Article

Allozyme variation of *Nuphar subintegerrimum* (Nymphaeaceae) at Kita-Nagaike pond, Kakogawa City, Hyogo Prefecture, Japan.

Takeshi SUZUKI*, Keiko MURAYAMA**, Yasuro KADONO** & Teruto NAGAYOSHI*

Abstract

A natural population of *Nuphar subintegerrimum* in an irrigation pond was threatened by highway construction and its transplantation was planned. Allozyme variations of 60 plants belonging to three separate patches were examined in order to make a proper design assessment of transplantation including the conservation of genetic diversity of the population. Based on the variations of *Pgi-2* and *Tpi-3*, a total of four genotypes were recognized. The distribution patterns of each genotype indicated the dominance of vegetative reproduction by creeping rhizomes. However occurrence of different genotypes in one population suggested occasional establishment of seedlings produced sexually. Duplicated isozyme number of PGI and TPI suggested that *N. subintegerrimum* is genetically polyploid.

Key Words: Allozyme, clonal propagation, genetic diversity, *Nuphar subintegerrimum*

Nuphar subintegerrimum (Casp.) Makino (Photo. 1) is a perennial aquatic plant endemic to Japan (Kadono, 1994) . It grows in irrigation ponds and slow-flowing rivers in central to western Japan. Recently, however, its population has decreased due to reclamation, repairment construction and water pollution and been listed as an endangered species in Red Data Books (JPRDBC, 1989; Hyogo Pref., 1995).

In 1996, a relatively large population of *N. subintegerrimum* (ca.0.2 ha) was found to exist in the central area of Kita-Nagaike Pond, an irrigation pond for rice fields, located in Kakogawa City, Hyogo Prefecture, Japan (N34° 51', E134° 50'; ca. 1.3 ha, depth ca.2m) (Harima Wetland Research, unpublished) . However, in 1996, this pond was dried and dredged for construction of the Sanyo Highway, and a large part of the population was under the threat of extinction. In May of 1996, Kakogawa City Hall and the Himeji Office of Japan Highway decided to help conserve the population of *N. subintegerrimum* by temporarily transplanting the plants into other ponds and restoring the population after the construction was completed.

For the conservation of endangered species, it is important to preserve genetic diversity (Hamrick et al., 1991) . At the start of the present study, the population of *N. subintegerrimum* in Kita-Nagaike Pond consisted of three patches, apparently including more than 1000 plants. It was difficult, however, to distinguish each individual because *N. subintegerrimum* reproduce vegetatively by the extension of underground rhizomes. So it was necessary to distinguish "clones" to know the genetic diversity of the population. Allozyme analysis is a useful method to detect genetic variation and is often applied to reveal clonal variation.

The purpose of the present study was to estimate the genetic variation of *N. subintegerrimum* in Kita-Nagaike Pond by allozymes and to propose a proper design of transplantation to maintain the maximum genetic variation of the population.

Materials and methods

Leaves were collected from 60 plants belonging to the three patches; 35, 8, 17 plants from patches A, B and C, respectively (Fig.1) . Collections were made at intervals of more than 1 m. Samples were kept cool in plastic bags until the experiment.

Leaves were ground with a mortar and pestle, using grinding buffer after Gottlieb (1981) : 0.1M Tris-HCl (pH 7.5) , 10mM KCl, 10mM MgCl2, 1mM

*Division of Biological Resources, Museum of Nature and Human Activities, Hyogo, Yayoigaoka 6, Sanda , 669-13 Japan

**Department of Biology, Faculty of Science, University of Kobe, Tsurukabuto 1-2-1, Nada-ku, Kobe 657 Japan

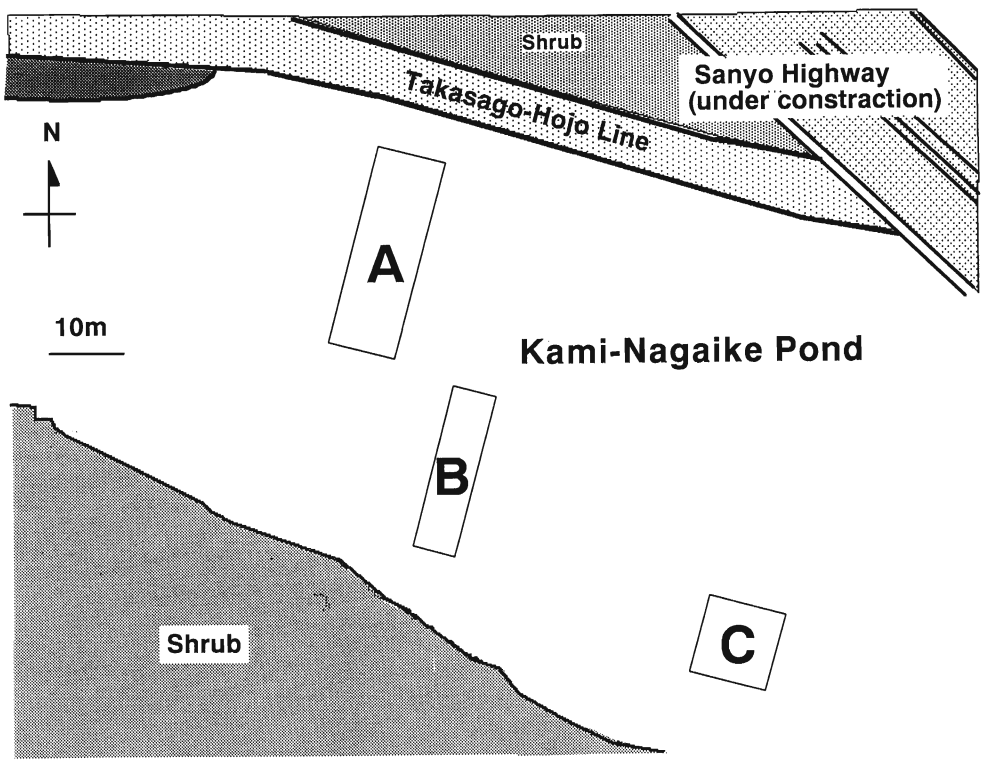

Fig. 1 Location of three patches of *N. subintegerrimum*. bar=10m

EDTA/4Na, 5% (w/v) PVP (Sigma 40T) and 0.2% (v/v) 2-mercaptoethanol. Crude extracts were centrifuged, and supernatants were used for electrophoresis.

Starch gel electrophoresis with a buffer system of histidine-citrate, pH6.5(Cardy et al., 1981) was employed. The following enzymes were stained: Malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), Phosphoglucoisomerase (PGI), Phosphoglucomutase (PGM), Triose-phosphate isomerase (TPI). Staining recipes were taken from Soltis et al. (1983).

Genetic interpretations of zymograms were referred from isozyme number and subunit structures of each enzyme (Gottlieb, 1982; Weeden and Wendel, 1989). Isozymes were numbered sequentially, beginning with the most anodally migrating one; allozymes were labeled alphabetically, also beginning with the most anodal form. The numbers and letters corresponded to inferred encoding alleles.

Results and discussion

Allozyme variations were observed and were interpreted in PGI and TPI. MDH and PGM were monomorphic. 6PGD were not interpretable.

PGI (Fig.2). This enzyme showed two regions of activity in *N. subintegerrimum*, as in other plant species (Gottlieb, 1982; Weeden and Wendel, 1989). More anodal region (PGI-1) was not clear. Variations were detected in more cathodal region: two patterns of three-band and one pattern of six-band. Based on dimeric structure of this enzyme, six-band pattern represented three kinds of alleles in two loci. Most cathodal band was expressed consistently, which suggests that *Pgi-3* (corresponding to more cathodal isozyme, PGI-3) were monomorphic. Central bands on three-band patterns were interpreted to be heterodimeric bands between anodal PGI-2, and cathodal PGI-3. Accordingly, we assumed that *Pgi-2* were polymorphic with two alleles and that six-band pattern showed heterozygous genotype in *Pgi-2*.

TPI (Fig.2): TPI is a dimeric enzyme typically with two compartmentalized isozymes (Gottlieb, 1982; Weeden and Wendel, 1989). This was the case in *N. subintegerrimum* of the present study. More anodal isozyme, TPI-1, were monomorphic. In more cathodal region, two isozymes, TPI-2 and TPI-3, were assumed as was in case of PGI. *Tpi-2* were monomorphic. In *Tpi-3*, two alleles were detected.

Isozyme duplication in more cathodal regions of PGI and TPI suggests that *N. subintegerrimum* is genetically polyploid, although the chromosome number of 2n=34 has been reported and was interpreted to be diploid based on x=17 in *N. subintegerrimum* (Okada and Tamura, 1981). Further study is necessary for chromosome number and gene duplication of more enzyme loci in *N. subintegerrimum* and other *Nuphar*.

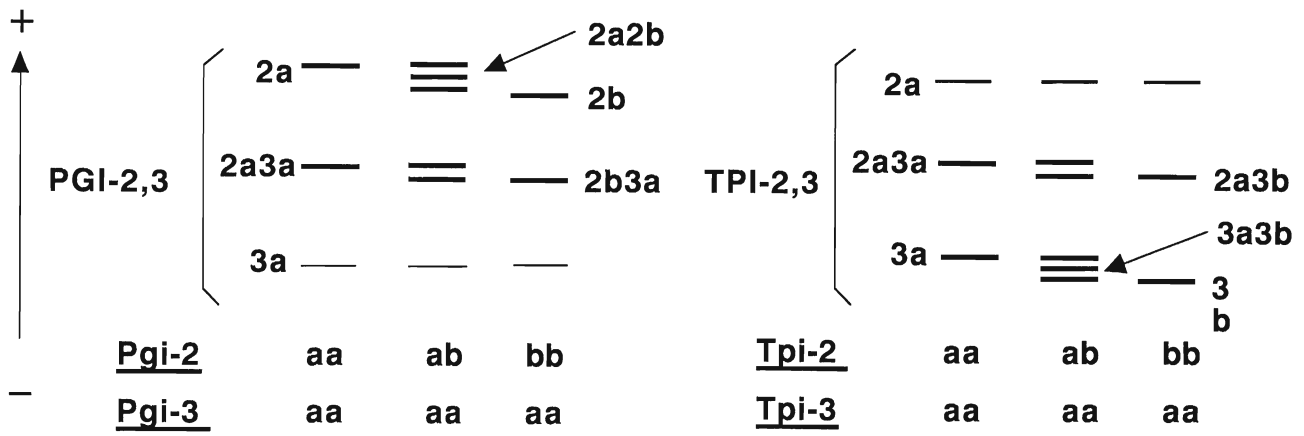

Fig. 2 Schematic zymograms of PGI (left) and TPI (right). See text for details.

Based on the allozyme variation of two loci, *Pgi-2* and *Tpi-3*, four genotypes were recognized in total. The distribution of each genotype in the three patches is shown in Fig.3. Patch A was comprised of the plants belonging to three genotype. The plants of *Pgi-2*^{ab}/ *Tpi-3*^{ab} occupied the northern half of the patch and the plants of *Pgi-2*^{aa}/*Tpi-3*^{ab} the southern half. The genotype *Pgi-2*^{bb}/ *Tpi-3*^{bb} was represented by only one sample that was growing near the center of patch. All the plants in patch B belonged to the genotype *Pgi-2*^{aa}/ *Tpi-3*^{ab} and patch C was exclusively composed of plants of the genotype *Pgi-2*^{aa}/ *Tpi-3*^{aa}.

These patterns of distribution of each genotype suggest the mode of colonization in this population of *N. subintegerrimum*. All samples from the patch B were represented by only one genotype with heterozygous *Tpi-3*^{ab}. If reproduced by sexually produced seeds in this patch, genotype segregation is expected. Genotypic uniformity suggests that they are most probably a clone vegetatively reproduced by creeping rhizomes. Thus far, no agamospermous seed formation has been recognized in *Nuphar*. The same situation occurred in the northern and southern halves of patch A. The southern and northern part of the patch were represented by two different clones respectively.

In contrast, the presence of four genotypes with different combination of alleles indicates the occasional occurrence of establishment from sexually produced seeds. One plant of *Pgi-2*^{bb}/*Tpi-3*^{bb} in patch A must have derived from a seedling. Homogeneity of *Pgi-2*^{aa}/ *Tpi-3*^{aa} in patch C is consistent with seed production and/or vegetative propagation.

In *Nuphar* populations, establishment from seedlings is rare and vegetative reproduction by rhizome extension is of greater importance (Heslop-Harrison, 1955; Barrat-Segretain, 1996). We confirmed that the plants isolated by more than 2 m connected to each other by a well-developed rhizome system (unpublished data). Although the flowers of *N. subintegerrimum* produce numerous seeds, our study indicates the dominance of vegetative reproduction and minimal role of seeds in the maintenance of the population of *N. subintegerrimum*. It should be noted that there is always the chance for them to establish new genotypes. Extensive survey of genetic variation among populations as well as in one population should be an interesting subject to evaluate the roles of sexual and vegetative reproduction in the colonization dynamics of *Nuphar* plants.

In the present study, it was proposed to include all four genotypes in order to conserve the genetic diversity of the population in a trial transplantation. They were transplanted to nearby pond in July, 1996 (Photo.2). From patch B and C, approximately 20 and 100 plants were transported, respectively. Regarding patch A, 50 plants from the north of the patch and 50 plants from the south, and ten plants around one plant of *Pgi-2*^{bb}/*Tpi-3*^{bb} were moved to another pond.

Acknowledgements

The authors thank Messrs. S. Matsumoto, K. Imadu, Y. Takashima for unpublished data for this pond, Mr. M. Yasukawa, Himeji office of Japan Highway, Messrs. H. Ishihara and F. Ito, Toyo-Kaneshita JV, for their helps for sampling and maintaining the natural population.

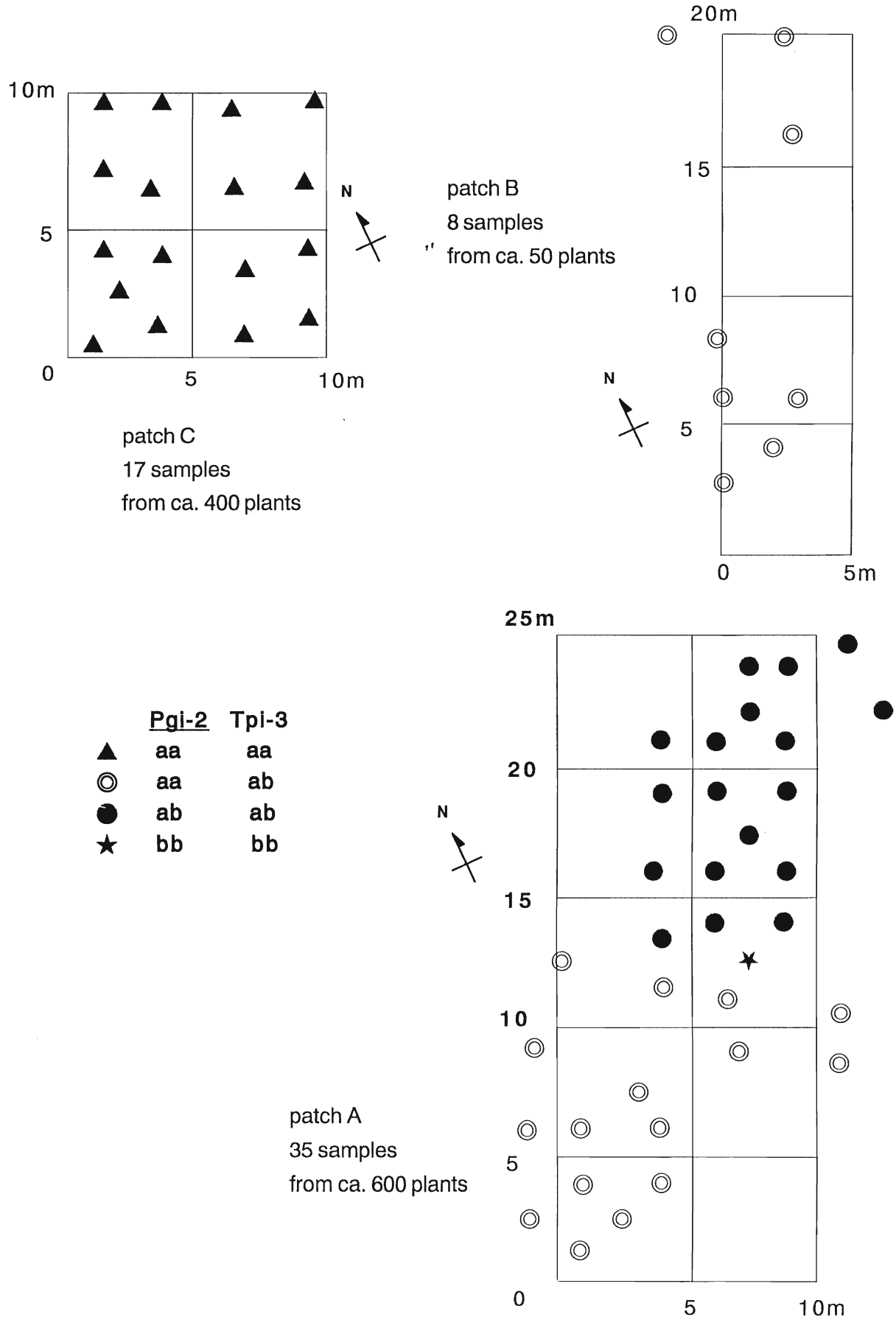

Fig. 3 Distribution of four genotypes of *N. subintegerrimum* in Patches A, B and C

References

Barrat-Segretain, M. H. (1996) Germination and colo-nization dynamics of *Nuphar lutea* (L.) Sm. in a form-er river channel. *Aquat. Bot.* 55: 31-38.

Cardy ,B.J., C.W.Stuber and M.N.Goodman (1981) Techniques for Starch Gel Electrophoresis of Enzymes from maize (*Zea mays* L.) . Inst. Statistics Mimeo-graph Series No1317 North Carolina State Univ., Raleigh, N.C.

Gottlieb, L.D. (1981) Elecrophoretic Evidence and Plant Populations. *Prog. Phytochem.* 7: 1-45

Gottlieb, L.D. (1982) Conservation and Duplication of Isozymes in Plants. *Sciencs* 216: 373-380

Heslop-Harrison, Y. (1955) *Nuphar* Sm. *J. Ecol.* 43: 342-364.

JPRDBC (Japan Plant Red Data Book Committee) (1989) Current Status of Endangered Species in Japan. Nature Conservation Society of Japan and World wild Fund for Nature Japan, Tokyo (in Japanese).

Harmrick, J.L., M.J.W. Godt, D.A. Murawski and M.D.Loveless (1991) Correlations between species traits and allozyme diversity: implications for conser-vation biology. In D.A.Falk and K. E.Holsinger (eds.) , *Genetics and conservation*, Oxford University Press, New York, USA

Hyogo Pref. (1995) Red Data Book in Hyogo Prefecture. (in Japanese)

Kadono, Y. (1994) Water Plants of Japan. Bunichi Sogo Shuppan, Tokyo (in Japanese).

Okada, H. and M. Tamura (1981) Karyomorphological study on the Nymphaeales. *Journ. Jap Bot.* 56: 367-374.

Soltis,D.E., C.H.Haufler, D.C.Darrow, and G.J. Gastony. (1983) A Compilation of Grinding Buffers, Gel and Electrode Buffers and Staining Schedules. *Am. Fern J.* 73: 9-27

Weeden, N.F. and J.F.Wendel (1989) Genetics of Plant Isozymes. In D.E.Soltis and P.S.Soltis eds., *Isozymes in Plant Biology*, Discorides Press, Portland, Oregon,USA, PP.46-72

Received: December 6, 1996
Accepted: January 30, 1997

Photo. 1 *Nuphar subintegerrimum* at Kita-Nagaike Pond, Kakogawa City in Hyogo Pref.

Photo. 2 Transplantation of *N. subintegerrimum* to other pond