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Molecular Phylogeny Determined Using Chloroplast DNA Inferred a New Phylogenetic Relationship of *Rorippa aquatica* (Eaton) EJ Palmer & Steyermark (Brassicaceae)—Lake Cress

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ABSTRACT

North American lake cress, *Rorippa aquatica* (Eaton) EJ Palmer & Steyermark (Brassicaceae), is listed as an endangered or threatened species. Lake cress shows heterophyllic changes in leaf form in response to the surrounding environment. Therefore, this species has received considerable attention from ecological and morphological perspectives. However, its phylogenetic position and taxonomic status have long been a subject of debate. To analyze the phylogenetic relationship of lake cress, we investigated chloroplast DNA sequences from 17 plant species. The results of phylogenetic reconstruction performed using *trnL* intron, *trnG* (GCC)-*trnM* (CAU), and *psbC-trnS* (UGA) indicated that lake cress is a member of *Rorippa*. Moreover, we found that the chromosome number of lake cress is 2n = 30. This result indicated that lake cress might have originated from aneuploidy of triploid species or via intergeneric crossing. Taken together, our results suggest an affinity between lake cress and *Rorippa* at the molecular level, indicating that lake cress should be treated as *Rorippa aquatica* (Eaton) EJ Palmer & Steyermark.

KEYWORDS

Lake Cress; *Neobeckia*; Phylogeny; *psbC-trnS*; *Rorippa*; *trnL* Intron; *trnG-trnM*

1. Introduction

North American lake cress, *Rorippa aquatica* (Eaton) EJ Palmer & Steyermark (Brassicaceae), is a perennial herbaceous plant. Flowers borne in racemes have white petals, tetradynamous stamens, and unilocular ovaries (**Figure 1(A)**). Lake cress is known to mostly reproduce vegetatively, and whole plants can be regenerated from leaf fragments (**Figures 1(B)** and **(C)**) [1], although some populations from the southern part of the United States are thought to produce viable seeds [2]. As the specific epithet of this species suggests, lake cress is character-rized by its semi-aquatic habit and is known to occur in a broad array of habitats. Lake cress has also been found in static waters, bays of lakes with rocky, sandy, or muddy soils, streams, muddy shores, and brooks. It appears to occur in hard and relatively cool water [3]. Before 1950, lake cress was known to be widespread throughout the northeastern United States. Currently, however, many of its habitats have been lost due to water pollutions [4-6]. Hence, conservation of this species is of concern in several states of the United States [5]. Additionally, the leaves of lake cress have an interesting feature: they show heterophyllic changes in response to the surrounding environment. They exhibit forms with weak serrate or deep lobed margins in terrestrial environments

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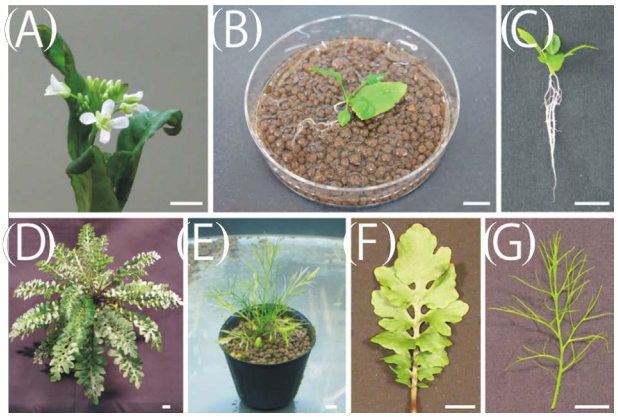

Figure 1. Gross morphology of *Rorippa aquatica*. (A) Flowers on inflorescence; (B) Regeneration of leaf fragments; (C) A regenerated plantlet; (D)–(G) Morphological variation in shoots and leaves. Shoots in terrestrial environments (D) and submerged aquatic environments (E). Leaves in terrestrial environments (F) and submerged aquatic environments (G). Bars = 1 cm.

and become pinnately compound in submerged aquatic environments (Figures 1(D)-(G)) [7,8]. Although heterophylly is widely noted among diverse plant species from ferns to angiosperms [8-10], little is known about the mechanism that regulates this phenomenon, because of the lack of an adequate model species. A previous study indicated that lake cress has some advantages as a model for studying heterophylly, such as plant size, easy maintenance, and systematic positioning (closely related to Arabidopsis) [8]. Hence, lake cress has received considerable attention from ecological, developmental, and physiological perspectives.

Although lake cress has such interesting characteristics, its phylogenetic position and taxonomic status are not yet known due to the considerable overlap of morphological characteristics of related genera [11]. Therefore, the nomenclature of lake cress has remained ambiguous thus far. Lake cress was originally described as a variety of horseradish (*Cochlearia armoracia* var. *aquatica* Eaton) [12]. However, later, Eaton [13] recognized it as a distinct species of *Cochlearia*. Subsequently, lake cress was treated as a species of *Nasturtium*, *Rorippa*, or *Armoracia* [14,15]. It was once also described as a monotypic taxon in the early history of its nomenclatural changes: *Neobeckia aquatica* [16]. Greene indicated many morphological and ecological differences and suggested that lake cress was sufficiently distinct to be recognized as a monotypic genus [16]. The systematic position of a species can be confirmed using molecular phylogenetic analyses. The only phylogenetic analysis reported in lake cress thus far was performed using the plastid gene *rbcL* [11]. The study suggested that lake cress is clearly distinct from *Armoracia* and could be treated as a monotypic sister genus to *Rorippa* [11]. However, the author sampled only 2 species of *Rorippa*, which comprise approximately 75 species [17]. Therefore, the number of operational taxonomic units (OTUs) was not sufficient to confirm the exact phylogenetic relationship. Additionally, the previous analysis was performed using *rbcL*, and hence, the author detected little variation in the region among species [11]. Hence, the phylogenetic relationship between lake cress and species belonging to the genus *Rorippa* and whether lake cress should be treated as a monotypic genus remain unclear. Indeed, the worldwide monograph of Brassicaceae presented by Appel and Al-Shehbaz [17] reduced *Neobeckia* to a synonym of *Rorippa*, and lake cress was treated as a species belonging to the genus *Rorippa* (*R. aquatica*) in the Flora of North America [18]. Thus, reconstructing the phylogenetic position of lake cress is an interesting aspect for clearly understanding its taxonomic status. Additionally, this analysis might be useful for understanding the processes for acquisition of heterophylly from the evolutionary perspective.

In this study, we reconstructed a relationship between lake cress and species belonging to the genus *Rorippa* to clarify their phylogenetic position by analyzing multiple regions of cpDNA. Moreover, we analyzed the chromosome number of lake cress to elucidate the evolutionary origin of the species. Our results indicated that lake cress is a member of *Rorippa* and confirmed that the species should be treated as *Rorippa aquatica* (Eaton) EJ Palmer & Steyermark, as indicated by previous taxonomic studies [17,18].

2. Materials and Methods

2.1. Plant Materials

Seventeen samples were investigated in this study (Table 1). *R. aquatica* were grown at the Kyoto Sangyo University, Japan, and planted in soil in growth chambers. Plants were grown at 20˚C with continuous light. Voucher specimens for all newly sequenced taxa were deposited in the following herbaria: Herbarium of the University of Tokyo (TI), Japan, and Herbarium of the National Museum of Nature and Science (TNS), Japan. *Nasturtium officinale* and *Cardamine hirsuta* were selected as outgroup according to the taxonomic classification of Les [11].

2.2. DNA Extraction, Amplification, and Sequencing

Total DNAs were isolated from 100 mg fresh leaves or 30 mg dried leaf specimens by using a DNeasy plant Mini Kit (QIAGEN, USA). The isolated DNA was resuspended in Tris-EDTA (TE) buffer and stored at −20˚C

Table 1. List of species, voucher numbers, and accession numbers of plant materials. Herbarium acronyms follow Index Herbariorum Part I [34].

Species	Locality	Voucher	*trnL* intron	*trnG-trnM*	*psbC-trnS*
R. aquatica	USA	Kyoto Sangyo Univ., (cult.)	AB871891	AB871892	AB871893
R. curvisiliqua	USA, Idaho	C. Read & W. R. Kathryn, 861143, (TI)	AB871894	AB871895	AB871896
R. sessiliflora	USA, Florida	R. K. Godfrey, 67613, (TI)	AB871897	AB871898	AB871899
R. obtusa	Canada, Montreal	F. Marie-Victorin & F. Rolland-Germain, 46806, (TI)	AB871900	AB871901	AB871902
R. pyrenaica	France, Loire	F. Schltz, (TI)	AB871903	AB871904	AB871905
R. barbareaefolia	Canada, Alaska	W. J. Cody & T. J. M. Webster, 5902, (TI)	AB871906	AB871907	AB871908
R. islandica	Nederland, Sleeuwijk	A. C. de Roon, (TI)	AB871909	AB871910	AB871911
R. dubia	Nepal, Kathmandu	G. Murata *et al*., 6303314, (TI)	AB871912	AB871913	AB871914
R. palustris	China, Rangtang	D. E. Boufford *et al*., 39061, (TI)	AB871915	AB871916	AB871917
R. elata	China, Baiyu Xian	D. E. Boufford *et al*., 37265, (TI)	AB871918	AB871919	AB871920
R. montana	China, Hunnan	H. Zhong-hui, 117, (TI)	AB871921	AB871922	AB871923
R. amphibia	Danmark, Jylland	A. Hansen, 198169, (TNS)	AB871924	AB871925	AB871926
R. nikkoensis	Japan, Tochigi	J. Haginiwa, (TNS)	AB871927	AB871928	AB871929
R. sylvestris	Japan, Fukui	S. Watanabe, 682661, (TNS)	AB871930	AB871931	AB871932
R. indica	Japan, Kyoto	Kyoto Sangyo Univ., (cult.)	AB871933	AB871934	AB871935
N. officinale	China, Derong Xian	D. E. Boufford *et al*., 30988, (TI)	AB871936	AB871937	AB871938
C. hirsuta	Japan, Kyoto	Kyoto Sangyo Univ., (cult.)	AB871939	AB871940	AB871941

until use. For phylogenetic analysis, *trnL* intron, *trnG* (GCC)-*trnM* (CAU), and *psbC-trnS* (UGA) were amplified using specific primers (5'-CGAAATCGGTAGACGCTACG-3': forward; 5'-GGGGATAGAGGGACTTGAAC-3': reverse), (5'-TCTCTTTGCCAAGGAGAAGA-3': forward; 5'-ATAACCTTGAGGTCACGGGT-3': reverse), and (5'-TGAACCTGTTCTTTCCATGA-3': forward; 5'-GAACTATCGAGGGTTCGAAT-3': reverse), respectively. These primers were designed on the basis of sequences published in previous studies [19,20]. Targeted DNA fragments were amplified using an AB 2720 thermal cycler (Applied Biosystems). The polymerase chain reaction (PCR) cycle conditions were incubation at 95˚C for 1 min, followed by 40 cycles of incubation at 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 40 s, with a final extension at 72˚C for 7 min. After the amplification, reaction mixtures were subjected to electrophoresis on 0.8% agarose gels to purify the amplified products by using QIAquick Gel Extraction Kit (QIAGEN). Cycle sequencing reactions were performed by using the purified PCR products, BigDye Terminator Cycle Sequencing Kit v 3.1 (Applied Biosystems), and the same primers used for the reactions. The products were resolved by capillary electrophoresis by using an ABI PRISM 3130 automated DNA sequencer (Applied Biosystems). All procedures were performed according to the manufacturers' instructions.

2.3. Data Analysis

Sequences of *trnL* intron, *trnG-trnM*, and *psbC-trnS* were aligned using the CLUSTAL W program [21] and adjusted manually as necessary. All positions containing gaps and missing data were excluded from the analysis. Phylogenetic trees constructed using neighbor-joining (NJ) [22] and maximum-likelihood (ML) methods [23] were obtained using MEGA5 program [24]. The bootstrap analyses [25] with 1000 replicates were performed using the same program for both the analyses. For the NJ analysis, nucleotide substitutions were used; the evolutionary distances were computed using the maximum composite likelihood (MCL) method [26], and the units were the number of base substitutions per site. Codon positions included were first + second + third + noncoding. For the ML analysis, nucleotide substitutions were used, and the evolutionary history was inferred using the ML method based on the Jukes-Cantor model [27], because the number of nucleotide substitutions per site was low (approximately 0.05 or less). The initial tree(s) for heuristic search were obtained automatically by applying NJ and BioNJ algorithms to a matrix of pairwise distances estimated using the MCL approach, and then selecting the topology with superior log likelihood value. Codon positions included were first + second + third + noncoding as were in the NJ analysis.

2.4. Chromosome Observation

Fresh root tips were pretreated with 0.05% colchicine for 3.5 h at 18˚C and fixed with 80% ethanol for 30 min. After repeated washing with distilled water, root tissues were macerated in an enzymatic mixture containing 4% cellulase (Onozuka R-10) and 2% pectolyase Y-23 for 60 min at 37˚C. After washing, root tips were placed on a glass slide and spread using 20 µl ethanol-acetic acid (3:1) solution. After overnight air drying, the chromosome preparations were stained with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) and observed under a microscope (Olympus BX51).

3. Results

The sequences of *trnL* intron, *trnG* (GCC)-*trnM* (CAU), and *psbC-trnS* (UGA) from 15 samples of *Rorippa* species and 2 samples from the outgroup species *N. officinale* and *C. hirsuta* were determined. All nucleotide sequence data obtained for the 3 noncoding regions in this study were deposited at DDBJ (**Table 1**). The nucleotide lengths of these regions were 517 - 527 bp for trnL intron, 224 - 228 bp for *trnG-trnM*, and 205 - 222 bp for *psbC-trnS*. The phylogenetic analyses involved 17 nucleotide sequences. There were a total of 922 positions in the final dataset. The relationship between the clades and localities of individuals is indicated in **Table 1**.

The optimal NJ tree with the sum of branch length = 0.09856586 is shown in **Figure 2(a)**. The numbers of percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of evolutionary distances used to infer the phylogenetic tree. The ML tree with the highest log likelihood (−1911.9178) is shown in **Figure 2(b)**. The percentage of statistical support of trees in which the associated taxa clustered together is shown next to the branches. In our phylogenetic tree generated using cpDNA region, all *Rorippa* samples (including *R. aquatica*) formed a monophyletic group. Two major clades were recognized in *Rorippa* (**Figures 2(a)** and **(b)**). One clade included many *Rorippa* species used in this study and a highly supported group (93% and 88% for NJ and ML analyses, respectively) with 10 closely related species. This group was divided into 2 subclades (**Figures 2(a)** and **(b)**). The other clade involved 5 species, and *R. aquatica* belonged to this clade.

Chromosomal analysis revealed 2n = 30 in the roots of *R. aquatica* (**Figure 3**). This result is different from that reported previously [5].

4. Discussion

Phylogenetic position and taxonomic status of lake cress have not yet been fully defined [11]. At present, lake cress is recognized as *R. aquatica* according to contemporary treatment in the Flora of North America [18]. However, molecular phylogenetic analyses of *Rorippa* species, including lake cress, are insufficient. Our molecular phylogenetic analysis confirmed that lake cress is a species belonging to the genus *Rorippa*, as indicated in the taxonomic studies by Palmer & Steyermark [14], and supported the treatment in the Flora of North America [18]. Moreover, the phylogeny of cpDNA suggested that *R. aquatica* is relatively close to *Rorippa sessiliflora* and *Rorippa pyrenaica*. *R. sessiliflora* is known to be distributed from eastern to central regions of the United States [28] and shows partial overlap of distribution with that of *R. aquatica* [29]. Therefore, speciation between these species might occur in the distribution range of these 2 species in the North American continent. Although these species might be closely related, various differences have been reported between the 2 species (e.g., annuals or perennials, presence of petals, and length of filaments). Hence, the relationship among *R. aquatica*, *R. sessiliflora*, and *R. pyrenaica* is still not clear. Revealing the detailed evolutionary processes of speciation among relatives of *R. aquatica* necessitates the analysis of more samples and molecular data.

Our phylogenetic study showed the evolutionary path of acquisition of heterophylly in this genus. According to previous studies, the leaves of *Rorippa amphibia* showed heterophyllic changes [30]. *R. amphibia* and *R. aquatica* did not form a monophyletic group in our cpDNA phylogeny. Hence, the result indicated that the acquisition of noticeable heterophylly occurred independently at least twice in the genus *Rorippa*. Thus, revealing and comparing the mechanisms of heterophylly in *R. amphibia* and *R. aquatica* would help determine whether the acquisition of heterophylly is a result of parallel evolution in the genus.

Information on chromosome numbers often helps delimit issues on systematics [31]. Chromosome number of *R. aquatica* was first reported as 2n = 24 by Les *et al*. [5]. In the species belonging to Brassicacea that are closely related to *Rorippa*, the base number of chromosomes (x = 8) is common, with only few instances of x = 7 or x = 6 [5]. Therefore, *R. aquatica* is thought to be triploid on the basis of the information of the base number of chromosomes [5]. However, our observation showed that the chromosome number of *R. aquatica* was 2n = 30. In fact, some plant species are known to have a variation in chromosome numbers [32]. A previous study also reported variation in chromosome numbers of *R. aquatica* (e.g., 2n = 23 - 26 [5]). However, 2n = 30 has not yet been reported in *R. aquatica*. If the chromosome number revealed in this study occurred from the normal base

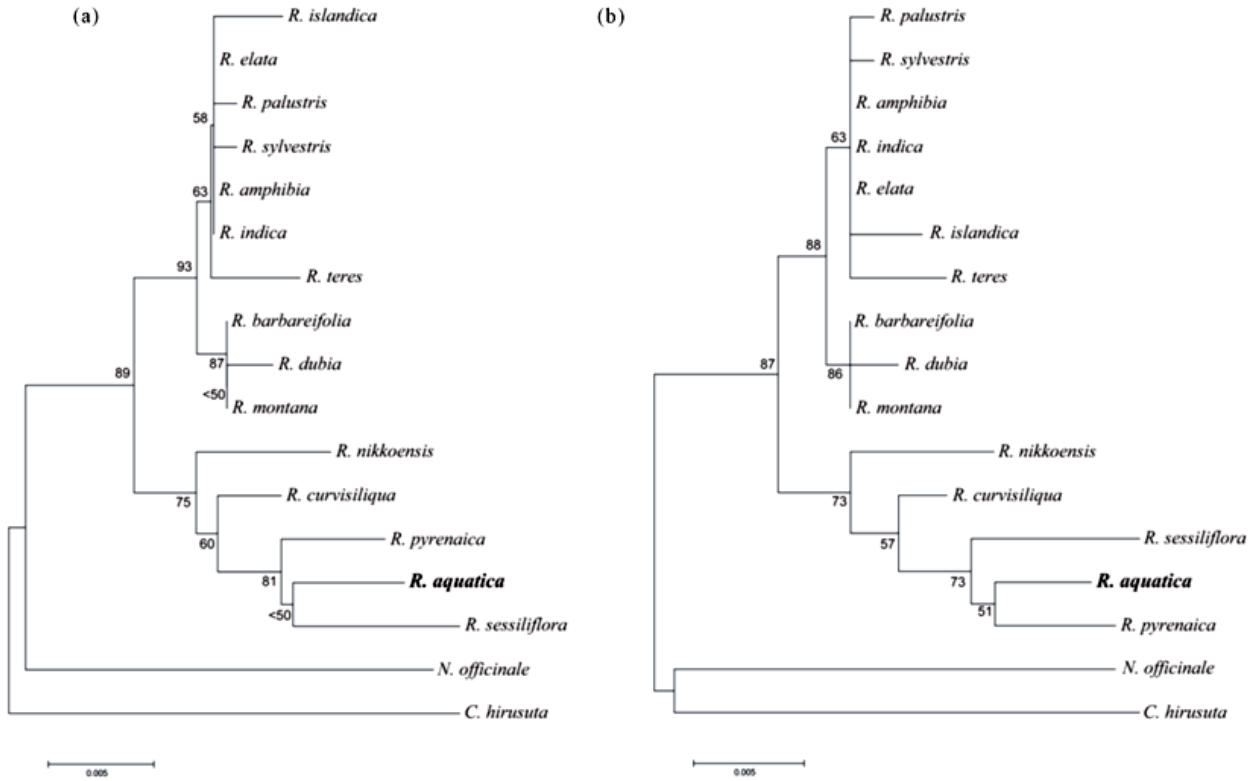

Figure 2. Phylogenetic trees constructed using cpDNA sequences. The evolutionary history was inferred using the neighbor-joining method (A) and maximum-likelihood method (B). Evolutionary analyses were conducted using MEGA5 [21]. The bootstrap values are indicated on branches (only those more than 50% are indicated on the tree).

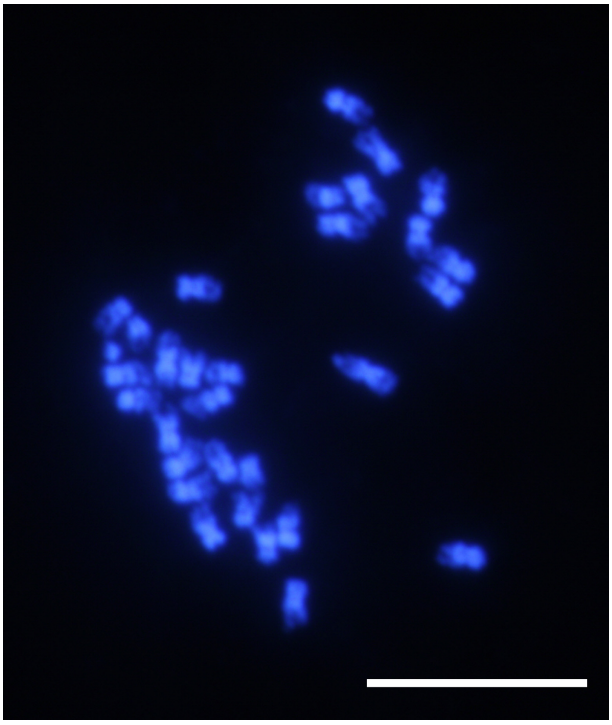

Figure 3. A photomicrograph showing chromosomes of *Rorippa aquatica*. *R. aquatica* (2n = 30). Bar = 10 µm.

number of chromosomes in *Rorippa* (x = 8), it might be attributed to aneuploidy of triploid or tetraploid species. This irregular chromosome number might also have occurred due to intergeneric crossing, although the parental group was unknown. Chromosome variations such as polyploidy or aneuploidy are known to play an important role in plant speciation [33]. In *R. aquatica*, some phenotypes have been reported to differ depending on the populations [2,6]; thus, some such differences might have occurred due to the differences in chromosome number.

In this study, we analyzed the phylogenetic relationship of the genus *Rorippa* while focusing on the systematic position of *R. aquatica*. However, we sampled only 15 species of *Rorippa*, and hence, many issues remained unresolved. To address these issues, phylogenetic analyses performed using a wider range of species and regions showing high variation, such as nuclear DNA sequences, might be effective. Such further analysis might allow better understanding of the phylogeny and evolutionary processes of the acquisition of heterophylly in the genus *Rorippa*.

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