Karyotype and RAPD Analysis to Elucidate Taxonomic Status in Two Morphological Forms of *Egeria densa* Planch. and *Hydrilla verticillata* (L.f.) Royle.

Nusrat Sultana, Syeda Sharmeen Sultana, and Sheikh Shamimul Alam*

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

Received February 27, 2013; accepted August 10, 2013

**Summary**  Three aquatic angiospermic plants, namely, *Hydrilla verticillata* (L.f.) Royle and two morphological forms of *Egeria densa* Planch. (dense leaf form and less dense leaf form), were studied cytogenetically and at the molecular level by using RAPD with five primer combinations. *Hydrilla verticillata* (2n=24) and *Egeria densa* (dense leaf form, 2n=16) showed distinct bimodal karyotypes. The smaller chromosome groups were metacentric in both the plants. The range of individual chromosome length for both bigger and smaller chromosomes groups was almost similar in these two plants. The total length of chromosome complement was about 1.5 times bigger in *Hydrilla verticillata* than in *Egeria densa* (dense leaf form). On the basis of RAPD finger-printing, these two plants were placed in the same cluster with very narrow genetic distance. This suggests that *Hydrilla verticillata* is actually an autotriploid of *Egeria densa* (dense leaf form). In contrast, the two forms of *Egeria densa* (dense leaf and less dense leaf form) were totally genomically different. *Egeria densa* (dense leaf form) was found to possess 2n=16 chromosomes, whereas 2n=24 chromosomes were found in *Egeria densa* (less dense leaf form). The "dense leaf form" has a distinct bimodal karyotype. In contrast, the "less dense leaf form" has a monomodal karyotype. The range of individual chromosome length of these two forms was also different. In *Egeria densa* (less dense leaf form), a pair of AT-rich secondary constriction bearing chromosomes was present, which was totally absent in the other form. Each specimen possessed distinct CMA-banding karyotypes. The RAPD fingerprinting placed these two forms in different clusters with a large genetic distance. The cytogenetical and RAPD data did not support placing the "dense leaf form" and "less dense leaf form" under the same species of *Egeria densa*. Therefore, a peer revisions for the taxonomic status of these three aquatic angiosperm is necessary.

**Key words**  Fluorescent karyotype, *Hydrilla verticillata*, *Egeria densa*, RAPD.

*Egeria densa* Planch. and *Hydrilla verticillata* (L.f.) Royle both belong to the family Hydrocharitaceae. They are submerged perennial species that usually root in mud. They are similar in appearance and have been recorded as weed. Early misidentification was reflected in the confusion of common names in the USA. For example, *Hydrilla verticillata* was called Florida Elodea, and *Egeria densa* was called Brazilian Elodea. Both have herbaceous stems and oblong to linear leaves in whorls of two to eight. *Egeria* and *Hydrilla* have quite different flowers, but are vegetatively very similar (Bowmer et al. 1995).

Inspite of vast similarities, these two species show some morphological differences. *Egeria* typically looks larger and leafier than *Hydrilla*. In the former genus, the majority of biomass is located near the water surface. The leaves are curved downwards in *Egeria*, while *Hydrilla* leaves are narrow and straight. No tuber formation occurs in *Egeria*, but potato-like tubers are found

*Corresponding author, e-mail: ssalam81@yahoo.com  
DOI: 10.1508/cytologia.78.277
in *Hydrilla*, which remain attached to the roots in the mud. Double nodes are present in *Egeria*, which produce lateral buds, branches and adventitious roots. The double nodes are typically spaced along the stems at 6–12 node intervals (Cook and Urmii-König 1984). Only fragments with a double node develop into new plants. In *Hydrilla*, double nodes are absent.

The occurrence of *Egeria densa* has not yet been recorded in the “Encyclopedia of Flora and Fauna of Bangladesh” (Siddiqui et al. 2007). However, a plant material has recently been collected from a remote natural lake called “Bogakain” situated in the hilly Bandarban district of Bangladesh, through a limnological expedition carried out by Khondker et al. (2010). In this expedition, they collected two morphological forms of *Egeria densa*: (i) dense leaf form and (ii) less dense leaf form. Both the materials have been maintained along with the locally collected *Hydrilla verticillata* in the Botanic garden, Department of Botany, University of Dhaka for more than three years. It has been noticed that these two forms sustained their characteristics, revealing that the characters are genetically controlled rather influenced environmentally. Therefore, an identification problem between the two forms of *Egeria densa* still exists.

It has been well established that where the morphological features are not sufficient, cytogenetical parameters often help to solve the identification problem (Alam et al. 2000). Karyotype analysis is one of the best cyogenetical parameters because it is stable, reliable, and specific to each individual organism. In addition to classical karyotype analysis, modern techniques for kryotype analysis have emerged in the last few decades. Fluorescent karyotype analysis with two common fluorochromes such as chromomycine A3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI) has been found suitable for characterizing karyotypes (Schweizer 1976, Kondo and Hizume 1982, Alam et al. 1998). With the help of the above mentioned techniques it was possible to characterize karyotypically the three forms of *Colocasia esculanta* (Alam and Deen 2002), two forms of *Typhonium trilobatum* (Huq et al. 2007), two forms of *Xanthosoma violaceum* (Deen and Alam 2002), three forms of *Colocasia fallax* (Begum and Alam 2009), and three forms of *Typhonium trilobatum* (Warasy and Alam 2009). Moreover, Sultana and Alam (2007) clearly distinguished between *Solanum nigrum* and *Solanum villosum* with the help of fluorescent chromosome banding.

DNA finger-printing by randomly amplified polymorphic DNA (RAPD) is another method for characterizing germplasms. The term DNA finger-printing/profiling describes the combined use of several single locus detection systems. This method has been used as versatile tool for investigating various genomic aspects of an organism. It includes characterization of genetic variability, genome finger-printing, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, etc. Genetic analysis using molecular marker technologies can provide a powerful approach to understanding the organization and distribution of genetic resources in nature and to manage populations. At the species level, the identification of taxonomic units and the determination of the uniqueness of a species are essential information for conservation, systematic, ecological, and evolutionary studies (Schierwater et al. 1994, Williams et al. 1990).

For the above reasons, a combined study of cytogenetical and molecular analysis has been undertaken for authentic identification of two forms of *Egeria densa* and *Hydrilla verticillata*. The aims of the present study was to:

1. find out the $2n$ chromosome number of these specimens,
2. compare the karyotypes after staining with Orcein, CMA and DAPI,
3. make a phylogenetic tree based on RAPD finger-printing, and
4. elucidate the taxonomic rank of these three specimens.

**Materials and methods**

Three specimens, *viz.* *Hydrilla verticillata* (L.f.) Royle, *Egeria densa* Planch. (dense leaf form), and *Egeria densa* Planch. (less dense leaf form), were investigated in this study. The two
morphological forms of *Egeria densa* Planch. were collected from the “Bogakain lake,” Bandarban district, Bangladesh. However, *Hydrilla verticillata* (L.f.) Royle was collected from around Dhaka city. These plants have been maintained in the Botanic garden, Department of Botany, University of Dhaka for more than three years.

**Cytogenetical study**

Healthy roots were collected and pretreated with 0.002 M 8-hydroxyquinoline for 4 h at 18°C followed by 30 m fixation in 45% acetic acid at 4°C. These were then hydrolyzed in a mixture of 1 N HCl and 45% acetic acid (2:1) at 60°C for 35 s. The root tips were stained and squashed in 1% aceto orcein. For CMA-banding, Alam and Kondo’s (1995) method was used with slight modification. After hydrolyzing and dissecting, the materials were squashed with 45% acetic acid. The cover glasses were removed quickly from dry ice and allowed to air dry for at least 24 h before analysis. The air-dried slides were first pre-incubated in McIlvaine’s buffer (pH 7.0) for 30 m, followed by a Distamycin A (0.1 mg/ml) treatment for 10 m. The slides were rinsed mildly in McIlvaine’s buffer supplemented with MgSO₄ (5 mM) for 15 m. One drop of CMA (0.1 mg/ml) was added to the materials for 15 m in a humid chamber and then rinsed with McIlvaine’s buffer with Mg²⁺ for 10 m. Slides were mounted in 50% glycerol and kept at 4°C for overnight before observation. These were observed under a Hund (WETZLAR) fluorescent microscope with a blue violet (BV) filter cassette.

**DNA isolation**

The leaves were harvested and their total genomic DNA was extracted by using a modified CTAB method (Doyle and Doyle 1987). The DNA concentration was quantified through a spectrophotometer (Analylykjen, Specord 50, Germany). The A 260/280 readings for DNA samples were 1.6–1.8.

**PCR amplification and primer survey**

The PCR reaction mixture contained 2 µl of the template DNA (25 ng), 18.8 µl of de-ionized distilled water, 2.5 µl of Taq buffer A 10X (Tris with 15 mM MgCl₂), 1.0 µl of primer (10 µM), 0.5 µl of dNTPs (2.5 mM), and 0.2 µl of Taq DNA polymerase (5 U/µl) for a total of 25 µl. PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denature at 94°C for 5 m, followed by denature at 94°C for 1 m, annealing at 36°C for 30 s, extension at 72°C for 3 m, and final extension at 72°C for 5 m. Five primers were used from Operon Technologies, USA: primer-1 (5′-GAA ACG GGT G-3′), primer-3 (5′-TGC CGA GCT G-3′), primer-8 (5′-GTC CTC GTA G-3′), primer-9 (5′-AAC GCG TAG A-3′), and primer-15 (5′-CCT TCC CTC T-3′) series.

**Gel electrophoresis**

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g of agarose powder containing 8 µl of ethidium bromide and 100 ml of 1×TAE buffer. Agarose gel electrophoresis was conducted in 1×TAE buffer at 50 V and 100 mA for 1.5 h. The DNA ladder (1 kb) was electrophoresed alongside the RAPD reactions as a marker. DNA bands were observed on a UV-transilluminator and photographed by a gel documentation system.

**Scoring and data analysis**

The PCR products were analyzed after gel electrophoresis. The photographs were critically examined for the presence (1) or absence (0), size of bands, and overall polymorphism of bands. These were carried out for further investigation. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei’s (1972) gene diversity, and genetic distance (D), and for constructing a UPGMA
Bimodality

In *Egeria densa* (dense leaf form), 2n=16 chromosomes were observed (Fig. 2). In this form, two distinct groups of chromosomes were found. One group contained five pairs of bigger acrocentric chromosomes. The range of individual chromosome length in this group was 3.76–5.26 μm. Gradual decrease in chromosomal length was observed. In contrast, the chromosomes of the other group were much smaller, and contained three pairs of metacentric chromosomes. The range of individual chromosome length of this group was 1.00–1.96 μm (Table 1). No gradual decrease in chromosome length was found in this group (Fig. 8).

In *Hydrilla verticillata*, 2n=24 chromosomes were found (Fig. 1). Two distinct groups of chromosomes were observed: one group with 15 larger chromosomes and another group with nine much smaller chromosomes. Each group has an uneven chromosome number. The morphology of the chromosomes in each group suggests arranging three chromosomes to make a homologue (Fig. 7). The range of individual chromosome length was 3.15–4.70 μm and 1.11–1.79 μm in the bigger and the smaller groups, respectively. The centromeric formula was 11ac+3sm+1m in the bigger group and 9m in the smaller group.

This feature clearly suggests the bimodal karyotype of these two specimens. Langeland (1989) reported eight groups of chromosomes in *Hydrilla verticillata*, in which there were 15 long and 9 short chromosomes. However, he did not mention the bimodality of the karyotype. According to Stebbins (1971), the bimodal karyotype is an extreme form of asymmetric karyotype, which ultimately indicates an advanced nature.

Meanwhile, no such bimodality of karyotype was found in *Egeria densa* (less dense leaf form) (Figs. 3, 9). Therefore, *Egeria densa* (dense leaf form) and *Hydrilla verticillata* are much more advanced than *Egeria densa* (less dense leaf form).

Karyotype of *E. densa* (less dense leaf form)

*Egeria densa* (less dense leaf form) contained 2n=24 chromosomes (Fig. 3). A gradual decrease in chromosome length was observed. No distinct group on the basis of chromosome length was observed (Fig. 9). The range of individual chromosome length was 1.49–3.6 μm. The centromeric formula of the chromosome was 10m+8sm+6ac. The total length of diploid complements of this specimen was 60.34 μm. In *Egeria densa* (less dense leaf form), a prominent secondary constriction was found on the long arm of each chromosome in pair II (Fig. 9). In CMA staining, this portion did not fluoresce well but the constriction was clearly observed (Fig. 12). This indicates that the secondary constrictions of this pair are rich in GC-repeats (Schweizer 1976). These chromosomes could be used as a marker chromosome of this specimen.

CMA banding pattern

In *Egeria densa* (dense leaf form), five chromosomes in the bigger group showed different CMA bands. A band was found on the terminal end of the long arm in a member of pair II. No such band was found in its homologue member (Fig. 11). The entire long arm of both the members of pair III showed a very thick CMA band. Both of the members of pair V fluoresced entirely with CMA. No bands were found in the smaller group in this specimen. The percentage of GC-rich segment was more than three times that of *E. densa* (less dense leaf form).

In comparison, four CMA bands were found in *Egeria densa* (less dense leaf form). Two bands were found on the upper arm that occupied almost the respective arms of the short arm in
A CMA positive band was found at the terminal region of a member in pair II. A thick band was found at the terminal region of the long arm in a member of pair VII. No such band was found in other homologue members (Fig. 12). This feature indicates the deletion of GC-rich repeats from the respective locus of their homologue members.

Therefore, the two forms of *E. densa* have different types of CMA-banding pattern. In contrast, no CMA band was found in *Hydrilla verticillata*.

**Karyotype similarity between Egeria densa (dense leaf form) and Hydrilla verticillata (L.f.) Royle**

Although these specimens belong to different genera, they show tremendous karyotype similarity as listed below.

i. Both of these specimens have bimodal karyotypes (Figs. 7, 8).

ii. The individual chromosomal lengths in the bigger- and smaller groups are similar in both specimens (Table 1).

iii. A gradual decrease in chromosomal length was observed in the bigger group but not in the smaller group for both specimens (Table 1).

iv. All the bigger chromosomes were acrocentric in *Egeria densa* (dense leaf form) and almost acrocentric (11 out of 15) in *Hydrilla verticillata* (Table 1, Figs. 7, 8).

v. The chromosomes of the smaller group were all metacentric in both specimens (Table 1).

vi. No secondary constriction was found in these two specimens.

The above data indicates close affinity between these two specimens.

**RAPD fingerprinting similarity between Egeria densa (dense leaf form) and Hydrilla verticillata (L.f.) Royle**

Five different primer combinations were used for RAPD fingerprinting pattern. *viz.* primer-1, primer-3, primer-8, primer-9, and primer-15. No bands were found in primer-15 in both cases, indicating the lack of 5'-CCT TCC CTC T-3' sequence in their genomes (Fig. 17, Table 1).

These two plants showed some common DNA fragments in the remaining four primer combinations (Figs. 13–16, Table 1). On the basis of RAPD fingerprinting, a phylogenetic dendrogram was produced (Fig. 18). These two plant specimens showed a very narrow genetic distance (maximum affinity), and therefore were placed in the same cluster.

**Table 1.** Comparative orcein and CMA-karyotype analysis of three specimens: *Hydrilla verticillata* (L.f.) Royle, *Egeria densa* Planch. (-dense leaf form), and *Egeria densa* Planch. (-less dense leaf form).

<table>
<thead>
<tr>
<th>Specimens</th>
<th>2n</th>
<th>Range of chromosomal length (μm)</th>
<th>Centromeric formulae</th>
<th>Total length of 2n chromosome complements (μm)</th>
<th>No. of satellites</th>
<th>No. of CMA bands</th>
<th>% of CMA bands</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hydrilla verticillata</em></td>
<td>24</td>
<td>Bigger group-15: 3.15–4.70</td>
<td>11ac+3sm+1m</td>
<td>74.65</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smaller group-9: 1.11–1.79</td>
<td>9m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Egeria densa</em> (dense leaf form)</td>
<td>16</td>
<td>Bigger group-10: 3.76–5.26</td>
<td>10ac</td>
<td>53.66</td>
<td>–</td>
<td>5</td>
<td>13.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smaller group-6: 1.00–1.96</td>
<td>6m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Egeria densa</em> (less dense leaf form)</td>
<td>24</td>
<td>1.49–3.61</td>
<td>10m+8sm+6ac</td>
<td>60.34</td>
<td>2</td>
<td>4</td>
<td>4.6</td>
</tr>
</tbody>
</table>

m=metacentric chromosome, sm=sub-metacentric chromosome, ac=acrocentric chromosome
2013 Karyotype and RAPD Analysis of *Egeria densa* and *Hydrilla verticillata* 283

What exactly are *Hydrilla verticillata* and *Egeria densa* (dense leaf form)?

*Egeria densa* (dense leaf form) was found to possess $2n=2x=16$ chromosomes with a sharp bimodal karyotype consisting of 10 bigger and 6 smaller chromosomes (Fig. 8). That is, the genome of this specimen would be $x=8$ (5 bigger and 3 smaller chromosomes). In contrast, *Hydrilla verticillata* was found to possess $2n=24$ chromosomes. It has also a distinct bimodal karyotype consisting of 15 bigger and nine smaller chromosomes (Fig. 7), which is five bigger and three smaller chromosomes more than the *Egeria densa* (dense leaf form). This indicates that an extra set of eight chromosomes (five bigger and three smaller) was present in the karyotype of *Hydrilla verticillata* (Fig. 7). The total length of the $2n$ chromosome complement of *Hydrilla verticillata* ($74.65 \mu m$) is about 1.5 times bigger than that of *Egeria densa* ($53.66 \mu m$) (Table 1). In addition, these two specimens showed tremendous similarity in karyotype features and DNA finger-printing pattern.

The above results clearly suggest that *Hydrilla verticillata* is an autotriploid of *Egeria densa* (dense leaf form).

Do the two forms (dense leaf form and less dense leaf form) of *Egeria densa* belong to the same species?

Although the morphological features placed these two specimens under the same species (Khondker *et al.* 2010), sharp genomic differences were observed between the two specimens as listed below.

i. The “dense leaf form” was found to possess $2n=16$ chromosomes whereas $2n=24$ chromosomes were observed in the “less dense leaf form.”

ii. The “dense leaf form” has a distinct bimodal karyotype. However, no such bimodality was found in the “less dense leaf form.”

iii. In the “less dense leaf form,” a pair of chromosomes (pair II) showed prominent secondary constriction on the long arm. No such chromosomes were found in the “dense leaf form” (Fig. 12).

iv. The two forms differed in respect of CMA banding pattern and percentage of GC-rich regions (Figs. 11, 12; Table 1).

v. The phylogenetic dendrogram based on RAPD analysis has placed the two forms in different clusters (Fig. 18).

The above data suggests that the two forms of *Egeria densa* are genomically totally different.

**Taxonomic status of these two specimens**

The foregoing discussion has clearly indicated *Hydrilla verticillata* as an autotriploid of *Egeria densa* (dense leaf form). Moreover, the two forms of *Egeria densa* (dense leaf form and less dense leaf form) are genomically totally different. Therefore, a peer revision for the taxonomic rank of these three specimens is necessary.
References


