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# Adaptive evolution of *rbcL* in *Conocephalum* (Hepaticae, bryophytes)

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ABSTRACT

An excess of nonsynonymous substitutions over synonymous ones has been regarded as an important indicator of adaptive evolution or positive selection at the molecular level. We now report such a case for *rbcL* sequences among cryptic species in *Conocephalum* (Hepaticae, Bryophytes). This finding can be regarded as evidence of adaptive evolution in several cryptic species (especially in F and JN types) within the genus.

Bryophytes are small land plants with simple morphology. We can therefore expect the existence of several biologically distinct units or cryptic species within each morphological species. In our previous study, we found three *rbcL* types in Asian *Conocephalum japonicum* (Thunb.) Grolle and also found evidence strongly suggesting that the three types are reproductively isolated cryptic species. Additionally, we examined *rbcL* sequence variation in six cryptic species of *C. conicum* (L.) Dumort. previously recognized by allozyme analyses. As a result, we were able to discriminate the six cryptic species based only on their *rbcL* sequences. We were able to show that *rbcL* sequence variation is also useful in finding cryptic species of *C. conicum*.

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## 1. Introduction

### 1.1. Cryptic species in bryophytes

Bryophytes are small land plants with a simple morphology. We can therefore predict the existence of several biologically distinct units or cryptic species within the morphological species of bryophytes. Cryptic species have also been reported in several bryophytes. Two cryptic species were recognized in both *Pellia epiphylla* and *P. endiviifolia* (Pelliaceae, Hepaticae) through allozyme analysis (Zielinski, 1987; Szweykowski and Odrzykoski 1990). Analyses of nuclear $tRNA^{Leu}$ intergenic sequences confirmed that *P. epiphylla* and *P. endiviifolia* can each be separated into two cryptic species (Fiedorow et al., 2001). Dewey (1988, 1989) and Szweykowski and Odrzykoski (1990) reported that *Riccia dictyospora* (Ricciaceae, Hepaticae) and *Aneura pinguis* (Aneuraceae, Hepaticae) consist of three and two cryptic species, respectively, also based on allozyme data.

### 1.2. Searching for cryptic species using rbcL sequence variation

In angiosperms, *rbcL* variation has been considered useful only for inter-generic or inter-familial level of phylogenetic analyses (Chase et al., 1993). In various ferns, however, *rbcL* variation has been shown to be a useful tool for finding cryptic species (Murakami et al. 1998a,b, Yatabe et al., 1998, 1999, 2001; Kato et al., 2001; Masuyama et al., 2002). We considered it highly possible that *rbcL* variation would also be useful for finding cryptic species within bryophytes. In our previous study (Miwa et al., 2003, 2004), we found cryptic species in *Conocephalum japonicum* Grolle using *rbcL* sequence variation rather than allozyme analysis as a preliminary indicator. In those studies, three distinct types of *rbcL* sequences (JN, JS and CS), differing by 6–10 nucleotides, were found in Asian *C. japonicum*. We found a mixed population of JN/JS in Abuta, Hokkaido, Japan, and JS/CS types in Chitou, Taiwan. At those sites, the two *rbcL* types were growing closely side by side. We also conducted allozyme analysis and detected polymorphisms in EST and TPI enzymes. Tight associations between the allozyme and the *rbcL* variants were observed. Individuals with genotypes of other combinations were not found in these populations. Thus, we concluded that these three *rbcL*

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types of *C. japonicum* represent three reproductively isolated cryptic species.

The purposes of this study are: 1) to detect cryptic species in *Conocephalum* (Hepaticae) using allozyme and *rbcL* variants: 2) to detect evidence of adaptive evolution or positive selection at the molecular level in several cryptic species within the genus through analyses using the program PAML (Phylogenetic Analysis by Maximum Likelihood) version 4 (Yang, 2007).

In this study, we examined *rbcL* sequence variation among the 6 cryptic species of *C. conicum* (L.) Dumort. formerly recognized by allozyme analyses (Odrzykoski and Szweykowski, 1991; Akiyama and Hiraoka, 1994). Allozyme variation was examined at 33 enzyme loci in 26 samples from throughout the geographical range of *C. conicum*. Variation was partitioned into 6 discrete groups, suggesting that this morphologically defined species is genetically heterogeneous. The degree of differentiation among these groups, as measured by genetic distances, is as large as was commonly reported between different vascular plant species, and much larger than the differences in conspecific populations. In Poland (Odrzykoski, 1987) and Japan (Akiyama and Hiraoka, 1994) respectively, two of these genetically distinct groups (S and L/S and J-type) occur sympatrically, but apparently do not interbreed. The geographical ranges of the other three groups (A, C, and F) are probably allopatric with the exception that the range of S, the most genetically divergent group, overlaps with group A in North America. Recently this group of S-type was also described as *C. salebrosum* (Szweykowski et al., 2005). It is suggested that these six natural assemblages constitute different cryptic species.

## 2. Materials and methods

### 2.1. Plant materials

Odrzykoski and Szweykowski (1991), and Akiyama and Hiraoka (1994) examined allozyme variation for 96, and 17 populations of *C. conicum* throughout the geographical distribution range of the species and found six discrete groups (Fig. 1). We selected 26 representative samples with remote localities in the Northern Hemisphere (Table 1) and cultivated part of them in IJO's laboratory. In addition, we prepared one more new material which was a dried sample (R1; Allozyme type unknown in *C. conicum*) from Sakhalin (Table 1). We generally collected one individual from each locality. Voucher specimens are in the Herbarium of the Institute of Experimental Biology, Adam Mickiewicz University (POZW), the Farlow Herbarium of Harvard University (FH), the Institute of Botany, Chinese Academy of Sciences (PE) and Graduate School of Science, Kyoto University (KYO). Voucher information and DNA Data Bank of Japan (DDBJ) accession numbers are in Table 1.

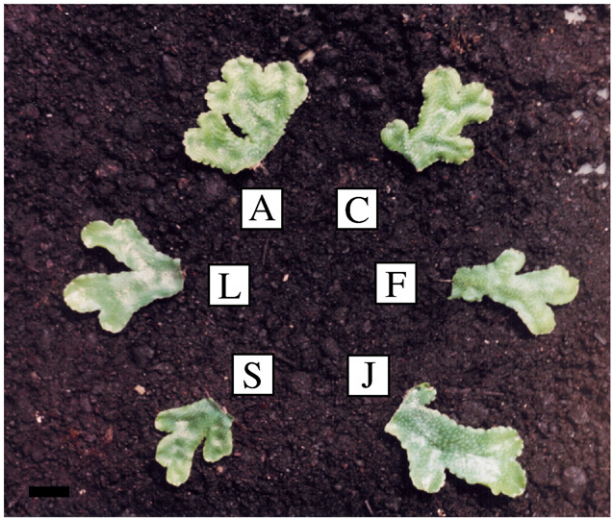


**Fig. 1.** General appearances of *Conocephalum conicum*. A, C, F, J, L and S respectively represent allozyme types (Odrzykoski and Szweykowski 1991, Akiyama and Hiraoka 1994). S-type was also described as *C. salebrosum* recently (Szweykowski et al., 2005). Bar; ca. 1 cm.

### 2.2. DNA extraction

A small amount of living material (approximately 50 mg) of each population was ground in liquid nitrogen in mortars to yield a fine powder. Total DNAs were extracted using the Nucleon PhytePure plant and fungal DNA extraction kit (Amersham), or alternatively the Plant DNeasy Kit (Qiagen).

### 2.3. PCR amplification

Partial *rbcL* gene segments were amplified by PCR using Ready-To-Go PCR Beads (Amersham). We used two sets of primers: 1-1 (ATGTCACCACAAACAGAGACTAAAGC), 2R (CTTCTGCTACAAATAAGAATCGATCTCTCCA), N2-1 (TGAAAACGTGAATTCCCAACCGTTTATGCG), NN3-2 (GCAGCAGCTAGTTCCGGGCTCCA) for the PCR (Hasebe et al., 1994). A typical PCR amplification included an initial denature (5 min, 94 °C) followed by 35 cycles with a 1 min denature at 94 °C, 1 min annealing at 50–55 °C, 2 min 30 s synthesis at 72 °C, and a final step of synthesis for 6 min at 72 °C. Products were visualized by running on a 1.0% ethidium bromide-stained agarose gel. PCR amplification products were prepared for sequencing by cleaning with a QIA quick PCR Purification Kit (Qiagen) following the manufacture's instructions.

### 2.4. DNA sequencing

The amplified fragments were sequenced with a Big Dye terminator cycle sequencing kit (Applied Biosystems) using the former primers, and run on an Applied Biosystems Model 377 or 3100 automated DNA sequencer (Applied Biosystems). The obtained sequences were handled by Sequence Navigator software (Applied Biosystems).

### 2.5. Molecular phylogenetic analysis

Taxon sampling consisted of those newly sequenced accessions listed in Table 1, plus previously published sequences (Miwa et al. 2003, 2004) from three cryptic species of *C. japonicum* (DDBJ No. JN-type AB046694, JS-type AB046695 and CS-type AB046719). Sequences were aligned with GENETYX-MAC program, version 14.0.9 (GENETYX). The Neighbor-joining phylogenetic tree was constructed using the CLUSTALX program, version 2.0.3 (Thompson et al., 1997). Bootstrap analyses were conducted on 1000 replicates using the same program. In calculating the distance matrix, the Neighbor-joining phylogenetic analyses were also inferred with the following three models of nucleotide substitutions; Jukes–Cantor method (Jukes and Cantor, 1969), Kimura's 2-parameter method (Kimura, 1980) and Tajima–Nei method (Tajima and Nei, 1984), using the MEGA software, version 4.0 (Tamura et al., 2007). We used sequencing data from two species of bryophytes, *Physcomitrella patens* subsp. *patens* (No. AP005672; Sugiura et al. 2003) and *Marchantia polymorpha* (No. X04465; Ohyama et al. 1986) as outgroups.

### 2.6. Analysis for detecting adaptive evolution

Comparison of synonymous and nonsynonymous substitution rates in protein encoding genes is important for studying the mechanism of DNA sequence evolution (Kimura, 1983). An excess of nonsynonymous substitutions over synonymous ones has been

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