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Establishment of Species-Specific DNA Markers to Identify Interspecific Hybrids of *Hibiscus*

Masaki Ochiai, Kensuke Nakagomi, and Hirokazu Fukui

Faculty of Applied Biological Science, Gifu University 1-1, Yanagido, Gifu, Gifu, 501-1193, Japan

ochiai.masaki.h3@f.gifu-u.ac.jp

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Summary

Confirmation of hybridization is important for interspecific crosses. This study established species-specific DNA markers to select hybrids from interspecific crosses among 4 *Hibiscus* species: *H. cannabinus* L., *H. acetosella* Welw. ex Fic. 'Black King', *H. coccineus* Walt., and *H. mutabilis* L.. Start codon targeted (SCoT) primers were used to establish species-specific DNA markers. Species identification was performed based on the electrophoresis band patterns of PCR products using SCoT primers. The results show that some SCoT primers could be used as species-specific DNA markers among the 4 *Hibiscus* species: SCoT7 primer could distinguish *H. cannabinus* L. (blue flower type) or *H. coccineus* Walt. from the other species, a combination of SCoT2 and ScoT3 primers could distinguish *H. cannabinus* L. (white flower type) or *H. mutabilis* L. from the other species, and a combination of SCoT3 and SCoT7 primers could distinguish *H. acetosella* Welw. ex Fic. 'Black King' from the other species. Accordingly, these DNA markers were applied to detect the pollen parents of interspecific crosses, and the results were consistent with the results from morphological evaluation.

INTRODUCTION

Interspecific crossing is an important breeding method that is used to develop new characteristics in horticultural plants. However, because interspecific crosses generally have low fertilization rates, the presence of self-pollinated individuals among the individuals obtained by crossbreeding is problematic. Morphological evaluation, DNA marker analysis, and ploidy analysis can distinguish between cross- and self-pollinated individuals. However, a suitable method must be selected according to the plant materials in question. *Hibiscus*, such as *Hibiscus rosa-sinensis* L. (a tropical plant), are well-known ornamental plants. Temperate *Hibiscus* plants, such as *H. mutabilis* L. and *H. syriacus* L., are also renowned in East Asia. The flowers of well-known *Hibiscus* are red, pink, orange, yellow, or white, whereas only a few cultivars and species have blue or purple flowers. Therefore, to breed new cultivars with blue or purple flowers, our group made interspecific crosses among four *Hibiscus* species: *H. cannabinus* L., *H. acetosella* Welw. ex Fic. 'Black King', *H. coccineus* Walt., and *H. mutabilis* L. Accordingly, this study aimed to establish species-specific DNA markers to identify pollen parents of progenies obtained by crossing.

MATERIALS AND METHODS

Plant Materials

Tetraploids of *H. cannabinus* L. (blue flower type), tetraploids of *H. cannabinus* L. (white flower type), *H. acetosella* Welw. ex Fic. 'Black King' (tetraploid cultivar), tetraploids of *H. coccineus* walt., tetraploids of *H. mutabilis* L., and their cross populations were grown in a greenhouse at the Gifu Field Science Center, Gifu University.

Selection of DNA extraction method

The following methods were evaluated to find a suitable DNA extraction method for *Hibiscus* species: a sodium dodecyl sulfate (SDS)-based method (Edwards et al., 1991), a cetyltrimethylammonium bromide (CTAB)-based method (Escaravage et al., 1998), a modified CTAB method using polyvinylpolypyrrolidone (PVPP) with a high-pH condition (Kasajima et al., 2013), DNA sui sui-VS (Rhizo, Japan), a Blood & Cell Culture DNA Mini Kit (QIAGEN, Germany), and a DNeasy Plant Mini Kit (QIAGEN, Germany). Each DNA extraction method was performed using young leaves of *H. cannabinus* L. (blue flower type). The presence of DNA and its purity were checked by electrophoresis.

Establishment of species-specific DNA markers

Start codon targeted (SCoT) primers were screened to identify species-specific DNA markers that can distinguish species based on the specificity of PCR and electrophoresis bands (Collard and Mackill, 2009). MightyAmp DNA Polymerase Ver.3 (Takara Bio, Japan) was used for PCR with the following program: 98°C for 2 min followed by 30 cycles of 98°C for 10 s, 55°C for 15 s, and 68°C for 2 min. SCoT primers were evaluated alone or in combinations of 2 in PCR.

Evaluation of hybridization in progenies

The species-specific DNA markers identified above were applied to detect the pollen

parents of the progenies obtained by crossing. Progenies were also subjected to morphological evaluation. Length of terminal leaflet, width of terminal leaflet, length of leaf, and attached angle of bottom lateral leaflets were measured in mature leaves. Flower color was subjectively evaluated in crosses between *H. cannabinus* L. (blue flower type) and *H. cannabinus* L. (white flower type).

RESULTS AND DISCUSSION

Selection of DNA extraction method

Hibiscus plants contain large quantities of polysaccharides, which interfere with DNA extraction. Therefore, 6 DNA extraction methods were evaluated to select a suitable method for *Hibiscus* plants. Extracts derived using the SDS-based method or DNA sui sui-VS did not show any DNA bands **(Fig. 1**).

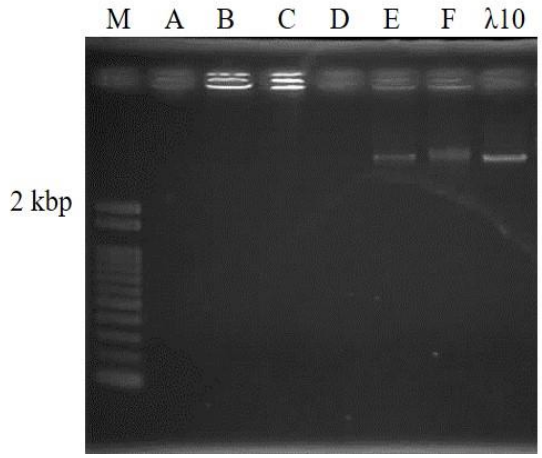

Figure 1. Electrophoresis of extracts derived using each DNA extraction method M: 100-bp DNA size marker, A: SDS-based method, B: CTAB-based method, C: modified CTAB method using PVPP and high-pH condition, D: DNA sui sui-VS, E: Blood and Cell culture DNA mini kit, F: DNeasy Plant mini kit, λ10 : λ-DNA as a positive control.

DNA could not be separated from unnecessary components in the intermediate step. Fluorescence was observed in the wells of agarose gels in the electrophoresis results of extracts derived using the CTAB-based method or modified CTAB-based method with PVPP and high pH. This phenomenon is observed when DNA extracts contain many high-molecular-weight compounds. Although these methods could extract DNA, they did not remove enough polysaccharides. Meanwhile, clear DNA binding was observed in the results electrophoresis results of extracts derived using the Blood & Cell Culture DNA Mini Kit and DNeasy Plant Mini Kit, suggesting their suitability for DNA extraction from *Hibiscus* plants. Accordingly, the DNeasy Plant Mini Kit was chosen for the following experiments owing to its simplicity of operation.

Establishment of species-specific DNA markers

PCR products with SCoT7 primer showed a band specific to *H. cannabinus* (blue flower type) between 1 and 2 kbp as well as a band specific to *H. coccineus* over 2 kbp (**Fig. 2**). SCoT7 primer distinguished *H. cannabinus* (blue flower type) or *H. coccineus* from the other samples. PCR products obtained using a combination of SCoT2 and SCoT3 primers showed an obvious band specific to *H. cannabinus* (white flower type) over 2 kbp and one for *H. mutabilis* over 2 kbp. The combination of SCoT2 and SCoT3 primers could distinguish *H. cannabinus* (white flower type) or *H. mutabilis* from the other species. PCR products obtained using a combination of SCoT3 and SCoT7 primers showed an obvious band specific to *H. acetosella* 'Black King' between 1 and 2 kbp.

Furthermore, the combination of SCoT3 and SCoT7 primers distinguished *H. acetosella* 'Black King' from the other species.

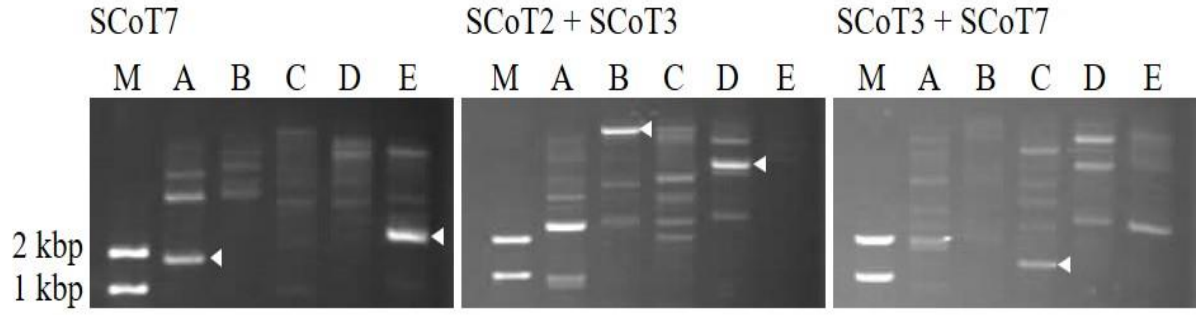

Figure 2. Electrophoresis of PCR products using SCoT primers. M: 100-bp DNA size marker, A: *H. cannabinus* (blue flower type), B: *H. cannabinus* (white flower type), C: *H. acetosella* 'Black King, D: *H. mutabilis* , E: *H. coccineus*. White arrowheads indicate deduced species-specific band.

Evaluation of hybridization in progenies

The species-specific DNA markers established above were applied to distinguish the progenies of interspecific crosses. Given that the embryonic parents are clear in the crossing populations, the species-specific DNA markers were used to identify pollen parents. Eleven progenies obtained by crossing between *H. cannabinus* (blue flower type) and *H. cannabinus* (white flower type) were examined using the marker specific to *H. cannabinus* (blue flower type) (**Fig. 3**).

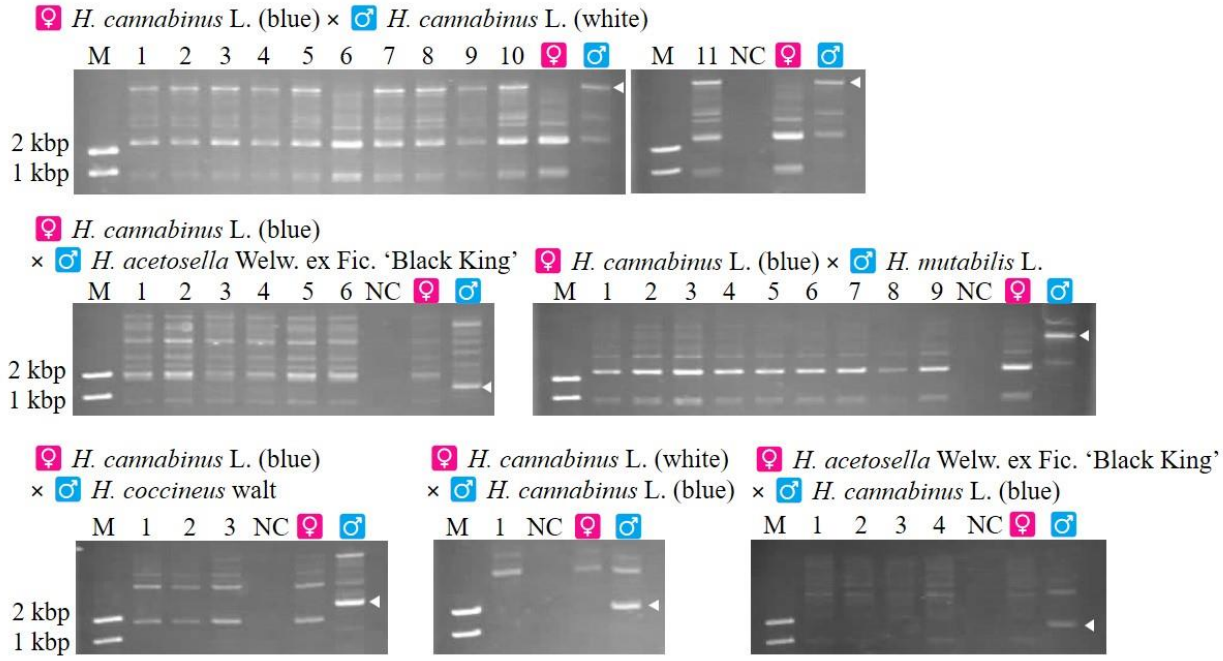

Figure 3. Detection of DNA markers in the crossing populations. M: 100-bp DNA size marker. Numbers indicate individual numbers of progenies in each crossing. NC: non-template control. White arrowheads indicate species-specific band of pollen parent.

Ten progenies exhibited a band specific to *H. cannabinus* (blue flower type), whereas 1 progeny did not. This suggests that the 10 progenies are hybrids between *H. cannabinus* (blue flower type) and *H. cannabinus* (white flower type); meanwhile, the remaining progeny is likely a self-crossing of *H. cannabinus* (blue flower type). Regarding flower color, *H. cannabinus* (blue flower type) and *H. cannabinus* L. (white flower type) exhibit dark purple and white petals, respectively. The 10 progenies that exhibited the band specific to *H. cannabinus* (blue flower type) had pale purple petals, whereas the 1 progeny that exhibited the band specific to *H. cannabinus* (white flower type) had white petals (**Fig. 4**). Thus, these morphological characteristics are consistent with the DNA marker results.

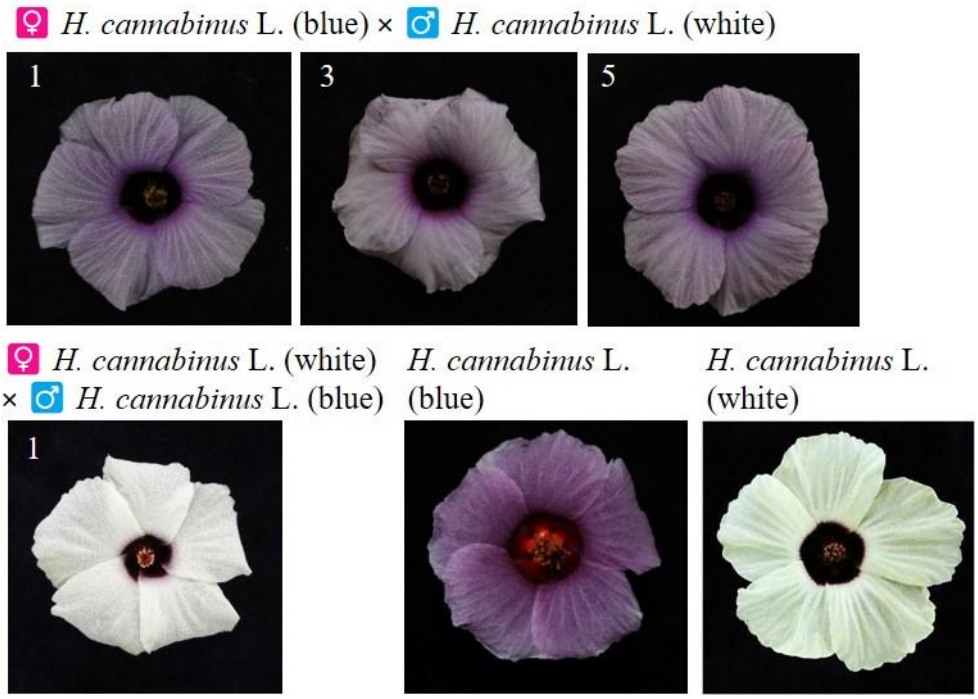

Figure 4. Flower morphology in crossing populations. Numbers in upper left corners represent individual numbers of progenies in each crossing.

Six progenies obtained by crossing *H. cannabinus* (blue flower type) and *H. acetosella* 'Black King' were examined using the marker specific to *H. acetosella* 'Black King' **(Fig. 3**). However, none of the progenies exhibited the band specific to *H. acetosella* 'Black King', suggesting that they are self-crossings of *H. cannabinus* L (blue flower type). The leaf shape of these progenies resembled that of *H. cannabinus* (blue flower type) (**Fig. 5**), which is consistent with the DNA marker results.

Nine progenies obtained by crossing *H. cannabinus* (blue flower type) and *H. mutabilis*. were examined using the marker specific to *H. mutabilis*. (**Fig. 3**). However, none of them exhibited the band specific to *H. mutabilis*, suggesting that they are self-crossings of *H. cannabinus* (blue flower type). The leaf shape of these progenies resembled that of *H. cannabinus* (blue flower type) (**Fig. 5**), which is consistent with the DNA marker results.

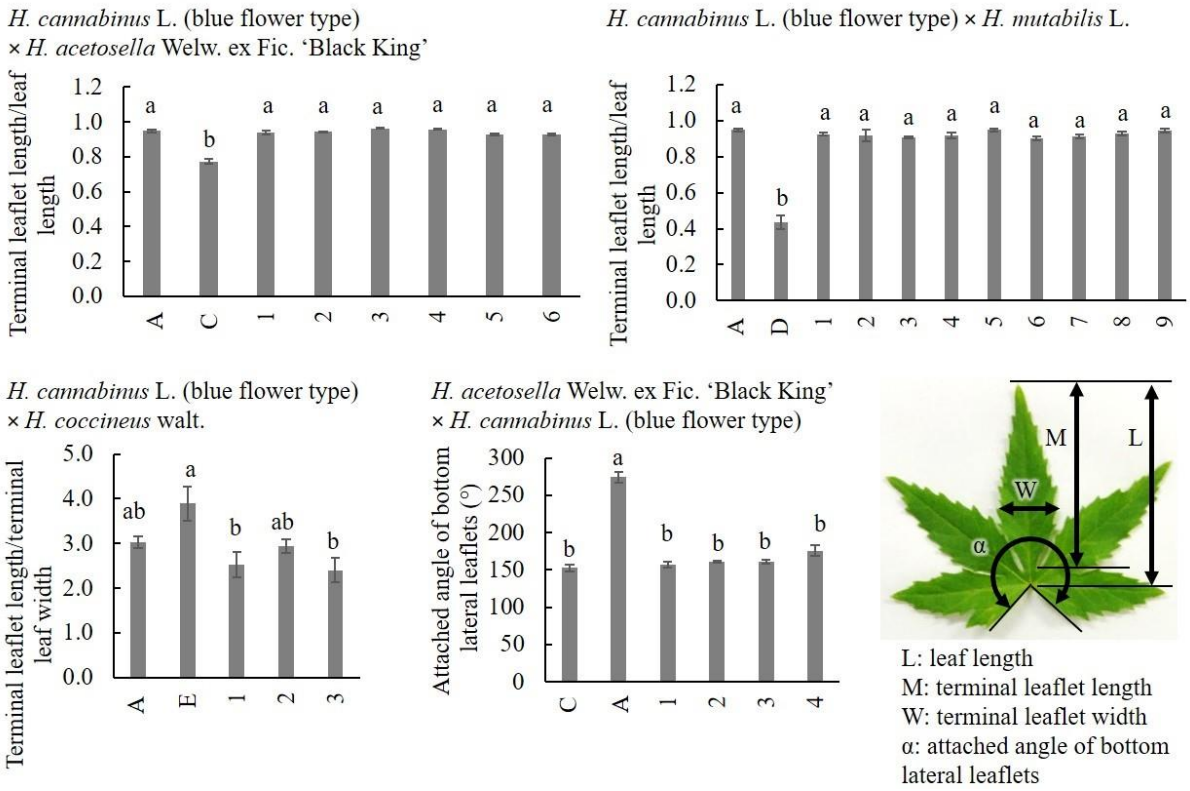

Figure 5. Leaf shape in crossing populations. A: *H. cannabinus* (blue flower type), C*: H. acetosella* 'Black King, D: *H. mutabilis*, E: *H. coccineus*. Numbers indicate individual numbers of progenies in each crossing. Error bars indicate standard deviation. Different lower-case letters in the same graph indicate a significant difference at $P < 0.05$ according to the Tukey–Kramer test.

Three progenies obtained by crossing *H. cannabinus*. (blue flower type) and *H. coccineus* were examined using the marker specific to *H. coccineus*. **(Fig. 3)**. However, none of them exhibited the band specific to *H. mutabilis*, suggesting that they are self-crossings of *H. cannabinus* (blue flower type). The leaf shape of these progenies resembled that of *H. cannabinus* L. (blue flower type) (**Fig. 5**), which is consistent with the DNA marker results.

Four progenies obtained by crossing *H. acetosella* 'Black King' and *H. cannabinus* (blue flower type) were examined using the marker specific to *H. cannabinus* (blue flower type) **(Fig. 3)**. However, none of them exhibited the band specific to *H. cannabinus* (blue flower type), suggesting that they are self-crossings of *H. acetosella*. 'Black King'. The leaf shape of these progenies resembled that of *H. acetosella* 'Black King' (Fig. 5), which is consistent with the DNA marker results.

In summary, this study established species-specific DNA markers for *H. cannabinus* (blue flower type), *H. cannabinus* (white flower type), *H. acetosella*. 'Black King', *H. coccineus* walt., and *H. mutabilis* The ability of these species-specific DNA markers to identify species was also confirmed by identifying the pollen parents of the progenies of crosses among the four *Hibiscus* species.

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