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Short communication

Can genetic bar-coding be used to identify aquatic *Ranunculus* L. subgenus *Batrachium* (DC) A. Gray? A test using some species from the British Isles

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ABSTRACT

Aquatic *Batrachium Ranunculus* species are a key component of river macrophyte communities selected for protection under European Union legislation. The group's simplified morphology and variable taxonomic interpretation often makes identification to species level very difficult. A genetic approach was trialled as an alternative, more reliable, means of identification. DNA barcoding using four markers (chloroplast and nuclear) was tested. The chloroplast sequence *trnH-psbA* worked best and allowed identification of three out of five species while nuclear sequences supported the identification of two hybrids. This method is amenable to simplification through techniques such as PCR-RFLP or RT-PCR. It has the potential to provide easy, rapid and inexpensive identification of *Batrachium Ranunculus* species.

1. Introduction

Accurate identification of species from the water crowfoots (*Ranunculus* L., subgenus *Batrachium* (D.C.) A. Gray), which are mainly (sub)aquatic, can be difficult to do using morphology (Preston and Croft, 2001). The taxonomic characters available are limited because members of the taxa have evolved a simplified morphology and characters are variable within species (Preston and Croft, 2001), further confounding identification. It is not surprising then that these plants are considered to be amongst the most taxonomically challenging group of aquatic plants found in Ireland and Great Britain (Preston and Croft, 2001).

The presence of *Batrachium Ranunculus* species can indicate an aquatic flora of conservation value and members of the group can also help to indicate the trophic status of rivers (Dawson et al., 1999; Holmes et al., 1999; JNCC, 2005). For these reasons, member species are commonly surveyed in the countries of the European Union where legislation requires protected habitats to be monitored and water quality assessed (European Commission, 1991, 1992, 2000). The practical demands of survey programmes mean sites are often visited when *Batrachium Ranunculus* species are not in flower, making it impossible to identify the plants to species level because flower parts may be the only definitive diagnostic characteristics (Holmes, 1983; Stace, 1991; Hughes, 2003). This can limit the usefulness of the survey data produced because conservation and water quality assessment require data resolved to species level. Hence there is a practical management need to identify these plants with certainty.

The group last underwent significant taxonomic revision in the 1960s with some additional work in the interim, (Cook, 1963; Webster, 1984, 1991; Johansson and Jansen, 1991; Diosdado et al., 1993). Since then a chromatographic investigation of flavonoids and a karyological study aimed to find reliable taxonomic characteristics (Webster, 1991; Diosdado et al., 1993). More recently genetic studies have been used with some success (Johansson and Jansen, 1991; Ku et al., 2007; Koga et al., 2008) with phylogenies indicating that the *Ranunculus* clade is well supported, nesting *Batrachium* within *Ranunculus* s. str (Johansson and Jansen, 1991; Horandl et al., 2005; Emadzade et al., 2010). Here we report on a pilot study which investigates the potential use of genetic barcoding to provide a rapid and dependable means of identifying *Batrachium Ranunculus* species collected from flowing water sites in the British Isles. The technique has been applied to resolve identification amongst other similarly difficult aquatic plants in recent years, such as the seagrass *Zostera asiatica* Miki and the marine Gracilariaceae (Talbot et al., 2006; Guillemin et al., 2008).

The search for an internationally accepted barcoding system in plants has been well documented (Chase et al., 2005, 2007; Kress

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et al., 2005; Cowan et al., 2006; Lahaye et al., 2008) and could lead to a means of rapidly and reliably identifying species as well as to a greater understanding of speciation and the relationships within complex groups of organisms. Mitochondrial DNA is used for barcoding in animals; however, the low substitution rate of mtDNA in plants makes it ineffective at identification to species level (Kress et al., 2005), consequently the chloroplast genome is targeted. Chloroplast DNA is a structurally stable non-recombining genome which evolves faster than the plant mitochondrial genome and yet slower than the nuclear genome, making it ideal for barcoding purposes. A recent paper by the Consortium for the Barcode of Life's (CBOL) Plant Working Group (Hollingsworth et al., 2009) selected a 2-locus barcode comprising chloroplast regions *rbcL* and *matK*. However this combination was found to discriminate in only 72% of cases. Therefore a quarter of species cannot be identified reliably using this method alone and it is pertinent to explore other genomic regions when barcoding a taxonomic group for the first time.

In this study we selected three chloroplast regions, the intergenic spacers *trnH-psbA* and *trnC-ycf6* (Shaw et al., 2005) and a section of the coding gene *matK* (Cuenoud et al., 2002). We also targeted one nuclear-encoded sequence. In phylogenetics, one of the most widely used nuclear genome sequences has been the internal transcribed spacer (ITS) region of the 18S-5.S-26S nuclear ribosomal cistron (Alvarez and Wendel, 2003). This region is subject to concerted evolution: a process which occurs throughout rDNA, whereby repeat copies of genes acquire mutations simultaneously, so in species of allopolyploid origin, such as some *Batrachium Ranunculus* (Dahlgren and Cronberg, 1996), this may effectively result in a single copy from either parent over time (Soltis et al., 2004). In hybrids this may possibly be detected as a copy from both parents. A previous *Ranunculus* phylogeny study based on ITS sequences noted that reticulate relationships within the genus could be elucidated using a combination of ITS and chloroplast sequences (Horandl et al., 2005) although all species within the group would need to be included.

In this pilot study we aimed to test the use of sequence barcoding in multiple individuals of *Ranunculus* subgenus *Batrachium* by comparing plastid and nuclear DNA for species resolving power and to test the ability of combined sequences to identify putative hybrids.

2. Materials and methods

2.1. Sampling

British aquatic *Ranunculus* specimens were sampled and identified during summer 2008 by Dr Nigel Holmes, retired referee for aquatic *Ranunculus* identification for the Botanical Society of the British Isles (Table 1). The samples were identified as belonging to five species and two hybrids and additional morphological observations were noted at the time of collection. Stem and leaf material was dried on silica gel and stored at −20 °C.

2.2. DNA extraction and PCR reactions

DNA extractions using *Ranunculus* leaf material were carried out using the DNeasy Plant mini kit (Qiagen) following manufacturer's instructions for frozen plant tissue. Polymerase chain reactions were performed in 25 μl volumes containing 2.5 μl 10X ThermoPol Reaction buffer (New England Biolabs), 200 μM each dNTP, 1.6% (v/v) bovine serum albumin (BSA), 0.2 μM each primer, 1 U Taq DNA polymerase (New England Biolabs) and 2 μl template DNA. Primers used are detailed in Table 2. Template DNA was diluted 1:10 with dH_2O for *matK* PCR following optimisation. Polymerase chain reactions were performed in Thermo Hybaid thermal cyclers using the following protocols: 94 °C initial denaturation for 4 min, 35 cycles of 94 °C denaturation for 45 s, 55 °C annealing for 45 s, and an extension of 72 °C for 1 min to amplify the intergenic transcribed spacer (including ITS-1 and ITS-2); 3 min at 94 °C, 40 cycles of 94 °C, 55 °C and 72 °C each for 1 min for the amplification of *trnC-ycf6*; 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min for *matK*. A standard protocol was followed for *trnH-psbA* (Shaw et al., 2005). All amplification protocols finished with a final extension of 72 °C for 10 min.

2.3. Sequence analysis

Successfully amplified regions were sequenced. Prior to sequencing, the PCR product was cleaned using 0.4 U Shrimp Alkaline Phosphatase (New England Biolabs) and 1 U Exonuclease I (New England Biolabs) to remove excess dNTPs and primers. Forward primers were added, apart from *matK* for which the reverse primer was used as the forward sequence was found to be of a consistently poor quality for all species, and samples were taken to the NERC Biomolecular Analysis Facility sequencing service (Genepool) at the University of Edinburgh. Sequence alignments were assembled using CodonCode Aligner version 3.5.4 (CodonCode Corporation) and manually checked for quality. Polymorphisms occurring in chromatograms with background noise were rare and ignored. Sequences were submitted to GenBank: accession numbers are listed in Table 1. All chloroplast sequences were trimmed and appended to each other to form one sequence over 1500 bp in length. Two multi-base indels (insertion-deletion events) and a 29 base pair (bp) complementary inversion were coded as single mutation events. To check for reticulate relationships between species the split decomposition network approach was used to analyse the sequences. This analysis was performed using the program SplitsTree 4.0 (Huson and Bryant, 2006). Uncorrected-P distances were used and gaps and ambiguous sites were ignored. A fit value (ranging from 0 to 100%) signifies the similarity between the pairwise distances generated by this method and the original distance; a high value indicates that the splits graph represents the phylogenetic signal in the data well. Additional analysis using ITS sequences from *Batrachium Ranunculus* species deposited in GenBank (Horandl et al., 2005) was performed but not included as the species identity of the sequences did not always agree with those generated in this study. This may be due to hybridisation or retained ancestral variation in separate populations and further investigation with increased sample size and species coverage is needed.

3. Results

3.1. Chloroplast sequences

The aligned region *trnH-psbA* was 340 bp in length with two single nucleotide polymorphisms (SNPs) and one indel as well as a 29 bp complementary inversion. This region can discriminate *Ranunculus circinatus* Sibth., *Ranunculus fluitans* Lam. and *Ranunculus trichophyllus* Chaix, but cannot distinguish between *Ranunculus peltatus* Schrank and *Ranunculus penicillatus subsp. pseudofluitans* (Dum.) Bab. The two purported hybrids have sequences which align most closely to *R. trichophyllus*. The 656 bp *trnC-ycf6* region has seven SNPs and one indel; these mutations resolve *R. circinatus* and *R. trichophyllus*, but not *R. fluitans*, *R. peltatus* and *R. penicillatus subsp. pseudofluitans*. Hybrid A has a unique five-nucleotide insertion but is otherwise identical to the *R. trichophyllus* haplotype. The *trnC-ycf6* region of Hybrid B amplified with difficulty and warrants further investigation, however