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Authors: Kondo, Toshiaki, Watanabe, Sonoko, Shiga, Takashi, and Isagi, Yuji

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Microsatellite Markers for *Nuphar japonica* (Nymphaeaceae), an Aquatic Plant in the Agricultural Ecosystem of Japan

Toshiaki Kondo\(^2\), Sonoko Watanabe\(^2\), Takashi Shiga\(^3\), and Yuji Isagi\(^4\)

\(^2\)Graduate School for International Development and Cooperation, Hiroshima University, Hiroshima, Japan; \(^3\)Faculty of Education, Niigata University, Niigata, Japan; and \(^4\)Graduate School of Agriculture, Kyoto University, Kyoto, Japan

**Premise of the study:** *Nuphar* species (Nymphaeaceae) are representative aquatic plants in irrigation ponds in Japanese agricultural ecosystems. We developed 15 polymorphic microsatellite markers for *N. japonica* and confirmed their utility for its close relatives *N. oguraensis* var. *akiensis* and *N. xaijoensis*, which originated from natural hybridization between *N. japonica* and *N. oguraensis*.

**Methods and Results:** Genetic variation was characterized in 15 polymorphic loci in three populations of *N. japonica*. The average number of alleles per locus was 3.47 (range = 2–9; \(n = 32\)), and the average expected heterozygosity per locus was 0.84 (range = 0.5–1.0); 11 loci were amplified in *N. oguraensis* var. *akiensis* and 15 in *N. xaijoensis*.

**Conclusions:** The polymorphic microsatellite markers developed in this study will be useful for investigating the levels of genetic diversity within remnant populations of *Nuphar* taxa and could provide a valuable tool for conservation genetics of these taxa.

**Key words:** aquatic plant species; cross-amplification; microsatellite; *Nuphar japonica*; Nymphaeaceae.

In recent years, aquatic plants growing in irrigation ponds, such as *Nuphar* species, are disappearing owing to land reclamation, repair work, water quality deterioration, and invasion of exotic species. Consequently, *N. oguraensis* is currently included in the Japanese Red Data Book (Ministry of the Environment, Japan, 2015). Thus, understanding the genetic diversity of *Nuphar* species will play a key role in its future management. Although microsatellite markers have been developed in two relatives of *N. japonica* (*N. lutea* [Ouborg et al., 2000] and *N. submersa* [Yokogawa et al., 2012]), their utility for *N. japonica* and *N. oguraensis* has been shown to be limited (Yokogawa et al., 2012). Therefore, we have developed polymorphic genomic microsatellite markers for use in genetic investigations of the three *Nuphar* taxa of the Saijo Basin.

**METHODS AND RESULTS**

We collected plant samples from three populations of *N. japonica* (Sawahara, Kouno, and Doinouchisako-shita ponds), one population of *N. oguraensis* var. *akiensis* (Rakan Pond), and one population of *N. xaijoensis* (Imori-shita Pond) in the Saijo Basin, Hiroshima Prefecture, Japan (Appendix 1); each population was from a separate pond. The geographic distance between individual ponds ranged from 2.3 to 8.4 km, with an average of 5.8 km. We selected Saijo Basin as the study site because it is unique in having three sympatrically distributed *Nuphar* taxa. Sample size was eight or 12 plants per population (48 plants in total). We allowed at least 10 m between sampled individuals to avoid duplicating samples from the same genet. Pond size and the clonal nature of the species led to small sample sizes for each population. Total genomic DNA was isolated from 30–50 mg of leaf tissue from each plant by using the DNA Suisui–VS extraction buffer (RIZO, Tsukuba, Ibaraki, Japan).

DNA extracted from one *N. japonica* plant collected in Kouno Pond was used for library preparation with a TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, California, USA). Sequencing was performed on a MiSeq.

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3 Author for correspondence: kondo@hiroshima-u.ac.jp

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Evaluation of genetic polymorphism within all 32 N. japonica plants showed that the 15 loci had moderate levels of polymorphism. The total number of alleles per locus in these populations ranged from two to nine (mean ± SE: 3.47 ± 0.48). Expected heterozygosity per locus was generally high, ranging from 0.50 to 0.78 (0.56 ± 0.02). At the population level, the number of alleles ranged from two to six (2.47 ± 0.13), observed heterozygosity from 0.50 to 1.00 (0.84 ± 0.02), and expected heterozygosity from 0.45 to 0.74 (0.54 ± 0.01). The levels of polymorphism were moderate in N. japonica and N. × saijoensis and relatively low in N. oguraensis var. akiensis (Table 2). Deviations from Hardy–Weinberg equilibrium in each population and linkage disequilibrium were tested using GENEPOP software, Web version 4.2 (Raymond and Rousset, 1995). Among all loci in all five populations, 11 locus–population combinations deviated significantly from Hardy–Weinberg equilibrium (P < 0.01; Table 2). Significant linkage disequilibrium (P < 0.05) was observed among six locus pairs in N. japonica populations (NJ03362 and NJ14763, NJ04340 and NJ14763, NJ04340 and NJ15714, NJ18322, NJ14763 and NJ15714, and NJ14763 and NJ18010). Such lack of equilibrium and significant linkage disequilibrium could be explained by the small number of samples in each population and clonal reproduction by rhizome growth in Nuphar taxa.

CONCLUSIONS

In this study, a total of 15 polymorphic microsatellite markers for N. japonica were developed. Eleven of them (five polymorphic) also amplified in N. oguraensis var. akiensis and 15 (14 polymorphic) amplified in N. × saijoensis. These markers will be useful both for investigating gene flow among the three taxa of Nuphar in the Saijo Basin and for determining the effects of habitat networks on levels of genetic diversity.
within the populations of these taxa. The results may have important implications for aquatic plant conservation and restoration.

**LITERATURE CITED**

ARONESTY, E. 2011. ca-utils: Command-line tools for processing biological sequencing data. Website https://expressionanalysis.github.io/ca-utils/ [accessed 26 October 2016].

BOLGER, A., M. LOMISE, and B. USHAEL. 2014. TRIMMOMATIC: A flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120.

COOK, C. D. K. 1996. Aquatic plant book. SPB Academic Publishing, Amsterdam, The Netherlands.

FAIRCLOTH, B. C. 2008. MSATCOMMANDER: Detection of microsatellite repeat arrays and automated, locus-specific primer design. Molecular Ecology Resources 8: 92–94.

MINISTRY OF THE ENVIRONMENT JAPAN, 2015. Red Data Book 2014–Threatened Wildlife of Japan, Vol. 8: Vascular Plants. Gyousei, Tokyo, Japan.

OUEBERG, N. I., W. P. GOODALL-CORREY, P. SAUMITOU-LAPRADE, I. BONNN, and J. T. EPFL. 2000. Novel polymorphic microsatellite loci isolated from the yellow waterlily, Nuphar lutea. Molecular Ecology 9: 497–498.

PAGETT, D. J. 2007. A monograph of Nuphar (Nymphaeaceae). Rhodora 109: 1–95.

PAGETT, D. J., M. SHIMODA, L. A. HORKY, and D. H. LES. 2002. Natural hybridization and the imperiled Nuphar of western Japan. Aquatic Botany 72: 161–174.

RAYMOND, M., and F. ROUSSET. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248–249.

ROZEN, S., and H. SKALETSKY, 1999. Primer3 on the WWW for general users and for biologist programmers. In S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SHIGA, T. 2007. A systematic study of Nuphar (Nymphaeaceae) in Japan with special reference to the role of hybridization. Ph.D. dissertation, Kobe University, Kobe, Japan.

SHIGA, T., J. ISHI, Y. ISAI, and Y. KADONO. 2006. Nuphar submersa (Nymphaeaceae), a new species from central Japan. Acta Phytotaxonomica et Geobotanica 57: 113–122.

SHIGA, T., and Y. KADONO. 2015. Nuphar saikoakensis (Nymphaeaceae), a new species from central to western Japan. Journal of Japanese Botany 90: 20–26.

SHIMIZU, T., and K. YANO. 2011. A post-labeling method for multiplexed and multicolored genotyping analysis of SSR, indel and SNP markers in single tube with bar-coded split tag (BStag). BMC Research Notes 4: 161.

YOKOGAWA, M., T. SHIGA, S. KANeko, and Y. ISAI. 2012. Development of nuclear microsatellite markers for the critically endangered freshwater macrophyte, Nuphar submersa (Nymphaeaceae), and cross-species amplification in six additional Nuphar taxa. Conservation Genetics Resources 4: 295–298.
**APPENDIX 1.** Voucher information for populations of *Nuphar* species used in this study. One voucher was collected from each population sampled.

| Species                                      | Pond name*                      | Geographic coordinates | Voucher collection no. b |
|----------------------------------------------|---------------------------------|------------------------|-------------------------|
| *N. japonica* DC.                           | Sawahara                        | 34°24′11″N, 132°44′13″E | Watanabe0001            |
| *N. japonica*                               | Kouno                           | 34°25′24″N, 132°41′24″E | Watanabe0002            |
| *N. japonica* Miki var. *akiensis* Shimoda   | Doinouchisako-shita             | 34°21′42″N, 132°44′04″E | Watanabe0003            |
| *N. oguraensis* Shimoda                     | Rakan                           | 34°26′11″N, 132°44′20″E | Watanabe0004            |
| *N. ×saijoensis* (Shimoda) Padgett & Shimoda| Imori-shita                     | 34°21′54″N, 132°42′32″E | Watanabe0005            |

*Populations are located in the Saijo Basin, Hiroshima Prefecture, Japan.

b All vouchers were deposited in the Herbarium of the Graduate School for International Development and Cooperation, Hiroshima University, Hiroshima, Japan.