British *Apium repens* (Jacq.) Lag. (Apiaceae) status assessed using random amplified polymorphic DNA (RAPD)

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ABSTRACT

Random amplified polymorphic DNA was used to assess the taxonomic status of the only known remaining British population of putative *Apium repens* (Jacq.) Lag., on Port Meadow, Oxfordshire. This study reveals that the population comprises both true *A. repens*, and also *A. nodiflorum* (L.) Lag. in a prostrate dwarf phenotype almost indistinguishable from *A. repens*. A cultivation experiment supports the molecular evidence.

KEYWORDS: Creeping Marshwort, Port Meadow, phenotypic plasticity, *Apium nodiflorum*, plastodeme, Umbelliferae.

INTRODUCTION

Creeping Marshwort, *Apium repens* (Jacq.) Lag., is listed in Annexes II and IV of the *E.U. Directive on the Conservation of Habitats and Wild Fauna and Flora* ("*Habitats Directive*") and also Appendix I of the Council of Europe's *Bern Convention*. The belief that *A. repens* occurs in Britain has led to its inclusion in Schedule 8 of the *Wildlife and Countryside Act*, 1981, giving it full protection. Under the *E.U. Directive*, Special Areas of Conservation must be designated for the species listed on Annex II, and species on Annex IV must be given full protection. Listing on Appendix I of the *Bern Convention* also means that full protection is required for this species in countries which are signatories.

The only known remaining location of putative British *Apium repens* is Port Meadow, Oxfordshire (v.c. 23). Port Meadow is grazed today by the horses and cattle of the Freemen of Oxford in the same way as was recorded in the Domesday Book of 1087. This continuity of land management may explain the survival of *A. repens* at the site. However, recent cultivation experiments have drawn the status of this population into question since cultivated plants have tended to revert, either completely or partially, to *A. nodiflorum* (L.) Lag. (M. Southam, pers. comm. 1994). In fact less recent cultivation of putative *A. repens* from older sites met with similar reversions "the plant becoming much larger, the leaves increasing to four inches . . . the number of the involucres is reduced" (Druce 1927). Such cultivation experiments highlight the continuum of form often observed between *A. repens* and *A. nodiflorum* which has confused botanists for over a century. Such confusion is exemplified by Professor Babington belatedly pointing out that "the *repens* of Engl. Bot., 1431 [Smith 1795], is a form of *nodiflorum*" (cited in Lees 1880) and also by the large number of infraspecific taxa of *A. nodiflorum* (e.g. var. *vulgare* Schultz (Schultz 1854); var. *depressum* Schultz (Schultz 1854); var. *longipedunculatum* Schultz (Schultz 1854); var. *ochreatum* DC. (De Candolle 1805); var. *pseudorepens* Watson (Watson 1867); var. *repens* Syme (Syme 1865); see Riddelsdell & Baker 1906).

The continuum of form between *A. repens* and *A. nodiflorum* may be a result of hybridization

between the two species and/or phenotypic plasticity. Indeed it appeared possible that *A. repens* was extinct in Britain, with hybrids or *A. nodiflorum* phenocopies of the *A. repens* habit causing taxonomic confusion.

Morphological studies cannot fully resolve this point. However, in the past, claims of hybridity have been made on the basis of morphology: "*Helosciadium [Apium] nodiflorum* Koch . . . growing with presumptive *H. repens* Koch., and apparently hybridising with it" (Riddelsdell 1917b), or refuted: "*A.* × *riddelsdellii* Druce, *nom. nud.* was reported doubtfully from Binsey Common and Port Meadow (v.c. 23), in 1917 but all the specimens seen appear to be variants of *A. nodiflorum*" (Tutin 1975). Fruit morphology has been suggested as the best diagnostic character (Riddelsdell & Baker 1906) but it is rarely accessible in the field, due to grazing (A. Roberts & C. Huxley-Lambrick, pers. comm. 1994), flooding or failure of fruits to mature (Lees 1880). Other characters suggested for identification (Tutin 1980), such as rooting at the nodes and the number of involucral bracts, overlap in the field. The character of rooting at the nodes is particularly poor as it merely distinguishes between upright *A. nodiflorum* and prostrate *A. repens*. Prostrate *A. nodiflorum* roots readily at the nodes.

Cytological evidence does not clarify the situation since both *A. repens* and *A. nodiflorum* are generally thought to have the same number of chromosomes, 2n = 22 (Rutland 1941; Baksay 1956; Hlavacek *et al.* 1984). The B.S.B.I. handbook count of 2n = 16 for putative *A. repens* (ex Witney; Tutin 1980) has been corrected from the original slides to give 2n = 18 (A. J. Richards, pers. comm. 1994). This count needs to be confirmed with other material but accords with the count of 2n = 20 for putative hybrid material in Cambridgeshire (Stace 1984). Putative *A. repens* is now extinct at Witney (R. Palmer, pers. comm. 1994).

In this paper we present a preliminary random amplified polymorphic DNA (RAPD) analysis of non-British (Frankfurt, Swiss and Moroccan) *A. repens*, putative *A. repens* from Port Meadow, and *A. nodiflorum* from two different British localities (Table 1). Two plants of *A. nodiflorum* came from ditch habitats at Port Meadow, and two further plants were collected at a sufficient distance from Port Meadow to be certain of representing another population (East Hagbourne, just on the Oxfordshire side of the Oxfordshire/Berkshire border, grid reference SU/525.879). The Frankfurt material came from the Frankfurt Botanic Garden via the Reading University Harris Botanic Garden, and the Swiss material came directly from the Neuchâtel University Botanic Garden. There is some doubt about the exact provenance of both these specimens, but they certainly represent wild origin non-British *A. repens*. The Moroccan *A. repens* was collected wild by Dr Stephen Jury in the High Atlas and was received via the Reading University Harris Botanic Garden.

RAPD techniques are a proven, relatively inexpensive and effective way of determining the taxonomic identity of specimens with only nanograms of extracted DNA (Hadrys *et al.* 1992; Marsolais *et al.* 1993; Crawford *et al.* 1993; van Buren *et al.* 1994). Short random sequence primers are added to total DNA extracted from leaf tissue, and the mixture subjected to thermal cycles that promote a polymerase chain reaction (PCR). The resultant amplification products (RAPDs) can then be separated on an agarose gel, and the polymorphic DNA bands used to determine taxonomic identity. RAPDs are thus ideal for determining whether pure *Apium repens* occurs on Port Meadow, and hence whether *Apium repens* occurs in Britain. This is important for the forthcoming

TABLE 1. *APIUM* OPERATIONAL TAXONOMIC UNITS (O.T.U.s) CULTIVATED AND USED TO ANALYSE RAPD VARIATION

O.T.U.	Taxon	Grid ref.	Locality
1,2	*A. nodiflorum*	SP/495.085	Port Meadow, Oxon. In ditch.
3,4	*A. nodiflorum*	SU/525.879	East Hagbourne, Oxon. S.W. of East Hagbourne church in shady ditch. *Grassly & Cronk* s.n. (**OXF**).
5-10	putative *A. repens*	SP/495.085	Port Meadow, Oxon.
11	*A. repens*	—	Morocco
12	*A. repens*	—	Frankfurt
13	*A. repens*	—	Switzerland

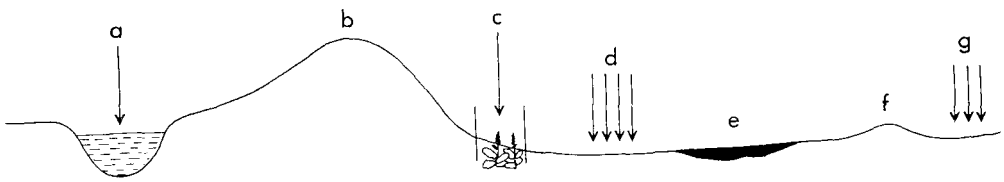

FIGURE 1. Highly schematic representation of the ecological distribution of *Apium repens* and *A. nodiflorum* plastodemes on Port Meadow. Illustrated is the ditch around Port Meadow (a), the Victorian rubbish dump (b), the *Rumex* sp. zone denoting the edge of winter flooding (c), the main area of *A. repens* distribution (d), the area heavily poached by horses and cattle (e), remains of English Civil War defences (f), and the southern site for *A. repens* (g). O.T.U.s 1 & 2 were sampled from habitat (a), O.T.U.s 5–9 were sampled from habitat (d), and OTU 10 came from the southern site (g).

statutory 5-yearly review of Schedule 8 of the *Wildlife and Countryside Act*, 1981, the imminent revision of the plant *Red Data Book* (Perring & Farrell 1983) and for actions to implement the *E.U. Habitats Directive*. For these reasons we are contracted by the Joint Nature Conservancy Council (J.N.C.C.) to undertake a study of *A. repens* at Port Meadow.

METHODS

Authentic *Apium repens* plants from Frankfurt, Switzerland and Morocco were cultivated in the Oxford Botanic Garden, along with *A. nodiflorum* collected from a ditch in East Hagbourne and on Port Meadow (Fig. 1). In addition, with an appropriate licence from English Nature under the *Wildlife and Countryside Act*, 1981, putative *A. repens* samples from Port Meadow were collected by one of us (Q.C.B.C.) and cultivated at the Oxford University Botanic Garden. Thus 13 operational taxonomic units (O.T.U.s) were available for study as listed in Table 1.

In order to carry out the RAPD analysis total DNA was extracted from 9 mm diameter leaf discs following Harris (1993). Discs were homogenised in a 1·5 ml Eppendorf tube using a disposable grinder (Anachem). To each homogenate 1 ml of 2 × CTAB extraction buffer (2% CTAB, 1·4 M sodium chloride, 100 mM Tris-HCl, pH 8·0, 0·2% β-mercaptoethanol, 1% PVP-40T) was added and the tubes incubated for 30 minutes at 65°C. Extracts were then purified using chloroform: isoamyl alcohol (24:1) before precipitating the CTAB-DNA complex, removing the CTAB and resuspending in 100 μl TE (10 mM Tris-HCl, pH 7·3, 1 mM EDTA). Seven ten-base-pairs-long primers [B1,2,5,7,11,12,20 (Operon Technologies Inc., Alameda, California) selected to give useful polymorphic genetic markers] were used in PCR amplifications with DNA extracts from the 13 O.T.U.s. Amplifications were done in 50 μl of reaction mixture containing: 17·5 μl distilled deionised water; 5 μl DNA; 5 μl 1 mM dATP; 5 μl 1 mM dCTP; 5 μl 1 mM dGTP; 5 μl 1 mM dTTP; 5 μl 10 × Dynazyme^{TM} buffer (100 mM Tris-HCl, pH 8·8; 15 mM $MgCl_2$; 500 mM KCl; 1% Triton-X-100); 2 μl 100 nM primer; 1 unit Dynazyme^{TM} (Finnzymes OY; Flowgen Laboratories). The reaction mixture was subjected to 45 thermocycles each consisting of 1 minute at 92 °C, 3 minutes at 35 °C and 2 minutes at 72 °C. A final cycle of 3 minutes at 72 °C ensured complete extension of the remaining products prior to holding the samples at 4 °C until analysis.

After the products were separated on 2% agarose gels in tris-acetate buffer containing 0·5 μg/ml ethidium bromide, 53 genetic markers were scored. Repetition confirmed the validity of these markers.

A 13 × 53 binary matrix indicating marker absence/presence for each O.T.U. was thus obtained (Appendix 1). Using Jaccard similarity (an asymmetric similarity measure suitable for binary data that makes no assumptions about the nature of the characters being scored), the shared genetic markers allowed the O.T.U.s to be related in 53 dimensional space, each dimension representing a genetic marker. The 53 dimensions were then reduced to two by the use of eigen-values as calculated by the program PCoord of the R-package (Legendre & Vaudor 1991). The two dimensions were then plotted to show the genetic similarity of all 13 O.T.U.s (Legendre & Vaudor 1991).

The morphological reversions of new leaves produced by the putative *Apium repens* after seven weeks of cultivation were assessed and a series of leaf silhouettes taken, using the second leaf down from the tips of shoots produced in cultivation.

RESULTS

The discrete nature of genetic markers obtained from RAPDs is shown in Fig. 2. This figure is a photograph of RAPD products separated on an agarose gel stained for DNA and illuminated with UV light. It clearly shows bands from primers that are genetic markers (primer 5) which distinguish

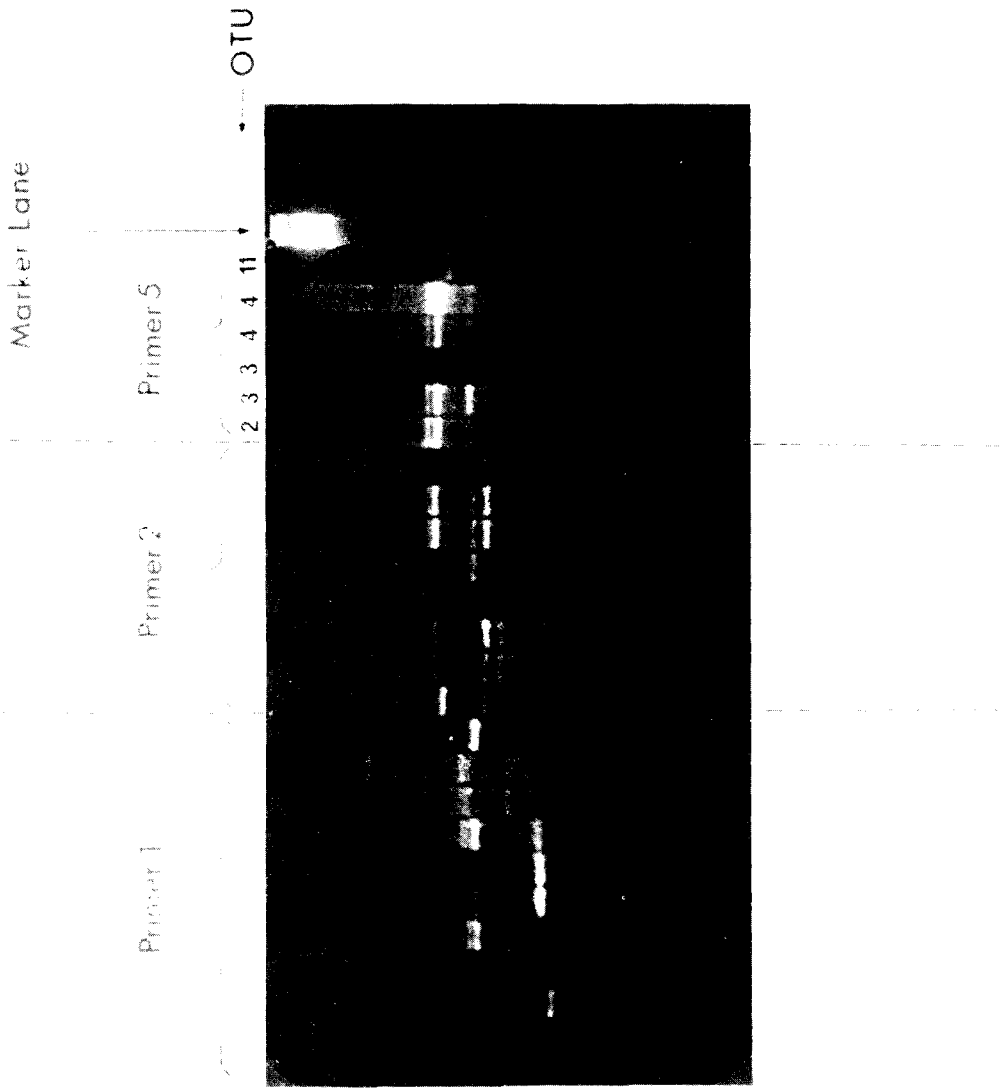

FIGURE 2. The photograph shows RAPD products obtained when Operon primers B1, 2 & 5 are (sequence 5'-TGCGCCCTTC-3') added to DNA extracts from the Port Meadow and East Hagbourne *Apium nodiflorum* and the Moroccan *A. repens* (O.T.U.s in order 2, 3, 4, 11). The genetic markers generated by Operon primer B5 (sequence 5'-TGCGCCCTTC-3') are identical for O.T.U.s 2, 3 & 4 but very different for O.T.U. 11 (*A repens* from Morocco). The second replicate of O.T.U. 3 has failed to amplify because of an excess of DNA.

Apium nodiflorum from Moroccan *A. repens*. When such markers (detailed in Appendix 1) were used to calculate genetic similarities of the 13 O.T.U.s by the Jaccard coefficient followed by Principal Coordinate analysis, Fig. 3 was obtained. This clearly distinguishes *A. nodiflorum* from the 'true' (non-British) *A. repens*. In addition, it shows that the Port Meadow putative *A. repens* plants (O.T.U.s 5–10) fall either into the *A. nodiflorum* genetic cluster (O.T.U.s 1–6) or the *A. repens* genetic cluster (O.T.U.s 7–10, 12, 13). The large RAPD divergence of the Moroccan plant is a point of interest and is discussed below.

The cultivation experiments support the RAPD evidence. New leaves produced after only two months cultivation in common garden conditions show that two of the Port Meadow putative *Apium repens* (O.T.U.s 5 & 6) revert partially to *A. nodiflorum* (Fig. 4c, d), not in leaf size but in leaflet toothing and shape. The remaining Port Meadow '*A. repens*' (O.T.U.s 7–10) retain or even increase the distinctiveness of their field morphology (Fig. 4e).

DISCUSSION

Although this is a preliminary study the data strongly indicate that the Port Meadow '*Apium repens*' population consists of 'true' *A. repens* (O.T.U.s 7–10) similar to the *A. repens* from Frankfurt and Switzerland, and also *A. nodiflorum* phenocopies of *A. repens* (O.T.U.s 5 & 6). The discrete nature of the *A. nodiflorum* and *A. repens* genetic clusters in Fig. 3 is inconsistent with high levels of hybridisation, where a genetic continuum would be expected. However, it is possible that some genetic interchange has occurred, as suggested by the slightly intermediate nature of the Port Meadow *A. nodiflorum* phenocopies of '*A. repens*' in Fig. 2 (O.T.U.s 5 & 6). Further experiments with more O.T.U.s, primers and a wider sampling of Port Meadow '*A. repens*', *A. nodiflorum* and European *A. repens* would allow a more exact assessment of this possible genetic exchange. In addition to the RAPD data, the chemistry of secondary products may provide further useful characters. The leaves of *A. repens* are pleasant tasting (resembling parsley) without the slightly peppery watercress-like aftertaste of *A. nodiflorum*. Riddelsdell (1917a) claimed they were more palatable to slugs, and in the Middle Atlas Mountains of Morocco *A. repens* is sought out and eaten by Barbary Apes (*Macaca sylvana*: G. Drucker, pers. comm. 1994).

The large RAPD divergence of the Moroccan *Apium repens* (Fig. 3) suggests that the population sampled is distinct from European *A. repens*, and has been for some time. This lack of inter-continental genetic exchange is unsurprising. However, much wider sampling of *A. repens* would be desirable before the implications of this observation can be assessed.

The RAPD data do, however, show the tight genetic clustering of *Apium nodiflorum* even though there is great phenotypic plasticity. The plasticity of Port Meadow *A. nodiflorum* can be seen to produce several discrete morphological types as recognisable plastodemes (sensu Gornall 1987; Gilmour & Heslop-Harrison 1954). These plastodemes (assemblages of plants phenotypically rather than genetically distinct) are the result of local environmental conditions and in cultivation the *A. nodiflorum* phenocopies of '*A. repens*' tend to revert towards the phenotype of typical specimens found in ditches (Fig. 4). The plasticity has long been apparent to workers on these species (Riddelsdell 1917a). The plastodemes observed on Port Meadow are shown schematically in Fig. 1.

The large erect form of *Apium nodiflorum* occurs in fresh-water ditches (Fig. 1a) such as those around the edge of Port Meadow and at East Hagbourne. These plants vary in size from 15 cm to 1 m; the main stems root only at the base, and each leaf bears 3–11 leaflets (2–4 cm long elliptic lanceolate or ovate lanceolate). Involucral bracts are usually absent. The ditch plastodeme accords with *A. nodiflorum* var. *vulgare* Schultz (1854).

On Port Meadow a very distinct (approx. 1–6 m wide) zone, characterised by *Rumex crispus* L., marks the edge of winter flooding (Fig. 1c). This zone occurs around that part of the edge of Port Meadow that was deliberately raised in the late 19th century by dumping city waste, both to dispose of rubbish and to provide a refuge for stock from the floodwaters. In this zone smaller prostrate plants of *A. nodiflorum* are found. Such variants approximate to *A. nodiflorum* var. *pseudorepens* H. C. Watson (1867).

On the meadow itself around the edge of areas heavily poached by cattle and horses (Fig. 1e), some almost perfect *Apium nodiflorum* phenocopies of *A. repens* are found together with true *A.*

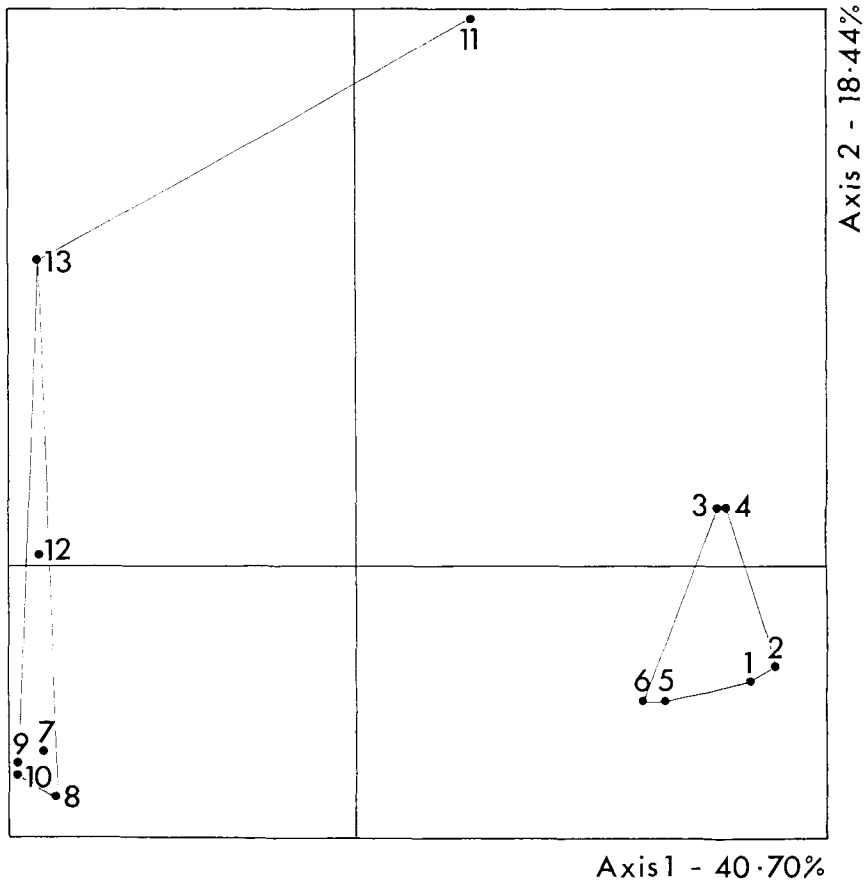

FIGURE 3. Two dimensional principal coordinates plot of the genetic relatedness of the 13 O.T.U.s (listed in Table 1) as calculated using the R package (Legendre & Vaudor 1991). The genetic clusters of *Apium nodiflorum* (O.T.U.s 1–6) and *A. repens* (O.T.U.s 7–10, 12–13) are visible, as is the divergent Moroccan *A. repens* (O.T.U. 11).

repens (Fig. 1d). The extent to which these *A. nodiflorum* phenotypes resemble true *A. repens* varies somewhat according to characters such as leaflet shape, size and number, rooting at nodes, degree of procumbency, and involucral bract numbers. These phenovariants broadly correspond to *A. nodiflorum* var. *longipedunculatum* Schultz forma *simulans* Riddelsdell. The existence of *A. nodiflorum* × *A. repens* hybrids (Riddelsdell 1917c = *A.* × *riddelsdellii* Druce, nom. nud.) has also been suggested. There is no definite evidence for the existence of such hybrids. It is also around the area heavily poached by cattle and horses that true *Apium repens* exists as identified by the genetic markers used in this study. Despite growing intermixed with *A. nodiflorum* phenocopies, *Apium repens* has retained a discrete genetic identity as a species in Britain and hence should remain in Schedule 8 of the *Wildlife and Countryside Act*, 1981. The Port Meadow collections which cluster with *A. repens* had the more incised leaflets in the field. O.T.U. 7 was the only plant collected in flower, and had an inflorescence with four involucral bracts. Probably the best characters for indicating *A. repens* in the field are deeply incised leaflets *and* four or more involucral bracts. Less well marked material should be confirmed by cultivation or a RAPD genetic test.

When such a range of phenotypes is assumed by a species such as *Apium nodiflorum*, allowing the occupation of a range of habitats, the selective advantage of phenotypic plasticity is obvious (Coleman *et al.* 1994). There are many examples of variable and widespread species assuming the

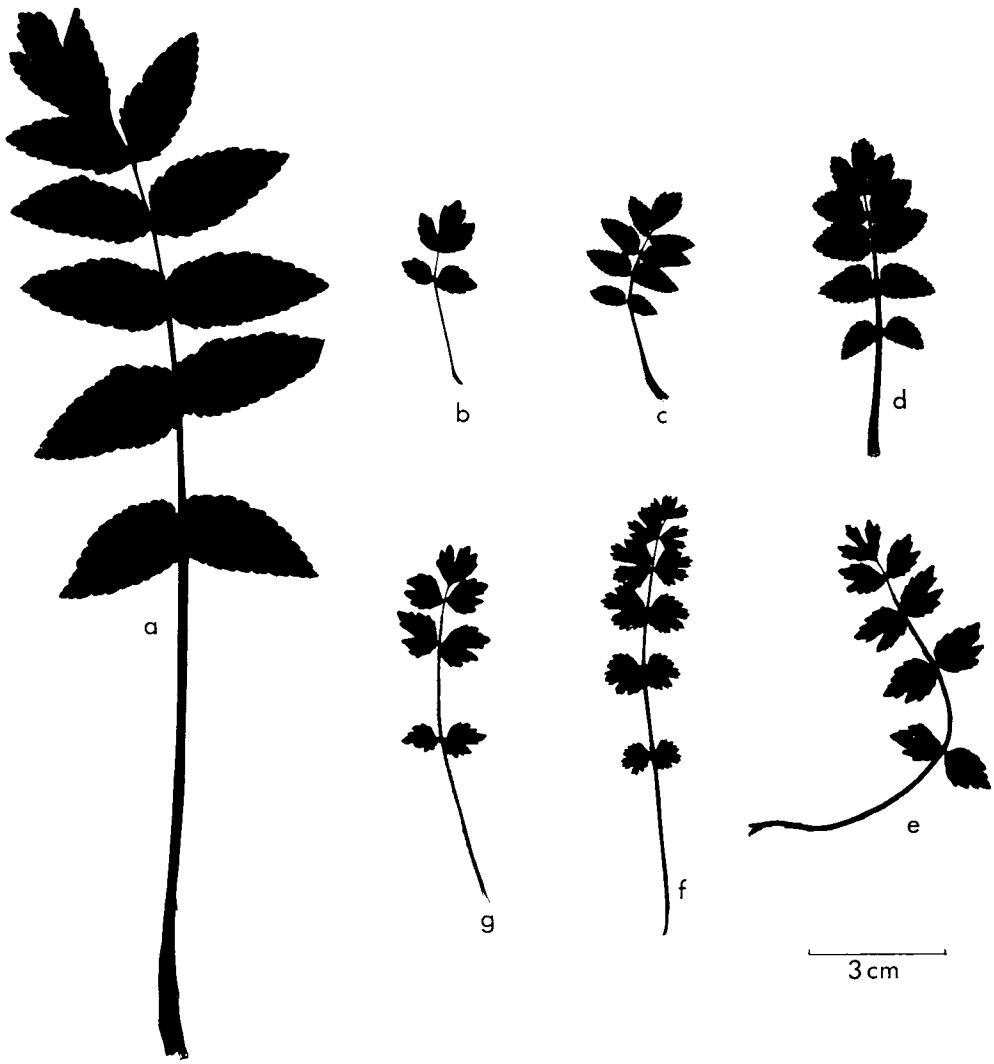

FIGURE 4. Leaf spectra for *Apium nodiflorum* and *A. repens* after seven weeks under cultivation. Operational Taxonomic Units (O.T.U.s) as in Table 1. a. O.T.U. 3 – East Hagbourne *A. nodiflorum*; b, c. O.T.U. 5; d. O.T.U.s – Port Meadow putative *A. repens* showing reversion to *A. nodiflorum*; e. O.T.U. 7 – Port Meadow putative *A. repens* not showing reversion; f. O.T.U. 12 – Frankfurt *A. repens*; g. Moroccan *A. repens*. O.T.U.s 5, 6 & 7 had very similar leaf form in the field.

general form of related ecologically specialised species in particular habitats, for instance *Trifolium occidentale* Coombe/*T. repens* L. (Coombe 1961) and *Ranunculus* × *levenensis* Druce ex Gornall/*R. flammula* L. (Gornall 1987). Stebbins (1950, p. 129) gives the case of *Camelina sativa* (L.) Crantz subsp. *sativa* which mimics *C. sativa* subsp. *linicola* Sch. & Sp. when grown in flax (*Linum usitatissimum* L.). He suggests that the directly genetic adaptation of subsp. *linicola* is selectively advantageous in the specialised habitat, over the phenotypic response of subsp. *sativa*. Conservation management on Port Meadow should aim to favour *A. repens* rather than the *A. nodiflorum* meadow plastodeme, now that the discrete genetic identity of British *Apium repens* has been demonstrated. There remain three broad questions to be answered: 1. What is the extent (if any) of

hybridisation that would be revealed by wider sampling in the population? 2. What are the ecological conditions that favour *A. repens*? What is the optimum level of grazing and poaching by stock, and could the plant survive in other parts of Port Meadow if introduced? 3. Does true *A. repens* occur in other Thames flood meadows? However, enough is presently known about *A. repens* to suggest that it would be an excellent candidate for an English Nature species recovery programme.

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APPENDIX 1

A. RAW DATA MATRIX

The numbering of the O.T.U.s follows that of the text and figures: *Apium nodiflorum* (1–4) 1 & 2 Port Meadow, 3 & 4 East Hagbourne; putative Port Meadow hybrid (5–10); *A. repens* (11–13) 11 Morocco, 12 Germany, 13 Switzerland.

MARKER	1111111111222222222233333333334444444444555
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OTU3	111000011000011000111000100000100010001000100010101000
OTU4	111000001000011000111000100001100010001000100011110000
OTU5	000011011000011000001010010001100010010011100000000000
OTU6	000001011000011000000010100000100010100011100100000000
OTU7	000000000000011000000100000010010010100000011000000000
OTU8	000010000000011000001100000010010010100000111000000000
OTU9	000010000110011000001100000110010010100000011000000000
OTU10	000010000110011000001100000110010010100000111000000000
OUT11	100000010001100111001001000000100110000010010000000000
OTU12	000010100010011000001000000110000000101000110000000001
OTU13	000000000010011100000000000001001000010110010000000111

B. GENETIC MARKERS (CHARACTERS)

The following list gives the primer number (Operon B[OPB] series) and the approximate number of base pairs in the order given in the matrix.

OPB1–970, 1335, 1015, 670, 3720, 790, 2680, 700, 980; OPB2–900, 965, 1335, 2680, 1630; OPB5–1215, 645, 1470, 1335, 1015; OPB7–780, 895, 445, 350, 1335, 2680, 1630; OPB7–895, 445, 350, 1060, 485, 1105, 660, 1470; OPB11–1550, 1045, 530, 420, 1335, 970, 1220, 660, 500; OPB12–1550, 1105, 1915, 930, 1410, 1160, 585; OPB20–1635, 1935, 1330, 670, 1120, 1035, 790, 682.