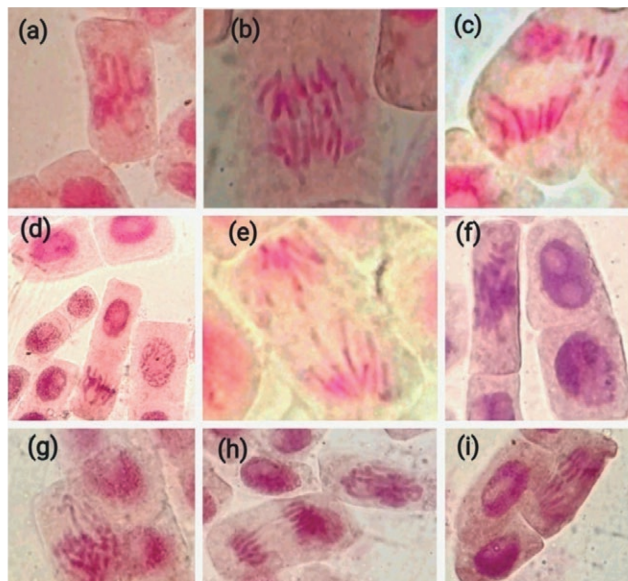

Figure 2. *Allium cepa* root tip cells exposed to the extracts of *Heliotropium keralense*(a)Metaphase clumping (b) Chromosome fragments.(c) Diagonal anaphase (d) Diagonal metaphase, giant cells (e) chromosome fragments (f)Diagonal metaphase and double lesions(g) Disoriented anaphase (h) Chromosome bridge and metaphase clumping(i) polar deviation.

DISCUSSION

In this study, the effect of *H. keralense* extracts was evaluated by root growth and cytology of root tip cells of*Allium cepa*. In *Allium cepa,* aqueous extracts of *H. keralense* reduces the root length and prevent root formation. .As the concentration of leaf, stem, and root extracts increased, the number and length of roots decreased. This growth gradation indicates an inhibitory effect of *H. keralense* on growth and cell division in *Allium cepa* roots and is similar to previous studies where in aqueous extracts of *Capparis spinosa* caused decreased mitotic index in *A.cepa* root tips(Sultan and Celik, 2009).The aqueous extract of *Campomanesia xanthocarpa* also showed the same effect in *A. cepa* root cells (Pastori et al., 2013).

Reduced mitotic index of *Allium cepa* root cells treated with different concentrations of extracts was observed in the present study. This indicates the inhibition of cell growth and cell death. It was found that the mitotic index decreased with increasing concentrations of the plant extracts. This result indicates the inhibition and suppression of mitotic division in the root cells of *A. cepa* by the chemical compounds present in the aqueous extract of plant parts. The reduction of cell division and cell differentiation in *A. cepa* root cells indicates the cytotoxic effect of the components in *H. keralense* aqueous extract.

The current findings demonstrate that as the concentration of leaf, stem, and root extracts increases, so do interphase and prophase lesions. Giant cells are generated as a result of endoreplication or endomitosis, while binucleated cells appear as a result of interrupted cytokinesis (Das et al., 2022). Chromosomal bridges, polar deviation during different mitotic phases and metaphase clumping, were the most frequent abnormalities: all of these aberrations are regarded as being notably cytotoxic (Askin and Aslanturk 2010, Barman et al., 2020, Roy et al., 2021). As treatment concentration increases, a variety of cytological abnormalities occurred throughout both metaphase and anaphase. At a 25% leaf extract concentration, the frequency of mitotic abnormalities such as diagonal metaphase and bridged anaphase is greater. The anaphase and metaphase abnormalities were low in different concentration of leaf, stem and root extracts compared to onion root tips treated with positive control. Chromosome stickiness can result from excessive elongation of chromatin filaments, which causes their improper condensation and can alter the physiochemical properties of nucleic acids, there by arresting the normal process of cell division and promoting cell death (Joti et al., 2012, Renjana et al., 2013, Moustafa et al., 2016, El-Ghamery and Mousa, 2017, Barman and Ray, 2022).This study found that the aqueous extract of *Heliotropium keralense* was lethal to the cell division of *Allium cepa* root tips.

Comparing extracts of leaf, stem, and root to negative control, a reduction in mitotic index was noted. A decrease in the mitotic index indicates that the extracts inhibit the DNA synthesis or block the G2 phase in the cell cycle (Akinpelu et al., 2019), thereby preventing the cell from entering mitosis (Sudhakar et al., 2001). Polar deviation of chromosome can occur due to intra- spindle filament distribution the distribution and indicates the presence of compounds that can interrupt the spindle fiber formation (El-Ghamery and Mousa, 2017). Anaphase bridges and sticky chromosomes are indicative of abnormal DNA condensation and destabilization of mitotic spindles (aneugenic effects) in *A. cepa* root tip cells (Barman et al., 2020, 2021 and 2022).

Mitotic index measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be interpreted as cellular death or a delay in the kinetics of cell proliferation (Rojas et al.1993). It is an acceptable measure of cytotoxicity in all living organisms (Smaka-Kinel et al., 1996). A decreased rate of mitotic index was determined because the extracts contained cytotoxic compounds. This result explains that the

extracts suppress cell division and proliferation. Other reports suggested that the occurrence of various chromosomal aberrations after treatment with plant extracts related to their cytotoxicity (Barman et al., 2020, Roy et al., 2021). The plant extract of *H. keralense* may contain chemicals that are capable of producing cytotoxic effects. Previous reports in *H. keralense* show two hepatotoxic compounds; iso-lycopsamine and intermedine and the plant can be considered as a toxic species (Subban et al., 1990). Ivana Boskovic et al. (2021) confirmed that plant extracts from the Boraginaceae family have cytotoxic potential on cancer cells. In the present study, it is evident that the leaf, stem and roots of the *Heliotropium keralense* induce chromosome aberrations and have a strong cytotoxic effect on other organisms.

To our knowledge, this is the first report of a cytotoxicity study of *Heliotropium keralense*. The plant prompted cytotoxic effects in *A. cepa* likely due to the phytochemicals that can interact synergistically and antagonistically on distinct activities of the genetic material in the test system. The uncontrolled use of this plant can cause negative physiological results to crucial organs. Therefore, further study should be conducted to standardize the concentration of this plant material for medicinal purposes. These phytochemicals from *H. keralense* may be potent anticancer agents. Further studies are needed for phytochemical profiling of this plant. Isolation of potential active compounds from plants and testing them against cancer calls would be of great importance.

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Chromosomal variations and genetic diversity in subpopulations of *Senna alexandrina* Mill. from Western Thar, India

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Abstract. Homologous recombination promotes genetic diversity by exchanging genetic material between homologs, ensuring unique combinations of alleles in offspring. Karyomorphology of the chromosomes can prove to be an efficient tool to reveal the true nature of plant species at genetic level. In this context, our study analyzed the karyomorphology and male meiosis in a medicinal herb, Senna (*Senna alexandrina* Mill., Syn. *Cassia angustifolia* Vahl.), family Fabaceae which is known for its significant polymorphic variations. By observing chromosomal variances, we aimed to shed light on the underlying genetic variations responsible for the observed polymorphism. All the accessions of Senna examined in this study exhibited a diploid chromosome number of 2n = 28. We found variations in the chiasma frequencies of almost all the accessions, particularly concerning the observed number of bivalents, quadrivalents, position of centromere and the presence of the B- chromosome at meiosis-I. Amongst the four accessions studied, two displayed reduced pollen stainability, which seems to be correlated with a lower frequency of chiasmata and the influences of the collection sites, that was confirmed by the regression analysis. Further, RAPD analysis also confirmed the variations in DNA homologous sequences recorded by the presence of variable length of the fragments in all accessions. All the results collectively underscored the existence of genetic diversity within the subpopulation of *Senna alexandrina* Mill. & may help to comprehend the broader evolutionary processes within the Fabaceae family.

Keywords: Cytogenetic analysis, Karyotype, Male meiosis, Mitosis, Polymorphism, *Cassia*, Subpopulation variations.

INTRODUCTION

Cytogenetic analysis particularly plays significant role in taxonomy, genetic abnormalities and genetic diversification, as karyotypes can examine genetic differences between subpopulations of the same species having distinct traits (Young et al. 2012; Jha and Halder 2023). Chromosomes, as the tangible carriers of Mendelian factors, have held a significant role in plant systematics because alterations in their number and structure have consistently been associated with the evolution and formation of plant species (Gill and Husaini 1982). The chromosomal variations reflect the sources of genetic

variation within populations at morphological, physiological, and biochemical level arise from gene expression influenced by the environment with root cause mutation (Chesnokov et al. 2020; Nonić and Šijačić-Nikolić 2021). Plant karyotype research holds significant importance in deciphering the origin and evolution of plant species, understanding molecular phylogeny, and elucidating floristic geography (Lucas et al. 2021). In medicinal plants, cytological analysis is crucial for ensuring the proper identification of herbs in medicinal drug preparations (Deakin et al. 2019; Nonić and Šijačić-Nikolić 2021) and for various other applications, including studying genetic disorders, understanding chromosomal abnormalities, and conducting research in fields such as evolutionary biology and genetics (Deakin et al. 2019; Vitales et al. 2020). In most cases, variations such as differences in chromosome length, arm ratio, position, and the presence of secondary constructions, provide enough information to differentiate individual chromosomes. However, at a more precise level, molecular markers allow us to assess and quantify genetic diversity within populations, species, or germplasm collection for understanding the genetic structure, evolutionary history, and potential adaptability of organisms (Marsjan and Oldenbroek 2006; Omondi et al. 2016).

Meiosis, a pivotal reproductive process, involves homologous chromosome pairing, synapsis, recombination, and segregation, effectively halving the chromosome number to maintain the species' diploid count in the zygote. In normal meiotic processes, pollen mother cells exhibit 100% pollen viability, regular bivalent formation, and normal cytokinesis (Pagliarini 2000; Kaur and Singhal 2019).

The paraphyletic genus *Senna* Mill., belongs to the subfamily Caesalpiniodeae of the family Fabaceae and comprises approximately 350 species that are distributed worldwide (Pellerin et al. 2019, Kumar et al. 2021). *Senna* species are extensively utilized in Africa, Asia, Europe, and Latin America for medicinal purposes and have gained recognition for their antimicrobial, anti-diabetic, anti-malaria, anti-inflammatory properties, which have been documented in traditional medicine practices (Resende et al. 2013; Oladeji et al. 2021). The genus *Senna* is predominantly characterized by a diploid chromosome number of 28, although alternative numbers such as 22, 24, 26,52, and 56 have been reported for specific species (Irwin and Truner 1960; Rasende et al. 2013; Cordeiro and Felix 2017; Nguyen et al. 2021).

Senna alexandrina Mill., syn. *Cassia angustifolia* Vahl. (Indian Senna or Egyptian Senna) is a native species to Saudi Arabia, and widely distributed in tropical and subtropical regions (Kumar et al. 2022). It is a under shrub plant height of 1-2 m having pinnately compound leaves with 4-8 pairs leaflets. Senna holds significant value in Ayurveda and is extensively used as a febrifuge, for splenic enlargement, typhoid, cholera, anemia and laxative purposes (Laghari et al. 2011; Shaily et al. 2023). India is the largest producer and exporter of Senna leaves, pods, and sennosides concentrated in the global market (Saudan 2018). Its leaves and pods possess important purgative properties used in medicine (Nayan et al. 2021).

Here, we present the karyomorphological and male meiosis studies on a subpopulation set of *Senna alexandrina* Mill. in four different accessions sites. Our aim is to gain insights into the genetic diversity within the species that is reflected and verified by the morphological variations in the species in its native environment.

MATERIALS AND METHODS

Plant materials

The germplasm of four accessions of *Senna angustifolia* Vahl. were obtained from different sources- the IHCM accession from NBPGR-CAZRI, Jodhpur (26.263611,72.995352), the IHGA accession from the private institute of Herbal Heritage, Sonamukhi Nagar, Jodhpur (26.193664-73.001885), and the RAU-1 and RAU-2 accessions were obtained from Swami Keshwanand Rajasthan Agriculture University, Jodhpur (28.075225,73.344524). Vouchers of these accessions were submitted to the Department of Botany, JNVU, Jodhpur, and BSI Jodhpur. The seeds were collected during the kharif season for two consecutive years and stored in a cool place. Following the experimental design, the seeds were treated and germinated in the nursery soil at the Botanical Garden, Department of Botany, JNVU, Jodhpur. Phenological records were made for all four accession numbers, including observations on the plant's habit, habitat, height, leaf size, shape, blooming period, cluster of pods, number of seeds per pod, and fruiting time.

Seed germination of F_0 generation

The seeds of *S. alexandrina* were obtained from wild populations (considered here as the F_0 generation). The seeds were first subjected to a fungicide treatment using 0.1% sodium Bavistin for 7-8 minutes to minimize fungal growth. Afterward, they were rinsed thoroughly four times with autoclaved distilled water to remove any residual fungicide. The sterilized seeds were then placed in disposable petri plates containing pre-moistened soil.

These plates were incubated in a controlled environment with a constant temperature of 25 ± 2°C and maintained in darkness to encourage germination.

Mitotic chromosome preparation and staining

After the seed germination, the root tips of appropriate length (0.5-1.0 cm) were excised in the morning between 7:30 am to 8:00 am and immediately pretreated with 0.025% colchicine (HiMedia © India) for three hours at room temperature to arrest cytological stages. After the pretreatment, the root tips were washed multiple times with distilled water, carefully dried by absorbing the moisture, and subsequently fixed in Carnoy's fluid (1 part of glacial acetic acid mixed with 3 parts of 95% ethanol (v/v)) for at least 24 hours at 4°C. The tips were stored in 10% ethanol at 10° C in a refrigerator for long-term use.

The stored root tips were hydrolyzed with 0.1 N HCl for 2-4 minutes at 60 ± 2°C and then washed with distilled water. The softened root tips were stained with 0.5% leuco-basic fuchsin (HiMedia) and subsequently squashed in 1% aceto-carmine (HiMedia) to obtain cytological observations.

At least five clear preparations of metaphase stage of each accession were analyzed to prepare karyotypes. The slides were observed under a light microscope (Olympus BX 60). The average length of the short (p) and long arm (q) of each chromosome was measured using the software Sigma (Pro v. 3 software and ImageJ software).

Male meiosis

Young flower buds were collected in the morning between 7:30 am to 8:00 am and immediately fixed in Carnoy's solution and kept for approximately 24 hours at room temperature. The fixed anthers were then separated from all non-anther parts by using a clean fine needle on the surface of alcohol-washed glass slide. The anther lobes were then squashed onto the glass slide using 1% acetocarmine. A total of 25-30 pollen mother cells (PMCs) at the diplotene/diakinesis and metaphase I stages were observed to record chromosome associations and chiasmata frequencies. It was noted that minor differences existed between the diakinesis and metaphase I stages in terms of associations and chiasma frequency. Therefore, observations from both stages were compiled together, considering an equal number of cells from each stage. Additionally, 15-20 PMCs were analyzed at anaphase I and II to study the distributional pattern of chromosomes/chromatids.

Microphotography

Microphotography was conducted using a Trinocular Research Microscope (Olympus, model BX60F) to capture photomicrographs of the cytological preparations. The photographs of the chromosomes were further analyzed using DRAWID software for ideogram development.

Genomic DNA isolation and PCR amplification for RAPD markers amplification

Total genomic DNA was extracted from 2 g fresh young leaves (2-3 weeks old) of F1 generation of all accession of *Senna alexandrina* by using a modified CTAB method (Lodhi et al. 1994). The extracted DNA was treated with RNase to eliminate RNA impurities and then DNA integrity of the isolated DNA was visualized on Agarose gel (0.8%). After quantification with a spectrophotometer (Thermo Scientific ND-2000), the purified DNA was served for PCR-based amplification. Six out of 20 applied arbitrary RAPD primers (Operon Biotechnologies, Alabama USA) were found suitable for the DNA fingerprint of two accessions IHCM and RAU 1 (Table 1).

PCR amplification was conducted using a programmable thermal cycler (Mycle Bio RAPD 96 Well Gradient Machine) for 35 cycles. The reaction mixture (25 µL) included 2 µl of template DNA (25 ng/µl), 2.5 µL of random primer (IDT Technologies, USA), 4.0 µL of 10 mM dNTPs (Biogene), 1 unit of Taq polymerase (Geni, Bangalore), 2.5 µL of 10X reaction buffer (GeNei), 0.3 µL of 1.5 mM $MgCl_2$, and 15.7 µL of nuclease-free sterile water. The amplification conditions were as follows: initial denaturation at 92°C for 1 minute, annealing at 37°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The reaction ended with an indefinite hold at 4°C.

The 25 µL of amplified PCR product were mixed with 5 µL of gel loading dye (0.25% bromophenol blue, 25% Xylene eynol and 30% glycerol) and then loaded on 1.5% agarose gel electrophoresis along with ladder of 1 kb in 1X TBE buffer at pH 8.2 containing 0.5 µL of Ethidium bromide. The results of the gel run were photographed at Gel-doc UV visualizer with kodak digital Camera. The electrophoretogram of the results of electrophoresis gel analysis was scored based on the presence (1) or absence (0) of bands for all RAPD primers with respect to two accessions.

Karyotype analysis

The karyotype comparison was followed according to Battaglia's (1955) classification as metacentric/

Table 1. Polymorphism detected by six RAPD arbitrary markers in *Senna alexandrina*.

Primer code	Sequence of oligo 5'–3'	Range of fragment Size (bp)		Total no. of bands per primer		Polymorphism (%)	Jaccard similarity coefficient
		IHCM	RAU1	IHCM	RAU2		
OPA10	GTGATCGCAG	300-1000	175-700	4	6	80	0.25
OPX-12	TCGCCAGCCA	325-900	175-750	7	6	92.85	0.077
OPX-18	GACTAGGTGG	200-700	350-1000	4	4	83.33	0.091
OPX-07	GAGCGAGGCT	300-650	225-500	3	3	85.71	0.17
OPX-04	CCGCTACCGA	200-525	100-900	6	7	86.67	0.143
OPB-18	CCACAGCAGT	325-1300	150-900	5	7	88.89	0.125

median [V], submetacentric/submedian [L], subtelocentric/ [J], and telocentric [I] based on an arm ratio of 1:1>1:1<1:3>1:3<1:0 and 0:1 respectively. For karyotype depiction, we used IdeoKar software (version 1.2) to generate ideograms. The degree of symmetry was estimated using Stebbins's (Stebbins 1971) scheme by recognizing three degrees of difference between the largest to the smallest chromosome of the complements, and four degrees with respect to the proportion of sub-telocentric chromosomes.

Statistical analysis

We employed various techniques to assess significant phenotypic and cytogenetic variations within the *Senna alexandrina* population. We selected 9-15 individuals from each accession sites. These individuals were then subjected to detailed phenological characterization, recording height, number of branches, leaves, leaflets, pods, seeds, and leaflet size. Additionally, we computed the mean frequency and range of chiasmata, the terminalization coefficient, and pollen stainability for all accessions. To identify statistically significant differences between sites, we compared the mean values of these measurements using one and two-factor ANOVA, multiple regression, and Tukey's post hoc statistics with the R programming language and related packages. Furthermore, we conducted PCA analysis for different RAPD primers, examining their clustering patterns within sites based on band size.

RESULTS

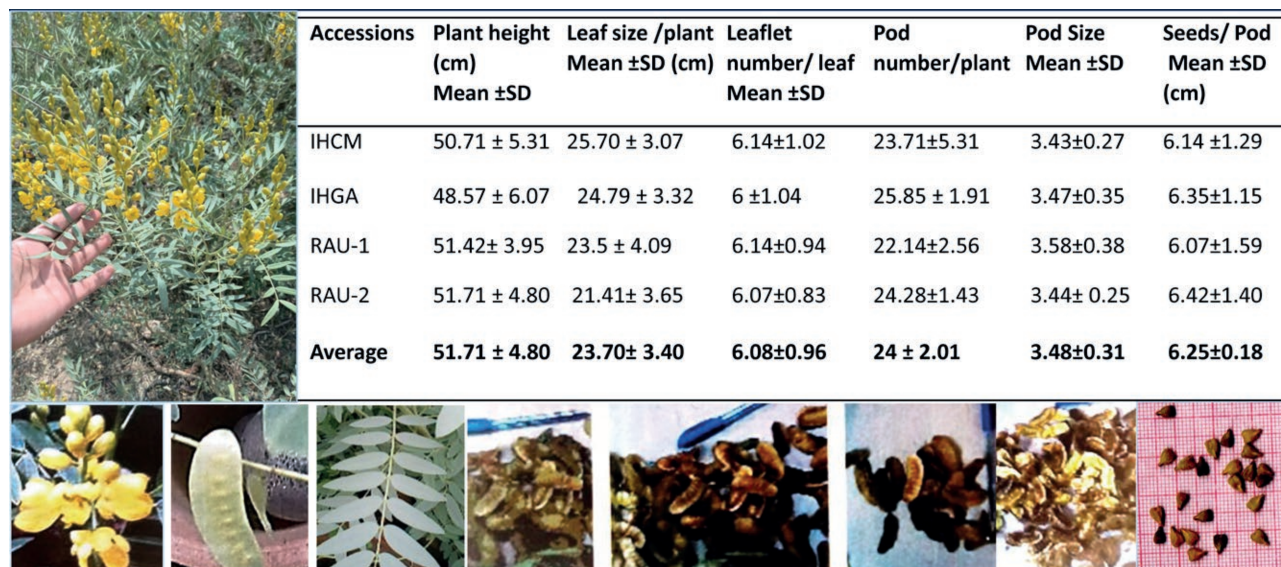
Morphological characteristics

Senna alexandrina is observed as a small shrub, reaching a height of approximately 51 cm. Accession IHGA showed a smaller height compared to others. The stem of the plant is erect, smooth, and pale green. It displays long spread branches bearing 5-8 jugate leaflets, which are usually oval or lanceolate in shape and glabrous (Fig 1). The pod size was ~ 3.5 cm (Fig. 1-table). The RAU 1 accession exhibited a larger pod size, while the IHGA accession had a higher number of pods. Seed counts per pod, however, did not show significant variation. Morphological differences appear to be influenced by environmental factors, which may also contribute to chromosomal and DNA sequence variations.

Karyotypic variations

The karyotypic analysis of different accessions of *Senna alexandrina* revealed notable variations in the number and type of chromosomes. All the studied accessions exhibited a chromosome count of 2n = 28, comprising fourteen pairs of homologous chromosomes arranged in descending order of length within the complements (Fig. 2).

Among the 28 chromosomes in the IHCM accession, eight pairs were metacentric, six pairs were submetacentric. No heteromorphic pairs or nucleolar chromosomes were observed. The resulting karyotypic formula was 16V+12L (Fig. 2). Whereas in the IHGA accession, out of the 28 chromosomes, 9 pairs metacentric, 4 pairs submetacentric, and 1 telocentric were found. No heteromorphic or nucleolar chromosomes were observed in any of the complements. The karyotypic formula obtained was 18V+8L+2I (Fig. 2). In the case of the RAU-1 accession exhibited 9 pairs of metacentric chromosomes, 4 pairs of submetacentric chromosomes, and one pair of telocentric chromosomes among its 28 chromosomes. No heteromorphic or nucleolar chromosomes were observed. The karyotypic formula obtained for this accession was 14V+12L+2I (Fig. 2). In the case of RAU-2, the number of chromosomes analyzed was 28, confirming the same chromosomal complement as the other accession. Among these chromosomes, seven



Accessions	Plant height (cm) Mean ±SD	Leaf size /plant Mean ±SD (cm)	Leaflet number/ leaf Mean ±SD	Pod number/plant	Pod Size Mean ±SD	Seeds/ Pod Mean ±SD (cm)
IHCM	50.71 ± 5.31	25.70 ± 3.07	6.14±1.02	23.71±5.31	3.43±0.27	6.14 ±1.29
IHGA	48.57 ± 6.07	24.79 ± 3.32	6 ±1.04	25.85 ± 1.91	3.47±0.35	6.35±1.15
RAU-1	51.42± 3.95	23.5 ± 4.09	6.14±0.94	22.14±2.56	3.58±0.38	6.07±1.59
RAU-2	51.71 ± 4.80	21.41± 3.65	6.07±0.83	24.28±1.43	3.44± 0.25	6.42±1.40
Average	**51.71 ± 4.80**	**23.70± 3.40**	**6.08±0.96**	**24 ± 2.01**	**3.48±0.31**	**6.25±0.18**

Figure 1. Phenotypical observations in *Senna alexandrina*. The attached table highlights morphological variations across accessions, including measurements of plant height, branch number, and leaflet size.

pairs were metacentric, five pairs were submetacentric, and two pairs were telocentric in nature. No heteromorphic or nucleolar chromosomes were observed in any of the complements. The karyotypic formula obtained was 14V+10L+4I (Fig. 2).

The karyotypic details of the examined accessions of *Senna alexandrina* revealed a combination of metacentric (V), submetacentric (L), and telocentric chromosomes. Across all four accessions examined, in IHGA and RAU-I, the longest pair of chromosomes in the karyotype (designated as the I pair) exhibited submetacentric, and in IHCM and RAU–2 it was observed to be metacentric (Fig. 2).

Male meiosis, associations and chiasma frequency

The accessions of *Senna* from the different locality sites (Table 2 and Fig. 3) showed variations in chromosome patterns at different meiosis stages. RAU-1 site accession showed highest numbers of bivalent (96.67±2.18) and pollen viability in form of stainability % (97.71±0.45) of pollen grain. We observed chromosome associations in the range of 89% to 96%. The accessions RAU1 and RAU2 displayed higher associations compared to IHCM and IHGA. There was the occurrence of quadrivalent formation, which happened in about 3.33% of cases in RAU1 but was absent in RAU2. Additionally, RAU2 showed higher pollen stainability, indicating potentially lower genetic diversity compared to RAU1 and the other two. A one-way ANOVA showed that the differences in pollen viability across the levels of % occurrence of Chiasmata are highly significant ($F(1, 10) = 34.6$, $p < 0.001$). This result provides strong evidence that pollen stainability is influenced by the level of Chiasmata (Fig. 4a). These findings indicate that pollen stainability varies significantly across different levels of Chiasmata per PMC per accession collection sites (Fig. 4a-c). Comparatively, a high mean value of chiasmata frequency has been observed in RAU-2 which 27.03 chiasmata per PMC was recorded. The lowest mean value for chiasmata was recorded as 24.8 in IHGA. The remaining accessions had values ranging between these two. No. of chiasmata generally observed per bivalent was one or two. The maximum association of chromosome was found as 12 ± 2 II + 2 ± 2 I in maximum cells however quadrivalent occurrence was frequent in IHCM and IHGA (Table 2).

Meiosis configuration

S. alexandrina- IHCM

The meiotic configuration of *S. alexandrina* IHCM was characterized by a predominant presence of bivalents in the Pollen Mother Cells (PMCs) while few shows both bivalents and univalents. However, a few cells exhibited quadrivalent associations, suggesting chromosomal pairing anomalies or structural rearrangements. The gametic number reported in all the cells of this accession was n=14. The mean percentage of total bivalents was 91.42±1.42 with the range 12.73±0.11 (12-13

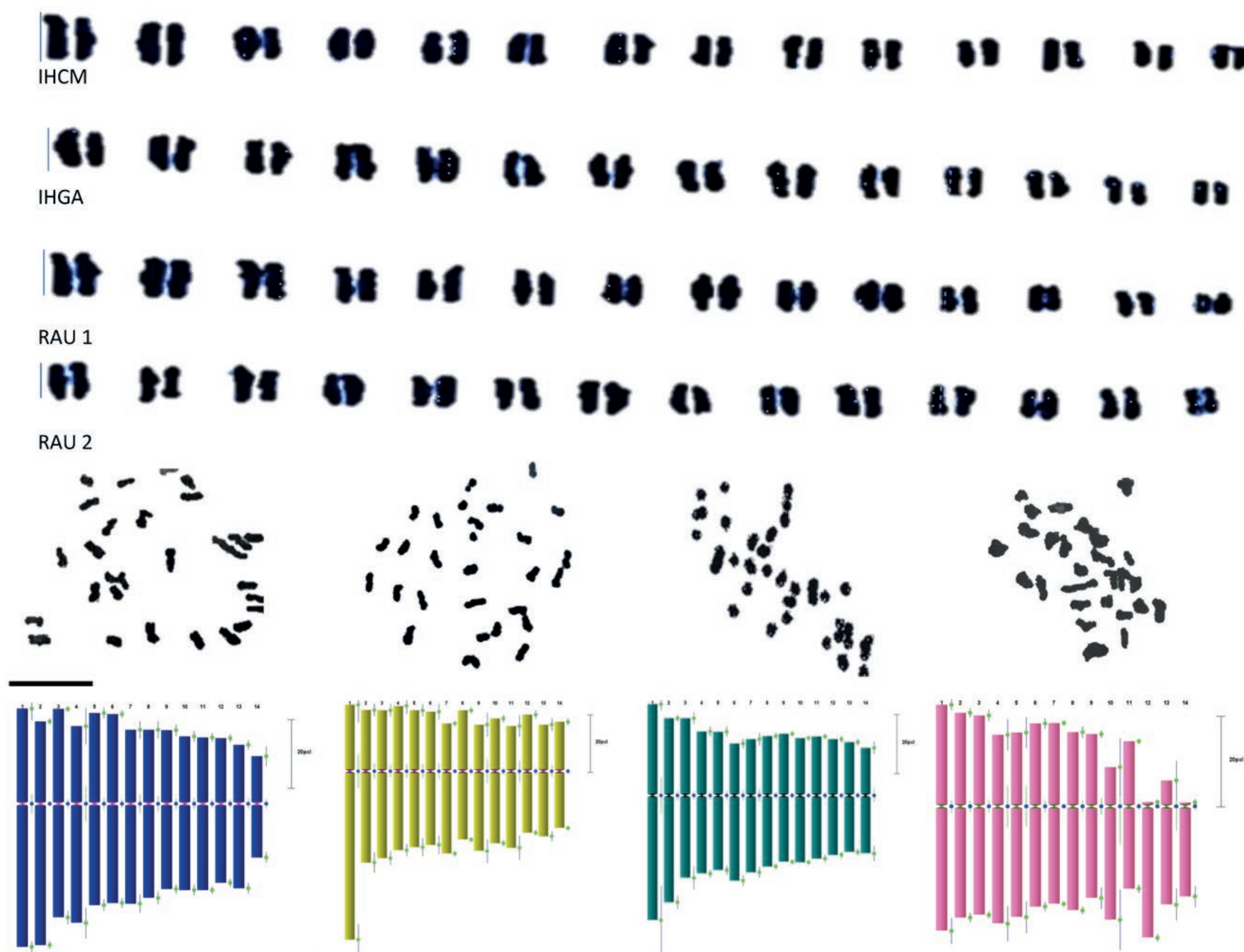


Figure 2. Mitotic karyotype of *Senna alexandrina* from four accession site. Mitotic spread and ideogram show that all four accession have the majority of the metacentric and submetacentric chromosome. RAU-2 had telomeric chromosomes as ideogram showed. Scale bar= 10 μm.

bivalents per cell), out of which 11.93±0.23 were of ring and 0.8 ±0.2 ~ 1 were rod-type bivalents. On average, there were 9.5±2.06 univalents per cell. The approximate chromosomal association per cell recorded was 1 IV+12 II+2 I (Table 2).

S. alexandrina- IHGA

In the IHGA accession, the mean percentage value of total bivalents was ~ 77%, out of which ~69% ring and ~ 25% were rod-type of bivalents, their number ranged between 8-14 in the observed PMCs. Few PMC cells also showed the presence of 1 or 2 quadrivalents (21.43±7.14 % abundance in per cell where quadrivalent present) with 5% occurrence in total observed PMCs. On average there were 13.09±4.12 % univalent per cell. Each cell on average may show chromosome associations 1 IV+11 II+ 2 I out of 14 gametic number. (Table 2).

S. alexandrina- RAU–1

The majority of the PMCs analyzed had shown a high frequency of bivalents along with a few cells showing a mixture of the quadrivalents or multivalent and univalents. The pollen viability was about 92% in this accession. The occurrence of total bivalents was 11-13 out of 14 maximum possible bivalents, out of which 10-13 were ring (12.8±1.58) and 0-3 (1.76±0.35) were rod type. On average 1.26±0.94 univalents (5.66±0.99%) were seen in the cells with 0-4 range. Each cell on average showed chromosome associations with 1 IV+12 II+1 I (Table 2, Fig. 3).

S. alexandrina- RAU–2

In this particular accession site, all of the examined PMCs exhibited almost fourteen bivalents (Fig. 3), with no observed quadrivalent or multivalent associations. The mean total number of bivalents was 13.53±0.31,

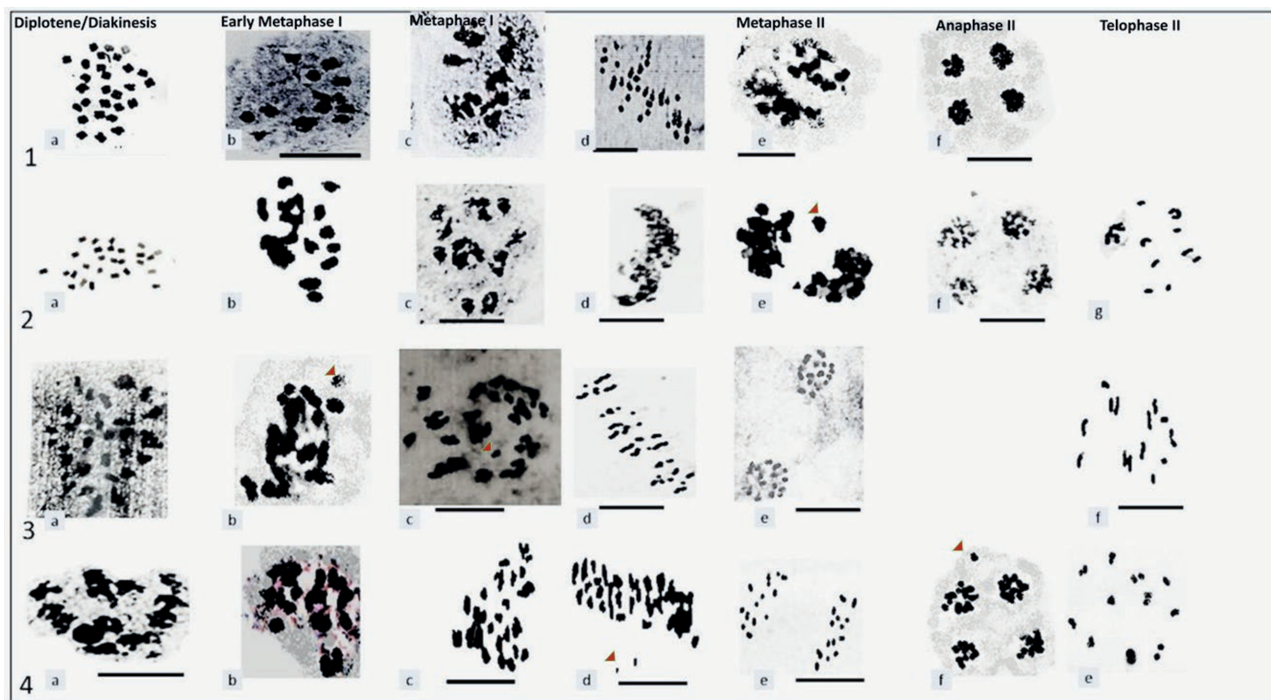


Figure 3. Meiotic chromosome behavior: Different stages of Meiosis in Pollen Mother Cells of *Senna angustifolia*. 1. IHCM, 2. IHGA, 3. RAU-1 and 4. RAU-2. B chromosome can be observed as a separated non-homologous part of DNA (arrow). Some chromosome was attached to nucleolus as a nucleolus organizing chromosomes (diakinesis stage 4a). One PLC at telophase (chromosome no. 14) showed nonseparated chromosome. Scale bar= 10 μm.

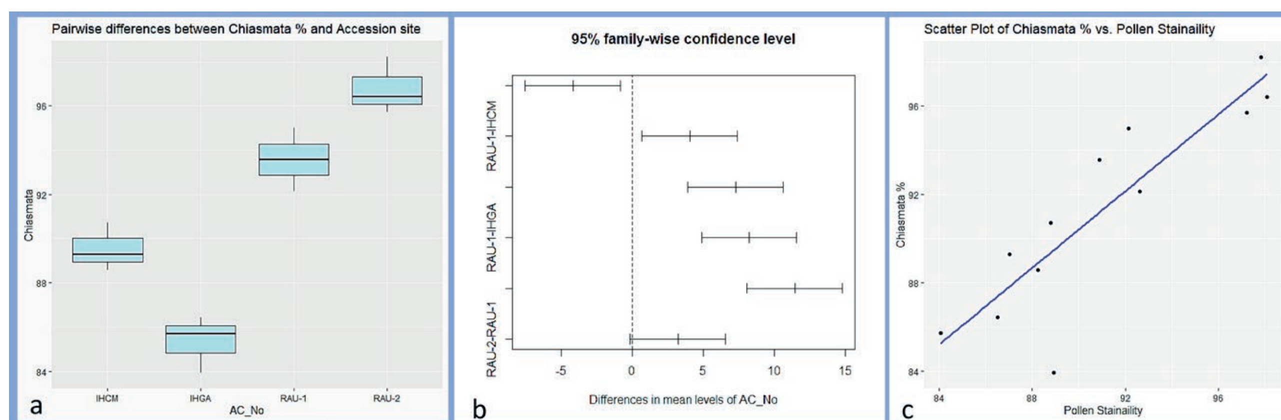


Figure 4. Post hoc analysis of Chiasmata frequency, terminalization and pollen stainability in *Senna alexandrina* with accession sites.: a. Pairwise comparison between chiasmata frequency and accession sites. Variation in chiasmata frequency significantly grouped as per site. b. Confidence level of Tukey HSD among groups. c. Correlation between pollen stainability and chiasmata frequency. Analysis: Software R using ANOVA (aov), regression (lm), plot (ggplot2). P value =0.05.

with a range of 13-14. Out of these bivalents (mean 96.67±2.18%), 13.63±0.32 (97.38 ± 2.29%) were ring type and 1.31±0.23 (11.90±2.97%) were identified as rod type. On average, 3.38±0.75% univalents were present per cell. Each cell demonstrated a chromosome association of 13-14 II+1-2 I (Table 2).

Meiotic association in anaphase I/II and pollen stainability

The no. of chiasmata ranged between 23-28 per PMC with an average of 25.9 out of which about 20 were terminalized with coefficient of 0.78. The percentage of pol-

Table 2. Meiosis chromosome observation in *Senna alexandrina* : Chiasmata %, terminalization co-efficient, chromosomes configurations, and pollen viability.

Ac. No.	Chiasmata Occurrence Mean ± SD %	Chromosome no. with Chiasmata/ cell Mean ± SD	Terminalization Coefficient (Tc) Mean ± SD	Quadrivalent/ PMC Mean ± SD % (Range)	Bivalent / PMC Mean ± SD % (Range)	Univalent /PMC Mean ± SD % (Range)	Pollen viability / flower Mean ± SD %
IHCM	89.52±1.09	25.20±0.26	0.72±0.07	1.90±0.82 (1.33±0.57)	90.95±0.82 (12.73±0.11)	9.52±1.19 (2.66±0.33)	88.01±0.91
IHGA	85.36±1.29	24.47±0.59	0.78±0.06	4.76±1.64 (3.33±1.15)	76.67±7.86 (10.73±1.10)	13.09±4.12 (4.05±0.41)	86.51±2.43
RAU-1	93.57±1.43	25.50±0.85	0.79±0.02	3.38±0.82 (2.67±0.57)	88.22±3.81 (12.35±0.53)	5.66±0.99 (1.59±0.27)	91.89±0.91
RAU-2	96.79±1.29	27.20±0.30	0.82±0.00	0	96.67±2.18 (13.53±0.31)	3.38±0.75 (1.23±0.30)	97.71±0.45

Single and two factor ANOVA and multiple-regression among the groups of IHCM, IHGA, RAU-1 and RAU-2.

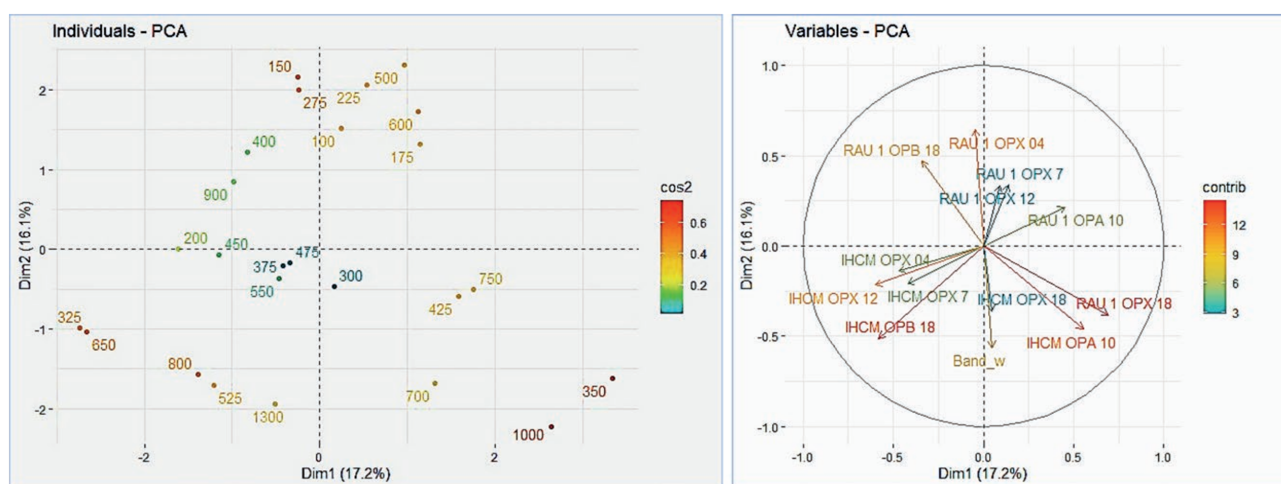


Figure 5. RAPD arbitrary markers-based polymorphism in two accessions of *Senna alexandrina*. -RAU 1 and IHCM. a), shows the clustering of all bands resulted RAPD markers. b), PCA cluster analysis significantly grouped accession and bands as per their variability. Cos2 = quality of representation of the variables of the principal components, contrib= contribution-based clustering.

len stainability was highest (97%) in RAU-2, and it was lowest (85%) in IHGA (Table 2).

Polymorphic bands amplified by RAPD markers

Out of four accession two were selected for RAPD analysis. The analysis of genetic variability in *Senna alexandrina* accessions IHCM and RAU-2 using six arbitrary primers (Table 1) resulted in the amplification of a total of 34 and 33 polymorphic bands, respectively. Polymorphic bands indicate genetic variability between the two accessions. The number of bands varied across different primers in IHCM and RAU-2 (Fig. 5).

Among the primers used, the highest number of polymorphic bands were amplified with primer OPX 12 and OPX 04 in the IHCM accession, and OPX 04 and OPB 18 in the RAU-2 accession. On the other hand, the lowest number of bands were amplified with primer OPA 10 and OPX 07 in IHCM, and OPX 18 and OPX 07 in RAU-2 (Table 2). The highest polymorphism was found for primer OPX-12 for these two accessions. The PCA analysis showed that the arbitrary RAPD primers OPX 04, OPX07, OPX12, and OPB 18 can be used to identify the intra species variations. However, advanced versions of DNA sequencing may give high potential results, but at preliminary level RAPD can provide evidence about genetic changes in a population. Dendrogram (Fig. 6) based on these primers were clustered RAUI for OPB18 and OPX04 against remaining band showing high similarity and less magnitude of the dif-

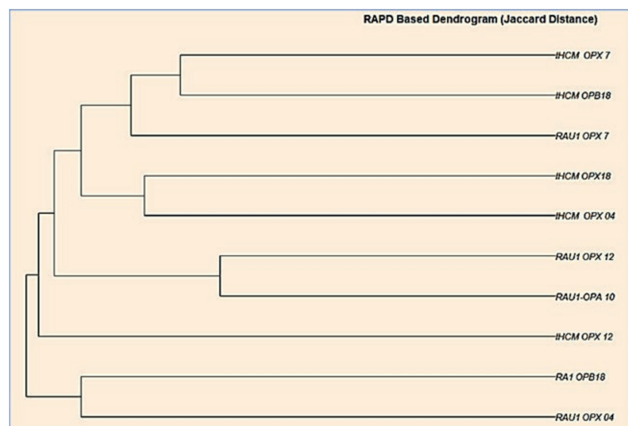


Figure 6. UPGMA Hierarchical clustering of *Senna alexandrina* accessions (RAU 1 and IHCM) based on RAPD band presence/absence patterns (binary data). Jaccard Distance –Jaccard distance metric used to measure similarity (proportion of shared bands). Shorter branch lengths indicate higher similarity in the proportion of shared bands.

ference as per the Jaccard coefficient. The amplified bands might share a high degree of sequence similarity.

DISCUSSION

Cytological evidence strengthens the understanding of the evolutionary origin of a species, it provides chromosome number, length, type, ploidy, and distribution pattern of specific sequences in the whole genome within a population of a species. Such information is useful in identifying the phylogenetic relationship among the related species (He et al. 2022). The present investigation involves a representative collection of four accessions of a sub-population Senna (*Senna alexandrina* Mill.) a natural laxative medicinal plant from different areas. All confirmed the somatic chromosome number as 28, without any indication regarding the existence of polyploidy/aneuploidy or any numerical variation in the natural populations. In our observations except for a few exceptional cases (±1), almost all the cells analyzed had shown the 2n number of chromosomes as 28 which confirms previous observations published on *Cassia angustifolia* Vahl. (Irwin and Turner 1960; Elaine et al. 2005; Cordeiro and Felix 2017; Nguyen et al. 2021). However, a chromosome number=26 for *Senna* plants has also been reported (Kumar et al. 2024a).

Distinctive variations have been observed in the karyotypes of multiple accessions of *Senna alexandrina*. These differences primarily involve the presence of metacentric (V) or submetacentric (L) chromosomes, although three accessions also displayed the presence of telocentric (I) chromosomes. There is a correlation between the level of ploidy and total haploid chromatin length (Doyle & Coate 2019). Across all four accessions examined, in IHGA and RAU-I, the longest pair of chromosomes in the karyotype (designated as the I pair) exhibited submetacentric, whereas in IHCM and RAU–2 it was metacentric. Variations in total haploid chromatin length or karyotype morphology within different diploid or tetraploid taxa may possibly be attributed to chromosomal rearrangements involving the loss or gain of segments, paracentric inversions, and translocations. Such alterations in the karyotype represent significant evolutionary mechanism that plays a role in the diversification and speciation of angiosperms for ecological adaptation (Weiss-Schneeweiss and Schneeweiss 2012; Lavania and Lavania 2021).

In the meiotic study, the occurrence of meiotic abnormalities in a species that is normally fertile and productive indicates the existence of some homoeostatic mechanism related to survival. The increase in the frequency of chiasmata also points out the capacity for the release of variability by the organism (Osman et al. 2021). The Meiotic behavior, reported in *Cassia flexuosa, Cassia vestita, and Cassia desvauxii* at diakinesis and metaphase I showed chromosome disjunction and segregation was over 99% and pollen fertility was over 92% (Biondo et al. 2006). The distal chiasma was predominant over interstitial chiasma, and they were terminalized at early metaphase I. The total chiasma frequency in PMCs of plants of one species is a stable index of recombination potential which is not dependent on the growing conditions (Strelnikova et al. 2019).

Meiotic behavior is generally regular with a predominance of bivalent pairing in diakinesis and metaphase I, and normal chromosome segregation at anaphase I and II. Some irregularities, such as quadrivalents, multivalents, and univalents at diakinesis and metaphase I, and bridges and unequal segregation at anaphases were observed in some accessions of *Senna splendida, S, multijuga, S. corymbosa* and *S. occidentalies* (Elaine et al. 2005). Interestingly, all the accessions of *Senna angustifolia* had a maximum of bivalents, with rare quadrivalent formation. Some earlier findings also confirmed the fact that chromosomal numerical changes in the genus Cassia (Irwin and Turner 1960; Biondo et al. 2006). A high frequency of multiple chromosomes pairing (multivalent) suggested strong similarities between chromosomes, indicating autopolyploidy whereas 'allo' or 'auto' polyploidy depends on degree of difference between parental genome (the level of bivalent paring) (De Storme et al. 2014). Collection site significantly affecting the chiasmata frequency in chromosomes. Which indi-

rectly correlated with environmental effect on genetic composition.

Another interesting observation is that except for one accession (RAU–2), all the remaining three accessions showed a mixture of bivalent, univalent, and quadrivalent associations. The highest percentage of PMCs with fourteen bivalents (98%) was observed in RAU–2. The least number of bivalents per PMC (75%) was observed in IHGA. Similarly, the highest percentage (17%) of univalents was recorded in IHGA followed by 16% in IHCM, 9% in RAU–1, and 4.5% in RAU–2. Such behavior of chromosomes with regard to their associations at metaphase I is reported earlier in a number of plants from arid regions i.e. *Salvadora, Capparis decidua, and Prosopis cineraria* (Rawat et al. 2007). Environmental factors rather than genetic and epigenetic factors have supposedly played a role in partial disruption of synapses among bivalents. The presence of univalents in various PMCs anyhow did not influence the distributional pattern of bivalents at anaphase I in 3 out of the 4 accessions analyzed. However, in one accession, the distribution was affected leading to the occurrence of lagging univalent.

The presence of extra chromosomal bodies may be linked with meiotic abnormalities. The unpaired and the B chromosomes are reported to be the main cause of the abnormal distribution of chromosomes at anaphase I and that of chromatids in anaphase II (Stebbins 1971). Similarly, the presence of B chromosomes was also observed *Salvadora, and Prosopis cineraria* (Rawat et al. 2007).

Random Amplified Polymorphic DNA (RAPD) is a versatile genetic analysis technique that does not require any prior knowledge of the DNA sequence of the target organism and can be used to study population differentiation and phylogenetic relationships (Ahmed et al. 2012). Nowadays RAPD markers are not popular due to the difficulties in reproducibility rate, however, RAPD markers are still used to access clone fidelity in *in vitro* and *ex vitro* grown plants because of low cost and rapid analysis (Kader et al. 2022). The smallest and the largest size presence of bands in a DNA sample compared to others, may be linked to there is alteration in that particular sequence which further may reflect in the time of DNA homologous pairing. However, to draw a more definitive conclusion and determine the nature of the addition or insert or delete, further investigation and sequencing of this specific DNA region would be necessary. As reported recently, the application of SCOT markers on DNA of *Senna alexandrina Mill.* confirmed the morphological variations at a targeted part of genome (Kumar et al. 2024b).

CONCLUSION

The present study reports chromosome karyomorphology in a subpopulation of *Senna alexandrina*. We observed that all the accessions of the plant had a maximum of bivalents, with rare quadrivalent formation. Our analysis revealed significant differences among accession types in chiasma formation. The regression model of the study showed that pollen stainability is influenced by the total formation of bivalents and chiasma frequency in pollen mother cells. These findings shed light on the factors influencing chiasma formation and contribute to our understanding of genetic recombination in plants.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, we used [https://chatgpt.com/sciSpace] in order to make the sentences clearer and for grammatical correctness. After using this tool/service, we reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Application of Genomic *In Situ* Hybridization (GISH) and tandem repeat sequence amplification for identification of *Erianthus – Saccharum* introgression

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Abstract. In our experiment a F1 hybrid (GU (04)-28-EO2) obtained from *Erianthus procerus* (IND 90-776) x *Saccharum officinarum* (PIO 96-435) was crossed with a commercial variety, Co 06027. Resulted BC1 hybrid (GU 12-25) was crossed with a commercial cane Co 12009. From this cross ten BC2 progenies were selected and analysed for introgression of *Erianthus* genome into *Saccharum*. F1 resulted from 2n+n chromosome transmission and was having the whole 40 chromosomes of *E. procerus* in it. The BC1 and BC2 resulted from n+n transmission. The introgression of *E. procerus* chromosomes into BC2 ranged from 8-10. Amplification of *Erianthus* specific tandem repeat (ESTR) sequences was successfully utilized in identification of genuine hybrids of *E. procerus* x *Saccharum*. No recombination events between *Erianthus* X *Saccharum* could be observed in F1, BC1 and BC2 clones. The current study forms a basis for targeted introgression breeding with a different unexploited species of *Erianthus, E. procerus* in sugarcane improvement programme.

Keywords: *Erianthus*, *Saccharum*, introgression, Genomic *in situ* hybridization (GISH), sugarcane, intergeneric hybrid.

INTRODUCTION

Modern sugarcane cultivars (2n=100-130) are developed from interspecific crosses made one century ago among few parent clones of *Saccharum officinarum* L. (2n=80) the sugar producing species and *S. spontaneum* L. (2n=40-128), a wild species. Due to interspecific hybridization involving the frequent utilization of a limited number of parental clones the genetic base has become narrow and the cultivars are showing limited resilience to biotic and abiotic stresses. It has been realized that an efficient method to broaden the genetic diversity for increased productivity and better adaptability as well as for providing more disease resistance the responsible genes of wild relatives have to be transferred through sugarcane breeding.

As one of the most important wild relative of sugarcane, genus *Erianthus* has vital role in contribution of desirable characters to sugarcane culti-

vars. Different species of *Erianthus* have been the focus of several sugarcane breeding programmes as a valuable contributor with many desirable characters like excellent vigour with strong root system, high fiber content, good ratooning ability and tolerance to biotic and abiotic stresses (Ram et al., 2001, Jackson and Hentry, 2011, Fekuhara et al., 2013). In spite of importance of different species of *Erianthus* in sugarcane breeding, the major constraint in generating intergeneric hybrids is the cross-incompatibility due to high genetic distance between *Saccharum* and *Erianthus*. Another constrain is the difficulty in distinguishing genuine intergeneric hybrids and self-progeny. The recent development of efficient molecular tools helped in identification of intergeneric hybrids such as PCR based analysis of 5 Sr DNA, SSRs, AFLPs and genomic slot blot hybridization have greatly enabled the identification of intergeneric hybrids *Saccharum* x *Erianthus* (D'Hont et al., 1995, Cai et al., 2005, Aitken et al., 2006, Besse et al., 1997) from *S. officinarum* (female) and *Erianthus* as male as well as its back crossed variant (D'Hont et al., 1995, Cai et al., 2005, Krishnamurthy et al., 2007, Nair et al., 2006, Piperidis et al., 2000, Piperidis et al., 2010). In order to detect the alien chromosomes and chromosomal segments in a putative hybrid, advanced cytological methods are widely used. Genomic in situ hybridization (GISH is a powerful cytological tool for identifying the introgression status of alien chromosomes in sugarcane (Alix et al., 1998, Jakson and Hentry, 2011).

At ICAR-Sugarcane breeding Institute, Coimbatore, India, *Erianthus* introgression programme has been going on for the last two decades and most of the cases the wild species E. arundinaceus has been used as female parent as a source of potentially valuable traits. In the present study we used a different species of *Erianthus* i. e. *E. procerus* as a female parent to cross with *S. officinarum* (male parent). Cytologically and also at the molecular level we analysed the F1 and back cross progenies of *E. procerus* x *S. officinarum*. In sugarcane during back crosses the problem of shy flowering and non-synchronous flowering makes the breeder to use different sugarcane clones rather than using one of their parents. Such type of crossing methods is recognised as modified back crosses and resulted progenies are referred as back cross progenies. Here we analysed ten BC2 progenies of *E. procerus* x *S. officinarum* through Genomic in situ hybridization (GISH) and also analysed the amplification of *Erianthus* specific tandem repeat sequences to confirm its hybridity. The genuine hybrid identification and molecular cytogenetic characterization will be helpful for the planning of the breeding strategies for further utilization of transferred traits from *E. procerus*.

MATERIALS AND METHODS

The plant materials used for the study are ten BC2 progenies of a F1 hybrid, GU 04(28) EO2. This F1 hybrid was derived from a cross involving *E. procerus* (female parent) and *S. officinarum* (male parent). *E. procerus* clone, IND 90-776, was collected from Arunachal Pradesh and *S. officinarum* clone, PIO 96-435, was an atypical clone derived from interspecific cross undertaken at the place of origin. The F1 hybrid, GU 04 (28) EO2, was back crossed with a commercial sugarcane cultivar, Co 06027, and obtained BC1 progenies. Among this a confirmed BC1 clone, GU12-25, was crossed with another commercial variety, Co 12009. From this cross BC2 progenies were raised and analysed for introgression of *Erianthus* chromosomes.

From the BC2 population ten clones were randomly selected and its hybridity has been confirmed by amplifying the *Erianthus* specific tandem repeat (ESTR) sequences (Yang et al., 2019). A PCR reaction mixture was prepared (Table 1) and amplification was carried out on a Master Cycler (Eppendorf-Nexus gradient) using the primer pairs ESTR-F and ESTR-R (ESTR F: 5'-AGGAAGTTATGGTGGTGGAGTAT-3'; ESTR R: 5'_CGCCATTCCTATTGC-3'). The PCR programme was performed as follows: Pre denaturation at 94°C for 3 min, 34 cycles of 94°C for 1min, 55°C for 35 sec, 72°C foe 30s and 72°C for10 min. PCR products were run in the 1.5% agarose gel electrophoresis

Single budded cuttings of *E. procerus*, *S. officinarum*, F1, BC1, and BC2cloes were collected from the experimental fields of ICAR-Sugarcane breeding institute and planted in the pots. Root tips were collected after 15 days of planting and the somatic chromosome number has been determined according to Sobhakumari and Asmita, 2014.

For GISH analysis of BC2 progenies the mitotic chromosome preparations were performed as described by Sobhakumari et al. (2020). The mitotic slides were freeze dried in liquid nitrogen and dehydrated by dipping in ethanol. These slides were stored in moisture free

Table 1. PCR reaction mixture.

Component	Volume (μl)
Distilled water	15.0
10xPCR buffer	2.0
dNTP mix (10mM)	1.0
ESTR F (primer)	0.5
ESTR R (Primer)	0.5
DNA (50ng/μl)	0.5
Taq enzyme (5U/μl)	0.5

slide boxes in room temperature. For GISH analysis the genomic DNA from *E. procerus* was isolated, fragmented to 500-1000bp size and labelled with biotin-16dUTP (thermo Scientific-USA) and used as GISH probe. The methodology followed for GISH analysis was as described previously by Sobhakumari et al., 2021. The hybridization mixture consists of 50 ng of labeled probe of *E. procerus*, 50% deionized formamide, 10% dextran sulphate, 0.5ng of labeled salmon sperm DNA. After post hybridization washes and FITC incubation the slides were mounted in Vectasheild (Vector labs, UK) mounting medium with DAPI (4,6-diamino 2-phenylindole). GIAH signals were captured using an Axioscope A1 imager fluorescent microscope with Axicam 202 (Carl Zeiss, Gottingen, Germany). Images were processed using Zen 3.0 software (Carl Zeiss, Gottingen, Germany). For each clone 10-15 cells in metaphase were analysed to calculate the number of *E. procerus* chromosomes.

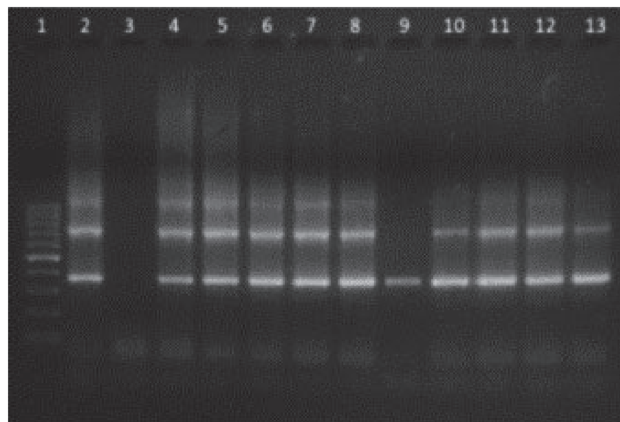

Figure 1. Electrophoretogram of ten BC2 progenies with their parents for amplification of ESTR primer in genomic DNA: 1) 100bp ladder, 2) GU 12-25 (Female parent). 3) Co 12009 (Male parent), 4) GU 19-222 5) GU 19-223 6) GU 19-224 7) GU 19-225 8) GU 19-226 9) GU 19-227 10) GU 19-228 11) GU 19-230 12) GU 19-231 13) GU 19-234.

RESULTS AND DISCISSION

We reported for the first time the chromosome composition of fertile *E. procerus* x *S. officinarum* F1, BC1 (F1 x sugarcane cultivar, Co 06027), and BC2 (BC1 x sugarcane cultivar, Co 775) hybrids *via* genomic *in situ* hybridization (GISH) (Sobhakumari et al., 2020). In this study we utilized a BC1 progeny, GU 12-25 with *20 E. procerus* chromosomes to raise BC2 clones. From the BC2 population obtained from GU 12-25 x Co 12009, a set of clones were randomly selected for the classical and molecular cytogenetic analysis. Identifying the genuine hybrids in the intergeneric hybrid population of *Erianthus* x *Saccharum* is difficult due to the high selfing rate (Besse et al., 1997). As a preliminary evaluation of these BC2 clones for confirmation of its hybridity, PCR amplification was done using *Erianthus* specific 5S rDNA sequences. The results showed that 5S rDNA sequence amplification was not obtained in some of the genuine hybrids (data not shown). As 5S rDNA has one locus per set of basic chromosomes, it is present only in few chromosomes in the *Erianthus* genome. Due to unequal segregation and elimination of *Erianthus* chromosomes at different stages, the advanced back cross progenies may not inherit the chromosome that carry the 5S rDNA loci, this may be the reason for not getting amplification of 5S rDNA sequences in BC2 progenies. This showed that *Erianthus* specific 5S rDNA sequences may not be reliable for the identification of genuine hybrid progenies. Hence the *Erianthus* specific tandem repeat sequences (ESTR) reported by Yang et al. (2019) was used as a marker to confirm the hybridity of randomly selected BC2 progenies. This marker was reported earlier as an *E. arundinaceus* specific marker and it was showing hybridization sites in the sub telomeric regions at one or both ends of 60 chromosomes of *E. arundinaceus* during FISH experiment (Yang et al., 2019). For the first time this marker is used as a hybrid identification tool in the progenies of *E. procerus* x *Saccharum*. In *E. procerus* parent as well as in its true progenies it has amplified successfully around 380bp (Fig. 1). The amplification was not obtained in the sugarcane varieties. The intergeneric population generated from *E. arundinaceus* x *S. officinarum* was initially confirmed with isozyme markers (Deng et al., 2002). However, they could not identify the two hybrids because of absence of banding patterns in different parents. Following this work many reports have come to confirm genuine hybrids with SSR markers, 5S rDNA sequences and internal transcribed spacer (ITS) sequences etc. (Cai et al., 2005a, Cai yet al., 2005b, Zheng et al., 2004). In 2019 Yang et al. reported the AGPR 52/53 sequences for identification of hybrids from *Saccharum* spp. and *E. arundinaceus* and the same sequences worked well in our experiments to identify *E. procerus* x *S. officinarum* backcross hybrids with modifications in the PCR reaction mixture and programme.

In recent years many cytogenetic research has been carried out on chromosome transmission of different generations of intergeneric hybrids involving *Saccharum* and *E. arundinaceus* (Wu et al., 2014, Huang et al., 2015, Piperidis et al., 2000, 2010, Yang et al., 2019). From these reports it was found that elimination of chromosomes appeared to be a common and non-random event during

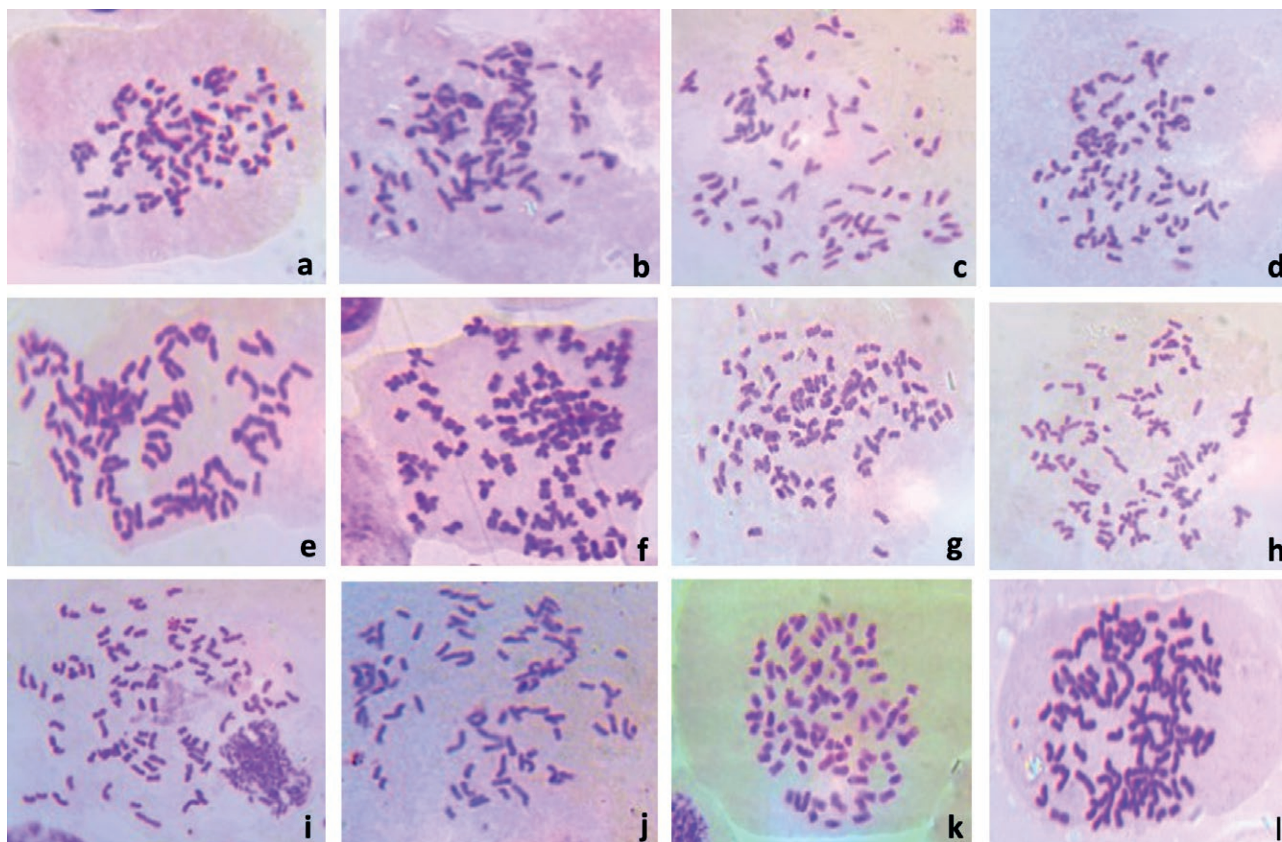

Figure 2. Somatic chromosome number of different clones in BC2 generation and its parental clones: a) GU 19-222 (2n=102), b) GU 19-223 (2n=100), c) GU 19-224 (2n=92), d) GU 19-225 (2n= 100), e) GU 19-226 (2n=92), f) GU 19-227 (2n=92), g) GU 19-228 (2n=100), h) GU 19-230 (2n=96), i) GU 19-231 (2n=96), j) GU 19-234 (2n=88), k) GU 12-25 (2n=92) (Female parent l) Co 12009 (2n=104) (Male parent).

intergeneric hybridization in *Saccharum*. We analysed by genomic in situ hybridization (GISH) the chromosome composition in three generations of *E. procerus* x *Saccharum* intergeneric hybrids: F1 (*E. procerus* x *Saccharum*), BC1 (F1 x sugarcane cultivar) and BC2 (BC1 x sugarcane cultivar). Classical cytological studies in ten BC2 clones showed that the 2n number ranged from 88-102 (Fig. 2). In our earlier *in situ* hybridization studies, it was reported that the F1, GU (04)28 EO2, was showing 2n+n chromosome segregation with 40 chromosomes of *E. procerus* (Sobhakumari et. al., 2020). BC1, GU 12-25, was contained 20 *Erianthus* chromosomes with n+n transmission. When GU-12-25 crossed with a commercial variety, Co 12009, we could raise BC2 population from which 10 clones were randomly selected for GISH analysis with *E. procerus* biotin labelled probe. We found that out of 10 BC2 clones studied two clones were with 8 *E. procerus* chromosomes and only one clone with 9 *E. procerus* chromosomes whereas the majority of them were with 10 *E. procerus* chromosomes (7 clones) (Fig. 3). These results revealed that the number of *E. procerus* chromosomes in transmission of BC1 to BC2 progenies were approximately reduced by half, but we also observed the transmission where reduction was less than half. The details of the parentage, somatic chromosome number and *E. procerus* introgression pattern in F1, BC1 and BC2 generations of *E. procerus* x *S. officinarum* are given in Fig. 4.

In earlier reports GISH analysis allows the visualization of recombination between the species of *Saccharum* i.e., *S. officinarum* and *S. spontaneum* (D'Hont et al., 1996). Different frequencies or abilities of different chromosomes to form homologous recombination among *S. spontaneum* and *S. officinarum* was reported by Wang et al., in 2021. These species are closely related and they showed interspecific recombination. In spite of that in our study the GISH result from F1, BC1 and BC2 clones did not reveal chromosome exchange between *Erianthus* and *Saccharum* chromosomes. The absence of recombination may be due to the genetic distance between *Erianthus* x *Saccharum* which did not allow the gene transfer by chromosome pairing and chiasma formation

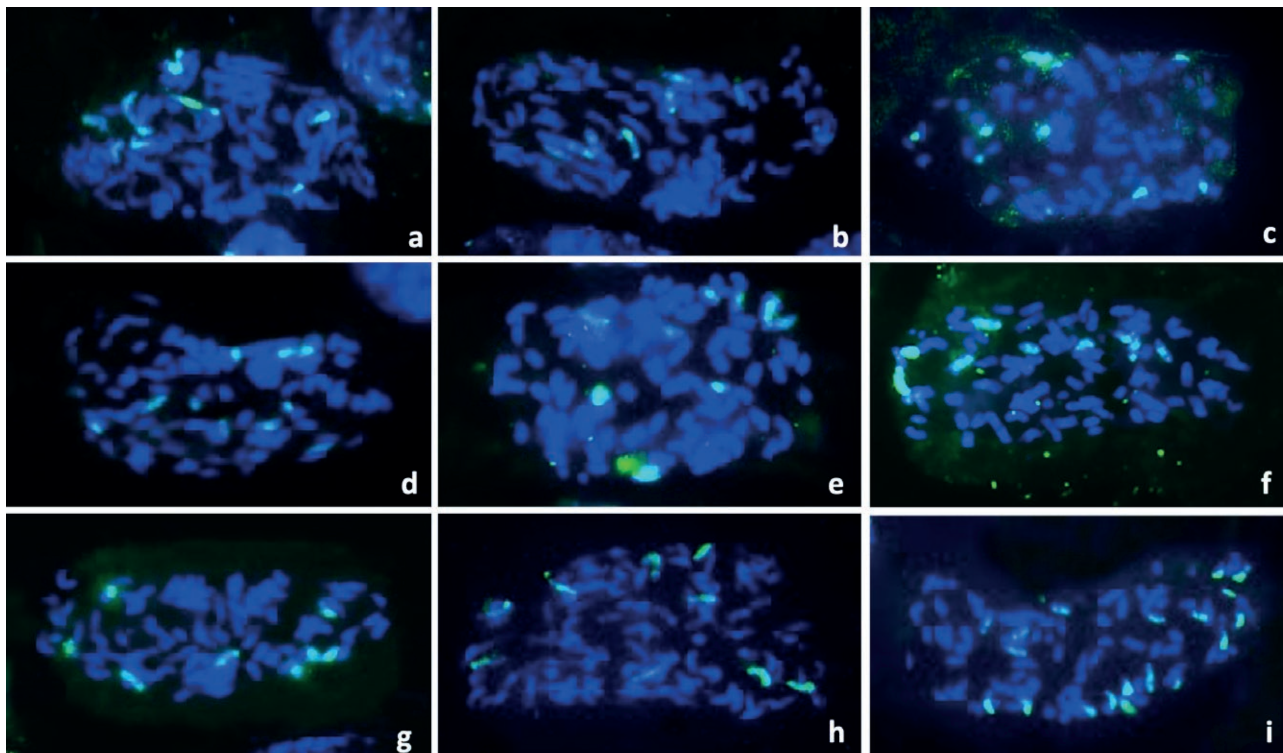

Figure 3. GISH analysis of BC2 (a-h) clones and its female parent (i): a) GU 19-222, b) GU 19-223, c) GU 19-224, d) GU 19-225, e) GU 19-226, f) GU 19-227, g) GU 19-228, i) GU 19-231, j) GU 12-25.

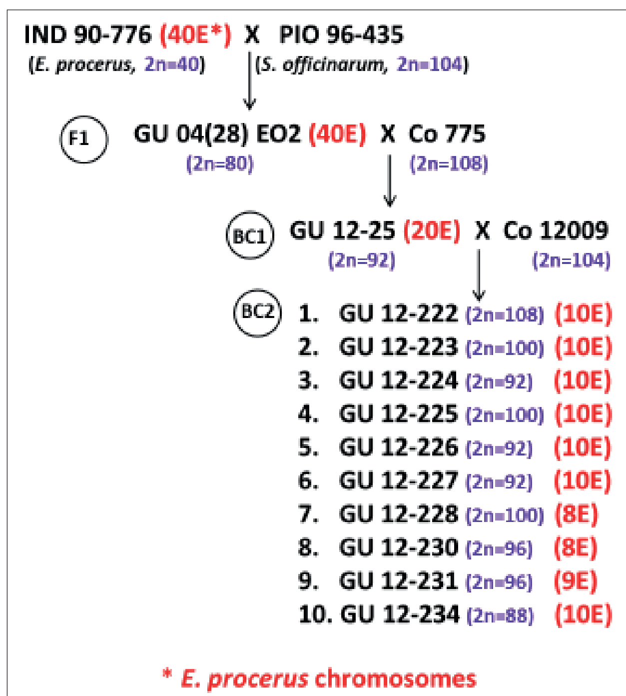

Figure 4. The parentage, somatic chromosome number and *E. procerus* introgression pattern in F1, BC1 and BC2 generations of *E. procerus* x *S. officinarum.*

(Piperidis et al., 2000, 2010). In wheat the role of 'pairing homeologous 1' (ph1) gene has been described in preventing the pairing between related genera (Hauhar and Chibbar, 1999). In sugarcane no such genes have been reported so far.

Chromosome transmission in introgressed population without inter chromosomal exchange/ recombination revealed that *E. procerus* genome has been introgressed into sugarcane cultivars only by whole chromosomes. Such chromosomes in the advanced back cross generations are potential source for gene sequencing and SNP marker production after sorting out them separately. Further back crossing with commercial clones improves the cane traits and juice quality in *Erianthus* x *Saccharum* hybrids along with the biotic and abiotic stress tolerance.

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Mapping of five classes of repetitive DNAs and microsatellite repeats in the genome of the Rainbow Shark, *Epalzeorhynchos frenatum* (Fowler, 1934)

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Abstract. The karyotype and chromosomal characteristics of *Epalzeorhynchos frenatum* (Fowler, 1934) were examined using different staining techniques (Conventional banding, C-banding and Ag-NORs banding) and the use of fluorescent *in situ* hybridization (FISH) to detect 18S, 5S rDNA sites and microsatellite sequences ($(CA)_{15}$, $(GA)_{15}$ and $(CGG)_{10}$ as markers). The result revealed karyotypes with 2n = 50 chromosomes (NF=86), consisting of 12 metacentric, 12 submetacentric, 12 acrocentric, and 14 telocentric chromosomes; there were no heteromorphic sex chromosomes. The NORs site was a noticeable proximal heterochromatic block on the short arm of pair No. 13, and C-positive heterochromatin was detected in the centromeric sections of the chromosomes as well as the telomeric regions of other pairs. On the short arm of pairs No. 13 and 15, the telomeric regions contained the 18S and 5S rDNA sites, respectively. Several chromosomes bearing these repetitive DNA sequences were shared, alongside with some exclusive chromosomal markers especially CGG rich segment (No. 13). This means that, as verified by two methods, it involves a syntenic condition for the 18S rDNA, NORs, and $(CGG)_{10}$ microsatellite probes.

Keywords: *Epalzeorhynchos frenatum*, Rainbow Shark, Chromosome, Repetitive sequences, Fish cytogenetics.

INTRODUCTION

Freshwater fish of the family Cyprinidae (Cypriniformes) are found in Thailand's main hydrographic basins and are distributed widely throughout Southeast Asia. One of this family's biggest subgroups is the subfamily Cyprininae that belong to the major large tribes such Labeonini, Poropuntiini, and Smiliogastrini (Phimphan et al., 2020). In Thailand, freshwater fish from over 200 different species are utilized as ornaments. The family Cyprinidae is responsible for over half of Thailand's ornamental fish population. The most well-liked species include *Epalzeorhynchos frenatum* (Fowler, 1934), that

one of the several fish species that are important to Thailand's economy as ornamentals (Sermwatanakul, 2005).

Several cytogenetically examined of *E. frenatum*, exhibit conservative karyotypic diversification and are readily distinguished in terms of size, shape, and chromosomal number, indicating the existence of separate species (Bertollo et al., 1986, 2000). Numerous fish species have been subjected to molecular cytogenetic analysis employing repetitive DNAs, such as rDNA repeats, satellite DNAs, telomeric sequences, and other classes of microsatellite sequences. These investigations have shown how much potential there is to learn more about karyotype differentiation through the study of repetitive DNAs. These genomic elements have the ability to alter the molecular makeup of chromosomes and slow down the rate of recombination between them, two essential processes in chromosome differentiation.

This study presents a chromosomal characteristic analysis of *E. frenatum*, using different staining techniques (Conventional banding, C-banding, and Ag-NORs banding) and fluorescent *in situ* hybridization (FISH) with repetitive DNA probes (18S rDNA and 5S rDNA probs) and microsatellite sequences ($(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ as markers). The distribution of repeated DNA sequences within the chromosomes serves as a key indicator of the comprehensive karyotype characterization that this approach provided.

MATERIAL AND METHODS

Specimens collected and conventional methods

Ten male and ten female of Rainbow Sharks (*Epalzeorhynchos frenatum* Fowler, 1934) from the Mae Klong River in the Ratchaburi area of Thailand were subjected to cytogenetic analysis (Figure 1). A hand net was used to collect the specimens, which were then brought to the study station in sealed plastic bags with oxygen and clean water inside. In order to reduce animal suffering, the trials adhered to ethical guidelines and used clove oil as anesthetic before slaughtering the animals. The process was approved by the Ethics Committee of Muban Chombueng Rajabhat University and by the RGJ Committee under no. U1-04484-2559. Mitotic chromosomes were extracted from anterior kidney cell suspensions by standard air-drying techniques. Additionally, the distribution of C-positive heterochromatin was found using the C-banding approach, and the Ag-NOR position on chromosomes was found using silver staining. The specimens were deposited in the fish collection of the Cytogenetic Laboratory, Department of Biology, Faculty of Science and Tecnology, Muban Chombueng Rajabhat University.

Figure 1. General characteristic of *Epalzeorhynchos frenatum* (Fowler, 1934).

Chromosome probes and FISH experiments

Two tandemly arrayed DNA sequences isolated from the genome of an Erythrinidae fish species, *Hoplias malabaricus*, were used as probes. The first probe contained a 5S rDNA repeat and included 120 base pairs (bp) of the 5S rRNA transcribed gene and 200 bp of the non-transcribed spacer (NTS) sequence. The second probe contained a 1400 bp segment of the 18S rRNA gene obtained via PCR from the nuclear DNA. The 5S and 18S rDNA probes were cloned into plasmid vectors and propagated in DH5a *Escherichia coli* competent cells (Invitrogen, San Diego, CA, USA). The 5S and 18S rDNA probes were labeled with Spectrum Green-dUTP and Spectrum Orange-dUTP, respectively, using nick translation according to the manufacturer's recommendations (Roche, Mannheim, Germany).

The microsatellites $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ were synthesized. These sequences were directly labeled with Cy3 at the 5' terminus during synthesis by Sigma (St. Louis, MO, USA).

Fluorescence *in situ* hybridization (FISH) was performed under high stringency conditions (Yano, et al. 2017). Metaphase chromosome slides were incubated with RNAse (40 μg/ml) for 1.5 h at 37°C. After the denaturation of the chromosomal DNA in 70% formamide/2x SSC at 70°C for 4 min, 20 μl of the hybridization mixture (2.5 ng/μl probes, 2 μg/μl salmon sperm DNA, 50% deionized formamide, 10% dextran sulphate) was dropped on the slides, and the hybridization was performed overnight at 37°C in a moist chamber containing 2x SSC. The first post-hybridization wash was performed with 2x SSC for 5 min at 65°C, and a final wash was performed at room temperature in 1x SSC for 5 min. Finally, the slides were counter-

stained with DAPI and mounted in an antifade solution (Vectashield from Vector Laboratories).

Image processing

Approximately 20 metaphase spreads were analyzed to confirm the diploid chromosome number, karyotype structure and FISH results. Images were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) with CoolSNAP and the Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified according to their arm ratios as metacentric (m), submetacentric (sm), acrocentric (a) or telocentric (t).

RESULTS

The Rainbow Shark (*Epalzeorhynchos frenatum*) has 2n=50. All four types of chromosomes (metacentric, submetacentric, acrocentric, and telocentric chromosome) make up its karyotype structure, which has a fundamental number (NF) of 86. The C-positive heterochromatic bands were observed in the centromeric and telomeric regions of many chromosomes. The Ag-NOR sites are located in the telomeric region of the biggest acrocentric pair's short arms (pair 13), the exclusive location of major ribosomal sites in these regions was confirmed by *in situ* hybridization with 18S rDNA probes. The 18S rDNA sites are found in the telomeric position of the short arms of pair 13, but the 5S rDNA genes are only found in the telomeric area of acrocentric pair 15 (Figure 2).

The chromosomal mapping of all microsatellite sequences indicates a different dispersed distribution. It was observed that the $(CA)_{15}$ microsatellite was primarily concentrated in the telomeric regions of each chromosome. Although the sequence $(GA)_{15}$ was broadly distributed on the chromosomes, it displayed weaker signals than the sequence $(CA)_{15}$. It also shows a dispersed distribution without preferential accumulations on the centromeric and telomeric regions in any of the chromosomal pairs. While the sequence $(CGG)_{10}$ showed hybridization signals on just one of the short arms of the largest acrocentric pair (pair 13), it looked to be weakly accumulated in several chromosomes (Figure 3), no differential hybridization patterns were detected between males and females.

The idiogram of *Epalzeorhynchos frenatum* represents gradually declining length of the chromosomes (Figure 4). The karyotype is asymetrical karyotype with metacentric, submetacentric, acrocentric and telocentric chromosomes. The karyotype formula of *Epalzeorhyn-*

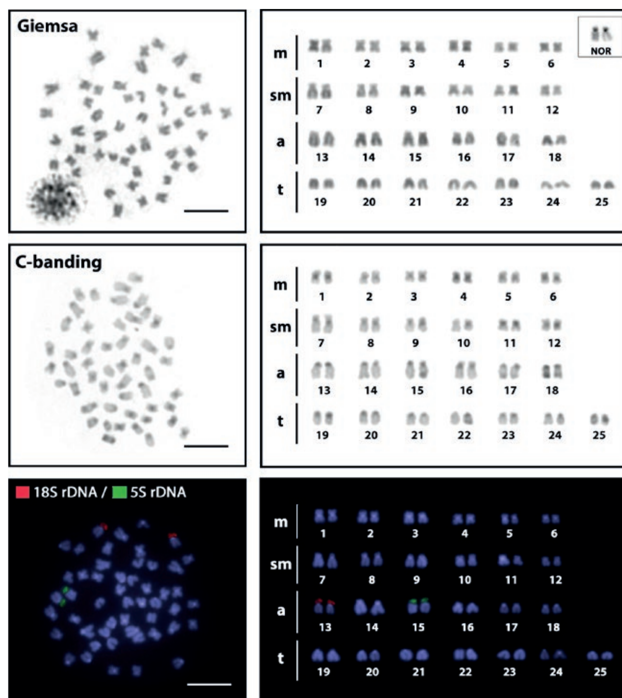

Figure 2. Mataphase and karyotypes of *Epalzeorhynchos frenatum* arranged from conventionally Giemsa-stained, Ag-stained (highlighted in the boxes), C-banded and after fluorescence *in situ* hybridization with an 18S rDNA probe. Bar 5 μm.

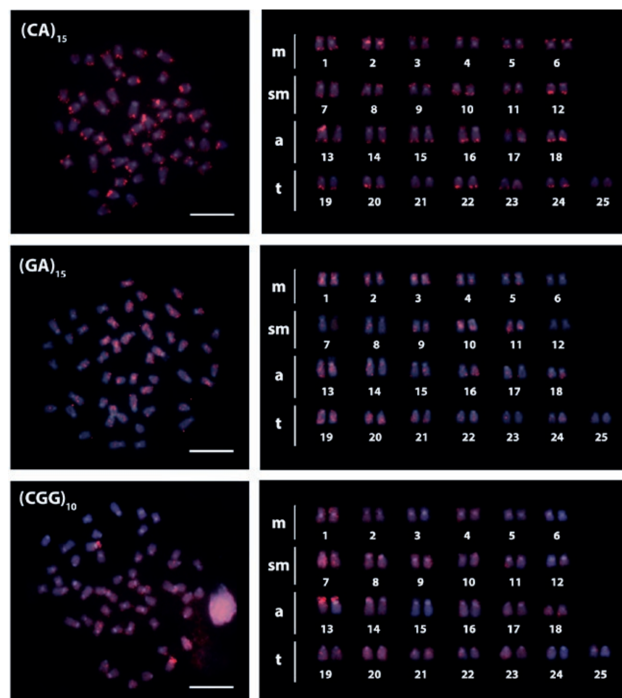

Figure 3. Chromosomal mapping of di- and tri-nucleotide microsatellites in the chromosomes of *Epalzeorhynchos frenatum* by fluorescence *in situ* hybridization. The general distribution pattern of $(CA)_{15}$, $(GA)_{15}$ and $(CGG)_{10}$ microsatellites as probe. Bar = 5 μm.

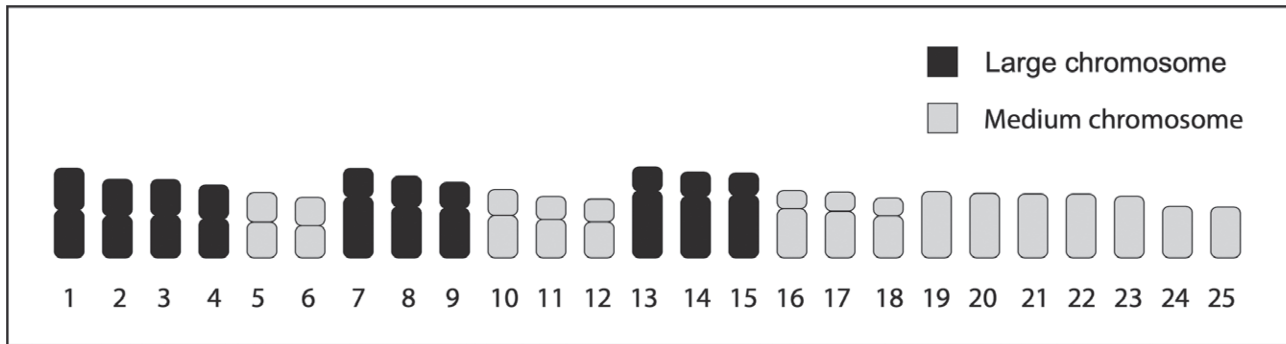

Figure 4. Standardized idiogram showing lengths and shapes of chromosomes of *Epalzeorhynchos frenatum* (2*n*=50) by conventional staining techniques.

chos frenatum is as follow: 2n (50) = L^{m}_{8}+L^{sm}_{6}+ L^{a}_{6}+ M^{m}_{4}+M^{sm}_{6}+ M^{a}_{6}+ M^{t}_{14}

DISCUSSIONS

Karyotype uniformity among Epalzeorhynchos *species*

Epalzeorhynchos frenatum from Mae Klong River, Ratchaburi province, Thailand, was subjected to cytogenetic analysis. All specimens of *E. frenatum* have 50 chromosomes, and their karyotypes are asymmetric (Figure 2, 3 and Table 2). On the other hand, a few species of the genus *Epalzeorhynchos* have been the subject of extensive cytogenetic study (Table 1). The karyotype of *E. frenatum*, *E. bicolor*, and *E. munensis* is 2n=50, which is in line with other species' findings. However, there is a difference in *E. frenatum* (2n = 48), as described by Magtoon and Donsakul (1993). It implies that their karyotypes continue to be conserved even after speciation. However, each species' greater adaptive divergence and substantial chromosomal rearrangement variability cause them to display an asymmetrical karyotype.

In addition, karyotypes with essentially comparable structural patterns were seen in *Epalzeorhynchos* species; majority of these showed the formation of asymmetric karyotypes and 2n = 50. All of the *Epalzeorhynchos* species that have been examined to date (Magtoon and Donsakul, 1993; Donsakul et al., 2012; Phimphan et al., 2020; current study) share these traits. Furthermore, these fishes have somewhat different karyotypes due to changes in NFs. These discrepancies might stem from population-level species-specific changes or from incorrectly identifying one species as another because of species complexity.

Additionally, similar to every other species in the genus *Epalzeorhynchos*, it was not possible to observe the physically distinct sex chromosome (Magtoon and Donsakul, 1993; Donsakul et al., 2012; Phimphan et al., 2020). Numerous species in this family exhibit the same phenomena (Arai, 2011).

Chromosome markers of E. frenatum

This is the first report on the distribution of C-positive heterochromatins at the centromeric and telomeric sites of the majority of the *E. frenatum* chromosomes (Figure 2). This distribution pattern resembles those of other fish that have had their C-banding documented (Cioffi, et al., 2009).

Table 1. Reviews of cytogenetic reports in the genus *Epalzeorhynchos*. (2n = diploid number, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric and NORs = nucleolar organizer regions, NF = fundamental number, – = not available).

Species	2n	NF	Formula	NORs	Reference
E. bicolor	50	74	20m+4sm+2st+24a	-	Magtoon and Donsakul (1993)
E. munensis	50	84	22m+12sm+2st+14a	-	Donsakul et al. (2012)
E. frenatum	48	72	14m+10sm+8st+16a	-	Magtoon and Donsakul (1993)
	50	78	18m+10sm+10st+12a	2	Phimphan et al. (2020)
	50	86	12m+12sm+12a+14t	2	Present study

Table 2. Mean length of short arm chromosome (Ls), length long arm chromosome (Ll), length total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphase cells of the male and female the Picasso triggerfish (*Epalzeorhynchos frenatum*), 2*n*=50.

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome type
1	0.672	0.769	1.441	0.051±0.005	0.528±0.051	metacentric
2	0.587	0.677	1.264	0.045±0.002	0.531±0.049	metacentric
3	0.577	0.683	1.260	0.044±0.003	0.543±0.034	metacentric
4	0.545	0.627	1.172	0.041±0.004	0.534±0.049	metacentric
5	0.472	0.577	1.049	0.037±0.005	0.546±0.057	metacentric
6	0.453	0.519	0.972	0.034±0.002	0.529±0.081	metacentric
7	0.460	0.978	1.438	0.051±0.004	0.680±0.047	submetacentric
8	0.492	0.822	1.314	0.046±0.004	0.633±0.056	submetacentric
9	0.415	0.799	1.214	0.044±0.003	0.648±0.040	submetacentric
10	0.420	0.681	1.102	0.039±0.002	0.614±0.028	submetacentric
11	0.370	0.618	0.988	0.035±0.003	0.621±0.038	submetacentric
12	0.368	0.579	0.947	0.033±0.003	0.612±0.043	submetacentric
13*	0.388	1.076	1.464	0.052±0.003	0.734±0.045	acrocentric
14	0.397	0.986	1.383	0.048±0.004	0.718±0.044	acrocentric
15	0.359	1.007	1.366	0.049±0.001	0.731±0.039	acrocentric
16	0.294	0.790	1.084	0.039±0.003	0.719±0.058	acrocentric
17	0.309	0.749	1.057	0.037±0.003	0.709±0.026	acrocentric
18	0.287	0.676	0.964	0.034±0.002	0.702±0.005	acrocentric
19	0.000	1.066	1.066	0.038±0.004	1.000±0.000	telocentric
20	0.000	1.040	1.040	0.037±0.002	1.000±0.000	telocentric
21	0.000	1.029	1.029	0.036±0.003	1.000±0.000	telocentric
22	0.000	1.027	1.027	0.036±0.002	1.000±0.000	telocentric
23	0.000	0.992	0.992	0.035±0.002	1.000±0.000	telocentric
24	0.000	0.831	0.831	0.029±0.002	1.000±0.000	telocentric
25	0.000	0.819	0.819	0.029±0.001	1.000±0.000	telocentric

Remark: * NOR-bearing chromosome.

Ag-NOR/18S rDNA sites are the sole pair of relevant chromosomal markers that all *E. frenatum* share. NORs are chromosomal landmarks made up of tandemly repeated ribosomal gene sequences (rRNA). Three genes that code for 18S, 5.8S, and 28S ribosomal RNA make up each unit in eukaryotes (Sharma et al., 2002). Since these chromosomal features are frequently species-specific, the number and location of rDNA clusters have been extensively exploited in systematics and phylogenetic reconstructions (Britton-Davidian et al., 2012). The outcome here is similar to that of earlier research on the chromosome-bearing nucleolar organizer area (Phimphan et al., 2020). This trait is shared by a variety of fish species and other vertebrates (Supiwong et al. 2012, 2013).

Additionally, there were discernible nucleolar organizer areas in the telomeric region of the short arms of *E. frenatum*'s biggest acrocentric chromosome pair 13. This aligns well with the Phimphan et al. (2020) research on the same species' karyotype. According to their research, there is a noticeable secondary constriction in the areas next to the telomere of chromosome pair 10 (submetacentric), which corresponds to the regions known as nucleolar organizer regions and is distinguished by Ag-NOR sites.

The majority of fishes typically only have one pair of NORs on their chromosomes; only a small number of fishes have more than two pairs, such as two pairs (*Cyclocheilos enoplos* (Bleeker, 1849): Magtoon and Arai, 1993), three pairs (*Cyclocheilichthys apogon* (Valenciennes, 1842): Chantapan, 2015) and four pairs (*Puntius denisonii* (Day, 1865), *P. semifasciolatus* (Günther, 1868): Nagpure et al., 2004; *P. filamentosus* (Valenciennes, 1844): Nagpure et al., 2003), which may be caused by translocation between certain regions of the chromosomes that have NOR and another chromosome (Sharma et al., 2002). The current analysis demonstrates that the studied species has a NOR site on a single pair of chromosomes. In fish, this is seen as a straightforward isomorphic requirement (Almeida-Toledo, 1985).

Organization of repetitive DNAs in the chromosomes of E. frenatum

This is the first report of the presence of repetitive DNAs on *E. frenatum*, the 5S rDNA sites are found in the telomeric area on the short arm of pair 15, the 18S rDNA sites are evenly distributed on the telomeric location on the short arm of pair 13. These genes frequently display a non-syntenic arrangement, which may indicate a plesiomorphic state in fish.

The identifiable organizational patterns seen in the heterochromatin of *E. frenatum*'s microsatellite sequences. On all chromosomes, the $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ microsatellites have a weak and diffuse distribution; yet, in certain regions of the chromosomes, they also exhibit a few prominent clusters with strong signals (Figure 3). It may appear from earlier and ongoing research that microsatellites make up every heterochromatin in fish genomes (Cioffi and Bertollo, 2012). Nevertheless, microsatellites have also been discovered in noncentromeric areas; a large number of these were found in or close to genes (Rao et al., 2010). This is consistent with the microsatellite $(CGG)_{10}$ pattern found in this investigation. As a result, this information is helpful for analyzing the phylogenetic proximity of this genus, which may have similar microsatellite sequence distribution patterns, indicating separate evolutionary routes that result in homoplastic chromosomal features. However, because to the rapid changes in these sequences, there could be a noticeable evolutionary divergence in their distribution (Cioffi et al., 2011; Molina et al., 2014a; 2014b). Actually, the way repeating DNAs are arranged in different species is demonstrated by the structure of microsatellite sequences.

Chromosome evolution of the genus Epalzeorhynchos

The primary factor causing karyotype diversification in several Cypriniformes species is chromosomal rearrangements. With diploid chromosome numbers ranging from 2n=48 to 50 in the tribes Labeonini and Smiliogastrini, while the tribe Poropuntiini is more conserved at 2n = 50 (Arai, 2011), the various Cyprinidae species underwent an extremely diversified karyotype evolution when considering the numerical and structural aspects of their complements. There were also notable differences in the NF, possibly as a result of the occurrence of pericentric inversions (Getlekha et al., 2018). The results of the analyses demonstrate the combined significance of the various chromosome rearrangements such as centric fission fusion and particularly pericentric inversions in the evolutionary modeling of their karyotypes (Getlekha et al., 2016a; 2016b).

The majority of members of the genus *Epalzeorhynchos* have asymmetrical karyotypes with metacentric submetacentric acrocentric and telocentric chromosomes, and nearly all of them have 2n = 50. In the current investigation, same karyotypic pattern was also seen in *E. frenatum* (2n=50). Pericentric inversions, which appear to be frequent in other freshwater fish species, particularly in the family Cyprinidae, are thought to be the source of the diploid chromosomal numbers in these species (Arai & Nagaiwa, 1976; Marques et al., 2016).

CONCLUSION

This research can verify diploid chromosome, fundamental number, and distribution patterns of microsatellites on the chromosomes based on the chromosome study of the Rainbow Shark (*Epalzeorhynchos frenatum*) using conventional analyses (Giemsa staining, Ag-NOR, and C-banding) and molecular analysis (*in situ* mapping of five classes of repetitive DNAs and microsatellite repeats, including 18S rDNA, 5S rDNA, $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ as markers). According to the data, *E. frenatum* has an asymmetric karyotype with 2n=50. 86 was the fundamental number (NF). The centromeric and telomeric regions of certain chromosomal pairs are where the C-positive heterochromatic blocks are more commonly found. The unique position of the major ribosomal sites in these pairs was confirmed by *in situ* hybridization with 18S rDNA probes, and the Ag-NORs sites were discovered on the telomeric region of the short arms of the largest acrocentric chromosome pair 13. Nevertheless, the 5S rDNA genes are only found in the telomeric region of the short arms of the acrocentric chromosome pair 15. Moreover, microsatellites $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ have been sparingly mapped throughout all chromosomes, with the CGG rich section being the exception (No. 13). This means that, as verified by two methods, it involves a syntenic condition for the 18S rDNA, NORs, and $(CGG)_{10}$ microsatellite probes.

Three species in the genus *Epalzeorhynchos* have undergone cytogenetically analyzed research thus far. Significant karyotype traits are provided by the *Epalzeorhynchos* species for the debate of chromosomal and genetic conservatism. It is anticipated that more research on different species and more data from molecular chromosomal analysis will clarify the chromosome evolution and karyotype pattern in these fishes. Gaining a rudimentary understanding of cytogenetics can help in the future development of potentially marketable species and stains. Research on karyotypes aids in examin-

ing the genetic makeup of aquatic animal species within each habitat, allowing for the precise identification of species relationships. This could potentially aid in future hybridization efforts aimed at improving strains (Sofy et al., 2008), managing chromosome sets in organism breeding (Na-Nakorn et al., 1980), and choosing brood stocks (Mengampan et al., 2004).

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Natural hybridization between *Iris minutoaurea* Makino and *Iris odaesanensis* Y. N. Lee in Korea: evidence from cytological traits

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Abstract. Interspecific hybridization resulting in diploid-homoploid hybrids is relatively rare in natural populations. The *Iris* genus, which comprises numerous economically important species, is a taxonomically complex group in which polyploidization and hybridization frequently contribute to taxon diversification and speciation in natural populations. In Korea, populations of the diploid *Iris odaesanensis* (2*n* = 28) and *Iris minutoaurea* (2*n* = 22) come into contact with each other, leading to homoploid hybrids, as recently demonstrated by molecular phylogenetic and morphological surveys. In this study, we aimed to confirm whether the chromosome number, genome size, and pollen viability of the putative hybrids corresponded to the hybrid nature of their diploid parental species. As previously demonstrated, the hybrids exhibited intermediate tepal color traits between the parental taxa. *Iris minutoaurea*, *I. odaesanensis*, and the putative hybrids were consistently diploid (2*n* = 22, 28, and 25, respectively), confirming that homoploid hybridization mainly occurred in natural populations. The genome size of the putative hybrids (mean: 3.84 pg, range: 3.80–3.86 pg) was additive when compared with those of the parental diploid species (i.e., 3.72 pg in *I. odaesanensis* and 3.95 pg in *I. minutoaurea*). No fertile pollen grains were found in the putative hybrids, which may have prevented the establishment of hybrid lineages and backcrosses with the parental species *I. odaesanensis* or *I. minutoaurea*. Together, these data confirm the existence of natural homoploid *Iris* hybrid populations in Korea and shed light on the dynamics of interspecific hybridization in the *Iris* genus.

Keywords: chromosome number, genome size, homoploid hybrid, *Iris*, pollen abortion.

INTRODUCTION

Hybridization between closely related angiosperm taxa contributes to the diversification and speciation of natural populations (Soltis and Soltis 2009; Whitney et al. 2010; Yakimowski and Rieseberg 2014; Goulet et al. 2017; Kim et al. 2023). Natural hybridizations are collectively regarded as a crucial evolutionary mechanism, resulting in the most complex diversification of the Iridaceae family (Arnold et al. 1990; Young 1996; Makarevitch et al. 2003; Son et al. 2015; Samad et al. 2016; Niketić et al. 2018). A new hybrid lineage may form through allopolyploidy accompanied by the duplication

of chromosome sets or through homoploid hybrid speciation between species of the same ploidy level (Soltis and Soltis 2009; Kadereit 2015; Schumer et al. 2014). To date, numerous homoploid hybrid species from natural populations have been recognized in many economically important plant families (Asteraceae–Lipman et al. 2013; Poaceae–Jiang et al. 2013; Amaryllidaceae–Smirnov et al. 2017; Lamiaceae–Arabaci et al. 2021; Iridaceae–Xiao et al. 2021; Isoëtaceae–Suissa et al. 2022). An increasing number of interspecific hybrids of the genus *Iris* have occasionally been reported, e.g., *I. nelsonii*, a natural hybrid between *I. hexagona* and *I. fulva* (Arnold 1993), *I. verticolor*, a hybrid between *I. virginica*, and *I. setosa* (Lim et al. 2007), and *I.* ×*ampliflora*, a hybrid between *I. japonica* and *I. wattii* (Xiao et al. 2021). However, little is known regarding the morphological, ecological, genetic, and cytological context between natural homoploid hybrid species (Yakimowski and Rieseberg 2014; Feliner et al. 2017).

Iris L. ser. *Chinense* (Diels) Lawrence includes eight species that are mainly distributed in Korea, China, and Japan according to a recent taxonomic study (Wilson 2020). Four *Iris* species (*I. odaesanensis* Y. N. Lee, *I. minutoaurea* Makino, *I. koreana* Nakai, and *I. rossii* Baker) are recognized in Korea (Lee 2003; Sim 2007; Choi et al. 2020a, 2022a, b, c). *Iris odaesanensis* is known to be an endemic species in Korea (Sim 2007). However, some researchers have considered that the species are sub-endemic in Korea, with populations also being found in Jilin, China (Zhao et al. 2000; Son et al. 2015; Choi et al. 2020; Wilson 2020). However, whether the *Iris* population in China represents *I. odaesanensis* requires further investigation. Among the Korean *Iris* species within the *Chinenses* series, *I. koreana* was hypothesized to be of hybrid origin based on cytological data (i.e., chromosome number and genome size variation; Choi et al. 2020a). Subsequent molecular cytogenetic data substantiated *I. koreana* as a putative allopolyploid species, with genomic contributions from two closely related species, *I. minutoaurea* and *I. odaesanensis* (Park et al. 2022). Interestingly, putative hybrid plants between *I. minutoaurea* and *I. odaesanensis* have been documented in Gasan, Korea, based on both molecular analysis and morphological data, revealing intermediate characteristics in tepal color and position of the ovary when compared with the parental species (Son et al. 2015; Yang et al. 2020). Despite increasing interest in these putative hybrid plants from natural populations, debate continues regarding whether these plants show conserved chromosome numbers, genome sizes, and karyotype features compared with their diploid parents, *I. odaesanensis* and *I. minutoaurea*.

We recently investigated sympatric populations of *I. odaesanensis* ($2n$ = 28) and *I. minutoaurea* ($2n$ = 22) using molecular cytogenetic and complete plastome data (Choi et al. 2020a; Park et al. 2022). In those investigations, we identified sympatric populations of putative hybrid individuals that appeared to be derived from two distinct genetic clusters corresponding to diploid *I. odaesanensis* and *I. minutoaurea*. To verify the plausible hybrid origin and relationship with *I. odaesanensis* and *I. minutoaurea*, we compared the chromosome numbers, genome size values, and pollen characters with those of the putative parental species using both light microscopy and scanning electron microscopy (SEM). In addition, we tested the pollen viability of the putative hybrid.

MATERIALS AND METHODS

Plant materials

We selected two mixed-growing natural populations of *I. minutoaurea* and *I. odaesanensis* and putative hybrids between them for this study (Table 1; Figure 1A–D). Living plants were transplanted at Chungnam National University for cytological observations. We examined one to six different individuals of each species and the putative hybrids to check their chromosome numbers and pollen viabilities and estimate their genome sizes (Table 1). All voucher specimens were deposited in the Herbarium of Chungnam National University (CNUK, Daejeon, Korea).

Chromosome numbers, karyotypes, and pollen viabilities of putative hybrid plants

We determined chromosome numbers in root tip metaphasic plates using the standard Feulgen staining technique as described by Choi et al. (2020a). We placed pollen grains from two anthers per hybrid individual into aniline blue dye solution to distinguish between fertile and sterile pollen grains (Choi et al. 2020b; Kim et al. 2021). To make detailed palynological observations, we dehydrated mature anthers from putative hybrid individuals in an ethanol series (70%, 90%, 95%, and 100%) at room temperature over 30 min. The dehydrated anthers were immersed in liquid carbon dioxide for critical point drying (Leica Microsystems, EM CPD300, Germany). All dried samples were mounted on aluminum stubs and coated using an ion-sputtering device (Hitachi, E-1010, Japan). The pollen grains in the dried anthers were gently removed using a needle and analyzed using SEM (Hitachi, S3000N, Japan) at 20 kV

Table 1. Plant material used for cytological analysis of *I. odaesanensis*, *I. minutoaurea*, and their putative hybrids in natural populations in Korea.

Taxon; collection number	Locality: global positioning satellite (GPS) coordinate	Chromosome number	Genome size 1C ± S.D. (pg)	Reference
*I. odaesanensis**				
JCKC1932271	Gasan, Kyungsang	$2n = 28$	3.76 ± 0.021	Choi et al. 2020a
JC041904	Gasan, Kyungsang	$2n = 28$	3.74 ± 0.042	Choi et al. 2020a
JC041903	Gasan, Kyungsang	$2n = 28$	3.67 ± 0.015	Choi et al. 2020a
JC041940	Gasan, Kyungsang	$2n = 28$	3.73 ± 0.018	Choi et al. 2020a
Mean			3.72	
I. minutoaurea Makino				
JC041913*	Gasan, Kyungsang	$2n = 22$	4.09 ± 0.042	Choi et al. 2020a
Gasan_8*	Gasan, Kyungsang	$2n = 22$	3.92 ± 0.008	Choi et al. 2020a
Che-01	Mt. Cheonma, Gyeonggi; N37°41’24”, E127°24’36”, 157 m	$2n = 22$	3.85 ± 0.009	This study
Che-02	Mt. Cheonma, Gyeonggi; N37°41’24”, E127°24’36”, 157 m	$2n = 22$	3.94 ± 0.008	This study
Go-1	Go-Nam, Gyeonggi; N37°40’22”, E127°15’17”, 157 m	$2n = 22$	3.93 ± 0.009	This study
Hwa-3	Mt. Hwa-Ya, Gyeonggi; N36°56’25”, E127°16’47”, 162 m	$2n = 22$	4.02 ± 0.004	This study
Mean			3.95	
I. minutoaurea × *I. odaesanensis**				
JC041927	Gasan, Kyungsang	$2n = 25$	3.86 ± 0.017	This study
JC041906	Gasan, Kyungsang	$2n = 25$	3.85 ± 0.009	This study
JC041924	Gasan, Kyungsang	$2n = 25$	3.80 ± 0.051	This study
Mean			3.84	

* The GPS coordinates, latitude, and longitude of the collection sites are not indicated for protection purposes, although the latter species has recently been excluded from the list of endangered Korean taxa.

with a working distance of 10 mm, as described by Kim et al. (2021).

Genome-size measurements

The genome sizes of four *I. minutoaurea* individuals and three putative hybrids were measured via flow cytometry, with *Solanum pseudocapsicum* L. (1C = 1.2946 pg; Temsch et al. 2010) serving as an internal standard. Genome-size estimations for *Iris* species were carried out as described by Choi et al. (2020a). Due to the variable genome-size data in our previous research, ranging from 3.70 pg/1C to 4.09 pg/1C with *I. minutoaurea* (Choi et al. 2020a), we included additional 1C values for the same species from another different natural population to clarify the range of genome-size data (Table 1).

RESULTS

Confirmation of homoploid hybridization based on the chromosomal number and genome size

Despite floral color variations among the investigated taxa (e.g., *I. odaesanensis* in Figure 1A, *I. minutoaurea* in Figure 1B, and their putative hybrids in Figure 1C–D), cytological analysis showed that *I. minutoaurea*, *I. odaesanensis*, and their putative hybrids were consistently diploid, with $2n = 22$, 28, and 25, respectively (Figure 2), confirming that homoploid hybridization occurred in natural populations (Figures 1–2). The karyotypes in the putative hybrid plants could not be directly compared with their homologous pairs because of their odd chromosome numbers ($2n = 25$); thus, they were analyzed with reference to their parental karyotypes. The karyotypes of the hybrid plants were mostly composed of metacentric, sub-metacentric, and acrocentric chromosomes, regardless of their parental chromosomes (Figure 2B). Although satellites and nucleolar-organizer regions did not normally show clear localization in mitotic cells due to high chromosome condensation (Figure 2A–B), they were occasionally visible in hybrid plants (Figure 2C).

Flow cytometric analyses yielded high-resolution histograms with distinct sample peaks and internal standards for all seven individuals investigated (Table 1). The results of our genome-size (1C) analysis are shown in Table 1, together with our previous data. The genome size of *I. minutoaurea* was higher (range: 3.85–4.09 pg) than that of *I. odaesanensis* (range: 3.67–3.76 pg). The

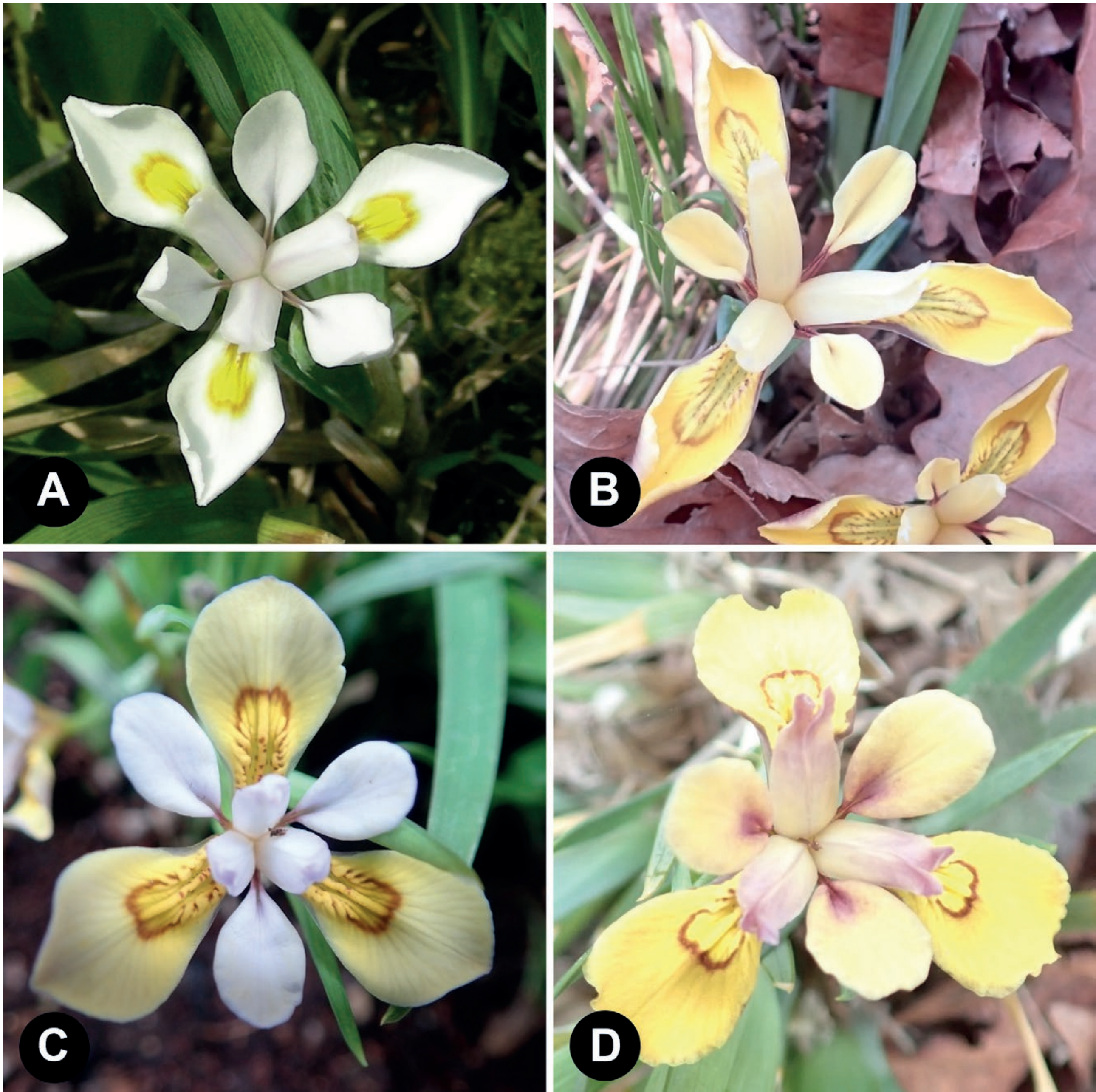

Figure 1. Floral morphological variations in the studied *Iris* taxa. **A.** *Iris odaesanensis*: standards and styles were white, and falls were white with lower central half highlighted in yellow. **B.** *Iris minutoaurea*: standards and styles were yellow, and falls were yellow with its central lower half highlighted in dark purple. **C–D.** Putative hybrids between *I. odaesanensis* and *I. minutoaurea*. The putative hybrids showed tepal colour variations: with white standards and styles and yellowish falls with its lower central half highlighted in dark purple **(C),** yellow standards and falls with their lower basal part highlighted in dark purple and yellow styles with its apical margin coloured in purple **(D)**. Photographs were taken by T.-S. Jang.

1C mean value of the putative hybrids (mean: 3.84 pg, range: 3.80–3.86 pg) was additive with respect to the diploid parental species (i.e., 3.72 pg in *I. odaesanensis* and 3.95 pg in *I. minutoaurea*).

Pollen fertility in the putative hybrids

Mature anthers from all hybrid plants were removed, and all pollen grains were stained with

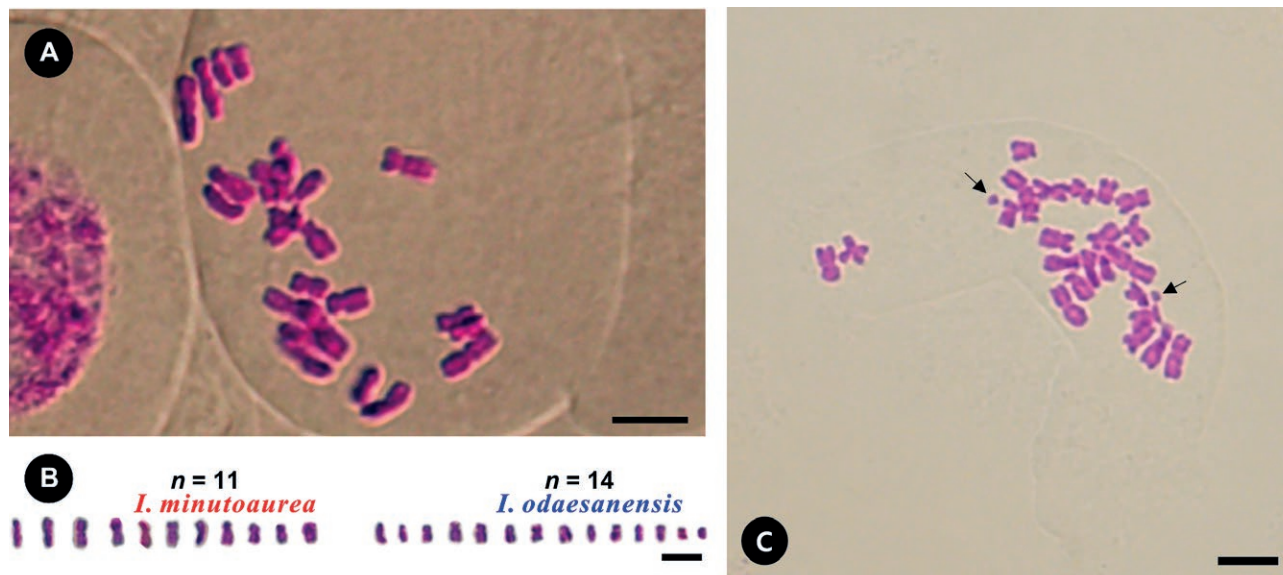

Figure 2. Chromosome numbers and karyotypes of putative hybrids ($2n = 2x = 25$) of *I. minutoaurea* ($2n = 2x = 22$) and *I. odaesanensis* ($2n = 2x = 28$). **A, C.** Mitotic metaphase chromosomes with $2n = 25$ (arrows indicate satellites of putative hybrids). **B.** Karyotype of a putative hybrid including a haploid chromosome of $n = 11$ (*I. minutoaurea*) and $n = 14$ (*I. odaesanensis*). Scale bars = 5 µm (*n* indicates a haploid chromosome number; karyotypes were analyzed in comparison to previous chromosome-counting data [Choi et al. 2020a]).

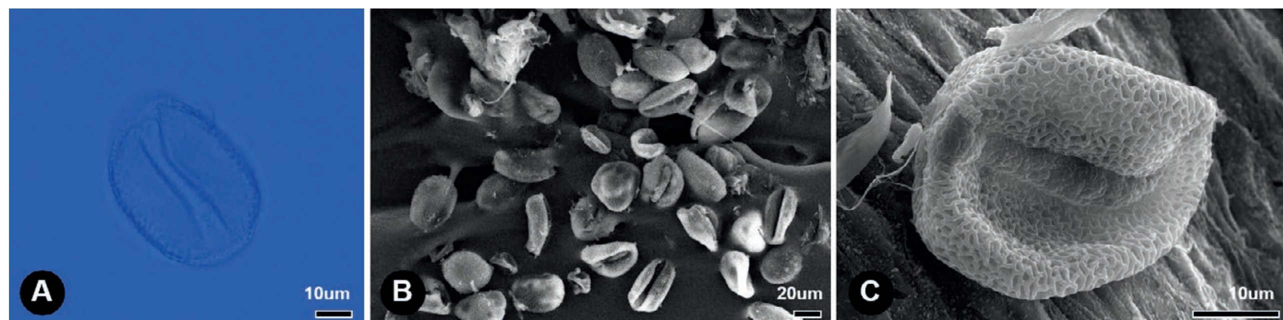

Figure 3. Light and scanning electron microscopic photographs (LM and SEM) of sterile pollen grains in putative hybrids between *Iris odaesanensis* and *I. minutoaurea*. **A.** An unstained pollen grain (sterile pollen grains) in aniline blue dye solution. **B, C**. Sterile pollen grains observed by SEM. **B.** Stamens of putative hybrids produced sterile pollen grains. **C.** Enlargement of a sterile pollen grain by SEM.

aniline blue dye solution to assess the fertility of the putative hybrid plants (Figure 3A). However, despite staining, no fertile pollen grains were found, indicating that most or all pollen grains were infertile (Figure 3). SEM analysis performed on pollen samples from the putative hybrid individuals revealed that the pollen grains were monosulcate and mostly curled up or shriveled with folded aperture membranes (Figure 3B–C). The exine ornamentation of the putative hybrids was reticulate with continuous muri (very rarely discontinuous).

DISCUSSION

Here, we provide cytological and palynological evidence for the presence of homoploid hybrids between *I. minutoaurea* and *I. odaesanensis* (*I.* ser. *Chinense*) in natural Korean populations, in agreement with previous results based exclusively on molecular and morphological evidence (Son et al. 2015; Yang et al. 2020). Both diploid parental species differ in terms of morphology, chromosome number, genome size, and complete chloroplast genome composition (Sim 2007; Choi et al. 2020a; Park et al. 2022). Based on molecular phylogenetic analyses of their *mat*K and nrITS sequences, the

putative hybrids did not form a clearly separated monophyletic group when analyzed together with their parental species (*I. minutoaurea* and *I. odaesanensis*; Son et al. 2015). The present chromosome number and genome-size data for the homoploid hybrids appeared to be more conclusive (Table 1). Thus, our results are consistent with the hypothesis that hybridization in the *Iris* genus of the Iridaceae family may have been important for taxon diversification and speciation (Arnold et al. 1990; Young 1996; Lim et al. 2007; Gao et al. 2021; Park et al. 2022).

Natural homoploid hybrid speciation accompanied by reproductive isolation from putative parents has often been reported for the Iridaceae family (Arnold et al. 1990). The intermediate morphological variations of the *Iris* putative hybrid were not influenced by environmental factors. This lack of influence was attributable to a sympatric distribution in their parental diploid species (Table 1). Instead, these variations seemed to correlate with cytological differences, as frequently observed with other homoploid hybrid species (Peruzzi et al. 2012; Musiał et al. 2020; Xiao et al. 2021; Pellicer et al. 2022). Hybridization in plants can be affected by overlapping flowering periods in sympatric areas (Paun et al. 2009; Abbott et al. 2010; Jiang et al. 2013; Kim et al. 2023) and is mainly documented for apomictic groups (Paun et al. 2006; Hörandl 2010; Hojsgaard and Hörandl 2019). Our results confirmed that homoploid hybrid species of Korean irises may have evolved partial intrinsic reproductive isolation from both diploid parents considering their different base chromosome numbers (x = 11 in *I. minutoaurea* and x = 14 in *I. odaesanensis*; Table 1). Such different base chromosome numbers and odd chromosome number could lead to infertility in individuals and prevent the establishment of hybrid lineages and back-crosses with the parental species (Carnicero et al. 2023). However, only limited data are available regarding the emergence of *Iris* hybrids from natural populations, their potential for sexual and asexual reproduction (e.g., by rhizome disruptions), or morphological divergence from the parental taxa (Arnold et al. 1990; Niketić et al. 2018; Yang et al. 2020; Gao et al. 2021).

Efforts to maintain homoploid hybrids from natural populations are likely to fail because of their infertile offspring (Stebbins 1958; Yakimowski and Rieseberg 2014). Pollen abortion in putative hybrid *Iris* plants could be reflected by impaired chromosomal pairing during meiosis between the two unequal parental genomes (x = 11 and 14). These hybrid plants are not amenable to sexual reproduction, suggesting that the hybrids probably represent a natural first-generation hybrid species, as has been reported for other species (Denduangboripant et al. 2007; Saito et al. 2007; Tseng and Hu 2014). Although such hybrid taxa may not eventually produce well-differentiated hybrid species, they can facilitate the testing of key predictions from models of hybrid speciation, as observed with other plant groups (Barton 2001; Chen et al. 2022). Further investigation using molecular cytogenetic techniques and genomic *in situ* hybridization of the putative hybrid and its parental species will shed light on the speciation and diversification of *Iris* ser. *Chinense*.

CONCLUSION

In summary, our comprehensive data demonstrate the existence of populations of natural homoploid *Iris* hybrids. Natural hybrids of *I. odaesanensis* ($2n$ = 28; 3.72 pg/1C) × *I. minutoaurea* ($2n$ = 22; 3.95 pg/1C) were additive in terms of the chromosome number ($2n$ = 25) and genome size (i.e., 3.84 pg/1C). The odd chromosome number in the homoploid hybrid could affect the formation of infertile pollen grains, thereby preventing the establishment of hybrid lineages and backcrosses with the parental species *I. odaesanensis* or *I. minutoaurea*.

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