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GENOME SIZE OF THE DIPLOID HYBRID SPECIES *HIPPOPHAE GONIOCARPA* AND ITS PARENTAL SPECIES, *H. RHAMNOIDES* SSP. *SINENSIS* AND *H. NEUROCARPA* SSP. *NEUROCARPA* (ELAEAGNACEAE)

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Hybridization may induce rapid genomic changes, including gain or loss of DNA. We examined nuclear DNA content of a diploid hybrid species, *Hippophae goniocarpa* and its two parental species. The nuclear 2C DNA value was 2.95±0.08 pg for *H. goniocarpa*; for the parental species it ranged from 2.54±0.09 pg to 2.66±0.12 pg for *H. rhamnoides* ssp. *sinensis* and from 3.15±0.19 pg to 3.49±0.06 pg for *H. neurocarpa* ssp. *neurocarpa*. The nuclear DNA content of *H. goniocarpa* was intermediate between those of the two parental species, confirming that this species is of diploid hybrid origin and further suggesting that it is in an early stage of the process of speciation.

Key words: Nuclear DNA content, flow cytometry, *H. goniocarpa*, hybrid species, interspecific and intraspecific variation.

INTRODUCTION

Hybridization can induce rapid genomic changes and thereby cause changes in DNA content (Baack et al., 2005). For example, Price et al. (1983) showed that DNA content differed significantly between *Microseris* F_1 hybrids and those of their parental species, and Bureš et al. (2004) found that the DNA values of most interspecific hybrids of *Cirsium* were between those of their putative parents. Three *Helianthus* hybrid species showed a marked increase in DNA content as compared with the parental species (Baack et al., 2005). However, Rayburn et al. (1993) found that the DNA content of the F_1 maize hybrids did not significantly differ from that of the parents. In other taxonomic groups it remains unclear how the DNA content of hybrids or hybrid species changes as compared with their parents.

Here we report changes of DNA content in a diploid hybrid species, *Hippophae goniocarpa* (Elaeagnaceae). This species is distributed sympatrically with two other species of the same genus (*H. rhamnoides* ssp. *sinensis*, *H. neurocarpa*) in the northeast Qinghai-Tibetan Plateau of China, without spatial separation (Lian et al., 1995, 2000). Besides these three species, several shrub or tree species have been described under the genus *Hippophae* (sea buckthorns) (Rousi, 1971; Lian et al., 1996; Bartish et al., 2002), all of which have received a great deal of attention as important nitrogen-fixing species for afforestation (Lian et al. 1996, Tian et al. 2004) or their fruits as raw materials in soft-drink production (Lian et al. 1996). These species are wind-pollinated and dioecious, with gender determined genetically (Rousi, 1971; Lian et al., 1998; Bartish et al., 2000). So far all of them have been shown to be diploid with 2n = 24 (Rousi, 1971; Lian et al., 1998). Most morphological characters of *H. goniocarpa* are intermediate between those of the two putative parental species (Lian et al., 1997). However, this species shows distinct reproductive isolation from those two species (Lian et al., 2000; Wang et al., 2008). Based on these morphological traits and isozyme data, Lian et al. (1995) suggested that *H. goniocarpa* originated from hybrid speciation between *H. rhamnoides* ssp. *sinensis* Rousi and *H. neurocarpa* ssp. *neurocarpa* S.W. Liu & T.N. He. This hypothesis was subsequently confirmed by

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analysis of random amplification of polymorphic DNA (RAPD) markers, internal transcribed spacer (ITS) sequence data and morphological data (Bartish et al., 2000, 2002; Sun et al., 2003). In addition, *H. goniocarpa* was found to have double maternal origin from both putative parental species, and this homoploid hybrid species is probably in an early stage of speciation following initial reproductive isolation (Wang et al., 2008); some ecophysiological traits superior to both parents have been established in this species (Ma et al. 2009).

Flow cytometry (FCM) has been widely used to quantify DNA content in both animal and plant species (Arumuganathan and Earle, 1991; Bennett and Leitch, 1995; Doležel and Bartoš, 2005; Doležel et al., 2007). Genome size and C-value are important characteristics of all living organisms. These measures may be very helpful in clarifying the origin and evolution of present-day species (Thalmann et al., 2000; Zonneveld, 2001; Šiško et al., 2003; Bureš et al., 2004).

In this study we estimated DNA content in *H. goniocarpa* and its parental species by flow cytometry (FCM). These estimates may be useful in understanding the speciation process in this species and the ensuing changes in nuclear DNA content.

MATERIALS AND METHODS

PLANT MATERIALS AND CULTURE

Seeds of the populations sampled for this study were collected within the typical distribution ranges of three species in the field (Tab. 1). All plants used for DNA content estimation were grown from seeds. The seeds were sterilized in 0.3% $KMnO_4$ for 15 min, rinsed several times with sterile water until the purplish red color faded, immersed in water at 60°C for 24 h (water was changed once during that period), placed in lidded Petri dishes and germinated in a tissue culture box under an 8 h photoperiod at variable 20–30°C (Tayier et al., 2006). Young healthy leaves of each individual were randomly collected. We tried several internal reference species as suggested by Dr. Jaroslav Doležel (Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic) and found *Vigna radiata* to be more suitable than the other species because its DNA content is closest to the sampled species; ~0.50 g leaf of this species (2C = 1.40 pg; Bennett and Smith, 1976; Johnston et al., 1999) was used as the standard reference.

SAMPLE PREPARATION

Nuclei suspensions were prepared according to Otto (1990) because Otto buffer provides FCM histograms exhibiting G0/G1 peaks with unequalled resolution for most plant species, especially species with smaller genomes (Doležel and Bartoš, 2005; Loureiro et al., 2006). Young healthy leaves of the sample (~500 mg) were excised and fresh leaves of *Vigna radiata* were added simultaneously as the internal standard, washed in distilled water, then placed in a 10 mm Petri dish on ice and chopped with a sharp scalpel in 1.5 ml freshly prepared ice-cold solution of Otto I buffer (0.1 M citric acid monohydrate, pH 2–3) containing 0.5% (v/v) Tween 20. The solution containing nuclei was mixed well with a pipette and the suspension was filtered to remove large debris (through 42 μm nylon mash soaked in Otto I buffer for 20 min before use) into a microfuge tube. After incubation at 4°C for 10 min, suspensions were pelleted by spinning at 1000 rpm for 8 min at 4°C. The supernatant was removed and the pellet containing the nuclei resuspended in the residual buffer, 100 μl mixture of 1:2 Otto I and Otto II (0.4 M $NaHPO_4 \cdot 12H_2O$) was added, and the nuclear suspension was stained with 200 μl mixture of 100 μg/ml propidium iodide (PI; Sigma P-4170, U.S.A.) and 50 μg/ml DNase-free RNase (Sigma, U.S.A.) to prevent staining of double-stranded RNA.

FLOW CYTOMETRY ANALYSIS

After retaining for 10–30 min in the dark (Abreu et al., 2008; Rossi et al., 2008), the samples were analyzed with an Elite flow cytometer (Epics XL, Coulter Beckman, U.S.A.) equipped with an air-cooled argon-air laser tuned at 15 mw and operated at 488 nm. The mean position of the G_0/G_1 (nuclei) peak of the sample and the internal standard were analyzed with Multicycle software. The mean DNA content of each sample, measured in picograms, was calculated based on 5000 scanned nuclei. At least nine measurements were obtained from each group, and are presented as means and standard deviations of the sample. The formula used for converting fluorescence values to DNA content was as follows: nuclear DNA content = (mean position of sample peak/mean position of peak of standard) DNA content of the standard.

SAMPLE DESIGN AND ANALYSIS

We quantified the nuclear DNA content of three individuals for each population collected for each species. Each individual was analyzed independently three times. Differences in DNA content between species were tested with the Tukey HSD post hoc test in SPSS for Windows ver. 16.0, and one-way ANOVA was used to determine the significance of differences between populations and species.

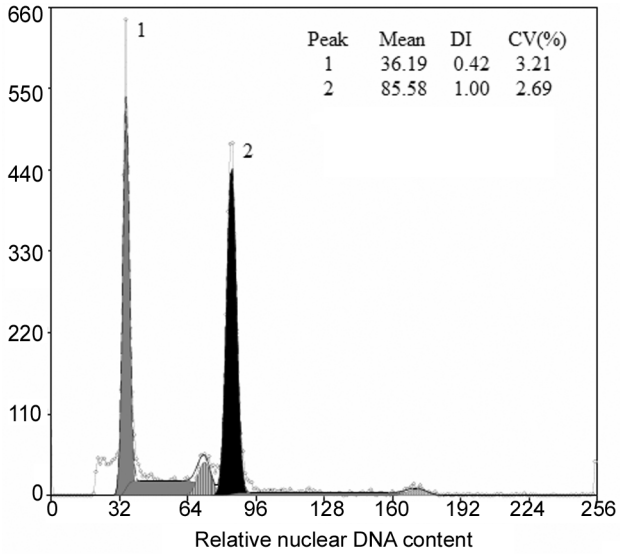

Fig. 1. Selected histogram of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from young leaves of *Vigna radiata* cv. Berken (peak 1) (2C = 1.40 pg DNA, as internal reference standard) and *Hippophae* L. species (peak 2). The mean channel number, DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation (CV, %) of G_0/G_1 (peaks 1 and 2) are given

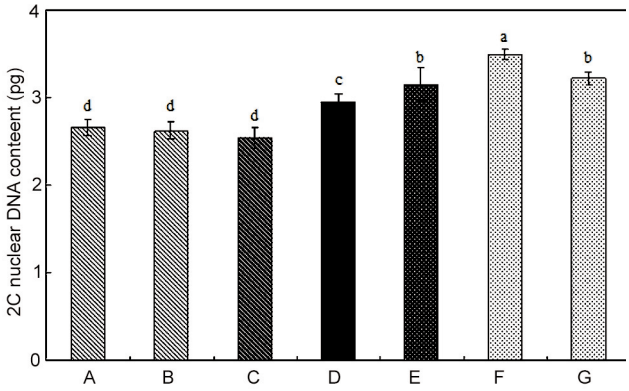

Fig. 2. Nuclear DNA content of seven populations of three sampled species. Different letters indicate significant differences between populations ($P < 0.01$). Left to right: Diebu (A), Huzhu (B) and Qilian (C) populations of *H. rhamnoides* ssp. *sinensis*; *H. goniocarpa* (D, solid bar); Qilian (E), Yushu (F) and Hongyuan (G) populations of *H. neurocarpa* ssp. *neurocarpa*.

RESULTS

CVs below 3.50% were achieved for both the standard *Vigna radiate* and the sample in all measurements (standard CV = 2.89±0.08; sample CV = 3.24±0.10; Fig. 1). Doležel and Bartoš (2005) suggested that CVs below 5% are acceptable for FCM assessments. We found that fluctuations of CV% were less than 5% when we mixed the samples and internal standard leaves. Therefore, all estimations reported here should represent the actual DNA amount in each population of each species.

The 2C DNA values of each sampled population varied from 2.54±0.09 pg in *H. rhamnoides* ssp. *sinensis* in Qilian to 3.49±0.06 pg in *H. neurocarpa* ssp. *neurocarpa* located in Yushu, Qinghai, representing 1.37-fold variation. The three populations of *H. rhamnoides* ssp. *sinensis* species had similar DNA content ($P = 0.562$), with 2C values of 2.54±0.09 pg, 2.62±0.10 pg and 2.66±0.12 pg, representing only ~3% interpopulation difference. One-way ANOVA indicated striking differences ($P < 0.01$) between the three populations of *H. neurocarpa* ssp. *neurocarpa*, with DNA content estimated at 3.49±0.06 pg, 3.15±0.19 pg and 3.22±0.07 pg.

The 2C DNA value for the hybrid species *H. goniocarpa* was estimated at 2.95±0.08 pg (only a single population of this species was found). The nuclear DNA C-value for this hybrid species differed significantly from those of the parental species ($P < 0.01$), and its DNA amount was intermediate between those of the putative parents (Tab. 1, Fig. 2). Overall our results suggest that the three *Hippophae* species investigated here have typically small genomes (Tab. 1).

DISCUSSION

When the samples were mixed with the standard the CV values increased. When estimated separately both of the CVs were smaller (~2.0 or less); the internal standard had a lower 2C distribution (mean CV = 1.80). Interactions among the various substances in the samples and in the plants might be responsible for the slight variation of nuclear DNA content and CV%, through nucleus-cytoplasm interactions (Noirot et al., 2002).

We found distinct interspecific variation of DNA content within the same genus *Hippophae*. The three sampled species differed 1.37-fold in nuclear DNA content (2C = 2.54±0.09–3.49±0.06 pg). This range was slightly below the mean value found for other genera without polyploidization (Plant DNA C-values database; Bennett and Leitch, 2005). Variations in genome size between closely related species may reflect adaptive and evolutionary differences at the nuclear level (Ohri, 1998). This phenomenon was particularly obvious for *Pinus*, a genus with a broad distribution (Bogunic et al., 2003). These variations may derive from retroelements, primarily the LTR (long terminal repeat) retrotransposons (Vicient et al., 1999) and other repetitive DNA content (Flavell et al., 1974). Changes

TABLE 1. Geographic regions and nuclear DNA content of *Hippophae* species studied (2n=24)

Taxon	Location	2C DNA content ± s.d. (pg)	Peak CV (%)
H. rhamnoides subsp. *sinensis*	Qilian, Qinghai	2.54 ± 0.09 [d]	3.35
	Huzhu, Qinghai	2.62 ± 0.10 [d]	3.50
	Diebu, Gansu	2.66 ± 0.12 [d]	3.23
H. goniocarpa	Qilian, Qinghai	2.95 ± 0.08 [c]	3.49
H. neurocarpa subsp. *neurocarpa*	Qilian, Qinghai	3.15 ± 0.19 [b]	3.08
	Yushu, Qinghai	3.49 ± 0.06 [a]	3.02
	Hongyuan, Sichuan	3.22 ± 0.07 [b]	2.99

2C values are given as mean 2C DNA content (±SD). Coefficients of variation (Peak CV, %) of sample G_0/G_1 peaks are also given. Significant differences between accessions are indicated by different letters.

in DNA content through selective removal or increase of small amounts of DNA contribute greatly to interspecific differentiation (Ohri and Khoshoo, 1986; Wakamiya et al., 1993; Jeffrey et al., 2005). Frequent hybridization as well as genetic introgression in plants also changes the DNA content of populations within species within the local distribution (Hall et al., 2000; Bureš et al., 2004).

The nuclear DNA content of the hybrid species *H. goniocarpa* (2.95±0.08 pg) was approximately intermediate to those of the two parental species; the ratio of 2C DNA amounts of the parental and hybrid species were 0.88 for *H. rhamnoides* ssp. *sinensis* and 1.11 for *H. neurocarpa* ssp. *neurocarpa*. This finding is inconsistent with the only case study of nuclear DNA content of natural hybrid species: mature hybrid species in the genus *Helianthus* have 50% higher DNA content than both parental species; however, the DNA content of synthetic hybrids in this genus was estimated to be intermediate between the two parental species (Baack et al., 2005). Natural hybrids of other genera (*Cirsium*, *Helleborus*, *Cucurbita*) have also been shown to have intermediate DNA content versus the parental species (Zonneveld, 2001; Šiško et al., 2003; Bureš et al., 2004). Intermediate trait values are commonly found for most hybrids because hybrids usually combine alleles from both parental species (Rieseberg et al., 2003; Campbell et al., 2005). Thus the intermediate DNA content of *H. goniocarpa* confirms its hybrid origin and suggests that it might be in an early stage of hybrid speciation, although with its high germination rates it has developed an effective reproductive barrier to the two parental species (Lian et al., 1997; Wang et al., 2008). This suggestion is also consistent with the molecular evidence that concerted evolution of nuclear ITS in this species has not been completed (Wang et al., 2008). It remains unclear whether this species will increase its DNA content with further gene interactions and range extension in the final speciation stage as found in *Helianthus* hybrid species (Baack et al., 2005).

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