MOLECULAR SYSTEMATIC STUDIES IN SOME MEMBERS OF THE GENUS SENECIO L. (ASTERACEAE)

Stephen Andrew Harris

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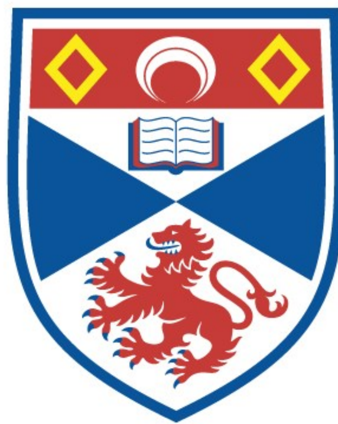

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Molecular systematic studies in some members of the genus *Senecio* L. (Asteraceae).

by

Stephen Andrew Harris.

A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy.

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October 1990.

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Abstract.

The large body of data which is available makes some members of the genus *Senecio* an ideal group on which to use molecular techniques to study biosystematic problems.

Three major problems have been addressed:- (i) What is the degree of intraspecific DNA variation present in *Senecio cambrensis*, *S. squalidus* and *S. vulgaris sl*? (ii) Did *S. vulgaris* ssp. *vulgaris* var. *hibernicus* originate via the introgression of *S. squalidus* genes into *S. vulgaris* ssp. *vulgaris* var. *vulgaris*? (iii) Is *S. cambrensis* the allohexaploid hybrid of *S. squalidus* and *S. vulgaris sl*?

These questions have been addressed using both the nuclear and chloroplast genomes. It has been demonstrated that molecular evidence can provide new insights into relationships, but can also produce results which are either contradictory to other evidence or inconclusive.

Intraspecific variation was encountered in *Senecio squalidus* and *S. vulgaris sl* for both the nuclear (ribosomal DNA) and chloroplast genomes. This variation has provided new insights into the relationship between the two subspecies of *S. vulgaris*. It is proposed that *S. vulgaris sl* may have originated via reciprocal crosses between *Senecio* species possessing different chloroplast genomes. The hybrid nature of the majority of *S. cambrensis* populations was confirmed, since most of the *Senecio*

species analysed could be distinguished on the basis of their ribosomal DNAs.

Molecular techniques have produced contradictory evidence regarding the relationship of *Senecio squalidus* to *S. vulgaris* ssp. *vulgaris sl*. In this case the two taxa have identical chloroplast genomes. This conflicts not only with the rDNA data but also with morphology, cytology and isozymes. The possible reasons for this conflict are discussed.

The ribosomal and chloroplast genomes have produced inconclusive evidence regarding the introgressive origin of *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus*.

In this thesis some of the exciting applications of molecular biology to biosystematics have been reviewed and the need for multidisciplinary approaches to biosystematic problems is emphasised.

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Chapter 1.

General introduction.

"None but those who have experienced them can conceive of the enticements of science. In other studies you go as far as others have gone before you, and there is nothing more to know; but in the scientific persuit there is continual food for discovery and wonder."

Frankenstein.
M. Shelley.

Biosystematic research is continually searching for new characters to use in the classification of organisms (Stace 1980). Over the past ten years methods developed in molecular biology for analysing DNA have become available to systematists. This availability has led to an explosion of interest in the application of these techniques to a wide range of plant and animal material. The rationale for studying DNA is simple. Since DNA is passed from generation to generation it should provide the best insight into the phylogeny of an organism (Giannasi and Crawford 1986). One should, however, bear in mind the caution expressed by Stace (1980) with regard to phytochemical evidence in systematics, ie. the apparently more fundamental nature of the data may not make it more important in a classification. *A priori* the usefulness of a data source is unknown. Within the group of interest, molecular characters may be useless because they are either invariable or show very high degrees of parallelism and convergence (Hillis and Moritz 1990, Moritz and Hillis 1990). Similar arguments have been used by Stace (1980) for many other character sources, eg. pollen grain structure, flower colour and chromosomes.

Within a plant cell three genomes are available for analysis:- (i) The mitochondrial genome (mtDNA), (ii) the chloroplast genome (cpDNA) and (iii) the nuclear genome (nDNA). Each of these genomes have their own modes and tempos of evolution (Palmer 1985b). Therefore, they each have their own potential value in answering particular questions posed by plant systematics.

Three areas in which they have considerable potential are:- (i) The description of the degree of variation present within and between taxa, (ii) taxon identification and (iii) reconstruction of plant phylogenies. The research presented in this thesis uses molecular markers to tackle some of these aspects in the genus *Senecio*.

1.1 The genus *Senecio*.

1.1.1 General taxonomic background.

Senecio L. is, probably, the largest genus of Angiosperms (Jeffrey *et al* 1977). As many as 3000 species have been recognised (Willis 1973), although recent estimates have placed the number closer to 1500 by the removal of some species into closely related genera (eg. Mabberley 1987, Jeffrey 1978). This cosmopolitan genus occurs in a wide range of habitats and as a number of different life forms, from desert succulents in South Africa to temperate annuals (Jeffrey *et al* 1977). *Senecio*

sl has centres of diversity in the Andes, the West Indies and South and tropical Africa (Nordenstam 1977).

The wide range of morphological and ecological variation has led to a heterogeneous concept of the genus. Jeffrey *et al* (1977) highlight the varying generic concepts that different workers have. Jeffrey (1978), in a synthesis of available morphological, phytochemical and cytological evidence has suggested a framework within which generic concepts in the tribe Senecioneae may be tested (Table 1.1). *Senecio sensu stricto* falls into group IX of Jeffrey's system. This is the largest group recognised and the one in which the delimitation of the genera are particularly problematic.

Cytological studies have been of little help since within the Tribe Senecioneae, two base numbers are found in those genera that are closely related to *Senecio sl* ($x=5$ and $x=10$, Nordenstam 1977). This has led to considerable debate regarding the base number of the genus *Senecio*. This debate has been reviewed by Lawrence (1980) and she suggested that either base number may have been ancestral in the genus. For all practical purposes, however, the base number of the genus is usually taken to be $x=10$, and this is the convention which will be followed in this thesis. Thus *S. vulgaris* is a tetraploid ($2n=4x=40$).

Diversification of the genus has been accompanied not by base number change but by polyploidy, where at least three major 'ploidy levels are found on each Continent (Lawrence 1980), and Lawrence (1980) has described the

genus *Senecio* as a very good example of the secondary cycle of polyploidy described by Stebbins (1971).

Table 1.1 Classification of part of the Tribe Senecioneae based on morphological, cytological and phytochemical evidence. According to Jeffrey (1978).

A. 'Cacalioids'

I. 'Insulares'

II. 'Woody Cacalioids'
Senecio plus *Traversia*, *Bedfordia*, *Luina*, and *Tetradymia*. Possibly *Faujasia*, *Alciope*, *Gynoxys*.

III. 'Herbaceous Cacalioids'
Senecio plus *Adenostyles*, *Psacalium*, *Petasites*, *Homogyne*, *Tussilago*, *Doronicum* and *Arnoglossum*.

B. 'Tephroseroids'

IV. 'Type 1'
Senecio.

V. 'Type 2'
Senecio.

C. 'Senecionoids'

VI. 'Palustres'
Senecio.

VII. 'Austroamericanae'
Senecio plus *Synosma*, *Culcitium* and *Microchaete*. Possibly *Werneria*.

VIII. 'Neotropicae'
Senecio.

IXa. 'Eusenecionoids'
Senecio. Possibly *Kleinia*.

IXb. 'Gynuroids'
Senecio plus *Gynura*, *Emilia*, *Kleinia ss* and *Crassocephalum*. Possibly *Cineraria*, *Steirodiscus*, *Lopholaena* and *Kleinia*.

X. 'Othonnoids'
Othonna and *Euryops*.

XI. 'Synotoids'
Senecio and *Mikaniopsis*.

1.1.2 The genus in the British Isles and Europe.

Sixty seven species of *Senecio* have been recorded, as either native or established, in Europe (Chater and Walters 1976). Seventeen species are recorded as occurring in the British Isles (Clapham *et al* 1987) either as natives or established aliens. Crisp (1972) records a further 22 species that are occasionally introduced or have been recorded at least once in the British Isles. Other species are horticulturally valuable (Jeffery 1980).

Ten species are considered native in the British Isles, two of which are or are nearly, extinct (*Senecio congestus* and *S. paludosus*). Of the remaining eight native species, only *S. vulgaris sl* and *S. cambrensis* have been studied in any detail here. Of the seven established introductions, only *S. squalidus* has been studied here. The species that have been used in this study are shown in Table 1.2, along with their chromosome numbers. A body of biosystematic data has now been amassed on a number of *Senecio* species in both the British Isles and Europe. Some of the most notable of these studies are Crisp (1972), Alexander (1975, 1979), Kadereit (1984a, 1984b), Taylor (1984) and Ashton (1990). In view of the close relationships postulated between the British species and some European species, it has been necessary to include some non-native species in this study.

Table 1.2. Chromosome numbers and status in the British Isles of the *Senecio* taxa used in this study.

Taxon[¨].	Chromosome number (2n)[°]	Status in Britain.■
S. aethnensis Jan ex DC.	20	N/A
S. cambrensis Rosser.	60	Native
S. chrysanthemifolius Poiret.	20	N/A
S. jacobaea L.	40	Native
S. paludosus L.	40	Native
S. squalidus L.	20	Naturalised.
S. vernalis Waldst. & Kit.	20	Casual.
S. vulgaris L.		
ssp. *vulgaris*		
var. *vulgaris*.	40	Native
var. *hibernicus* Syme.	40	Native
ssp. *denticulatus* (O.F. Muell.)P.D. Sell.	40	Native

¨ Authorities are only presented for the taxa which were used in this study. Other taxa mentioned in the thesis text are not given authorities. *Senecio vernalis* is recognised here as a species, rather than a subspecies of *S. leucanthemifolius* as proposed by Alexander (1979).

° Chromosome numbers are taken from reports in Clapham *et al* (1987), Crisp (1972) or Alexander (1979).

■ N/A - not recorded in the British Isles. *Senecio aethnensis* is a Mt. Etna endemic and *S. chrysanthemifolius* has a Mediterranean distribution.

1.1.2.1 *Senecio vulgaris sensu lato*.

Senecio vulgaris is a tetraploid (2n=4x=40), self-compatible, monocarpic ephemeral which reproduces predominantly via self-fertilisation (Trow 1912, Hull 1974, Marshall and Abbott 1982). The taxonomy of the species was clarified by Allen (1967), who recognised three varieties;

(i) var. *vulgaris*, the common non-radiate type.

(ii) var. *hibernicus*, an inland radiate type which is morphologically similar to var. *vulgaris*, except for the presence of ray florets.

(iii) var. *denticulatus*, a predominantly radiate type which is confined to maritime habitats in the British Isles.

Sell (1968) went further and elevated var. *denticulatus* to subspecific status. In this study the recognition of two subspecies is accepted; ssp. *vulgaris* and ssp. *denticulatus*. Within ssp. *vulgaris* two varieties are recognised; var. *vulgaris* and var. *hibernicus*.

The recent literature has revealed some dispute over the origin of *Senecio vulgaris*. Weir and Ingram (1980), on the basis of chromosome pairing at meiosis in hybrids, considered *S. vulgaris* to be an allopolyploid, with half its genome homologous to that of *S. squalidus*. Kadereit (1984a) suggested that *S. vulgaris* ssp. *denticulatus* is an autopolyploid of *S. vernalis* (2n=2x=20), from which *S. vulgaris* ssp. *vulgaris* was derived.

The ray floret polymorphism has fascinated geneticists for many years. Trow (1912) showed that the polymorphism was controlled by a single gene, the two alleles of which

showed incomplete dominance. *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris* is homozygous for the non-radiate allele and var. *hibernicus* is homozygous for the radiate allele. The effect on outcrossing in *S. vulgaris* ssp. *vulgaris sl*, of the radiate allele has been studied by Marshall and Abbott (1982, 1984a, 1984b), Warren (1987) and Irwin (1990).

Two proposals have been advanced for the origin of the var. *hibernicus*: (i) Introgression of the radiate gene from *Senecio squalidus*. (ii) Mutation of the ray floret locus.

The evidence relating to these two modes of origin has recently been reviewed by Ashton (1990) and Irwin (1990). However, briefly the evidence for the introgressive origin is based on three lines of enquiry:

(i) The broadly parallel spread of *Senecio squalidus* and *var. hibernicus* (Crisp 1972, Stace 1977).

(ii) Intermediacy of var. *hibernicus* between *Senecio squalidus* and var. *vulgaris* (Richards 1975, Monaghan and Hull 1976, Oxford and Andrews 1977).

(iii) Artificial synthesis of hybrids and backcross products (Harland 1954, Gibbs 1971, Ingram 1977, Ingram *et al* 1980, Taylor 1984).

Recently, Ashton (1990) has provided evidence, from an isozyme study, that var. *hibernicus* may have gained an AAT (aspartate aminotransferase) allozyme from *Senecio squalidus*. This considerably strengthens the argument for introgression.

Stace (1977) however, considered that the possibility of an introgressive origin of var. *hibernicus* had been advanced to the exclusion of the alternative, equally

likely, mutation hypothesis. Within the Asteraceae mutations are known which result in a change of the ray floret character, ie. radiate to non-radiate (eg. *Aster tripolium*, *Leucanthemum vulgare*) and non-radiate to radiate (eg. *Bidens cernua*). Indeed, such mutations are known to occur in the genus *Senecio* [eg. non-radiate *S. squalidus* (Taylor 1984) and non-radiate *S. sylvaticus* in Sweden (R. Ingram, Pers. Comm.)].

On the basis of the fertility of artificial triploids, Ingram *et al* (1980), accepted the view of Stace (1977) that var. *hibernicus* probably had a restricted origin and that subsequent spread was via fruit dispersal. However, Kadereit and Briggs (1985) and Abbott (1986) have questioned the restricted origin of var. *hibernicus*. The variation found in life history traits, they argue, suggests that there may have been a multiple origin of the variety.

Senecio vulgaris ssp. *denticulatus*, an ecologically distinct winter annual, is separated from *S. vulgaris* ssp. *vulgaris sl* by the possession of shorter ray florets (2.3-3.0mm vs 3.5-5.5mm in var. *hibernicus*), a densely arachinoid indumentum (usually) and leaf shape (Allen 1967, Kadereit 1984a). In Europe, ssp. *denticulatus* has been recorded from Börnholm, Lolland, the Freisian Islands, the Baltic coasts of Sweden and Denmark and the Normandy Coast (Allen 1967). In the Mediterranean, Kadereit (1984a) states that this taxon becomes a montane element. In the British Isles, ssp. *denticulatus* is apparently restricted to a few locations on maritime dunes (Ainsdale Beach in Lancashire

and the Channel Islands, Ashton 1990). Early records of ssp. *denticulatus* (Allen 1967, Perring and Sell 1968, Crisp 1972) from other coastal sites in the British Isles (Devon, Cornwall, Cheshire and the Isle of Man) have not been confirmed in recent years (Ashton 1990). This may be due to extinction of the subspecies at these sites or the early confusion surrounding the nomenclature of the intraspecific ranks of *S. vulgaris* (Allen 1967).

1.1.2.2 *Senecio squalidus*.

Senecio squalidus is a diploid (2n=2x=20), largely self-incompatible alien which is widespread, and common as an annual or short-lived perennial in the British Isles. *Senecio squalidus* spread from the Oxford Botanic Garden in the 19th century, via the railway network, following its introduction into the Gardens prior to 1690 (Druce 1927).

The spread of this species and its establishment in the British Isles has been chronicled by Kent (1956, 1957, 1960, 1963, 1964a, 1964b, 1964c, 1964d, 1966) and more recently by Ashton (1990). Today the species is common in Scotland, in suitable habitats, south of the Forth-Clyde line. Above this line Ashton (1990) states that apparently stable populations occur in Fife (Kirkcaldy and Methil) and Angus (Kirriemuir). Other reported populations (eg. Aberdeen and Dundee) are apparently ephemeral.

Two features are of importance in the historical spread of *Senecio squalidus* in Britain. (i) The delay of over a century between its introduction to the Garden and

its spread to the city. (ii) The colonisation success of the species in suitable habitats.

It is not known whether one or more introductions of *Senecio squalidus* were made to Oxford, although Ashton (1990) states (on the basis of the sporophytic self-incompatibility system) that at least two different introduced plants must have been cultivated. These could, however, have been derived from a single seed accession.

Crisp (1972) has speculated about the nature of the introduction. He suggests that it may have been introduced from a hybrid swarm between two Mediterranean species; *Senecio aethnensis* and *S. chrysanthemifolius*. However, as Walters (1964) has pointed out, the taxonomy of the plants related to British *S. squalidus* is very confused, despite two revisions of the European *Senecio* species (Chater and Walters 1976, Alexander 1979). Ashton (1990) states that;

> '..., the possibility exists that further introductions into Britain of *S. squalidus*, or morphologically similar inter-fertile relatives, have occurred, and the close similarities of the British plant to its European relatives, most notably *S. rupestris*, increases the prospect that such introductions would go undetected.'

1.1.2.3 *Senecio cambrensis*.

Senecio cambrensis, an endemic British species, was first described by Rosser (1955) from a specimen collected from Ffrith in Wales. Rosser considered this plant to be an allohexaploid (2n=6x=60) hybrid between *S. vulgaris* (2n=4x=40) and *S. squalidus* (2n=2x=20). Other locations for this species have been recorded on the Wirral (near the

Ness Botanic Gardens), at Mochdre in Wales and at Leith in Edinburgh (Ashton 1990).

In a review of the history and evidence relating to the allopolyploid nature of the species, Ashton (1990) suggests that the population on the Wirral may be an introduction. This however leaves three disjunct populations at Wrexham, Mochdre and Leith. Recently, Ashton (1990), using isozymes, has shown that *Senecio cambrensis* has had at least two origins. Once in Wales at Ffrith, and again in Scotland at Leith. A third possible origin was suggested for the population at Mochdre.

1.2 Thesis outline.

The research reported in this thesis is directed towards the following biosystematic problems in some British *Senecio* species:-

i) What is the degree of intraspecific DNA variation present in *Senecio vulgaris sl*, *S. squalidus* and *S. cambrensis*? This has been addressed using chloroplast DNA, nuclear ribosomal DNA and random nuclear DNA sequences.

ii) Did *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus* originate via the introgression of *S. squalidus* genes into *S. vulgaris* ssp. *vulgaris* var. *vulgaris*? This question has been addressed using chloroplast DNA, ribosomal DNA and random nuclear sequences.

iii) Is *Senecio cambrensis* the allohexaploid hybrid of *S. squalidus* and *S. vulgaris* ssp. *vulgaris sl*, and if so

which was the female parent? In addition, the possibility of reciprocal crosses at the different sites of origin is investigated. This question has been addressed using chloroplast DNA and ribosomal DNA sequences.

The data relating to each of these questions is presented according to the genome studied. The chloroplast genome (Chapter 3) and nuclear genome [ribosomal DNA (Chapter 2) and random nuclear sequences (Chapter 5)] have been used. The mitochondrial genome was not used in this study because of the complexity of its arrangement (Palmer 1985b) and the lack of suitable probes. Throughout this thesis the need for multidisciplinary approaches to biosystematic problems is emphasised.

Chapter 4 is a review of the literature relating to two assumptions in the biosystematic use of chloroplast DNA; the low level of intraspecific variation and uniparental plastid transmission.

Chapter 6 concerns the identification of *Senecio squalidus*-specific nuclear DNA probes.

Finally, in Chapter 7 the data as it applies to *Senecio* biosystematics is discussed.

Chapter 2.

Biosystematics of some British and European *Senecio* species: - Ribosomal DNA evidence.

" ... Of secrets kept, strength like a tower,
And trust unbroken, freedom, escape;
Of changing and of shifting shapes,
Of snares eluded, broken traps,... "

The Silmarillion
J. R. R. Tolkien.

Introduction.

Two families of ribosomal RNA (rRNA) genes are encoded in the nucleus, the 18S-28S rRNA genes and the 5S rRNA genes. Both of these families have been the subject of considerable research in animals and plants however, only the former rRNA gene family will be considered here.

In this introduction I will briefly describe the structure, organisation and evolution of the various different regions of ribosomal DNA (rDNA). This has frequently been reviewed; most recently by Appels and Honeycutt (1986), Gerbi (1986) and Rogers and Bendich (1987). This will be followed by a consideration of rDNA as a biosystematic marker and its application in the study of plant hybridisation. Finally rDNA in the Asteraceae will be considered.

2.1.1 Structure, organisation and evolution of nuclear ribosomal DNA.

2.1.1.1 Ribosomal DNA structure and organisation.

Nuclear ribosomal RNA genes in Angiosperms are arranged as tandem arrays at one to four loci (Rogers and Bendich 1987). They are 7.5kb to 18.5kb in length (Appels and Honeycutt 1986) and are composed of a number of regions which have varying functional constraints and, therefore,

varying evolutionary rates (Gerbi 1986). Within diploid plant cells 500 to 40,000 rDNA repeats may be found (Rogers and Bendich 1987). Each rDNA tandem array behaves effectively as a single locus (May and Appels 1987, Saghai-Maroof *et al* 1984, Snape *et al* 1985 and Zimmer *et al* 1988). The general features of a rDNA repeat unit are shown in Figure 2.1.

(i) The coding regions are those segments of the rDNA which ultimately form the mature rRNAs. They are expected to have a relatively conserved sequence evolution and to vary relatively little between taxa. Exceptionally, parts of the coding region are variable (Wheeler and Honeycutt 1988), but such difficulties have not usually been encountered in restriction enzyme studies (Jorgensen *et al* 1987).

(ii) Internal transcribed spacers (ITS) are those portions of the transcribed RNA that are excised during RNA maturation. These show an intermediate level of variation which is consistent with the view that they are under an intermediate level of functional constraint. This conservation may be a reflection of the presence of processing signals (Schaal and Learn 1988). Some transcribed spacer regions have been sequenced, eg. *Lupinus luteus* (Rafalski *et al* 1983) and *Sinapis alba* (Rathgeber and Capesius 1989). A comparison of such sequences has shown that the two ITS regions (Figure 2.1, regions a and b) evolve at different rates, both of which are greater than the 5.8S rRNA gene (Jorgensen and Cluster 1988).

(iii) The intergenic spacer (IGS). This has also been known as the nontranscribed spacer (NTS), but is something of a misnomer since it is now known that this region of the rDNA is transcribed but that the transcript is very short lived (Rogers and Bendich 1987). The IGS is expected to be the most variable region of the rDNA, in both length and sequence (Jorgensen and Cluster 1988). The structure of the IGS, both from sequencing studies (eg. Appels and Dvorak 1982b) and fine-scale genetic analysis (eg. Appels and Dvorak 1982a), indicates that the IGS is apparently composed of at least three functionally distinct regions; a) the subrepeats, b) the 3′ region and c) the 5′ region.

IGS subrepeat variability results in variation in rDNA repeat length. These subrepeats have been found in all organisms for which sequences are available and have been infered to exist in many others on the basis of periodic length variability (Rogers and Bendich 1987). The numbers of these subrepeats vary both within and between species, but are between 100bp and 200bp in length (Jorgensen and Cluster 1988). The function of these subrepeats is not known, but it has been proposed that they may represent either rDNA transcription "enhancers" (Flavell *et al* 1986c) or rDNA transcription terminators (Rogers and Bendich 1987).

The region 5′ of the subrepeat region is variable in length but has no known function. The region 3′ of the subrepeats is variable in both length and sequence. It is thought to contain the rDNA transcription promotors and the

binding sites for species-specific transcription factors (Gerbi 1986, Schaal and Learn 1988).

2.1.1.2 Ribosomal DNA evolution.

Jorgensen and Cluster (1988) have identified four modes of rDNA evolution:- (i) Copy number changes, (ii) methylation changes, (iii) site changes and (iv) length changes.

(i) Copy number changes. The variation in the number of rDNA repeats within and between taxa has been recently reviewed by Rogers and Bendich (1987).

(ii) Methylation changes. Plant nuclear DNA is extensively methylated at CG dinucleotides and CNG trinucleotides (where N is any nucleotide, Hepburn *et al* 1987). 5-Methylcytosine, the principle methylated base, may make up as much as 32% of all cytosine residues in the plant genome (Vanyushin *et al* 1960). Many studies have shown that ribosomal RNA genes are extensively methylated (Blundy *et al* 1987, Delseny *et al* 1984, Ellis *et al* 1983, von Kalm *et al* 1986, Steele-Scott *et al* 1984). DNA methylation has been associated with the suppression of gene activity (reviewed by Hepburn *et al* 1987). The effect of methylation in restriction enzyme studies is to change restriction pattern phenotypes due to apparent site loss.

(iii) Site changes (base substitution). Comparisons of aligned restriction maps show that base substitutions which affect restriction sites, are rare events in the coding region compared to the spacer regions (Jorgensen *et al*

1987, Gerbi 1986). When they do occur they alter restriction pattern phenotypes.

(iv) Length changes. Insertions and deletions may occur over much of the rDNA. But as a consequence of differential functional constraints, they tend to be more common in the IGS compared to either the coding regions or ITS. The IGS accumulates length variation either within the subrepeats or outside of them. In *Avena barbata* 17 different length variants have been analysed, all based on incremental length changes of a presumed subrepeat (length 115bp, Cluster *et al* 1984). In *Pisum sativum* a 100bp substitution has been shown to occur outside of the subrepeat region (Jorgensen and Cluster 1988). This type of change can have dramatic effects on restriction pattern phenotype, particularly if intra-individual rDNA repeat length variation occurs.

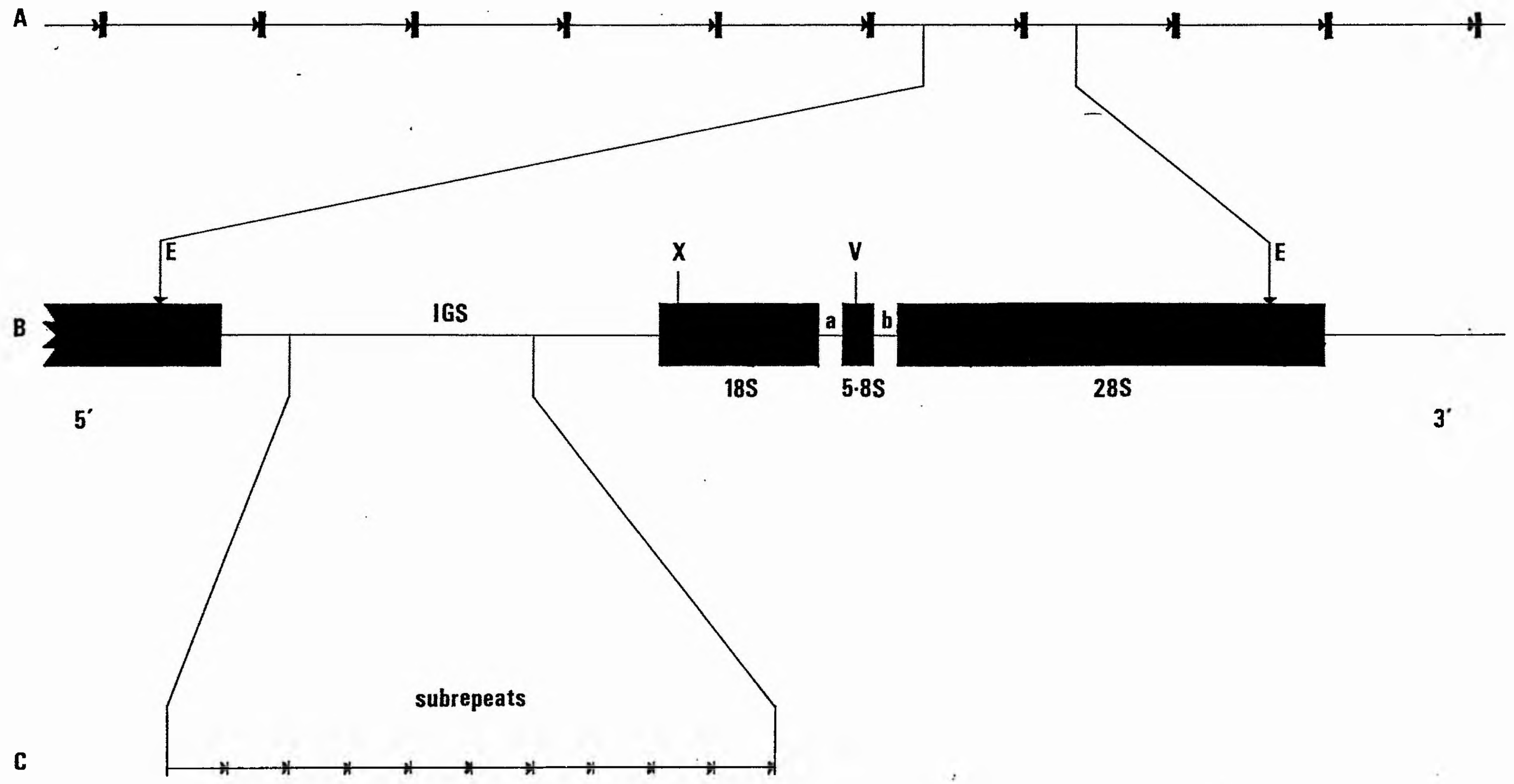
Early comparisons between different rRNA gene copies within a species (paralogous comparisons) showed greater homogeneity than between "homologous" loci in different species (orthologous comparisons). If rRNA genes were evolving independently then it would be expected that paralogous and orthologous comparisons would show the same degree of divergence. Thus a homogenising mechanism must exist within taxa. This led Zimmer *et al* (1980) and Arnheim (1983) to develop the general concept of concerted evolution of multigene gene families (Futuyama 1986). Two mechanisms are thought to be important in the concerted

evolution of rDNA; unequal crossing-over (Smith 1976) and gene conversion (Szostak and Wu 1980).

The relative contributions of unequal crossing over and gene conversion can lead to fixation of one variant or another as a result of genetic drift, though some evidence is available that rRNA gene length variants may have selective advantages. Rocheford *et al* (1990) have argued that their study, which showed a reduction in frequency of a short rRNA gene variant (3.4kb) and the concomitant increase of a long variant (5.2kb) in a mass-selected population of *Zea mays* 'Hays Golden', is good evidence of natural selection acting directly on the rRNA locus. Other studies which may indicate natural selection of rDNA length variants include Saghai-Maroof *et al* (1984, *Hordeum vulgare*) and Flavell *et al* (1986a, *Triticum dicoccoides*)

In addition to selection and drift, Dover (1982) has proposed that a third force (molecular drive) is important in the fixation of multigene family variants (reviewed in Dover *et al* 1982).

Figure 2.1. The general structure of ribosomal DNA (rDNA) illustrated with the wheat ribosomal clone (pTA71). **A.** The tandem arrangement of rRNA genes. **B.** Detail of a single rRNA gene; IGS - intergenic spacer, 18S-5.8S-28S - coding regions, a and b internal transcribed spacer. **C.** Detail of the IGS subrepeat region. The letters, in **B**, refer to restriction sites; X and V are the conserved *Xba*I and *Eco*RV sites respectively, E is the *Eco*RI site used in the cloning of pTA71.


A
B
C
E
E
X
V
IGS
a
b
18S
5·8S
28S
5′
3′
subrepeats

2.1.2 Ribosomal DNA as a biosystematic marker.

Ribosomal RNA genes have three features that make them ideal biosystematic markers.

A) They are present in all plants as a high copy number, mid-repetitive component of the genome (see Chapter 6). This means that these genes are detectable even in small quantities of genomic DNA (Doyle *et al* 1984, Doyle 1987).

B) The varying functional constraints on different regions of the molecule means that many levels of the taxonomic hierarchy can be analysed with this single gene; by a judicial choice of region. Jorgensen and Cluster (1988) have identified 11 types of rDNA variation that may be used to analyse 5 different ranks of the taxonomic hierarchy (from within Angiosperms to within species).

C) Ribosomal DNA probes are available from a number of different plant families, eg. *Triticum aestivum* (Gerlach and Bedbrook 1979), *Linum usitissimum* (Goldsbrough and Cullis 1981) and *Taraxacum officinale* (King and Schaal 1990).

In this Section I shall briefly describe some of the methods that have been used and the types of rDNA data that have been obtained. A brief consideration of the various levels of the taxonomic hierarchy at which rDNA data has been used will then follow.

2.1.2.1 Ribosomal DNA data: Methods and types.

Three broad approaches to rDNA data collection, in plants, have been used. (i) Thermal elution studies. (ii) Restriction enzyme analysis. (iii) Sequence analysis.

(i) Thermal elution studies. This technique, which derives a measure of similarity between two sequences, has largely been superceded by restriction enzyme analysis. However, Appels and Dvorak (1982a) have used thermal elution analysis in studies of the genus *Triticum* to great effect, and this paper should be consulted for more details of the method. The method requires that homologous (or nearly homologous) probes are available for the taxa of interest. Since only heterologous probes were available in this study (*Triticum aestivum* and *Linum usitissimum*) this technique was not used (see Chapter 6).

Doyle (1987) has used this technique to infer the degree of sequence divergence between the IGS of six *Glycine* species. Appels and coworkers have made extensive use of this technique, as an adjunct to restriction enzyme analysis, in the Poaceae (Appels and Dvorak 1982a, 1982b). Indeed Appels and Dvorak (1982b) argue that this technique provides a rapid method of assessing the degree of sequence similarity between particular regions of the rRNA gene for a large number of taxa. For a critical discussion of the technique, particularly with regard to phylogeny reconstruction, the review by Springer and Krajewski (1989) should be consulted.

(ii) Restriction enzyme analysis. At the present time this is the most common technique of rDNA analysis applied

to biosystematic studies. Two restriction enzyme based approaches have been adopted; either restriction fragment or restriction site comparison. A general description of these approaches is given in Chapter 3, Section 3.1.2.2. For both types of comparison radiolabelled probes are used, either cloned rRNA genes (or parts of them) or purified 18S and 28S rRNAs, to identify fragments of similar sequence in the genome of interest. As an initial step for site comparisons the construction of a rDNA restriction map is necessary, either from total genomic DNA (Sytsma and Schaal 1985) or from cloned rDNA (Doyle 1987). Ribosomal DNA site changes (either losses or gains) are then used as biosystematic characters in studies at all levels of the taxonomic hierarchy (Jorgensen and Cluster 1988). Restriction fragment analysis is less satisfactory because specific changes cannot be identified.

(iii) Sequence analysis. Many regions of the rDNA have been sequenced for a number of taxa, for example, coding region (*Oryza sativa*, Takaiwa *et al* 1984, 1985a), ITS (*Lupinus lutea*, Rafalski *et al* 1983) and IGS (*Triticum aestivum*, Barker *et al* 1988, *Oryza sativa*, Takaiwa *et al* 1985b). However, relatively few studies have used sequencing as a biosystematic tool. Hamby and Zimmer (1988) have used direct rRNA sequencing to look at phylogenetic relationships in the Poaceae, while McIntyre et al (1988a) have looked at eight species of the Triticeae (Poaceae). However, in the future, application of the polymerase chain reaction (PCR, Erlich 1989) to initiate primer extension in defined regions of the rDNA appears to have considerable

potential for the application of sequencing, at a reasonable cost, to all levels of the taxonomic hierarchy.

2.1.2.2 Application of ribosomal DNA in the taxonomic hierarchy.

An impressive body of data has been built up concerning the structure, expression and evolution of plant rRNA genes (Flavell *et al* 1986c, Flavell 1986a, 1986b, Rogers and Bendich 1987, Appels and Honeycutt 1986), but relatively little regarding the use of rDNA in biosystematics.

Although Jorgensen and Cluster (1988) recognised five taxonomic ranks at which ribosomal DNA may be of use, only three of these have been used to any great degree; namely the tribal, specific and intraspecific levels. Work at the tribal level has been dominated by studies in the Poaceae, probably as a result of its economic importance. Appels and colleagues have done much work in the Tribe Triticeae, looking at generic and species relationships (Appels and Dvorak 1982a,b, Gill and Appels 1988, McIntyre *et al* 1988a).

The majority of studies have, however, been conducted at the specific level and below (Table 2.1). Of particular interest to the present investigation are those studies which have been used to provide insights into hybridisation and introgression.

Ribosomal DNA has not been used extensively as a marker for introgressive hybridisation, due to the importance attached to the chloroplast genome in these studies (Chapter 3) and the small size of the sequence.

Tremousaygue *et al* (1988) have suggested that a rDNA probe composed of *Raphanus* IGS subrepeats may be a useful marker to follow the introduction of *Raphanus* nuclear DNA into *Brassica* species. A similar use for IGS subrepeats has been proposed by Appels and coworkers for the Triticeae (Appels *et al* 1986, McIntyre *et al* 1988a). Springer *et al* (1989) using a maize rDNA were able to detect a 2% *Sorghum halapense* 'contamination' of *S. bicolor* ssp. *drummondii*.

The introgressive origin of a taxon might be expected to have one or more of the following effects, with respect to the rDNA. (i) An increase in the level of length and/or site variation in the population compared to the putative non-introgressive progenitor. (ii) The presence of at least some individuals in the population with additive restriction profiles between the two presumptive parents. (iii) No effect at all, due to the loss of rDNA loci from the introgressing species, in the introgressant.

In wild species few molecular studies of introgression have been made. Schaal *et al* (1987) in a study of two subspecies of *Phlox divaricata* rejected an introgressive origin of ssp. *laphami*, since the presumed introgressant (ssp. *laphami*) had a lower rDNA diversity than the presumed progenitor (ssp. *divaricata*).

The most extensive studies of introgression, using rDNA molecular markers, which have been published are those of Rieseberg and colleages in the genus *Helianthus*. Rieseberg *et al* (1988) studied the presumed introgressive origin of weedy *Helianthus bolanderi* from serpentine *H. bolanderi* via the introduction of *H. annuus* genes. In this

case a recent introgressive origin was rejected since none of the presumptive introgressants had additive rDNA patterns as might be expected (in addition to other characters), though additive patterns were located in a hybrid swarm of *H. annuus* and *H. bolanderi*. Rieseberg *et al* (1990) report evidence of the presence of rDNA restriction fragments from *H. debilis* ssp. *cucumerifolius* in *H. annuus* ssp. *texanus* and propose that this may be the result of the introgression of genes from *H. debilis* ssp. *cucumerifolius* into *H. annuus*, following the introduction of *H.annuus* into Texas. In a study of the *Oryza sativa* complex, the presence of additive rDNA phenotypes led Cordesse *et al* (1990) to propose that introgression may be occurring. In general, however, rDNA has either not been used very frequently or has not proved successful as a marker for introgression.

Ribosomal DNA variation at the population level has been the subject of a number of investigations, in particular the extensive studies of Schaal and coworkers (Schaal and Learn 1988, Schaal *et al* 1987, Learn and Schaal 1987, King and Schaal 1989, King and Schaal 1990).

Such studies indicate that extensive intraspecific variation (both length and site) may occur (see Schaal and Learn 1988 for a review). Rogers *et al* (1986) found *Vicia faba* plants that had up to 20 different length variants and Schaal and Learn (1988) report work by Baum showing that *Lupinus texensis* has up to 11 length variants per individual (see also Chapter 3). Intra-individual variation on this scale is apparently exceptional. Studies with other species suggest that more moderate intra-individual length

variation occurs (1.98-2.65 length variants per individual, Schaal and Learn 1988, Schaal *et al* 1987, Saghai-Maroof *et al* 1984). Indeed some taxa show no length variation, for example, *Solidago altissima* shows no length variation (except for a rare deletion) but extensive site variation (Schaal and Learn 1988). On the basis of this and other data Schaal and Learn (1988) concluded that there was no consistent pattern between rDNA variation and population size or species range.

Table 2.1 Some published studies of ribosomal DNA variation, excluding Asteraceae.

Family	Taxon	Level§	Reference.
Apiaceae	Ferula communis	I	Olmedilla et al (1985)
Betulaceae	Betula	S	Bousquet et al (1989)
Brassicaceae	Brassica	S	Tremousaygue et al (1988)
Cucurbitaceae	Cucurbita	S	Ganal and Hemleben (1986)
Gentianaceae	Lisianthius	S	Sytsma and Schaal (1985)
"Leguminosae"	Glycine	S	Doyle and Beachy (1985)
	Pisum satium	I	Jorgensen et al (1987)
	Vicia	S	Lamppa et al (1984)
Liliaceae	Scilla peruviana	I	Carmona et al (1984)
	Trillium	S	Yakura et al (1983)
Poaceae	Hordeum	S	Saghai-Maroof et al (1984)
	Hordeum	S	Molnar et al (1989)
	H. vulgare	I	Saghai-Maroof et al (1984)
	H. spontaneum	I	Saghai-Maroof et al (1984)
	Sorghum	S	Springer et al (1989)
	Oryza	S	Cordesse et al (1990)
	Pennisetum glaucum	I	Gepts and Clegg (1989)
	Triticum diccocoides	I	Flavell et al (1986a)
	Zea	S	Zimmer et al (1988)
	Z. mays	I	Rocheford et al (1990)
Polemoniaceae	Phlox divaricata	I	Schaal et al (1987)
Portulacaceae	Claytonia	S	Doyle et al (1984)
Ranunculaceae	Clematis fremontii	I	Learn and Schaal (1987)

§ S - Specific level. I - Intraspecific level.

<u>2.1.3 Ribosomal DNA in the Asteraceae</u>.

In the present investigation, studies carried out in the Asteraceae are of particular interest, though they are relatively sparse. Ribosomal DNA restriction maps have been published for ten taxa (King and Schaal 1989, King and Schaal 1990, Tucci and Maggini 1986, Choumane and Heizmann 1988) and these have been useful for comparison with the results obtained in *Senecio*.

Some of the most detailed studies have been conducted in the genus *Helianthus*. Choumane and Heizmann (1988) analysed 61 genotypes from 39 species with restriction enzyme and thermal elution analyses of the IGS. Emphasising the complementary aspects of these approaches, they were able to show the presence of extensive rDNA length variation, apparently due to a 200bp subrepeat (it was suggested that these subrepeats may make useful species-specific probes). Another study in the genus enabled Rieseberg *et al* (1988) to reject an introgressive origin of weedy *H. bolanderi* (Section 2.1.3).

Studies by Schaal and collegues (King and Schaal 1989, Schaal and Learn 1988, King and Schaal 1990) in *Rudbeckia missouriensis*, *Solidago altissima* and *Taraxacum officinale* indicate that patterns of rDNA variation in the Asteraceae are very diverse, reflecting the range of rDNA variation that has been found in other Angiosperms.

The aims of the experiments reported in this Chapter were: (i) To assess the level and type of intraspecific

rDNA variation that occurs in *S. vulgaris sl.* and *S. squalidus*. (ii) To determine if rDNA could be used as a marker to look at the putative introgressive origin of *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus*. This was done by the construction of a rDNA restriction site map for each putative parental taxon (*S. squalidus* and *S. vulgaris* ssp. *vulgaris* var. *vulgaris*) and then surveying populations of all three taxa for rDNA restriction enzyme phenotypes. (iii) To confirm the hybrid nature of *S. cambrensis* at the nuclear DNA level.

Materials and Methods.

2.2.1 Plant material.

Achenes from single individuals of *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris*, *S. vulgaris* ssp. *vulgaris* var. *hibernicus*, *S. vulgaris* ssp. *denticulatus*, *S. squalidus*, *S. cambrensis*, *S. vernalis*, *S. aethnensis* and *S. chrysanthemifolius*, representing 133 accessions were sown under standard glasshouse conditions (Appendix A, Section A1). The locations from which these taxa were collected are given in Table 2.2

-Y

Table 2.2 Locations of the taxa used in the ribosomal DNA study.

Taxon.	Location.	Grid Ref.	No. of Indiv.	Source§
S. vulgaris ssp. *vulgaris* var. *vulgaris*.	Monomorphic populations.			
	Bo'ness, Scotland.	NS990810	1	SAH
	Brooklands Av., Cambridge.	TL460580	4	SAH
	Grantham St., Lincoln	SK970710	1	SAH
	Migvie, Aberdeenshire.	NJ437068	1	RJA
	Puffin Island, Wales.	SH653824	2	RJA
	Strathkinness Low Rd., St. Andrews.	NO484162	8	SAH
	York.	SE605508	4	PA
	Polymorphic populations.			
	Brymbo, Wales.	SJ296539	3	PA
	Central Station, Lincoln.	SK970710	1	SAH
	Devon Street, Grangemouth.	NS977814	5	SAH
	Leith Docks, Edinburgh.	NT268765	3	PA
	Mochdre, Wales.	SH822791	2	PA
	Salamander Street, Edinburgh.	NT276763	4	PA
S. vulgaris ssp. *vulgaris* var. *hibernicus*.	Central Station, Lincoln.	SK970710	1	SAH
	Devon Street, Grangemouth.	NS977814	5	SAH
	Leith Docks, Edinburgh.	NT268765	3	PA
	Mochdre, Wales.	SH822791	1	PA
	Newcraighall, Edinburgh. (Bank).	NT270730	5	JAI
	Newcraighall, Edinburgh. (Road).	NT270730	1	JAI
	Salamander Street, Edinburgh.	NT276763	7	PA
	York.	SE590510	11	RJA
	York.	SE605508	3	Warr

-Y

Table 2.2 Cont.

Taxon.	Location.	Grid Ref.	No. of Indiv.	Source§
S. vulgaris ssp. denticulatus.	Ainsdale, Lancashire.	SD295124	2	PA
	Quennevais, N of Pulente.	-	3	PA
S. squalidus.	Brymbo, Wales.	SJ296539	1	PA
	Cardiff, Wales.	ST173733	3	JAI
	Devon Street, Grangemouth.	NS977814	3	SAH
	Leith Docks, Edinburgh.	NT268765	11	PA
	Mochdre, Wales.	SH822781	2	PA
	Portland Street, Lincoln.	SK971710	4	SAH
	Sheffield.	SK350870	2	PA
	York.	SE590510	7	PA
S. cambrensis.	Brymbo, Wales.	SJ296539	3	PA
	Leith Docks, Edinburgh.	NT268765	1	PA
	Mochdre, Wales.	SH822791	3	PA
	Salamander Street, Edinburgh.	NT276763	7	SAH
S. vernalis.	Schlusslacker Weide, Eppelheim, nr. Heidelberg, Germany.	-	3	PA
S. aethnensis.	Mt. Etna, Sicily.	-	1	RJA
S. chrysanthem-ifolius.	Mt. Etna, Sicily.	-	1	RJA

§ Initials refer to collectors; JAI - Judith Irwin, PA - Paul Ashton, RJA - Richard Abbott, SAH - Stephen Harris, Warr - John Warren. Ainsdale ssp. denticulatus, Jersey ssp. denticulatus and Salamander Street S. cambrensis were determined by C. A. Stace, C. Preston and R. Ingram repectively.

2.2.2 DNA extraction, hybridisation and autoradiography.

Total DNA was extracted from individual plants (Appendix A, Section A2) and further purified by DEAE-Sephacel column chromatography (Appendix A, Section A2.3.2).

For construction of the restriction maps the number of restiction enzymes that were used was taxon dependent, but eleven enzymes were tried in various combinations (*Bam*HI, *Bgl*II, *Bst*EII, *Eco*RI, *Eco*RV, *Hin*DIII, *Pst*I, *Sal*I, *Taq*I, *Xba*I, and *Xho*I. See Section 2.2.4). For the survey of rDNA variation only three enzymes were used (*Bam*HI, *Eco*RI, *Eco*RV) for all accessions, since reliable cutting with the others was not obtained. A list of the cutting sites for the enzymes used are given in Table A1 (Appendix A).

The digestion, electrophoresis, blotting and probing of the DNA accessions are described in Appendix A, Sections A2.5 - A2.9. DNA from different taxa digested with a single enzyme were run on the same 1% agarose gel so that, where possible, direct comparisons between taxa could be made. When unusual restriction patterns were obtained for a sample, DNA from this sample was reanalysed.

The wash conditions for filters were:- a brief rinse in 2xSSC at room temperature followed by a 20 minute wash in 1xSSC + 0.1% SDS at 65°C and a subsequent 25 minute wash in 0.3xSSC + 0.1% SDS at 65°C.

2.2.3 Probe characteristics.

A cloned ribosomal DNA repeat from *Triticum aestivum* 'Chinese Spring' was used to locate ribosomal sequences in the *Senecio* nuclear genome. The probe, pTA71, is a complete rDNA repeat of 9.1kb cloned into an EcoRI site of pUC19 (Gerlach and Bedbrook 1979, gift of M. O'Dell, Cambridge Laboratory, John Innes Institute, Norwich). A diagram of the repeat is shown in Figure 2.1. The probe was labelled as in Section A2.8 (Appendix A).

2.2.4 Construction of ribosomal DNA restriction maps.

Restriction maps of *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris* (Migvie, Aberdeenshire A), *S. squalidus* (Cardiff 23/24, 6), *S. chrysanthemifolius* (2/N13/B) and *S. aethnensis* (26/17/B) were constructed using a double and single digest protocol of total DNA.

Nine enzymes were used in the construction of the *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris* rDNA map: eight hexanucleotide cutting enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*DIII, *Pst*I, *Sal*I, *Xba*I, *Xho*I) and one heptanucleotide cutting enzyme (*Bst*EII).

In the case of *S. squalidus* eight hexanucleotide cutting enzymes were used (*Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*DIII, *Pst*I, *Xba*I, *Xho*I).

For both *S. aethnensis* and *S. chrysanthemifolius* seven hexanucleotide cutting enzymes were used (*Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Pst*I, *Xba*I, *Xho*I).

Double digests were carried out in all combinations (for those useful enzymes that cut) as double digest mixtures. All digests from a single taxon were run on the same 1% agarose gel, in order to allow direct comparison between fragments. To confirm the fragment patterns for the double digests, gels were run on three separate occasions. Using this mapping strategy putative rDNA restriction maps were constructed for *S. aethnensis*, *S. chrysanthemifolius*, *S. squalidus* and *S. vulgaris* ssp. *vulgaris* var. *vulgaris*.

Results.

2.3.1 Ribosomal DNA restriction maps of *Senecio* species.

Restriction maps were constructed for four *Senecio* species (*S. aethnensis*, *S. chrysanthemifolius*, *S. squalidus* and *S. vulgaris* ssp. *vulgaris* var. *vulgaris*) using the double and single digest method. The maps produced are shown in Figure 2.2, while the fragment data on which these are based are given in Appendix F, Table F1. The restriction sites were mapped to two conserved sites in the rDNA (Figure 2.1):- (i) A *Xba*I site located approximately 166bp 3′ of the 5′ end of the 18s rRNA coding region (Eckenrode *et al* 1985) and (ii) an *Eco*RV site located in the 5.8s rRNA coding region (Rafalski *et al* 1983). These maps should be treated as provisional since a number of problems were encountered with the use of the heterologous wheat rDNA probe (see Discussion). The positions of the coding region and IGS were not determined precisely, but the coding region was assumed to be approximately 5.5kb in length for each taxon (Jorgensen and Cluster 1988).

(i) *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris* rDNA. A single repeat size of approximately 15kb was found in the rDNA of *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris*. Six restriction enzymes (*Bam*HI, *Bst*EII, *Eco*RI, *Eco*RV, *Xba*I, *Xho*I) were used to map the rDNA and a total of 18

restriction sites are suggested. Although only a single repeat size was found, two repeat types occur (Type A and Type B). These repeat types differ in the relative positions of *Eco*RI and *Eco*RV sites within the IGS (Figure 2.2i, Position D). Three other enzymes were also tested in this mapping study (*Hin*DIII, *Pst*I and *Sal*I). Both *Hin*DIII and *Sal*I did not cut within the rDNA region of *S. vulgaris* var. *vulgaris*. Although *Pst*I cut at least once in single digests, the digestion was very poor. This was particularly marked in double digests and, therefore, made mapping of this enzyme impossible.

(ii) *Senecio squalidus* rDNA. Two repeat lengths, of approximately 14.1kb and 12.8kb, were found in *S. squalidus*. Four enzymes were used to map (*Bam*HI, *Eco*RI, *Eco*RV, *Xba*I) and a total of 13 restriction sites are suggested (Figure 2.2ii). The difference between the repeats (approximately 1.2kb) is probably due to insertions into the IGS, although the relative positions of some of the restriction sites vary between the two repeat length classes. Four other enzymes were also tested in this mapping study (*Bgl*II, *Hin*DIII, *Pst*I, and *Xho*I). *Bgl*II, *Pst*I and *Xho*I did not cut within the rDNA region of *S. squalidus*. *Hin*DIII appeared to cut once but the pair of bands produced were difficult to distinguish from a prominent background smear.

(iii) *Senecio aethnensis* and *S. chrysanthemifolius* rDNA. As with *Senecio squalidus* both *S. aethnensis* and *S. chrysanthemifolius* gave two repeat lengths of approximately 14.1kb and 12.8kb. Indeed the four enzymes

that were mapped (*Bam*HI, *Eco*RI, *Eco*RV, *Xba*I) showed an identical site distribution to those of *S. squalidus* (Figure 2.2ii). Similarly the three enzymes that did not digest the rDNA of *S. squalidus* (*Bgl*II, *Pst*I, and *Xho*I), were unable to digest the rDNA of either *S. aethnensis* or *S. chrysanthemifolius*.

Figure 2.2. Restriction maps of (i) *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris* and (ii) *S. squalidus* ribosomal DNA. The filled box below each map represents the approximate position of the coding and internal transcribed spacers. b - *Bam*HI, h - *Xho*I, i - *Eco*RI, v - *Eco*RV, s - *Bst*EII, x - *Xba*I, h~ - methylated *Xho*I site and i• - *Eco*RI site of uncertain position. In (i), the region D highlights the area of difference between Type A and Type B repeats. In (ii), Q indicates the putative region of length variation between the two repeats.

(i)

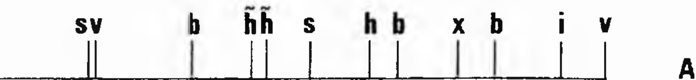
x b i v b b i v b i

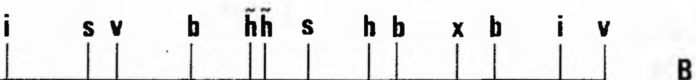
x b i v b b i v b

(ii)

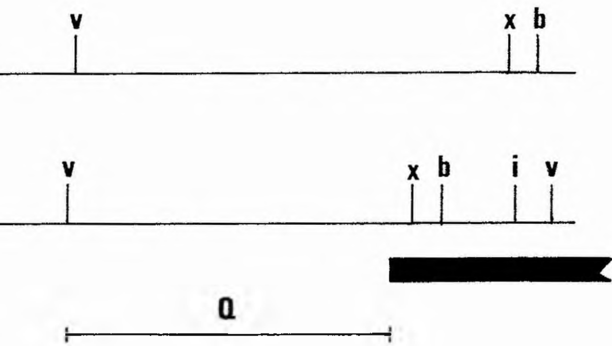
x b i v i b b i b v i

x b i v i b b i b v i


s v b h̃ h̃ s h b x b i v
A


i s v b h̃ h̃ s h b x b i v
B

D
1Kb


v x b
v x b i v
Q

2.3.2 Identification of ribosomal DNA phenotypes.

When total DNA from different *Senecio* taxa was digested with either *Bam*HI, *Eco*RI or *Eco*RV and probed with the heterologous wheat probe, pTA71, one or more fragment patterns (phenotypes) were produced. The enzymes *Eco*RI and *Eco*RV distinguished six and seven phenotypes respectively, these phenotypes were relatively simple compared to the eight phenotypes identified by *Bam*HI (Figure 2.3). Most of these phenotypes proved difficult to categorise on the basis of either site or length mutations as a result of complexities in the fragment pattern, which was especially the case with the *Bam*HI phenotypes. In the following description of the different rDNA phenotypes, the phenotypes of the mapped taxa are taken as a baseline for the purpose of comparison.

EcoRI Phenotypes (Figure 2.3A). Six *Eco*RI phenotypes (I-1, I-2, I-3, I-4, I-5, I-6) were found in the *Senecio* taxa examined. Phenotypes I-1 and I-3 are the baseline *Eco*RI phenotypes for *Senecio vulgaris* var. *vulgaris* (including *S. vernalis*) and *S. squalidus* respectively.

Phenotype I-2 is found only in *Senecio vulgaris* ssp. *denticulatus* and is apparently the result of two length mutations, an insertion and a deletion, in the *Eco*RI fragment that covers part of the IGS region of the two *S. vulgaris* repeat types.

Phenotype I-4 has a 3.8kb fragment additional to the baseline *Senecio squalidus* phenotype (I-3) which may be due a site mutation in some, but not all, of the rDNA repeats.

The result is two fragments, one of which is sufficiently similar to the heterologous wheat probe to hybridise, whereas the other is either very short and not detected or does not show sufficient sequence similarity to the probe to hybridise.

Phenotype I-5 is found only in *Senecio squalidus* and is apparently the result of a site mutation in one of the repeat length variants since excess enzyme does not lead to the disappearance of the 12.8kb fragment.

Phenotype I-6 is the expected additive phenotype of I-1 and I-3, and only occurs in the allohexaploid *S. cambrensis*.

EcoRV Phenotypes (Figure 2.3B). The seven *Eco*RV phenotypes (V-1, V-2, V-3, V-4, V-5, V-6, V-7) are similar to those of *Eco*RI. Phenotypes V-1 and V-2 are taken as the baseline *Eco*RV phenotype patterns for *Senecio vulgaris* var. *vulgaris* and *S. squalidus* respectively.

Phenotype V-3 occurs only in *Senecio squalidus* and is, compared to the baseline phenotype, apparently the result of a site mutation in some but not all of the rDNA repeats.

Phenotypes V-4 and V-5 are restricted to *Senecio vernalis*, for which no restriction map is available.

Phenotype V-6 is found in *Senecio vulgaris sl* and is apparently the result of a similar events to those proposed for phenotype V-3.

Phenotype V-7 is restricted to *Senecio cambrensis* and is the expected additive phenotype between phenotypes V-1 and V-2.

<u>**BamHI Phenotypes**</u> (Figure 2.3C). Eight *Bam*HI phenotypes (B-1, B-2, B-3, B-4, B-5, B-6, B-7, B-8) are found in the *Senecio* taxa studied. Phenotypes B-1 and B-4 are considered the baseline *Bam*HI phenotypes of *Senecio vulgaris* var. *vulgaris* and *S. squalidus* respectively. All of these phenotypes were difficult to interpret and to map (Section 2.4.1). These phenotypes could, however, be used to identify each taxon and are, therefore, very useful.

<u>Phenotype B-1</u> occurs in *Senecio vulgaris* ssp. *vulgaris sl* and *S. cambrensis* from Salamander Street, Edinburgh.

<u>Phenotypes B-2 and B-3</u> are found in *Senecio vulgaris* ssp. *vulgaris sl*.

<u>Phenotype B-4</u> is found only in *Senecio squalidus*.

<u>Phenotype B-5</u> is found only in *Senecio vernalis*.

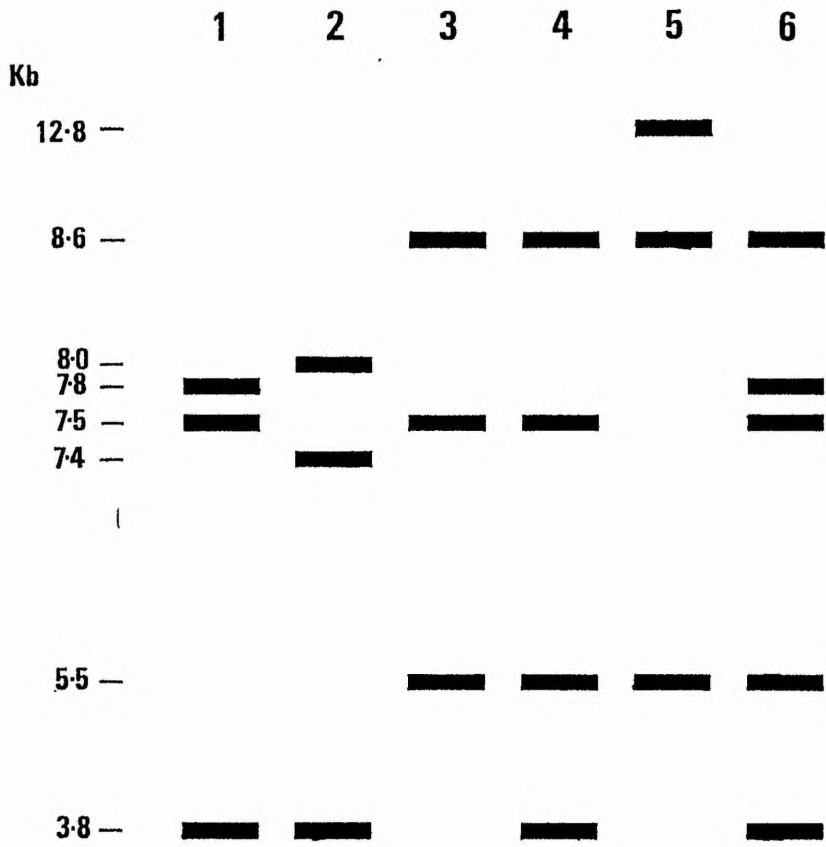
<u>Phenotype B-6</u> is found only in *Senecio vulgaris* ssp. *denticulatus*.

<u>Phenotypes B-7 and B-8</u> are found in *Senecio cambrensis*.

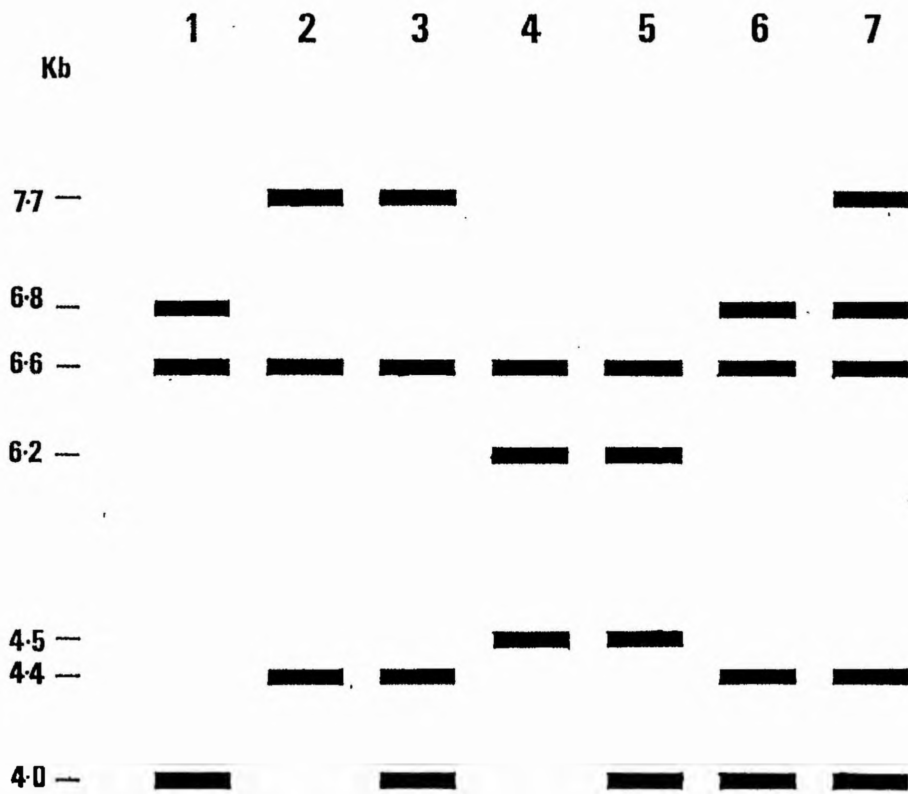
Each of the accessions used in the study was classified according to these phenotypes (Appendix F, Table F2) and the data summarised in Tables 2.3 to 2.4. All of these Tables exclude *S. chrysanthemifolius* and *S. aethnensis*, since only single accessions of each of these taxa were used for mapping studies (Section 2.3.1).

Taxa in Table 2.3 are arranged as combined phenotypes for all of the enzymes, those accessions that have data for one or more enzymes missing are excluded. While, in Table 2.4 the data (including the excluded accessions) are rearranged to consider single enzyme phenotypes.

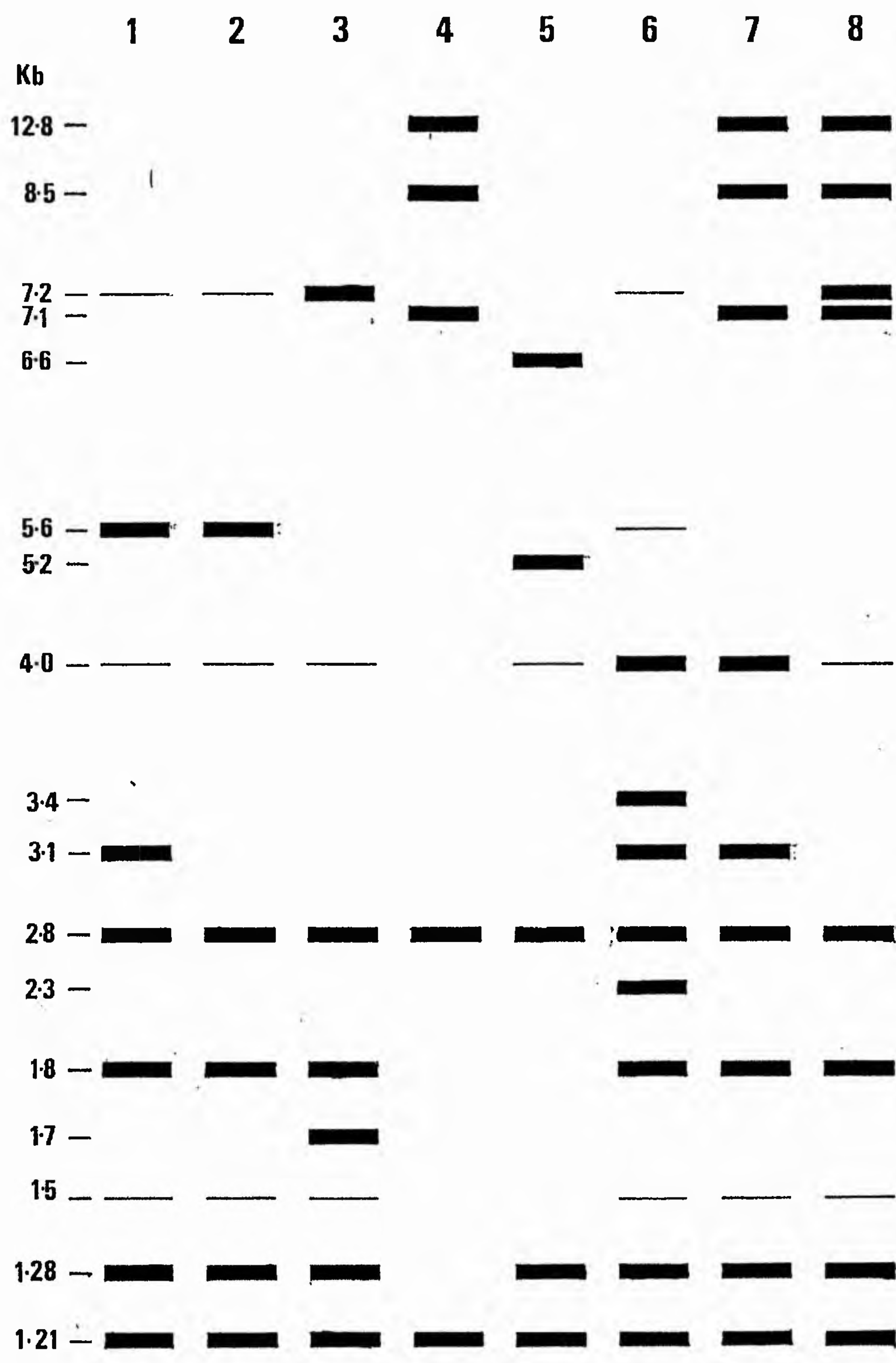
Figure 2.3. Representations of the different ribosomal DNA (rDNA) phenotypes identified in this thesis. Numbers above each phenotype are the numbers used in the text. **A.** rDNA phenotypes produced by the enzyme *Eco*RI. **B.** rDNA phenotypes produced by the enzyme *Eco*RV. **C.** rDNA phenotypes produced by the enzyme *Bam*HI, the thin line indicates fragments which were present, but were faint and may represent either partial digests or fragments from rare repeats.



A



B



1
2
3
4
5
6
7
8
Kb
12·8
8·5
7·2
7·1
6·6
5·6
5·2
4·0
3·4
3·1
2·8
2·3
1·8
1·7
1·5
1·28
1·21
C

-Y

Table 2.3. Frequencies of rDNA phenotypes in Senecio species, using only those accessions for which all three enzymes cut.

Taxon.§	N	Frequency of rDNA phenotypes (EcoRI:EcoRV:BamHI)*.													
		4:7:8	4:7:7	4:7:1	1:1:1	3:2:4	4:2:4	3:3:4	4:3:4	2:1:6	1:4:5	1:6:1	1:6:2	1:1:3	1:1:2
S. cambrensis.	11	0.455	0.091	0.091	0.363	-	-	-	-	-	-	-	-	-	-
S. squalidus	17	-	-	-	-	0.235	0.353	0.353	0.059	-	-	-	-	-	-
S. vernalis.	2	-	-	-	-	-	-	-	-	-	1.000	-	-	-	-
S. vulgaris ssp. denticulatus.	4	-	-	-	-	-	-	-	-	1.000	-	-	-	-	-
S. vulgaris ssp. vulgaris var. vulgaris. (M)	9	-	-	-	0.667	-	-	-	-	-	-	0.111	-	0.111	0.111
S. vulgaris ssp. vulgaris var. vulgaris. (P)	12	-	-	-	0.750	-	-	-	-	-	-	-	-	-	0.250
S. vulgaris ssp. vulgaris var. hibernicus.	22	-	-	-	0.409	-	-	-	-	-	-	0.091	0.045	0.045	0.409
S. vulgaris ssp. vulgaris var. vulgaris sl.	21	-	-	-	0.714	-	-	-	-	-	-	0.048	-	0.048	0.190
S. vulgaris ssp. vulgaris sl	43	-	-	-	0.558	-	-	-	-	-	-	0.070	0.023	0.047	0.302
S. vulgaris sl	47	-	-	-	0.510	-	-	-	-	0.085	-	0.064	0.021	0.043	0.277

§ M - Monomorphic population, P - Polymorphic population.
* Figure refers to the phenotype number for each enzyme (EcoRI:EcoRV:BamHI repectively) show in Figure 2.3.

-Y

Table 2.4. Frequencies of individual rDNA phenotypes in various Senecio species.

Taxon§	Number poplns.	No. indiv. examined. (EI:EV:BI)	EcoRI 1	2	3	4	5	6	EcoRV 1	2	3	4	5	6	7	BamHI 1	2	3	4	5	6	7	8
S. cambrensis.	4	13 13 12	0.385	-	-	-	-	0.615	0.385	-	-	-	-	-	0.615	0.500	-	-	-	-	-	0.083	0.417
S. squalidus.	8	26 22 30	-	-	0.500	0.385	0.115	-	-	0.682	0.318	-	-	-	-	-	-	-	1.000	-	-	-	-
S. vernalis.	1	2 3 3	1.000	-	-	-	-	-	-	-	-	0.667	0.333	-	-	-	-	-	-	1.000	-	-	-
S. vulgaris ssp. denticulatus.	2	4 5 5	-	1.000	-	-	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	1.000	-	-
S. vulgaris ssp. vulgaris var. vulgaris. (Mono)	7	11 19 16	1.000	-	-	-	-	-	0.895	-	-	-	-	0.105	-	0.813	0.125	0.063	-	-	-	-	-
S. vulgaris ssp. vulgaris var. vulgaris. (Poly)	6	14 16 18	1.000	-	-	-	-	-	0.938	-	-	-	-	0.062	-	0.778	0.167	0.055	-	-	-	-	-
S. vulgaris ssp. vulgaris var. hibernicus.	9	29 31 33	1.000	-	-	-	-	-	0.806	-	-	-	-	0.194	-	0.545	0.452	0.003	-	-	-	-	-
S. vulgaris ssp. vulgaris var. vulgaris sl.	13	25 35 34	1.000	-	-	-	-	-	0.914	-	-	-	-	0.086	-	0.764	0.147	0.089	-	-	-	-	-
S. vulgaris ssp. vulgaris sl.	22	54 66 67	1.000	-	-	-	-	-	0.864	-	-	-	-	0.136	-	0.672	0.284	0.044	-	-	-	-	-
S. vulgaris sl.	24	58 71 72	0.931	0.069	-	-	-	-	0.873	-	-	-	-	0.127	-	0.625	0.264	0.042	-	-	0.069	-	-

2.3.3 Ribosomal DNA variation in *Senecio squalidus* and *S. vulgaris sensu lato*.

Two trends are immediately apparent when Tables 2.3 and 2.4 are examined.

(i) Not all of the probe enzyme-combinations are equally polymorphic in or between taxa. For example, three *Eco*RI phenotypes characterise *Senecio squalidus* rDNA (I-3, I-4, I-5), but only one is found within *S. vulgaris* ssp. *vulgaris sl* (I-1). However, *Bam*HI reveals only one phenotype in *S. squalidus* rDNA (B-4) but three phenotypes in *S. vulgaris* ssp. *vulgaris sl.* (B-1, B-2, B-3). Each taxon displays two *Eco*RV phenotypes.

(ii) *Senecio squalidus* appears to be more variable than *S. vulgaris* ssp. *vulgaris sl.* (Table 2.3).

2.3.3.1 Ribosomal DNA variation in *Senecio squalidus*.

Senecio squalidus is apparently fixed for two repeat lengths of 14.1kb and 12.8kb (Figure 2.2b). No accessions were found which showed evidence of any other number. Some accessions of *S. squalidus* showed differential digestion of repeat size classes at low enzyme concentration (5 units/μg) but when the enzyme concentration was raised (25 units/μg) both repeat classes were digested. The three enzymes used to screen the individuals (*Bam*HI, *Eco*RI and *Eco*RV) sampled 66bp (approximately 0.47%-0.52%) of the rDNA repeat.

When population samples are large enough for such analyses, *Senecio squalidus* populations show differing

frequencies of *Eco*RI phenotypes. For example, in the population from Leith Docks in Edinburgh, three phenotypes occur (I-3, I-4, I-5), while in other populations only single phenotypes are found (eg. Cardiff and Devon Street, Grangemouth; Appendix F, Table F2).

2.3.3.2 Ribosomal DNA variation in *Senecio vulgaris sl*.

All accessions of *Senecio vulgaris sl* were apparently fixed for a single repeat length of approximately 15kb (Figure 2.2a). No accessions were found with more repeat lengths. Approximately 0.48% (72bp) of the *S. vulgaris sl* rDNA has been sampled.

The two *Senecio vulgaris* subspecies; ssp. *vulgaris* and ssp. *denticulatus*, could be distinguished on the basis of their *Eco*RI and *Bam*HI phenotypes. It was, however, not possible to differentiate var. *hibernicus* from var. *vulgaris* with the three enzymes surveyed. However, var. *hibernicus* did show some phenotype frequency distributions which were of interest.

Two populations of var. *hibernicus* were analysed from York (RJA and Warr, see Table 2.2). Although these two populations are represented by different sample sizes, they have distinctive *Bam*HI phenotype frequencies. York (Warr) had only one *Bam*HI phenotype (B-1). York (RJA) had phenotype B-1 at a low frequency (0.091) but the most common phenotype was B-2 (0.909). In no other population examined was this the most common phenotype.

2.3.4 Ribosomal DNA variation in other *Senecio* species.

Four other *Senecio* species have been studied (*Senecio aethnensis*, *S. cambrensis*, *S. chrysanthemifolius* and *S. vernalis*). *Senecio aethnensis* and *S. chrysanthemifolius* were analysed from single plants but both had identical rDNA phenotypes to *S. squalidus*. Of the two remaining species, *S. vernalis* was represented by three individuals and could be distinguished from the other taxa on the basis of *Eco*RV (V-4, V-5) and *Bam*HI (B-5) phenotypes. The *Eco*RI phenotype was identical to that of *S. vulgaris* ssp. *vulgaris* (I-1, Table 2.4).

The fourth taxon (*Senecio cambrensis*), the allohexaploid hybrid between *S. vulgaris* and *S. squalidus*, is of interest in that the expected additive rDNA phenotype patterns (I-6, V-7, B-7 or B-8) were found for all plants from three populations that were analysed (Brymbo and Mochdre in Wales and Leith Docks in Edinburgh). Phenotype I-6 is the additive pattern of phenotype I-1 (*S. vulgaris*) and either phenotype I-3 or I-4 (*S. squalidus*). When the EcoRV phenotypes are considered, V-7 is the additive result of phenotype V-1 (*S. vulgaris*) and either phenotype V-2 or V-3 (*S. squalidus*). Phenotypes B-7 and B-8 (*S. cambrensis*) appear to be the result of addition between phenotype B-1 (*S. vulgaris*) and phenotype B-4 (*S. squalidus*) or phenotype B-2 (*S. vulgaris*) and B-4 respectively. However, the *Bam*HI phenotypes are difficult to interpret because of the methylation sensitivity of the enzyme (Hepburn *et al* 1987) and the complex restriction patterns. Some of the fragments

are very probably partial digests (eg. the 1.5kb and 7.2kb fragments).

Particular interest is attached to the population from Salamander Street, Edinburgh where additive phenotypes were found for *Eco*RI and *Eco*RV in one out of six individuals examined. In all other cases the *S. vulgaris* ssp. *vulgaris sl* phenotype was found for *Eco*RI (I-1) and *Eco*RV (V-1). All individuals from this population showed *S. vulgaris* type rDNA phenotypes (B-I), ie. the *S. squalidus* rDNA was apparently absent.

Discussion.

Three aims were identified at the start of this research; (i) Assessment of the level and type of intraspecific rDNA variation present in *S. vulgaris sl* and *S. squalidus*. (ii) Applicability of rDNA to determining the introgressive origin of *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus*. (iii) Confirmation of the hybrid nature of *S. cambrensis*.

Two out of these three aims have been achieved. Restriction analysis of ribosomal DNA allowed the differentiation of most of the studied taxa but proved to be a poor marker for determining the origin of var. *hibernicus*. The ability to readily differentiate taxa allowed the hybrid origin of *S. cambrensis* to be confirmed at all sites, except Salamander Street in Edinburgh. Extensive levels of rDNA variability were encountered within *S. squalidus* and *S. vulgaris sl*.

2.4.1 Ribosomal DNA and the biosytematics of *Senecio vulgaris sl*.

The taxonomy and biosystematics of *Senecio vulgaris sl* in the British Isles is confounded by three related problems. (i) The origin of this tetraploid taxon, whether by autopolyploidy (Kadereit 1984b) or allopolyploidy

(Ingram *et al* 1980, Weir and Ingram 1980). (ii) The origin of *S. vulgaris* ssp. *vulgaris* var. *hibernicus*, whether by introgression (Ingram *et al* 1980) or mutation (Stace 1977). (iii) The relationship of ssp. *denticulatus* to ssp. *vulgaris*.

Ribosomal DNA has provided little data on the first issue in that it does not allow resolution of the auto- versus allo-polyploid origin of *S. vulgaris*. The contribution of rDNA data to the latter two issues is discussed later. The cpDNA evidence that bears on all three issues is presented in Chapter 3.

2.4.1.1 Ribosomal DNA and the introgressive origin of *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus*.

No additive phenotypes between *S. vulgaris* var. *vulgaris* and *S. squalidus* were found in any of the samples of var. *hibernicus* examined. The absence of additive phenotypes may be taken as evidence against an introgressive origin of this taxon. In isolation, this is a dangerous conclusion to draw from the data, since it is equally possible that introgression has no effect on rDNA phenotype. The tendency for rRNA genes to be located towards the termini of chromosomes in tandem arrays, means that large blocks of rDNA can be lost during single recombination events.

In rejecting the introgressive origin of weedy *Helianthus bolanderi* (via introgression of *H. annuus* into serpentine *H. bolanderi*), Rieseberg *et al* (1988) used data from isozymes and chloroplast DNA (in addition to the absence of additive rDNA phenotypes). Evidence presented by

Ashton (1990) suggests that an AAT allozyme from *S. squalidus* is present in var. *hibernicus*. This data supports the hypothesis of an introgressive origin for the taxon.

The possibility of gene flow between the two varieties of ssp. *vulgaris* may obscure any evidence of introgression using rDNA. The inclusion of one var. *vulgaris* sample (Migvie, Aberdeenshire) from beyond the present distribution of *S. squalidus* proved inconclusive as this had the most common rDNA phenotype (I-1:V-1:B-1). Therefore, rDNA (at least in this limited survey) provides no evidence either for or against the hypothesis of an introgressive origin of var. *hibernicus*.

Additional evidence that rDNA restriction analysis may not be the best approach to answering questions of introgression in the genus *Senecio* is provided by a survey of two populations of *S. vulgaris* var. *hibernicus* from York (RJA and Warr). The contrast between *Bam*HI phenotype frequencies (Section 2.3.3.2) in these two populations may be a reflection of differential fixation of rDNA (Section 2.1.1.2). 'RJA' is a collection that has been shown isozymically and morphometrically to be more '*squalidus*-like' than other var. *hibernicus* populations (Irwin 1990). Even this population, which presumably, has arisen in the recent past ('Seed' collected in 1979) shows no evidence of *S. squalidus* rDNA being present, which is in contrast to the isozyme and morphological data. If as Irwin (1990) suggests, 'RJA' is the result of fusion between an unreduced *S. squalidus* gamete and a haploid *S. vulgaris*

gamete one would expect an additive rDNA restriction pattern.

2.4.1.2 Ribosomal DNA and the relationship between *Senecio vulgaris* ssp. *vulgaris sl* and *Senecio vulgaris* ssp. *denticulatus*.

The two subspecies of *Senecio vulgaris* can readily be separated on the basis of their respective rDNAs (Table 2.3-2.4) with *Bam*HI and *Eco*RI. Similar subspecific differentiation of rDNA has been reported by Schaal *et al* (1987) between *Phlox divaricata* ssp. *divaricata* and *P. divaricata* ssp. *lamphi*. Differences between closely related, possibly conspecific taxa, are seen in the case of wild species and their cultivated relatives (Saghai-Maroof *et al* 1984, Cordesse *et al* 1990, Gepts and Clegg 1989). In such cases there is a trend for a reduction in the variability of the cultivated taxon. This is consistent with a taxon having gone through a genetic bottleneck (Section 2.4.4). In the Asteraceae, Rieseberg *et al* (1988) were able to distinguish weedy and serpentine races of *Helianthus bolanderi*. However, Tucci and Maggini (1986) were unable to distinguish two subspecies of *Cynara cardunculus*.

An absence of apparent ssp. *vulgaris* fragments in the ssp. *denticulatus* plants surveyed suggests that gene flow between these two subspecies may be a rare event (however, the sample sizes are very small). These two subspecies are known to be fully fertile when crossed artificially (Kadereit 1984a, Taylor 1984), but no plants of intermediate morphology have been reported from natural

populations at Ainsdale Beach in Lancashire (Ruth Ingram, Pers. Comm.).

2.4.2 Ribosomal DNA and the biosystematics of *Senecio cambrensis*.

Ribosomal RNA genes have been used to infer hybridity in a number of species. Doyle and Doyle (1988) were able to demonstrate the occurrence of natural interspecific hybridisation between *Claytonia virginica* and *C. caroliana* in eastern North America. Similar cases have been reported by Talbert *et al* (1990, *Tripsacum andersonii*) and Doyle *et al* (1985, *Tellima x Tolmiea*). In each case the supposed hybrid had the additive patterns of the two putative parents. Similar results have been found for artificial hybridisations, in fact many of the chloroplast DNA studies that have established the maternal/paternal parentage of hybrids, use rDNA as a confirmatory test of hybridity (Chapter 3).

The rDNA evidence supports a hybrid origin for *Senecio cambrensis* between *S. vulgaris* ssp. *vulgaris* and *S. squalidus;* such a conclusion has been reached from studies based on cytology (Rosser 1955), morphology (Taylor 1984) and isozymes (Ashton 1990).

This investigation of *Senecio cambrensis* has, however, generated a very interesting result for a population at Salamander Street in Edinburgh. In this population only one out of six accessions showed an additive rDNA phenotype for *Eco*RI and *Eco*RV. The other five accessions (and the *Bam*HI

phenotype of the above accession) showed profiles which were compatible with *S. vulgaris* ssp. *vulgaris* only (ie. I-1:V-1:B-1, Table 2.4). This result was unexpected and would lead one towards the suggestion that either the taxon described as *S. cambrensis* at this site is not a hybrid between the two putative parents or that the *S. squalidus* genome (at least the rDNA) in these plants is effectively absent. All the evidence that is available (eg. Ashton 1990, Taylor 1984) supports the view that this taxon is a hybrid. Moreover, one of the plants at this site did have additive *Eco*RI and *Eco*RV phenotypes (although the *Bam*HI phenotype was nonadditive).

A similar result to this has been reported by Zimmer *et al* (1988) for an artificial cross between *Zea mays* and *Z. luxurians*. The F_1 progeny of this cross were screened with the restriction enzymes *Eco*RI and *Sst*I. All of the plants had additive *Eco*RI phenotypes, but four out of the 12 individuals tested had only the *Z. mays* *Sst*I phenotype. That is, some of the F_1 hybrids apparently lacked the rRNA genes from *Z. luxurians* when tested with one enzyme (*Sst*I) but not the other (*Eco*RI). No explanation for this phenomenon was suggested other than that there was a need to know more about the inheritance of rDNA. Fabijanski *et al* (1990) report the presence of non-additive phenotypes in hexaploid *Avena* species using a random repeat sequence probe.

In *Senecio cambrensis* non-additive phenotypes have been obtained for most individuals, unlike the situation in *Zea*, where some additive phenotypes were found. There

is, however, no evidence to suggest that if more enzymes were used, additive phenotypes would not be obtained. If the hybrid nature of *S. cambrensis* from Salamander Street is accepted, then a number of suggestions may be made.

(i) Some *Senecio squalidus* plants at the Salamander Street site possessed *S. vulgaris* type rDNA phenotypes (either as a rare rDNA phenotype or to the exclusion of other phenotypes). Following the hybridisation event, rare rDNA phenotypes were amplified in some lines but not in others.

Nothing is known regarding the rDNA phenotypes of Salamander Street *Senecio squalidus*. It is, however, difficult to believe that accessions from this site would have drastically different rDNA phenotypes from the other *S. squalidus* accessions examined. The presence of a rare rDNA with a similar phenotype to that of *Senecio vulgaris* may be possible, but one would have to envisage a rapid amplification and fixation of an originally rare *vulgaris*-type *S. squalidus* rDNA variant (approximately 20 years, Abbott *et al* 1983).

(ii) The *Senecio squalidus* rDNA is heavily methylated and therefore not available for digestion by methylation sensitive enzymes. ie. the plant has no need for the rDNA transcripts from the *S. squalidus* rRNA genes. (Flavell *et al* 1986c). These digests (along with many others) revealed a smear of high molecular weight DNA (HMW-DNA) at the top of the autoradiograph. This probably indicates sequences that

share sequence similarity to the rDNA probe but are not cut due to methylation. It is not possible to distinguish those rDNA sequences that come from *S. vulgaris* or *S. squalidus*, without a more specific probe.

(iii) *Senecio squalidus* rDNA is present but at the limit of detection of Southern blotting technique.

Important questions with regard to these proposals include the inheritance of the rDNA in this system and the number of different loci which are involved and the distribution of rDNA repeat lengths and types between these loci. Cytological observations of nucleolar organiser regions (NORs) would suggest that there are six rDNA arrays (loci) in *S. cambrensis* (Ruth Ingram, Pers. Comm).

2.4.3 Ribosomal DNA and the biosystematics of *Senecio squalidus*.

The introduction of *Senecio squalidus* into the British Isles would be expected to result in a decrease in rDNA diversity, as a result of limited sampling of the wild rDNA gene-pool. A similar effect is reported when rDNA from a wild taxon is compared to a closely related domesticated taxon [eg. *Hordeum vulgare sl* (Saghai-Maroof *et al* 1984), *Oryza sativa* (Cordesse *et al* 1990), *Pennisetum glaucum* (Gepts and Clegg 1989)]. Thus it might be expected that a greater rDNA phenotype diversity would exist in Continental *S. squalidus*.

All of the *Senecio squalidus* accessions which were examined, were considered to have two repeat length variants (14.1kb and 12.8kb). These repeat length variants (length difference = 1200bp) are apparently not the result of a change in IGS subrepeat number since all subrepeats examined to date have been between 100bp and 200bp in length (Jorgensen and Cluster 1988). This suggests that either (i) the repeat length variants accumulated length mutations separately and are, therefore, probably associated with separate rDNA loci or (ii) that these variants are derived from different taxa, ie. *S. squalidus* is a hybrid between two taxa that possess different repeat length variants.

In the hexaploid *Triticum aestivum*, Gerlach and Bedbrook (1979) have shown that three rDNA repeat length variants are found (9.0kb, 9.1kb, 9.4kb), which have been shown to be associated with different haploid genomes (Appels and Dvorak 1982b).

Crisp (1972) has proposed that the British *Senecio squalidus* may be an introduction from a hybrid swarm formed between *S. aethnensis* and *S. chrysanthemifolius*. However, single accessions of the two putative parents which were analysed had identical rDNA length variants to *S. squalidus* (Figure 2.2). This raises the interesting possibility that an unknown taxon has been involved in the origin of *S. squalidus*.

A broad survey of *Senecio squalidus* in Britain and Europe, with a more "homologous" rDNA probe may provide some useful data on this point.

2.4.4 Ribosomal DNA in *Senecio* and other Asteraceae.

A comparison of the ten published Asteraceae rDNA maps (King and Schaal 1989, Choumane and Heizmann 1988, King and Schaal 1990 and Tucci and Maggini 1986) with those reported here show that there is a common distribution of *Bam*HI, *Eco*RI and *Eco*RV sites within the coding region (although fragment lengths may not be the same). Thus the coding region appears to be highly conserved (in terms of restriction site distribution in the Asteraceae) although there is considerable IGS variation between these taxa.

The majority of rDNA variation in *Senecio* species appears to be site variation. Within species site heterogeneity has been reported by other workers, eg. Doyle *et al* (1984) and King and Schaal (1989). However, rDNA length variation does exist between different taxa (eg. *S. vulgaris* ssp. *vulgaris* and *S. squalidus*). In the Asteraceae this type of variation has been reported in *Helianthus* (Choumane and Heizmann 1988) and in the Tribe Cynareae (Tucci and Maggini 1986).

The inability to find an enzyme that would either cut the rDNA repeat once reliably [*Xba*I will cut once in *Senecio vulgaris* ssp. *vulgaris sl* and *S. squalidus*, but not reliably] or had sites at either end of the IGS does not allow an accurate assessment of repeat length variation. The presence of single repeat length variants within a taxon (*Senecio vulgaris sl*) is not without precedence, eg. Rieseberg *et al* 1988, King and Schaal 1989, Rafalski *et al* 1983, Doyle *et al* 1984, Doyle and Beachy 1985, Sytsma and

Schaal 1985. Other studies have revealed extensive rDNA repeat length variation, eg. Delseny *et al* 1979, Oono and Suigura 1980, Appels *et al* 1980, Yakura *et al* 1983, 1984, Learn and Schaal 1987, Schaal *et al* 1987. The apparent fixation of two repeat length variants in *S. squalidus* across its range, is also found in *Gaura demareei*, a member of the Onagraceae (Schaal and Learn 1988).

2.4.5 Problems with ribosomal DNA in *Senecio*.

Three major problems were encountered with the construction of the maps shown in Figure 2.2.

(i) The heterologous wheat probe was unable to detect regions of the *Senecio* IGS. This was shown using a subcloned portion of the wheat rDNA IGS, which did not hybridise to *Senecio* genomic DNA (data not shown).

(ii) The occurrence of multiple repeat sizes or types made exact placement of restriction sites difficult.

(iii) The known presence of extensive methylation (Hepburn *et al* 1987) means that an apparent site loss may be the result of methylation or nucleotide substitution.

These problems have meant, that a number of assumptions were made during map construction. (i) Any fragment which occurs in the IGS will not be detected, or if it is detected then it will show a very poor signal intensity. (ii) A fragment that was very small or with only a small amount of coding sequence, would probably not be detected.

These difficulties have necessarily restricted the maps to the coding (ie. invariant) region and closely associated IGS. Similar constraints are imposed on those studies that use purified 18S and 28S rRNA as the hybridisation probe (eg. Carmona *et al* 1984). This limitation of the data could be overcome by two, not necessarily mutually exclusive, procedures.

(i) The construction of an rDNA map from a *Senecio* rDNA clone. This would overcome the problem of methylation (Jorgensen and Cluster 1988), but may underestimate the degree of rDNA variability that exists within an individual [without procedure (ii)].

(ii) An rDNA clone from another member of the Asteraceae (preferably the Senecioneae) or a *Senecio* species could be used as a probe. In this way the IGS containing fragments may be identified and a better indication of the degree and nature of rDNA variation in the genus obtained.

Such a map and probe set would be very useful in the light of the apparent high level of variation in *Senecio vulgaris sl* which has been obtained in this study (Tables 2.3 - 2.4).

Similar problems are also encountered with the different enzyme phenotypes that have been identified (Figure 2.3). Such phenotypes may be the result of partial methylation in one or more of the rDNA repeats, particularly since the three enzymes used in this survey are methylation sensitive. This problem may have been the cause of the observation that in some *Senecio squalidus*

accessions only one of the rDNA repeats was cut with *Bam*HI at low enzyme concentration (5 units/μg) but that both units were cut at higher concentrations (25 units/μg, Section 2.3.3).

The inability to differentiate some *Senecio* taxa in this study (eg. *S. aethnensis* vs *S. squalidus*) on the basis of their rDNAs may be due to a combination of factors:

(i) Extensive methylation of *in vivo* rDNA sequences (Hepburn *et al* 1987) may mean that only a limited number of restriction enzymes cut within the repeat. The choice of restriction enzyme can affect results as a consequence of the presence of methylated di- and tri-nucleotides (Greunbaum *et al* 1981). A greater number of restriction enzymes (preferably those that are relatively C and CNG methylation insensitive) plus a combination of 6bp and 4bp cutters may increase the amount of variation resolved.

(ii) The short length of the repeat may reduce the possible number of restriction sites that can be analysed, relative to chloroplast DNA (Chapter 3).

(iii) The rDNA probe itself may create problems, due to the presence of the IGS. The rapid divergence of much of the IGS (Section 2.1.1.1) means that heterologous probes, from widely divergent species, are unlikely to hybridise to this region of the rDNA. The exact nature of this problem is influenced by the taxonomic level at which the analysis is to be conducted.

In conclusion the results presented in this Chapter illustrate the degree, and types of variation which occurs

in the rDNA of some British and Continental *Senecio* species. No evidence to support or reject the introgressive origin of *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus* has been obtained although the data broadly supports the hybrid origin of *S. cambrensis*. Moreover, the difficulties of using rDNA as a biosystematic marker are illustrated.

Chapter 3.

Biosystematics of some British and European *Senecio* species: - Chloroplast DNA evidence.

"There is no branch of detective science so important and so neglected as the art of tracing footsteps"

A Study in Scarlet.
A. Conan-Doyle.

Introduction.

Over the past decade chloroplast DNA (cpDNA) has become the molecule of choice when studying plant biosystematic problems at the DNA level.

In this Introduction I shall provide some brief details about the general structure of cpDNA in Angiosperms and then go on to consider cpDNA as a phylogenetic marker; ie. the reasons for its widespread use, the methods used for data generation and analysis and the types of studies and information that have been obtained. At the species level cpDNA will be considered as a marker for the study of polyploid and introgressive speciation. Finally those studies that relate more specifically to the Asteraceae will be considered.

3.1.1 Structure and organisation of chloroplast DNA.

To understand the impact that cpDNA has had as an evolutionary marker it is necessary to understand its structure and organisation. Over the past few years many reviews have been published on various aspects of chloroplast genomes:- general chloroplast DNA structure and organisation (Bedbrook and Kolodner 1979)
organisation and structure of chloroplast genes (Whitfeld

and Bottomley 1983), comparative cpDNA organisation (Palmer 1985a, 1985b), cpDNA evolution (Palmer 1986a, 1987, Palmer *et al* 1988, Zurawski and Clegg 1987) and chloroplast DNA inheritance (Sears 1980, Tilney-Bassett 1978). These reviews form the basis of the following overview of Angiosperm cpDNA and should be consulted for more details and specific references.

Chloroplast DNA in Angiosperms is a circular molecule of between 120kb and 217kb in size. The most common organisation is for an invert repeat [IR, 0-76kb] to divide the molecule asymmetrically into a large single copy region [LSC, 80-100kb] and a small single copy region [SSC, 12-28kb] (Figure 3.1). The majority of the size variation is accounted for by differences in the length of the IR. The IR is considered to be a 'land-mark' feature in most chloroplast genomes because of its size, gene content, phylogenetic conservation and recombination properties. In the subfamily Papilionoideae of the family Leguminosae the IR is missing, as it is in many of the conifers that have been studied to date (Lidholm *et al* 1988, Strauss *et al* 1988, White 1990).

The invert repeat always contains a more or less complete set of chloroplast ribosomal RNA genes (rDNA). These are usually orientated such that the 23S rDNA is closer to the SSC region and the 16S rDNA closer to the LSC region. The wide variation in the size of the IR is correlated with changes in its gene content, for example, ten protein coding genes present as single copies in most land plants are duplicated in *Pelargonium x zonale hort.*

(IR=76kb, Palmer *et al* 1987). The IR is thought to be part of a copy correction mechanism in Angiosperms since the individual segments are identical within individual plants and naturally occurring or induced mutations occur in both segments.

The chloroplast genomes of *Oryza sativa*, *Nicotiana tabacum* and *Marchantia polymorpha* have been completely sequenced, providing valuable information about the organisation and genetic structure of the cpDNA in general. Two major groups of genes are encoded on the cpDNA. In rice and tobacco cpDNAs (Sugiura 1989) as many as 59 genes code for components of the chloroplast protein synthesis apparatus (including rRNA, tRNA, and RNA polymerase subunits) and up to 30 genes code for components of the photosynthetic apparatus (eg. large subunit of RuBisCO, ATPase genes, photosystem I and II components and NADH dehydrogenase).

3.1.2 Chloroplast DNA as a biosystematic marker.

3.1.2.1 Assumptions in the use of chloroplast DNA.

The widespread use of cpDNA is the result of a number of generalisations that have been made regarding the structure and evolution of this molecule (Palmer 1985b, 1986a, 1987, Palmer *et al* 1988).

A. Chloroplast DNA has a small size. The small, uniform size of the molecule, is such that resolution of all of the fragments resulting from digestion with

a 6bp-cutting restriction enzyme is possible but, at the same time, the molecule is also large enough to allow the rapid sampling of a large number of restriction sites by a moderate number of restriction enzymes (Palmer and Zamir 1982, Palmer 1985b).

B. Structural and sequence evolution are relatively conserved. This particular aspect of cpDNA has been the subject of a recent review by Birky (1988), which should be consulted for specific references, and more recently by Palmer (1990). Birky concluded that the lack of evidence for transposable elements (but see 'C' below) and importation of external DNA sequences into the chloroplast genome, plus low levels of recombination, contribute strongly to its conserved structural evolution. The alternative view is that because of the importance of chloroplasts, the cpDNA may not be able to sustain large amounts of structural variation except at specific sites. Sequence evolution is considerably slower in cpDNA than it is in either nuclear or mitochondrial DNA. Within the chloroplast genome, Zurawski and Clegg (1987) have pointed out that different genes accumulate base substitutions at different rates. This has lead Clegg (1990) to raise doubts about the broad application of a molecular clock to cpDNA.

C. Recombination is rare or absent. Intramolecular recombination occurs between the IR segments resulting in genetically identical, but physically distinct cpDNA isomers (Palmer 1983). The existence of

intramolecular recombination between IR segments and the formation of head-to-head dimers, via intermolecular recombination, does not necessarily indicate that a general recombination mechanism exists (Birky 1988), since a mechanism for plastid fusion would be required. However, in some taxa which have been studied there is evidence of extensive rearrangement of the cpDNA. For example, in *Trifolium subterraneum*, extensive sequence rearrangements are correlated with the occurrence of at least five dispersed repetitive sequences (Palmer *et al* 1988). Recombination has been recently identified in *Nicotiana* (Fejes *et al* 1990) and has been known for many years in the Alga *Chlamydomonas* (Gillham 1978).

D. Inheritance of chloroplast DNA is predominantly maternal. This point is the subject of the following Chapter and will be discussed there.

3.1.2.2 Methods of data generation.

Three main approaches are used to generate biosystematic data from cpDNA. These are restriction fragment comparison, restiction site comparison and DNA sequencing. These approaches have been comprehensively reviewed by Palmer and his colleagues (Palmer 1986b, 1987, Palmer *et al* 1988), but will be briefly outlined below.

A. Restriction fragment comparison. In this approach pure chloroplast DNA is digested with restriction enzymes and the fragments visualised by:- (i) Directly viewing an ethidium bromide stained gel (Palmer and

Zamir 1982, Hosaka 1986). (ii) Radioactive end-labelling of restriction fragments prior to running the gel (Lehväslaiko *et al* 1987, Baum and Bailey 1989). (iii) Using purified cpDNA as a radiolabelled probe (Ichikawa *et al* 1986, Neale *et al* 1986). All of these methods generate fragment patterns which are compared either to obtain a measure of genetic distance or to identify particular mutations. Specific mutations can be very difficult to identify and recourse is usually needed to cloned probes to resolve ambiguities. To have a reasonable chance of identifying specific mutations it is necessary that the cpDNAs have a low (0.5-1.0%) base sequence divergence (Palmer *et al* 1988).

<u>B. Restriction site comparison</u>. The use of restriction site comparisons to identify three types of cpDNA mutations:- base substitutions (point mutations), deletions/insertions (length mutations) and inversions, have been developed by Palmer and coworkers (Palmer 1985b, 1987, Jansen and Palmer 1988, Palmer *et al* 1988) from ideas originally proposed by Upholt (1977). The basic rational is to use cloned cpDNA as a probe, either from the same (homologous) or a different (heterologous) species to the one of interest, and so identify fragments of similar sequence. The three types of mutations are theoretically readily identified. Site mutations are identified by the loss of a large fragment in one cpDNA and its concomitant replacement by two smaller

fragments in a second cpDNA. Length mutations are identified as changes in the sizes of fragments which are mirrored in a number of different enzymes. Inversions are the most complex mutation to identify and are recognised by the construction of restriction maps using cloned probes to 'walk' around the chloroplast genome and locate fragments which are widely separated in one cpDNA but adjacent in a second cpDNA.

C. Chloroplast DNA sequence analysis. Using this approach a direct base-for-base comparison across a portion of the cpDNA is made. It is expensive and time consuming to undertake and is best suited to use at the family level and above (Palmer *et al* 1988). Not withstanding this drawback, sequencing particularly of the large subunit of the RuBisCO gene is being undertaken (Palmer *et al* 1988, Zurawski and Clegg 1987, Doebley *et al* 1990) and the recent application of the polymerase chain reaction (PCR, Erlich 1989) promises to revolutionise the collection of data from this source (Golenberg *et al* 1990).

3.1.3 Chloroplast DNA and speciation.

Palmer *et al* (1988) stated that over 40 studies had been published utilising cpDNA to look at relationships between congeneric species, this number is now rapidly rising (See the Abstracts for Meetings of the Botanical

Society of North America in the *American Journal of Botany* Supplements 1987-1990). The majority of these studies have used fragment comparison approaches and have included genera scattered throughout many different families (Table 3.1).

These studies, to a greater or lesser extent, have all generated important information about both the evolution of particular species groups and the evolution of cpDNA in general. However of particular interest to the present work are those studies into polyploid and introgressive speciation.

Chloroplast DNA has been used to address questions of both autopolyploid and allopolyploid speciation (Milo *et al* 1988, Soltis *et al* 1989a, 1989b, Soltis and Soltis 1989, Dally and Second 1990). Allopolyploid speciation is the most studied of these two modes, since until recently it was thought that cpDNA was an unsuitable molecule for use at the intraspecific level (see Chapter 4).

The identification of the maternal and paternal parentage of hybrids has been an important area where cpDNA has made a contribution, not only in the Angiosperms but also in lower plants, eg. the moss, *Plagiomnium medium* (Wyatt *et al* 1988) and the fern, *Hemionitis pinnatifida* (Ranker *et al* 1989). To identify the parentage of a hybrid two conditions must be met:- (i) The mode of cpDNA inheritance must be known. (ii) The two parental species must be distinguishable from each other on the basis of their cpDNA restriction profiles.

This approach has allowed Erickson *et al* (1983) and Palmer *et al* (1983) to independently identify the maternal parents of the *Brassica* amphidiploids, *B. carinata* and *B. juncea* as *B. nigra* and *B. campestris* (syn. *B. rapa*) respectively. In the case of the third amphidiploid, *B. napus*, the maternal parent was putatively identified as *B. oleracea*, since the cpDNA of the amphidiploid has diverged from both of the cytologically identified parents.

In *Tragopogon*, multiple origins of the allopolyploid species *T. miscellus* have been established. This species has had at least two independent origins, from reciprocal crosses between *T. pratensis* and *T. dubius* (Soltis and Soltis 1989). In the case of *Aegilops triuncialis* the racial differences in this tetraploid are the result of independent origins from reciprocal crosses between the diploids, *A. caudata* and *A. umbellulata* [Murai and Tsunewaki (1984) in Hosaka and Hanneman (1988a)].

Chloroplast DNA as an evolutionary marker for autopolyploidy has only recently been shown to be feasible, following extensive sampling of different cytotypes from within a species. Members of the family Saxifragaceae are apparently particularly amenable to this type of analysis. In the case of *Heuchera grossulariifolia* (Wolf *et al* 1990) and *Tolmiea menziesii* (Soltis *et al* 1989b) the tetraploid cytotypes have been shown to have had at least three independent origins. Such data gives an indication that cpDNA may be of value in studies at the intraspecific level.

In Angiosperms the ability to determine the maternal parentage of hybrids using cpDNA has been used to identify taxa which may have resulted from introgressive hybridisation. Rieseberg *et al* (1990) report evidence of the presence of *Helianthus debilis* ssp. *cucumerifolius* cpDNA in *H. annuus* ssp. *texanus* and propose that this may be the result of the introgression of cpDNA from *H. debilis* ssp. *cucumerifolius* into *H. annuus*, following the introduction of *H.annuus* into Texas (see Chapter 2).

However, the direct study of introgression has rarely been attempted using cpDNA, though introgression has been invoked to explain unusual cpDNAs present in some taxa. Examples of this type of study include the proposed introgressive origin of two cultivars ('Norin 31' and 'Altex') of *Brassica napus* (Palmer *et al* 1983) and the presence of W type cpDNA in *Solanum tuberosum* ssp. *andigena*, which may be the result of introgression with a wild *Solanum* species following the origin of ssp. *andigena* (Hosake and Hanneman 1988a). Similar explanations for unusual cpDNAs have been put forward in *Lycopersicon* (Palmer and Zamir 1982), *Pisum sativum* (Palmer *et al* 1985), *Dactylis glomerata* (Lumaret *et al* 1989) and *Zea perennis* (Doebley 1989).

The use of cpDNA has not always resolved hybridisation problems, rather on a number of occasions it has confounded them. For example, doubts have been raised about the introgressive origin of the weedy race of *Helianthus bolanderi* (Rieseberg *et al* 1988), since cpDNA mutations unique to this taxon were found. These, and other results,

which raise conflict in phylogenetic reconstruction between cpDNA analysis and other approaches to biosystematic problems have been reviewed in Sytsma and Smith (1988) and Sytsma (1990).

Table 3.1 Previous cpDNA studies of species relationships, excluding those in the Asteraceae.

Family.	Genus.	Reference.
Apiaceae	Daucus	DeBonte et al (1984)
Brassicaceae	Brassica	Erickson et al (1983)
		Kemble (1987)
		Palmer et al (1983)
Chenopodiaceae	Beta	Bonavent et al (1989)
		Ecke and Michaelis (1990)
		Fritzsche et al (1987)
		Mikami et al (1984)
Cucurbitaceae	Cucumis	Perl-Treves and Galun (1985)
Gentianaceae	Lisianthius	Sytsma and Schaal (1985)
Geraniaceae	Pelargonium	Metzlaff et al (1981)
"Leguminosae"	Glycine	Close et al (1989)
		Doyle et al (1990b)
		Shoemaker et al (1986)
	Hedysarum	Baatout et al (1985)
	Medicago	Rose et al (1988)
		Schlarbaum et al (1989)
	Pisum	Palmer et al (1985)
Linaceae	Linum	Coates and Cullis (1987)
Malvaceae	Gossypium	Wendel (1989)
Onagraceae	Clarkia	Systma and Gottlieb (1986b)
	Oenothera	Gordon et al (1982)
Orchidaceae	Oncidium	Chase and Palmer (1989)
Papaveraceae	Papaveraceae	Milo et al (1988)
Poaceae	Triticum/Aegilops	Tsunewaki and Ogihara (1983)
	Eleusine	Hilu (1988)
	Hordeum	Holwenda et al (1986)
		Baum and Bailey (1989)
	Oryza	Ichikawa et al (1986)
		Ishii et al (1986)
		Dally and Second (1990)
	Pennisetum	Clegg et al (1984)
	Zea	Doebley et al (1987)
		Timothy et al (1979
Rubiaceae	Coffea	Berthou et al (1983)
Solanaceae	Nicotiana	Rhodes et al (1981)
	Solanum	Buckner and Hyde (1985)
		Hosaka (1986)
		Hosake and Hanneman 1988a)
		Hosake and Hanneman (1988b)
	Lycopersicon	Palmer and Zamir (1982)

3.1.4 Chloroplast DNA in the Asteraceae.

At the tribal level the chloroplast genome of the Asteraceae has been the subject of considerable pioneering work (Jansen and Palmer 1987a, 1988), resulting in the resolution of the debate regarding the most primitive tribe of the Asteraceae. Traditionally, the Heliantheae have been considered the most primitive tribe, however, the identification of a 22kb inversion in all of the Asteraceae (tested to date) except one subtribe of the tribe Mutisieae has lead to the proposal that subtribe Barnadesiinae is the ancestral taxon. The absence of this inversion in all other Angiosperms tested, to date, and the support of independent restriction site data provides further evidence that this inversion marks a major split in the Asteraceae.

The Asteraceae have not been studied very extensively below the tribal level. Some work has been done, most notably in the genera *Coreopsis* (Crawford *et al* 1990), *Helianthus* (Rieseberg *et al* 1988, Rieseberg *et al* 1990), *Tragopogon* (Soltis and Soltis 1989), *Pyrrhopappus* (Turner and Kim 1990) and the 'Silversword' alliance (*Argyroxiphium*, *Dubautia* and *Wilkesia*; Baldwin *et al* 1990). Restriction maps of the cpDNA of some Composite species are available:- *Carthamnus tinctorius* (Smith and Ma 1985, Ma and Smith 1985), *Barnadesia caryophylla* (Jansen and Palmer 1987b), *Helianthus annuus* (Heyraud *et al* 1987) and *Lactuca sativa* (Jansen and Palmer 1987b). These indicate that an invert repeat is present in the cpDNA, and except for the

inversion already described, the molecule is very similar to other chloroplast genomes.

Within the Asteraceae little is known regarding the mode of cpDNA inheritance. In *Senecio vulgaris* (Bleyden 1988) and *Tragopogon* (Soltis and Soltis 1989) maternal cpDNA transmission has been confirmed at the molecular level. Genetic studies of the transmission of mutant chloroplasts have shown maternal inheritance of plastids in *Helianthus annuus* (Razorileleva *et al* 1970). The absence of plastid nucleoids in the pollen of *Artemisia absinthium*, *Cichorium intybus*, *Doronicum cordatum*, *Grindelia squarrosa*, *Helianthus annuus*, *Lactuca sativa* and *Solidago speciosa* has been used to infer maternal plastid transmission in these species (Corriveau and Coleman 1988). The absence of plastids in the male gametophyte of *Ambrosia psilostachya* has also been used to infer maternal plastid transmission (Hageman and Schroder 1989).

Senecio cpDNA has not been studied extensively, except in a study on the resistance of *S. vulgaris* ssp. *vulgaris sl* to the herbicide triazine, a character which is coded by the *psb*A gene on the chloroplast genome (Bleyden 1988). Jansen and Palmer (1987a) have looked at *Senecio mikanioides* cpDNA as part of a broader survey of the occurrence of the 22kb inversion in the Asteraceae. Palmer *et al* (1988) used an unidentified *Senecio* species as one taxon in a study of some of the generic relationships in the Asteraceae and have shown that the sister taxon to *Senecio* is *Cineraria* (in the restricted sample that was studied). At the present time, work is being conducted into

the cpDNA of the 'tree-Senecios' of Kenya (Palmer, Pers. Comm.).

The aims of the experiments reported in this Chapter were three-fold:- (i) Confirmation of the maternal transmission of plastids in *Senecio*. (ii) Assessment of the level of <u>intra</u>specific cpDNA variation within *S. vulgaris sl* and *S. squalidus* and determination of the levels of <u>inter</u>specific cpDNA variation in the genus *Senecio*. (iii) Determination of the plastid donor to *S. cambrensis* in Wales and Scotland.

Materials and Methods.

3.2.1 Plant material.

Achenes from single individuals of *Senecio aethnensis*, *S. cambrensis*, *S. chrysanthemifolius*, *S. jacobaea*, *S. paludosus*, *S. squalidus*, *S. vernalis*, *S. vulgaris* ssp. *denticulatus*, S. *vulgaris* ssp. *vulgaris* var. *hibernicus*, *S. vulgaris* ssp. *vulgaris* var. *vulgaris*, representing 24 accessions were sown under standard glasshouse conditions (Appendix A, section A1). The locations from which these taxa were collected and a sectional classification of the genus *Senecio*, according to Alexander (1979), are given in Table 3.2.

-Y

Table 3.2 Locations of the Senecio taxa used in the chloroplast DNA study.

Taxon.	Location.§		Grid Ref.	No. of Indiv.§§	Source*	Code No.
Section Senecio.						
S. vulgaris ssp.	Migvie, Aberdeenshire.	(M)	NJ437068	1	RJA	vMi
vulgaris var.	Puffin Island, Wales.	(M)	SH653824	1	RJA	vPu
vulgaris.	York.	(P)	SE590510	1	PA	vYo
	Mochdre, Wales.	(P)	SH822781	1	PA	vMo
	Brymbo, Wales.	(P)	SJ296539	4 c(3)	PA	vBr
	Salamander Street, Edinburgh.	(P)	NT276763	1	PA	vSa
S. vulgaris ssp.	Mochdre, Wales.		SH822781	1	PA	hMo
vulgaris var.	Brymbo, Wales.		SJ296539	1	PA	hBr
hibernicus.	Salamander Street, Edinburgh.		NT276763	1	PA	hSa
	York.		SE590510	1	RJA	hYo
S. vulgaris ssp.	Ainsdale, Lancashire.		SD295124	1	PA	dAi
denticulatus.	Quennevais, N. of Pulente, Jersey.		-	2 c(2)	PA	dJe
S. squalidus.	Salamander Street, Edinburgh.		NT276763	1	PA	sSa
	Brymbo, Wales.		SJ296539	3 c(2)	PA	sBr
	Stoke.		SP360780	1	PA	sSt
	York.		SE590510	1	PA	sYo
	Sheffield.		SK350870	1	PA	sSh
S. cambrensis.	Salamander Street, Edinburgh.		NT276763	1	SAH	cSa
	Brymbo, Wales.		SJ296539	3 c(2)	PA	cBr

Table 3.2 Cont.

Taxon.	Location.§	Grid Ref.	No. of Indiv.§§	Source※	Code No□.
Section Senecio.					
S. vernalis.	Schlusserlacker Weide, Eppelheim, nr Heidelberg, Germany.	-	1	PA	veGe
S. jacobaea.	Tentsmuir Forest, Fife.	NO499241	1	SAH	jTe
S. aethnensis.	Mt. Etna, Sicily.	-	1 t(1)	RJA	aEt
S. chrysanthem-ifolius.	Mt. Etna, Sicily.	-	1 t(1)	RJA	chEt
Section Doria.					
S. paludosus.	British material	-	1	CaBG	pCa

§ Monomorphic (M) and Polymorphic (P) S. vulgaris ssp. vulgaris sl populations.
§§ Those individuals marked with 't' were probed with total cpDNA, those marked 'c' were probed with cloned cpDNA. The numbers in brackets refer to the numbers of individuals used with each probe. Unless otherwise stated all of the individuals were probed with both total and cloned cpDNA
※ Initials refer to collectors; CaBG - Cambridge University Botanic Gardens, PA - Paul Ashton, RJA - Richard Abbott, SAH - Stephen Harris.
□ First letter of the code refers to the specific or varietal epithet, followed by the first two letters of the collection site.
Ainsdale ssp. denticulatus,, Jersey ssp. denticulatus and Salamander Street S. cambrensis were determined by C. A. Stace, C. Preston and R. Ingram respectively.

3.2.2 DNA extraction, hybridisation and autoradiography.

Total DNA was used in the cpDNA analysis because the isolation of purified cpDNA from *Senecio* species proved to be unreliable and the yields obtained were very poor. Total DNA was extracted from a pooled sample of ten plants, each having the same female parent (Appendix A, Section A2). The DNA was further purified on two sequential caesium chloride gradients (Appendix A, Section A2.3.1).

A total of 12 restiction enzymes were used; one tetranucleotide cutting enzyme (*Hae*III), ten hexanucleotide cutting enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*DIII, *Kpn*I, *Pst*I, *Sac*I, *Xba*I, *Xho*I) and one heptanucleotide cutting enzyme (*Bst*EII). However, only 11 enzymes were used in the final analysis with cloned probes because reliable cutting with *Xba*I was difficult, even after two cycles of caesium chloride purification. Because of similar problems with digestion (*Xba*I and *Xho*I) or the lack of resolution due to a large number of small fragments (*Hae*III), only nine enzymes were used when total cpDNA was used as a probe. A list of the cutting sites for the enzymes used are given in Table A1 (Appendix A).

Methods for digestion, electrophoresis, blotting and probing of sample DNA are described in Appendix A, Sections A2.5-A2.9. All of the taxa digested with a single enzyme were run on the same 1% agarose gel, so that direct comparisons between taxa could be made.

The conditions for washing the filters were:- two thirty minute washes in 2xSSC + 0.5% SDS at room

temperature followed by a further thirty minute wash in the same buffer at 65°C.

In the experiment to confirm maternal inheritance, DNA from the crosses was extracted from single plants as opposed to pooled plant material and purified using DEAE-Sephacel column chromatography (Appendix A, Section A2.3.2).

3.2.3 Probe characteristics.

Two types of chloroplast probes were used in this study; cloned *Lactuca sativa* cpDNA fragments (provided by J. D. Palmer, Indiana State University) and total *Lactuca sativa* cpDNA.

All of the probes were labelled as in Appendix A (Section A2.8) except that unincorporated nucleotides were not removed from the total cpDNA probe mixture prior to use, in order to retain all of the fragments in this heterogeneous mixture.

3.2.3.1 Cloned *Lactuca sativa* cpDNA.

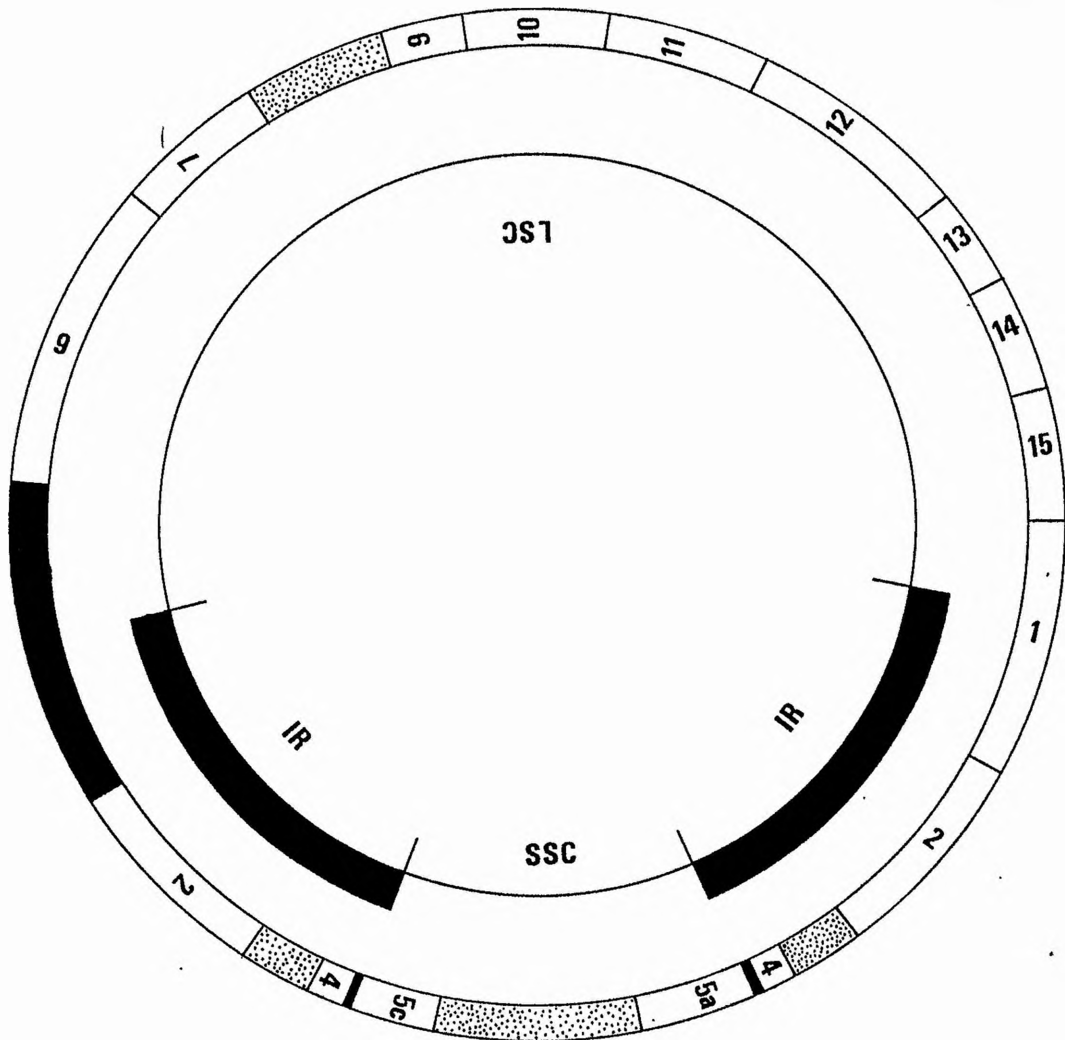
The *Lactuca sativa* cpDNA probes were created by cloning purified *L. sativa* cpDNA into the *Sac*I restriction site of the plasmid vector pUC18 (Jansen and Palmer 1987b). The total clone bank represents 96.4% of the *L. sativa* chloroplast genome. The sizes of the probes which were used in this study are given in Table 3.3 and their distribution around the chloroplast genome is shown in Figure 3.1. These

fragments will be referred to as pLsCx, where x is the number of the fragment in Figure 3.1. Cloned cpDNA probes were used either singly (pLsC1, pLsC2, pLsC4, pLsC6, pLsC7, pLsC9, pLsC15) or as a mixture (pLsC5a and pLsC5c; pLsC10, pLsC11 and pLsC12; pLsC13 and pLsC14).

3.2.3.2 Total *Lactuca sativa* cpDNA.

Total cpDNA was prepared from lettuce purchased from a local greengrocer (voucher not prepared), according to the method of Palmer (1986a). The purified cpDNA was digested with five units of *Eco*RI per microgram of DNA overnight at 37°C and then deproteinated, prior to labelling, with 'wet' chloroform (Appendix A, Section A2.8).

Figure 3.1. The general structure of chloroplast DNA, illustrated with *Lactuca sativa*. The abbreviations in the inner cicle refer to: IR - invert repeat, LSC - Large single copy region and SSC - Small single copy region. The numbers in the outer circle refer to the restriction fragments used as probes in the study, the filled-in region is a probe which has not been cloned, while the stippled areas are clones which were not used in this study.



LSC
IR
IR
SSC
6
7
9
10
11
12
13
14
15
1
2
4
5a
5c
4
2

Table 3.3. The sizes and cloning enzymes of the *Lactuca sativa* chloroplast DNA probes. All of the fragments are cloned into the plasmid vector pUC18.

PLASMID.	**SIZE(kb).**	**ENZYME.**
pLsC1	12.3	*Sac*I
pLsC2	9.9	*Sac*I
pLsC4	1.8	*Sac*I
pLsC5a	5.5	*Hin*DIII
pLsC5c	3.6	*Sac*I-*Hin*DIII
pLsC6	14.7	*Sac*I
pLsC7	7.0	*Sac*I
pLsC9	3.8	*Sac*I
pLsC10	6.9	*Sac*I
pLsC11	7.7	*Sac*I
pLsC12	10.6	*Sac*I
pLsC13	4.6	*Sac*I
pLsC14	5.4	*Sac*I
pLsC15	6.3	*Sac*I

All of these probes were the generous gift of J.D. Palmer (Indiana State University).

3.2.4 Chloroplast DNA inheritance.

To determine the mode of inheritance of the cpDNA, reciprocal crosses were made between *S. squalidus* (Devon Street, Grangemouth. Grid Ref. NS977814) and *S. vernalis* (Schlusserlacker Weide, Eppelheim, nr. Heidelberg, Germany). Individual, unopened capitula were bagged and allowed to develop to anthesis. Since both taxa are largely self incompatible (Crisp 1972, Kadereit 1984b), pollination was effected by rubbing the previously bagged capitula together and then rebagging these capitula. When fruit had been set, the achenes were collected and sown as in Section 3.2.1.

A total of seven potentially hybrid progeny were screened from the crosses. Six of the progeny were from the cross using *Senecio vernalis* as the maternal parent and one was from the cross with *S. squalidus* as the maternal parent. Vouchers of hybrid material have been deposited at StA.

In order to confirm the hybridity of the progeny from these crosses, total DNA was digested with *Eco*RV and probed with a heterologous nuclear ribosomal DNA (rDNA) clone, pTA71 (Chapter 2). Since both parents can be identified on the basis of their rDNA phenotypes, any hybrid individual would be expected to have an additive rDNA pattern.

The inheritance of the chloroplasts was followed using the probe-enzyme combination, pLsC6-*Bgl*II, since both parents could be distinguished using this probe-enzyme combination.

3.2.5 Data analysis.

3.2.5.1 Total chloroplast DNA probe.

Non-stoichiometric fragments were treated as single fragments, the reasons for this are given in Section 3.3.2

The data using total cpDNA as a probe was used to calculate S, an estimate of the proportion of fragments shared between any two cpDNA's, which is given by:

$$S = \frac{2n_{xy}}{(n_x+n_y)} \qquad \text{[Nei \& Li 1979, (Eq. 10)].}$$

Where n_{xy} is the number of shared fragments between the two cpDNAs, n_x is the number of fragments in cpDNA x and n_y is the number of fragments in cpDNA y.

An estimate of the sequence divergence, d, may be made by:

$$d = \frac{[-\mathrm{Ln}\ S]}{r} \qquad \text{[Nei 1987, (Eq. 5.42)].}$$

Where r is the number of bases in the oligonucleotide recognition sequence of the enzyme, in the case of this study r=6, for all nine enzymes studied.

The variance of the estimate, d, is given by:

$$V(d) = \frac{(2-S)(1-S)}{2r^2nS} \qquad \text{[Nei 1987, (Eq. 5.45)]}$$

Where n is estimated by $[n_x+n_y]/2$ and S is equal to $(1-d)^r$.

From the estimate of the variance, V(d), the standard error of the sequence divergence estimate was calculated as .V(d).

The values in the d matrix were clustered according to the method of Unweighted Pair Group Means (UPGMA, Sneath and Sokal 1973).

3.2.5.2 Cloned probes.

Data from the cloned probes was treated in a slightly different manner for two reasons. Firstly, many of the differences between the cpDNAs could be ascribed to restriction site mutations and were, therefore amenable to phylogenetic analysis (Sytsma and Schaal 1985, Jansen and Palmer 1988). Secondly, estimates of the percentage nucleotide substitution were made on the basis of enzymes having two different values of r, either r=4 (*Hae*III) or r=6 (all other enzymes). [The enzyme *Bst*EII, even though it has a 7bp recognition sequence, is treated as having a value for 'r' of r=6, since the central base of the recognition sequence may be any one of the four nucleotide bases, therefore, effectively only 6bp are involved in the specific recognition sequence (Nei 1987)].

A. Phylogenetic analysis. This was conducted on 18 site mutations that were present in two or more taxa, but which were not polymorphic within taxa. The PHYLIP package was used (Felsenstein 1985) and various

methods of analysis, with differing initial assumptions tried. A list of these follows:

The program MIX was used under the assumption of Wagner parsimony, ie. the loss or gain of a restriction site is assumed to be equally likely.

The program DOLLOP was used which makes an assumption of Dollo parsimony, ie. the loss of a restriction site is considered to be more likely than the gain of a site (DeBry and Slade 1985).

To place confidence intervals on the phylogenies, the programmes BOOT and DOLBOOT were used to perform a bootstrapping procedure (Felsenstein 1985). During this operation a random sample from the data matrix is drawn, with replacement, to create a new data matrix. This is then analysed using either MIX or DOLLOP and a record kept of those taxa that form monophyletic subsets in the estimated phylogeny. The operation is repeated, in this case 100 random sets of data were drawn from the original data matrix.

B. <u>Nucleotide substitution</u>. An estimate of the proportion of nucleotide substitutions per restriction site, p, was made using a maximum liklihood estimation (Nei and Tajima 1985, Nei 1987):

$$p = p_1 \frac{\dfrac{r_i(n_i - n_{xyi})}{[\{1-(1-p_1)^{r_i}\}\{2-(1-p_1)^{r_i}\}]}}{\dfrac{r_i n_i}{[2-(1-p1)^{r_i}]}}$$

[Nei 1987, (Eq 5.50)]

Where i is the ith type of enzyme with r_i recognition sequences, n_i is equal to $(n_x+n_y)/2$ and p_1 is the initial estimate of p or $p_1=(1-S^{1/r})$, where r=6.

When $p=p_1$, then p is the maximum liklihood estimate of p. This reiterative process was repeated five times using the computer package MINITAB.

Results.

3.3.1 Chloroplast DNA inheritance.

The results from the analysis of nuclear and chloroplast DNA in the putative hybrid progeny of the reciprocal *Senecio vernalis* x *S. squalidus* crosses are shown in Table 3.4.

Table 3.4 Inheritance of chloroplast DNA in the interspecific hybrid, *S. vernalis* x *S. squalidus*.

Cross (female x male)	n	rDNA	cpDNA
S. vernalis x *S. squalidus*.	6	V/S	V
S. squalidus x *S. vernalis*.	1	S	S

V - *S. vernalis*, S - *S. squalidus*.

These results show that in those progeny with a hybrid nucleus (as judged by an additive rDNA pattern), the cpDNA is that of the maternal parent. On the basis of this small sample it is suggested that cpDNA is maternally inherited in *Senecio* species (see Discussion, Section 3.4.1).

3.3.2 The size of *Senecio* chloroplast DNA.

The cloned cpDNA probes which have been used in this study represent approximately 80% of the *Lactuca sativa* chloroplast genome and are scattered throughout the large single copy, small single copy and invert repeat regions. Using the ten probes and eleven restriction enzymes, a total of 110 probe-enzyme combinations (PEC's) could be analysed, but on average 94 were used for each accession. This was possibly the result of poor digestion of some of the DNA samples and the lack of binding of some probes to some accessions. The former is likely to be the result of contaminating products that interfere with enzyme action, while the latter may be a function of poor sequence similarity of the probe to the test DNA and/or binding to small fragments that may not have been resolved during electrophoresis or partially lost during filter stripping procedures (Appendix A, Section A10). The total number of fragments generated, the number of PEC's used and the number of nucleotides sampled are shown in Table 3.5. A mean of approximately 94 PEC's and 1778bp were analysed for all of the accessions. Only those taxa for which all of the PEC's were at least tried, were used in this calculation; thus *S. vulgaris* var. *vulgaris* from Salamander Street is excluded since not all PEC's were used because of the very small quantity of DNA which was available. In an attempt to cover the remaining 20% of chloroplast genome, total *Lactuca sativa* cpDNA was used as a probe. The sizes of the

fragments that were generated for all of the PEC's and the total cpDNA for all of the taxa are shown in Appendix D.

During the course of this study multiple fragments of similar size have not been considered, since it was difficult to determine if any fragments which occurred in non-stoichiometric quantities were due to comigrating, non-homologous fragments or homologous fragments duplicated in the genome. The small size of the estimate for the invert repeat (IR) in the Asteraceae [25kb; Kolodner and Tewari 1979, Ma and Smith 1985, Jansen and Palmer 1988] means that non-stoichiometric fragments are likely to be the result of duplication of homologous fragments from this region of the molecule. Since the IR undergoes a type of concerted evolution (Section 3.1.1) treating comigrating fragments as single fragments is unlikely to have a serious effect. Therefore, the estimates of the size of the chloroplast genome for the various taxa (Table 3.6), obtained by summation of the visible fragments when total cpDNA was used as a probe, are probably under-estimates of the true size.

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Table 3.5 The mean number of fragments, PEC's used and base pairs (bp) sampled in the analysis of chloroplast DNA, using cloned probes.

Taxon.	No of indiv.	Mean No. of bp sampled.	Mean No. of PEC's used.	Mean No. of fragments.
S. vulgaris ssp. vulgaris var. vulgaris.	4	1796.0	94.0	315.0
S. vulgaris ssp. vulgaris var. hibernicus.	4	1844.0	97.0	323.0
S. vulgaris ssp. denticulatus.	1	1764.0	96.0	309.0
S. squalidus.	5	1798.0	94.0	315.0
S. cambrensis.	2	1705.0	91.0	290.0
S. vernalis.	1	1738.0	93.0	304.0
S. jacobaea.	1	1770.0	97.0	310.0
S. paludosus.	1	1552.0	80.0	274.0

Table 3.6. Estimates of the total size of the chloroplast DNA from various taxa of the genus *Senecio*, based on the size of the visible fragments when total *Lactuca sativa* cpDNA was used as a probe.

Taxon	No. of Indiv.	Mean No. enzymes.	Mean No. Frag.	Mean size(kb).
S. vulgaris ssp. *vulgaris* var. *vulgaris*.	3	8	119	93
S. vulgaris ssp. *vulgaris* var. *hibernicus*.	4	8	122	90
S. vulgaris ssp. *denticulatus*.	1	8	117	93
S. squalidus.	5	8	121	100
S. cambrensis.	2	8	118	98
S. vernalis.	1	8	118	95
S. jacobaea	1	7	93	73
S. paludosus.	1	5	81	74

3.3.3 Chloroplast DNA mutations in *Senecio* species.

In the taxa of *Senecio* studied using cloned probes both length and site mutations have been located. Those differences that could not be readily ascribed to either a site or length mutation are omitted.

The greatest problem in the analysis was the identification of the events responsible for particular fragment differences. The only length mutation was identified on the basis of a 350bp change that occurred with three enzymes (Plate 3.1). The site mutations (Table 3.7) were occasionally more problematic, since many of the fragment gains in one taxon were not exact sums of fragment losses in other taxa. This was probably a result of systematic measurement error (approximately 10%), inability to resolve very small fragments [this was found to depend very much on the enzyme used for digestion, *Eco*RI generated a lot of small fragments that could not be resolved, as did *Hae*III] and the use of heterologous *Lactuca sativa* cpDNA probes which may not have cross hybridised to the smaller fragments of *Senecio* cpDNA. As a result, site mutations were deduced on the basis of fragment differences that were not found with other PEC's.

A number of PEC's (Table 3.8) did not produce results that were readily interpretable as either length or site mutations. This may be a result of the probe spanning a region containing a number of mutations. Hence, the number of mutations that have been identified may underestimate those actually present in two ways:- firstly, since the

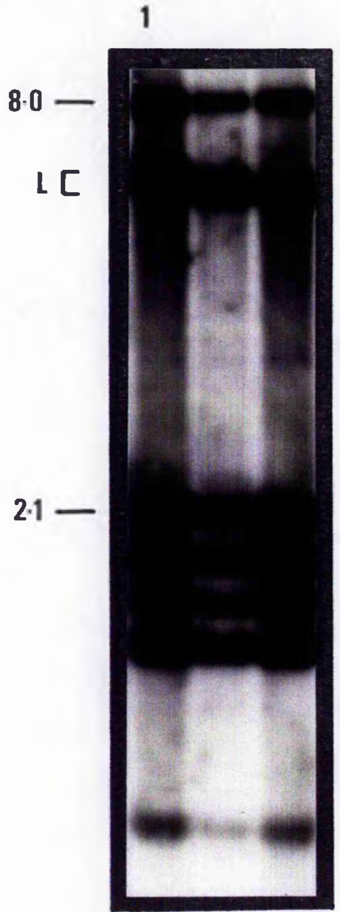
entire cpDNA has not been sampled with cloned probes some mutations may have been missed and secondly, only those mutations that could positively be identified have been used.

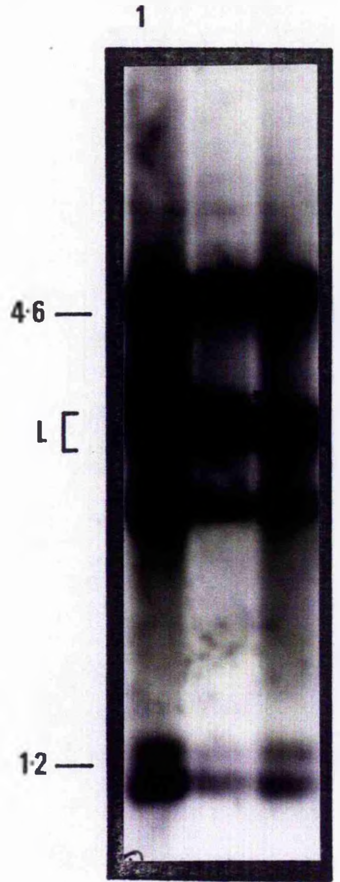
'Ghost' bands were present on some digests, which created an additional problem of interpretation and may have been the result of promiscuous DNA. Promiscuous cpDNA sequences are known in both the nuclear genome (Chesney and Scott 1989, Ayliffe *et al* 1988) and the mitochondrial genome (Stern and Lonsdale 1982, Stern and Palmer 1984, du Jardin 1990). Hence fragments which are being scored as cpDNA may represent nuclear or mitochondrial sequences with similarity to cpDNA (xenologous comparisons). The problem created by these sequences may not be as great as it first appears since, once they are inserted into the 'host' genome they evolve at the rates applicable to that genome (Birky 1988), and will quickly lose sequence similarity (Palmer 1990). These bands were ignored and only the strongly hybridising fragments were scored as real cpDNA sequences.

69 site mutations were located, of which 50 were autapomorphies (ie. mutations restricted to particular taxa), 18 were shared by two or more taxa and one was associated with a known polymorphism (Plate 3.2, Lanes 8, 11-13). The single length mutation which was identified was restricted to Welsh *Senecio cambrensis* (cBr). The evolutionary polarity for most of these mutations (ie. which are ancestral and which are derived) has not been identified since the putative outgroup, *Senecio paludosus*

proved to have a close similarity to *S. jacobaea*. The distribution of these mutations around the cpDNA is shown in Figure 3.2.

Plate 3.1. Digested *Senecio* DNA probed with the chloroplast DNA clone, pLsC6. A. *Bam*HI digest. B. *Bgl*II digest. C. *Sac*I digest. Lane 1 - *S. cambrensis* (cBr); Lane 2 - *S. vulgaris* ssp. *vulgaris* var. *vulgaris* (vMi); Lane 3 - *S. squalidus* (sSa). The asterisk in Lane 2 of B indicates the position of an unusual fragment. 'L' indicates the position of the 350bp length mutation which is found in cBr. All fragment sizes are measured in kilobases.


1
8·0
L
2·1
A


1
4·6
L
1·2
B

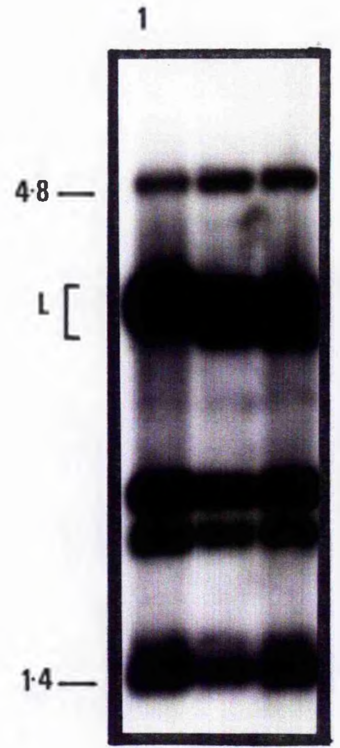

1
4·8
L
1·4
C

Table 3.7 Restriction site mutations and length mutations identified in the chloroplast DNA of *Senecio* species.

Enzyme.	Probe.	Character State. 0	1	Taxa.
Restriction site mutations.				
*Bam*HI	pLsC2	3.0	2.4 + 0.7	pCa,jTe
*Bam*HI	pLsC4	19.1 + 4.3	24.8	pCa,jTe
*Bam*HI	pLsC6	5.4 + (0.7)	6.3	pCa
*Bam*HI	pLsC6	5.4 + (0.6)	6.0	veGe
*Bam*HI	pLsC6	5.4	5.2 + (0.2)	dAi
*Bam*HI	pLsC6	0.91 + (0.04)	0.95	pCa
*Bam*HI	pLsC6	0.91	0.88 + (0.3)	jTe
*Bam*HI	pLsC9	7.4	6.3 + 1.8	pCa,dAi
*Bam*HI	pLsC9	2.3 + (0.1)	2.4	pCa
*Bam*HI	pLsC9	7.4	4.8 + 2.3	jTe
*Bam*HI	pLsC10-12	11.8 + 8.5	19.3	pCa
*Bgl*II	pLsC2	5.4	3.8 + 1.2	pCa,jTe
*Bgl*II	pLsC4	7.6 + (0.4)	8.0	pCa,jTe
*Bgl*II	pLsC5ac	8.4	8.2 + (0.2)	pCa,jTe
*Bgl*II	pLsC5ac	7.4 + (0.6)	8.0	pCa,jTe
*Bgl*II	pLsC6	1.3	1.2 + (0.1)	jTe
*Bgl*II	pLsC6	3.0	2.9 + (0.1)	jTe
*Bgl*II	pLsC6	3.0	2.8 + (0.2)	dAi
*Bgl*II	pLsC6	3.1 + (0.4)	3.5	veGe
*Bgl*II	pLsC6	3.0 + (0.3)	3.3	veGe
*Bgl*II	pLsC6	3.0 + (0.3)	3.3	pCa
*Bgl*II	pLsC7	1.6 + (0.1)	1.7	dAi
*Bgl*II	pLsC7	3.0 + (0.4)	3.4	veGe
*Bgl*II	pLsC7	1.3 + 1.6	2.8	pCa,jTe
*Bgl*II	pLsC9	3.3 + (1.1)	4.4	pCa,jTe
*Bgl*II	pLsC10-12	2.1	1.8 + (0.3)	jTe
*Bst*EII	pLsC1	1.6	1.1 + (0.5)	jTe
*Bst*EII	pLsC4	17.5	16.2 + (1.3)	pCa
*Eco*RI	pLsC4	2.1 + (0.1)	2.2	jTe
*Eco*RI	pLsC6	2.0 + 1.7	2.5	pCa,jTe
*Eco*RI	pLsC7	0.35 + (0.06)	0.41	jTe
*Eco*RI	pLsC9	5.4 + (0.3)	5.7	pCa
*Eco*RI	pLsC10-12	3.0	2.9 + (0.1)	vPu
*Eco*RI	pLsC10-12	3.0 + (0.1)	3.1	dAi
*Eco*RI	pLsC10-12	4.2	3.9 + (0.3)	jTe
*Eco*RI	pLsC10-12	2.0	1.9 + (0.1)	jTe
*Eco*RI	pLsC13-14	4.0	3.0 + (1.0)	pCa,jTe
*Eco*RI	pLsC15	0.4	0.37 + (0.03)	jTe
*Eco*RI	pLsC15	2.6 + (0.5)	3.2	jTe
*Eco*RI	pLsC15	2.0	1.4 + (0.6)	jTe
*Eco*RV	pLsC2	3.8 + (0.3)	4.1	jTe
*Eco*RV	pLsC5ac	22.4	19.0 + (3.4)	pCa
*Eco*RV	pLsC6	1.6	1.4 + (0.2)	jTe
*Eco*RV	pLsC6	7.8 + (0.9)	8.7	veGe

Table 3.7 Cont.

Enzyme.	Probe.	Character State. 0	1	Taxa.
Restriction site mutations.				
*Eco*RV	pLsC7	4.6 + (0.1)	4.7	dAi
*Eco*RV	pLsC7	1.5	1.4 + (0.1)	jTe
*Eco*RV	pLsC10-12	10.3	9.3 + (1.0)	pCa
*Hae*III	pLsC1	11.3	10.9 + (0.4)	pCa
*Hae*III	pLsC5ac	3.0	2.9 + (0.1)	pCa
*Hae*III	pLsC5ac	2.1 + (0.3)	2.4	pCa,jTe
*Hae*III	pLsC6	2.6	2.4 + (0.2)	dAi
*Hae*III	pLsC6	2.6	2.1 + (0.5)	jTe
*Hae*III	pLsC6	2.3	2.1 + (0.2)	sSh
*Hae*III	pLsC7	0.8	0.6 + (0.2)	jTe
*Hae*III	pLsC9	1.5	1.4 + (0.1)	pCa,jTe
*Hae*III	pLsC9	0.8	0.6 + (0.2)	pCa,jTe
*Hae*III	pLsC13-14	3.1	2.0 + 1.1	pCa,jTe
*Hae*III	pLsC13-14	1.6 + 1.1	2.2	pCa,jTe
*Hae*III	pLsC15	1.7	1.6 + (0.1)	pCa,jTe
*Hae*III	pLsC15	0.86 + (0.04)	0.90	pCa,jTe
*Hin*DIII	pLsC9	3.2 + (0.3)	3.5	jTe
*Hin*DIII	pLsC10-12	11.3 + (1.3)	12.6	jTe
*Hin*DIII	pLsC10-12	11.3	6.0 + 4.8	hBr,vBr, vSa,cSa
*Kpn*I	pLsC9	5.5 + (0.2)	5.7	dAi
*Pst*I	pLsC1	2.8 + (0.1)	2.9	pCa
*Sac*I	pLsC9	3.6 + (0.4)	4.0	pCa
*Sac*I	pLsC9	3.6 + (1.0)	4.6	jTe
*Xho*I	pLsC7	3.2 + (0.2)	3.4	dAi
*Xho*I	pLsC7	3.2 + (0.1)	3.3	jTe
Length mutation.				
*Bam*HI	pLsC6	5.38	5.70	cBr
*Bgl*II	pLsC6	2.95	3.31	cBr
*Sac*I	pLsC6	3.11	3.50	cBr

The use of '0' and '1' for the character states does not imply which character states are primitive and derived, it is merely a convenient method to indictate those taxa in which a particular change is present.
The figure in parentheses indicate fragment lengths which were not seen, but hypothesised to be present.
The code, in the taxa column, refers to those given in Table 3.2.

Table 3.8 Unidentified mutations in the chloroplast DNA of *Senecio* species.

Enzyme.	Probe.	Character State. 0	1	Taxa.
*Bam*HI	pLsC5ac	25.3	33.6	jTe
*Bam*HI	pLsC6	-	5.7	vMi
*Bgl*II	pLsC1	-	2.3	vPu
*Bgl*II	pLsC7	3.0	-	jTe,veGe
*Bst*EII	pLsC4	3.6	-	cSa,pCa, sSh
*Bst*EII	pLsC4	-	5.5	pCa
*Eco*RI	pLsC4	-	3.0,2.0,1.7	sSa
*Eco*RI	pLsC4	-	3.0,1.7	pCa,vYo
*Eco*RI	pLsC6	1.2	-	cSa,hBr, jTe, veGe
*Eco*RI	pLsC6	-	2.5,2.1	sSa
*Eco*RI	pLsC6	2.0,1.7	2.5,2.1	pCa
*Eco*RI	pLsC6	2.0,1.7	2.5	jTe
*Eco*RI	pLsC7	-	1.4,0.765	sSt,vBr
*Eco*RI	pLsC7	-	1.4	hMo
*Eco*RV	pLsC6	3.3	-	dAi,hBr, hMo,hYo, sBr,sSt, vBr,vSa
*Hae*III	pLsC4	3.0,1.4	3.2,1.7,1.3	pCa
*Hae*III	pLsC4	3.0,1.4	3.4,3.1	jTe
*Hae*III	pLsC5ac	-	2.6	jTe
*Hae*III	pLsC6	2.3	2.1,1.5	pCa
*Hae*III	pLsC6	1.3	-	jTe
*Hae*III	pLsC10-12	2.7	-	dAi,veGe
*Hae*III	pLsC15	1.4	-	cSa,jTe, pCa,sSh, vPu
*Hin*DIII	pLsC5ac	10.3,4.8,3.7	6.8,3.3	pCa
*Hin*DIII	pLsC5ac	-	6.7	jTe
*Hin*DIII	pLsC4	-	3.3	pCa
*Hin*DIII	pLsC6	17.1,7.6	18.3,16.1,8.1,7.4	pCa
*Hin*DIII	pLsC9	3.2	9.2,8.5,3.3	pCa
*Hin*DIII	pLsC10-12	11.3,7.1	11.9,9.2,5.4,3.2	pCa
*Sac*I	pLsC4	-	1.4	dAi,pCa, veGe
*Sac*I	pLsC6	-	4.8	cBr,jTe, pCa,sSa, sSh,vMi, vPu

Table 3.8 Cont.

Enzyme.	Probe.	Character State. 0	1	Taxa.
*Sac*I	pLsC6	-	2.6	sSh,vPu
*Sac*I	pLsC6	1.5,3.1	5.5,3.3,1.9,1.8	pCa
*Sac*I	pLsC6	3.1,1.4	3.1,2.6,	jTe
*Sac*I	pLsC10-12	8.7	6.9	jTe
*Xho*I	pLsC7	-	5.6	pCa
*Xho*I	pLsC7	-	2.3	hSa,vBr, vYo

The use of '0' and '1' for the character states does not imply which character states are primitive and derived, it is merely a convenient method to indictate those taxa in which a particular change is present.
The code, in the taxa column, refers to those given in Table 3.2.

Plate 3.2. *Hin*DIII-digested *Senecio* DNA probed with the chloroplast DNA clone, pLsC10/11/12 to show the polymorphism associated with triazine resistance (Lanes 8, 11-13). The lanes are:

1: *S. cambrensis* (cBr)
2: var. *vulgaris* (vMi)
3: *S. squalidus* (sSa)
4: *S. squalidus* (sBr)
5: *S. squalidus* (sSt)
7: var. *hibernicus* (hMo)
8: var. *hibernicus* (hBr)
9: var. *vulgaris* (vYo)
10: *S. squalidus* (sYo)
11: var. *vulgaris* (vBr)
12: var. *hibernicus* (hSa)
13: *S. cambrensis* (cSa)
14: *S. paludosus* (pCa)
15: *S. vernalis* (veGe)
16: ssp. *denticulatus* (dAi)
17: *S. squalidus* (sSh)
18 var. *hibernicus* (hYo)

All fragment sizes are measured in kilobases.

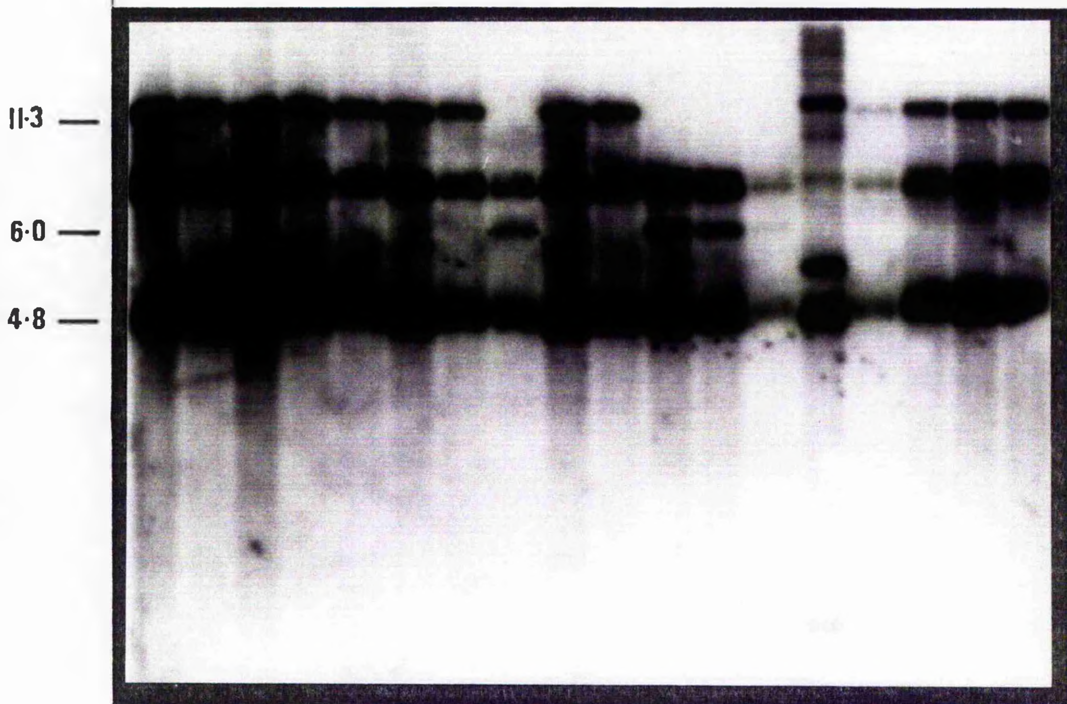
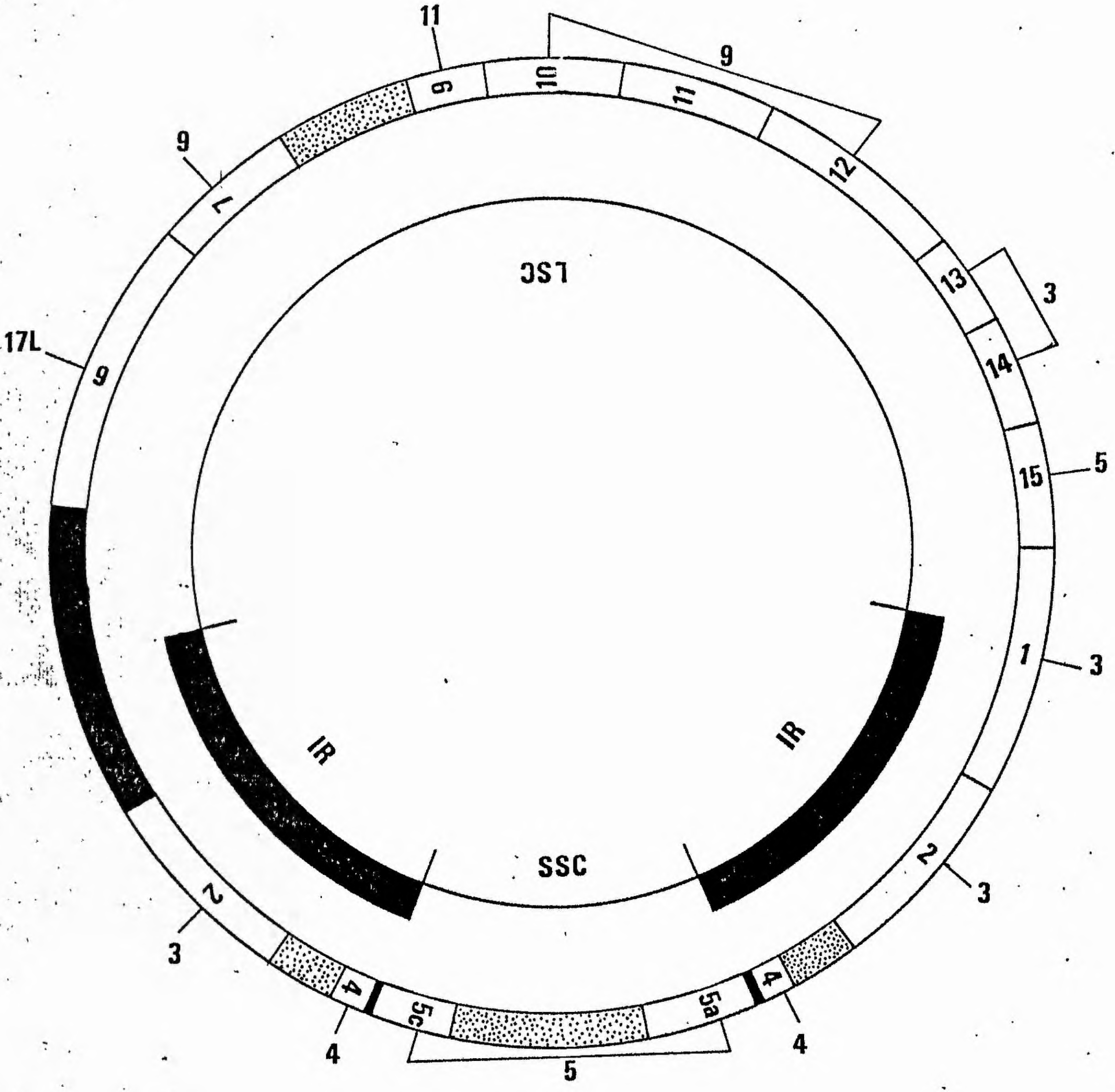

1
18
11·3
6·0
4·8

Figure 3.2. Distribution of the chloroplast DNA mutations, revealed in *Senecio* species, using *Lactuca sativa* chloroplast DNA probes. For a description of the inner and outer circle abbreviations see Figure 3.1. The numbers on the outside of the outer circle are the number of site mutations identified by that probe or probe cocktail (identified by a line joining two or more probes); L indicates the position of the identified length mutation.



LSC
SSC
IR
IR
17L
9
7
11
10
12
13
14
15
1
2
3
4
5a
5c
5

3.3.4 Intraspecific variation in *Senecio vulgaris* ssp.*vulgaris sl* and *S. squalidus*.

Within *Senecio vulgaris sl* and *Senecio squalidus* a number of polymorphic PEC's were identified (Table 3.9). This Table shows those PEC's that were polymorphic in terms of site mutations only, and also those which showed more complex patterns.

One feature of this Table is that, although polymorphism exists within both *Senecio vulgaris* ssp. *vulgaris sl* and *S. squalidus* these two taxa have essentially similar cpDNA due to shared polymorphism (in terms of the PEC's examined in this study).

The extremely close similarity of the cpDNA's from *Senecio vulgaris* ssp. *vulgaris sl* and *S. squalidus* is apparently repeated over the entire cpDNA, when total cpDNA was used as a probe (see Appendix D).

Table 3.9 Polymorphic (PEC's) in *Senecio vulgaris* ssp. *vulgaris sl*, *S. squalidus* and *S. cambrensis*.

Taxon	PEC
S. vulgaris ssp. *vulgaris* var. *vulgaris*.	Identified site mutations. *Eco*RI-pLsC10/11/12 *Hin*DIII-pLsC10/11/12 Unidentified mutations. *Bam*HI-pLsC6 *Bgl*II-pLsC1 *Eco*RI-pLsC4 *Eco*RI-pLsC6 *Eco*RI-pLsC7 *Hae*III-pLsC15 *Sac*I-pLsC6 *Xho*I-pLsC7
S. vulgaris ssp. *vulgaris* var. *hibernicus*.	Identified site mutations. *Hin*DIII-pLsC10/11/12 Unidentified mutations. *Eco*RI-pLsC6 *Eco*RV-pLsC6 *Xho*I-pLsC7
S. squalidus.	Identified site mutations. *Hae*III-pLsC6 Unidentified mutations. *Bst*EII-pLsC4 *Eco*RI-pLsC4 *Eco*RI-pLsC6 *Eco*RI-pLsC7 *Eco*RV-pLsC6 *Sac*I-pLsC6
S. cambrensis.	Identified site mutations. *Bam*HI-pLsC6 *Bgl*II-pLsC6 *Hin*DIII-pLsC10 *Sac*I-pLsC6 Unidentified mutations. *Bst*EII-pLsC4 *Eco*RI-pLsC6 *Hae*III-pLsC15

3.3.5 Chloroplast DNA in *Senecio cambrensis*.

3.3.5.1 *Senecio cambrensis* in Wales.

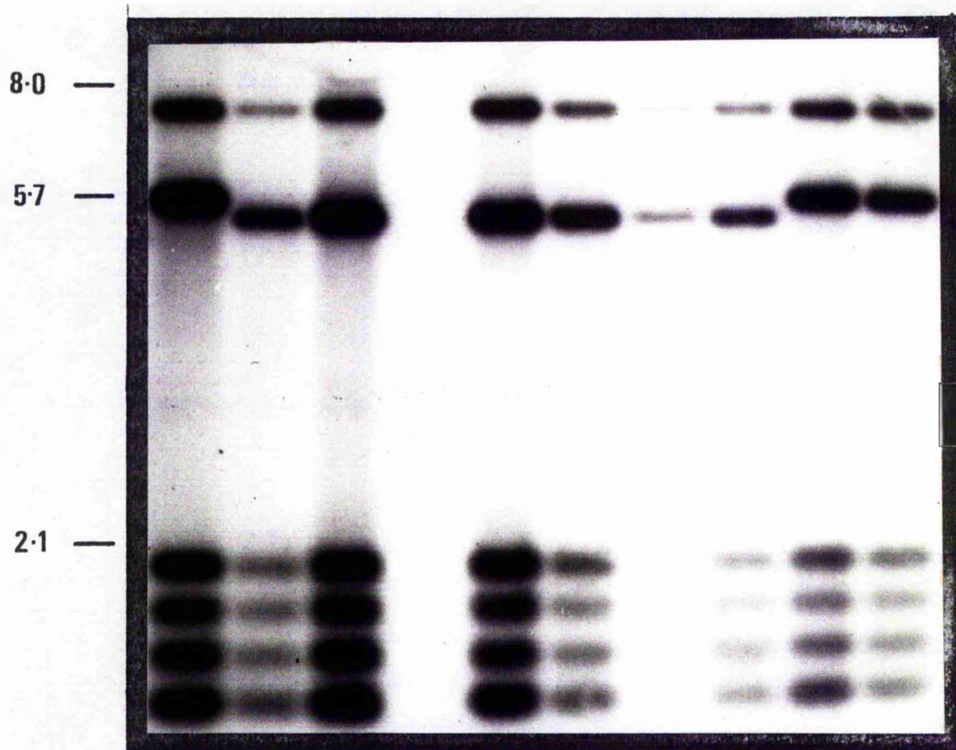
Senecio cambrensis was the only taxon for which there was conclusive evidence that a length mutation had occurred. In this case a length mutation of approximately 350bp was found in Welsh *S. cambrensis* (cBr), which was absent from Scottish *S. cambrensis* (cSa) (Table 3.7). Similarly, this length mutation was not found in any of the geographically separated populations of the two parental species which were analysed. Additional individuals of *S. vulgaris* var. *vulgaris*, *S. squalidus* and *S. cambrensis* were sampled from the Brymbo site and their cpDNA's screened (with pLsC6-*Bgl*II) for the presence of the length mutation (Plate 3.3). These results show that, in the three *S. vulgaris* var. *vulgaris* and three *S. squalidus* sampled, no evidence of the length mutation was found, but in Welsh *S. cambrensis* the length mutation was found in both additional individuals studied. The occurrence of this mutation is an important marker in *S. cambrensis* since it strongly suggests that the two populations had separate origins.

3.3.5.2 The origin of *Senecio cambrensis*.

The lack of variation between the cpDNA's of *S. vulgaris* ssp. *vulgaris sl* and *S. squalidus* made it impossible to identify the maternal parent of the allohexaploid hybrid, with the PEC's used in this study. Some insight may, however, be obtained from the observation that the pLsC10/11/12-*Hin*DIII polymorphism was found in

S. vulgaris ssp. *vulgaris sl* but was absent from *S. squalidus* (Plate 3.2, Lanes 8, 11-13). The presence of this polymorphism in Scottish *S. cambrensis* suggests that, at least, in this location the maternal parent was *S. vulgaris* ssp. *vulgaris sl*.

Plate 3.3. *Bam*HI-digested *Senecio* DNA probed with the chloroplast DNA clone, pLsC6 to show the distribution of the 350bp length mutation between Welsh and Scottish *S. cambrensis*. Lanes 1, 9, 10 - Welsh *S. cambrensis*; Lanes 2, 3 - Welsh *S. vulgaris* ssp. *vulgaris* var. *vulgaris*; Lanes 5, 6 - Scottish *S. vulgaris* ssp. *vulgaris* var. *vulgaris*; Lanes 7, 8 - Welsh and Scottish *S. squalidus* respectively.All fragment sizes are measured in kilobases.


1
10
8·0
5·7
2·1

<u>3.3.6 Chloroplast DNA in *Senecio vulgaris* ssp *denticulatus*.</u>

The presence of polymorphism in *Senecio vulgaris* ssp. *vulgaris sl* has been demonstrated in Section 3.3.4. However, when the two subspecies of *S. vulgaris* (ssp. *vulgaris sl* and ssp. *denticulatus*) were studied, a number of striking differences were found. Initially a single location at Ainsdale Beach in Lancashire was selected for study of the cpDNA from *S. vulgaris* ssp. *denticulatus*. Subsequently, two individuals from Jersey were examined, to look at two polymorphisms (pLsC6-*Bam*HI and pLsC6-*Bgl*II). The material from Ainsdale and Jersey gave different fragment patterns for these two PEC's. Jersey ssp. *denticulatus* produced patterns identical to those of ssp. *vulgaris*, while Ainsdale ssp. *denticulatus* produced a quite distinct pattern.

The analysis of the cpDNA of Ainsdale ssp. *denticulatus* and ssp. *vulgaris* with cloned probes showed these two taxa to have strikingly different cpDNAs (Plate 3.4, Lane 13 and Plate 3.5, Lane 13). On the basis of the identified site mutations (Table 3.8), nine site mutations separated ssp. *vulgaris* from Ainsdale ssp. *denticulatus*. If this is extended to include those patterns for which it was difficult to identify the exact changes, then two further differences are found. This result is fascinating in the light of the uniformity found within ssp. *vulgaris sl*.

Comparison of the rDNA of Jersey ssp. *denticulatus* and Ainsdale ssp. *denticulatus* with ssp. *vulgaris* using the PEC pTA71-*Eco*RV showed that both Jersey and Ainsdale ssp.

denticulatus had identical rDNA fragment patterns, but that these were quite distict from ssp. *vulgaris* (Chapter 2).

Plate 3.4. *Bgl*II-digested *Senecio* DNA probed with the chloroplast DNA clone, pLsC6 to show the differences between taxa. The lanes are:

1: *S. squalidus* (sBr)
2: *S. squalidus* (sSt)
3: var. *vulgaris* (vPu)
4: var. *hibernicus* (hMo)
5: var. *hibernicus* (hBr)
6: var. *vulgaris* (vYo)
7: *S. squalidus* (sYo)
8: var. *vulgaris* (vBr)
9: var. *hibernicus* (hSa)
10: *S. cambrensis* (cSa)
11: *S. paludosus* (pCa)
12: *S. vernalis* (veGe)
13: ssp. *denticulatus* (dAi)
14: *S. squalidus* (sSh)
15: var. *hibernicus* (hYo)
16: *S. jacobaea* (jTe)

All fragment sizes are measured in kilobases.

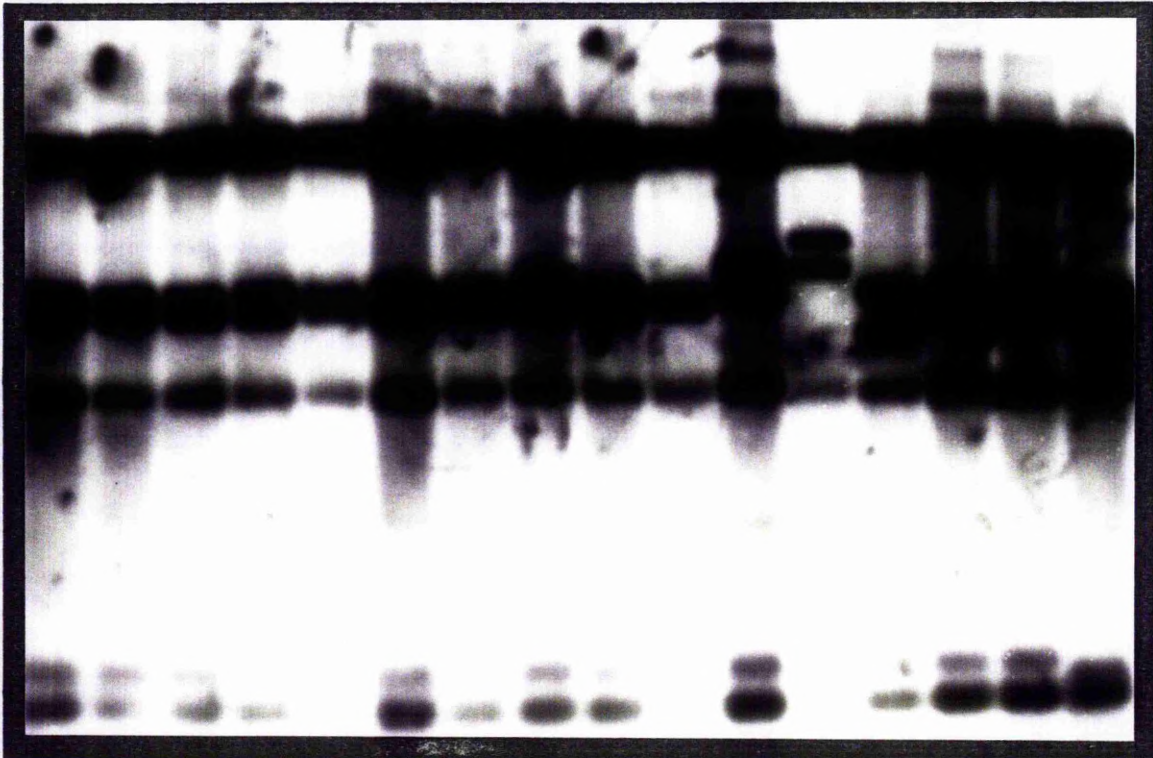
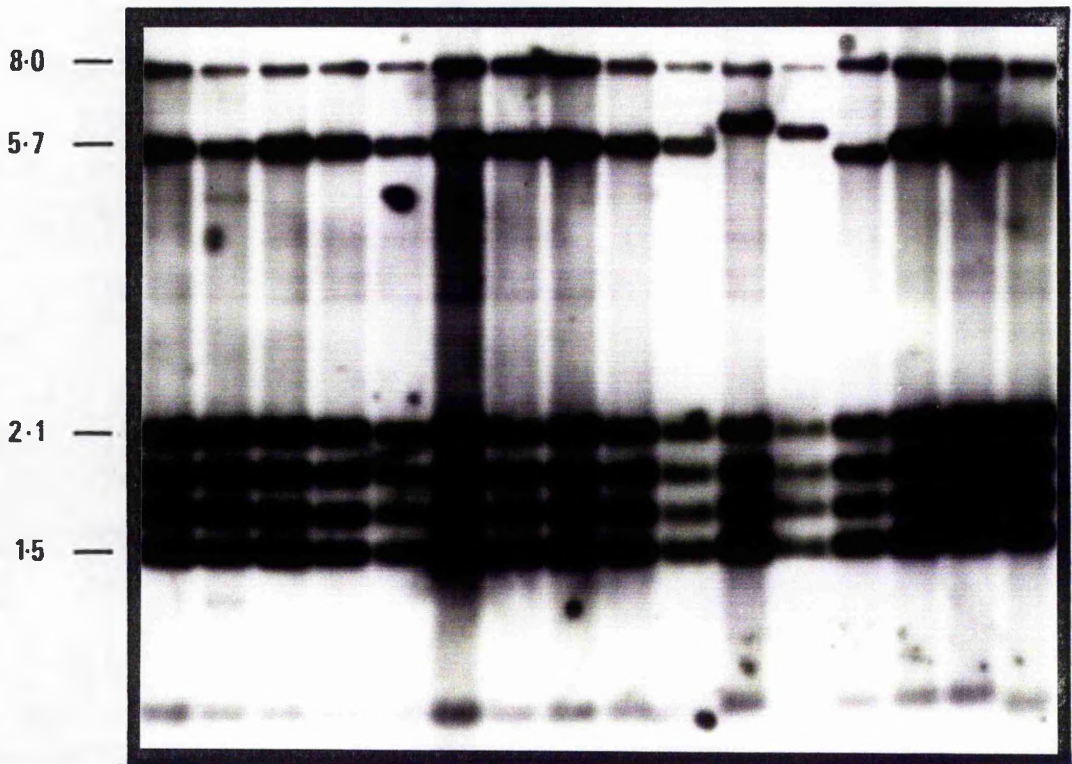

1
16
4·6
2·5
1·2

Plate 3.5. *Bam*HI-digested *Senecio* DNA probed with the chloroplast DNA clone, pLsC6 to show the differences between taxa. The lanes are:

1: *S. squalidus* (sBr)
2: *S. squalidus* (sSt)
3: var. *vulgaris* (vPu)
4: var. *hibernicus* (hMo)
5: var. *hibernicus* (hBr)
6: var. *vulgaris* (vYo)
7: *S. squalidus* (sYo)
8: var. *vulgaris* (vBr)
9: var. *hibernicus* (hSa)
10: *S. cambrensis* (cSa)
11: *S. paludosus* (pCa)
12: *S. vernalis* (veGe)
13: ssp. *denticulatus* (dAi)
14: *S. squalidus* (sSh)
15: var. *hibernicus* (hYo)
16: *S. jacobaea* (jTe)

All fragment sizes are measured in kilobases.


1
16
8·0
5·7
2·1
1·5

3.3.7 Chloroplast DNA in other *Senecio* species.

The cpDNA of five additional species of *Senecio* were analysed. In the case of *S. chrysanthemifolius* and *S. aethnensis* only six enzymes were used (*Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*DIII and *Sac*I) with total cpDNA as the probe. Comparison of the cpDNAs from these taxa revealed a number of putative differences, both relative to each other and to *S. squalidus*, which are summarised in Table 3.10. The identification of these mutations must be considered tentative due to the problems of using total cpDNA as a probe (see Discussion, Section 3.4.2). However, the general result is that *S. squalidus* is distinct in its cpDNA from both *S. aethnensis* and *S. chrysanthemifolius*.

Senecio vernalis cpDNA is distinct from that of *S. vulgaris sl* (Tables 3.7 and 3.8), while *S. jacobaea* and *S. paludosus* are similar to each other, but clearly distinguished from all the other *Senecio* species which have been used in this study.

Table 3.10 Variation in chloroplast DNA between *Senecio aethnensis*, *S. chrysanthemifolius* and *S. squalidus*, as judged from the comparison of total DNA probed with total *Lactuca sativa* cpDNA.

Enzyme.	Character State. 0	1	Taxa.
*Bam*HI	3.4	Absent	aEt,chEt
*Bgl*II	12.6	Absent	chEt
*Eco*RI	5.2	4.9 + (0.3)	chEt
	2.84	2.81 + (0.03)	chEt
	6.0	Absent	aEt
	12.0,11.0	Absent	aEt,chEt
*Eco*RV	Absent	2.5	aEt,chEt
*Hin*DIII	2.4	Absent	aEt

The use of '0' and '1' for the character states does not imply which character states are primitive and derived, it is merely a convenient method to indictate those taxa in which a particular change is present.
The figure in parentheses indicate fragment lengths which were not seen, but hypothesised to be present.
The code, in the taxa column, refers to those given in Table 3.2 and indicates those taxa that have character state 1, ie. S. squalidus has character state 1 for each PEC.

3.3.8 Chloroplast DNA divergence in the genus *Senecio*.

The two estimates of cpDNA divergence used, p (number of nucleotide substitutions per site) and d (sequence divergence), both make a number of assumptions:- (i) The nucleotide frequencies are equal for each DNA (ie. restriction sites are randomly arranged around the genome). (ii) The DNA diverges through the accumulation of single base substitutions. (iii) The method of detecting the differences between DNAs allows the separation of non-homologous sites or fragments (Section 3.4.2.2). Finally, for the estimation of p, the number of nucleotides in each genome is assumed to be constant. For a discussion of these assumptions, see Section 3.4.3.

Although all of the assumptions of the diversity statistics are not met they are sufficiently robust (if $d<0.3$) so as not to significantly affect the estimates (Nei 1987). However, as pointed out by Lehävasilo *et al* (1987) the actual values of these estimates may depend greatly on the enzymes which are used.

The percentage of nucleotide substitutions (p) in the genus varies between 0.027% and 8.56% (Appendix E, Table E1). Calculations of the range of percentage nucleotide substitutions within various *Senecio* species and Sections of the genus are shown in Table 3.11.

Sequence divergence, d, estimates when total cpDNA was used as a probe, vary between 0.00% and 7.35% (Appendix E, Table E2), which is roughly comparable to the value of p

estimated from the probe data. Calculations of the range of divergence within various *Senecio* species and Sections of the genus (Table 3.12) also show similar values to p.

Table 3.11. The range of values for percentage nucleotide substitution (100p) for species and Sections of the genus *Senecio* [excluding *S. vulgaris* (vPu)]. Abstracted from Appendix E.

Taxon.	**n**	**Range 100p.**	**Mean.**
S. vulgaris ssp. *vulgaris sensu lato*.	21	0.22-1.91	0.36
S. vulgaris sensu lato.	28	0.22-2.26	0.53
S. squalidus.	10	0.08-1.48	0.68
Senecio Section *Senecio* [*sensu* Chater & Walters 1976].	36	0.22-2.71	0.76
Senecio Section *Senecio* [*sensu* Alexander 1979].	136	0.03-4.96	1.10
Senecio Section *Jacobaea* [*sensu* Chater & Walters 1976].	28	0.08-4.49	1.43

Table 3.12. The range of values for percentage divergence of chloroplast DNA for species and Sections of the genus *Senecio* [excluding *S. vulgaris* (vPu)]. Abstracted from Appendix E.

Taxon.	**n**	**Range 100d.**	**Mean.**
S. vulgaris ssp. *vulgaris sensu lato*.	21	0.00-0.98	0.53
S. vulgaris sensu lato.	28	0.00-2.34	0.87
S. squalidus.	10	0.18-1.07	0.45
Senecio Section *Senecio* [*sensu* Chater & Walters 1976].	36	0.00-2.34	0.95
Senecio Section *Senecio* [*sensu* Alexander 1979].	136	0.00-4.20	1.09
Senecio Section *Jacobaea* [*sensu* Chater & Walters 1976].	28	0.18-3.86	1.22

3.3.9 Phenetic and phylogenetic relationships in the genus *Senecio*.

The estimates of percentage sequence divergence in Table E2 (Appendix E) were used to construct a UPGMA phenogram (Figure 3.3). Two widely separated clusters were found; in one cluster *Senecio jacobaea* and *S. paludosus* joined as a single group at a cpDNA divergence of 4.82%, while the other cluster was composed of all of the other taxa. This latter cluster was more heterogeneous than the former, with all of the taxa joined at a cpDNA divergence of 1.64%. The general pattern of the tree suggests that on the basis of overall cpDNA similarity, *S. squalidus, S. cambrensis* and *S. vulgaris ssp. vulgaris sl* are very similar (a single cluster formed at 0.91% cpDNA divergence), with *S. vernalis* and *S. vulgaris* ssp. *denticulatus* joining this cluster at 1.12% and 1.64% cpDNA divergence respectively.

The 18 restriction site mutations (characters) used to construct the phylogenies have been abstracted from Table 3.7 and are shown in Table 3.13, along with the character state distributions of the various taxa. Only 4 taxa were considered in the phylogeny reconstruction, since *S. squalidus, S. cambrensis, S. vernalis* and *S. vulgaris* ssp. *vulgaris sl* (Taxon 'X', Figure 3.4a) have an identical character state distributions. In this analysis *S. paludosus* was used as a baseline species for comparison. It must be stressed, however, that although *S. paludosus* has been used for rooting the tree the polarity of the

character states may not be those indicated as an ideal outgroup was not used (see Discussion, Section 3.4.1).

Using both the Wagner and Dollo parsimony criteria a single most parsimonious tree was generated (Figure 3.4a). The Wagner tree required a total of 19 steps to account for the distribution of the site mutations, while a single reversion was postulated in the Dollo parsimony tree. Both of the trees, therefore, displayed a single homoplasy for the same site mutation (number 11, pLsc9-*Bam*HI). A parallel loss of the site, relative to *Senecio paludosus*, was hypothesised in the lineage leading to *S. vulgaris* ssp. *denticulatus*. DOLBOOT and BOOT revealed that in 100% of the cases this branching pattern was found. Hence, the tree generated may be considered a good reflection of the data.

When the autapomorphic (mutations which are unique to one taxon) site changes and the single length mutation were placed on the tree (Figure 3.4b) it was found that the lineage 'X' could be resolved into separate lines, but it was impossible using this data set, to resolve the polytomy (multiple branch point) at this point into a proper branching pattern. Thus the data generated does not allow any suggestions to be made regarding phylogenetic hypotheses of relationships of *Senecio vulgaris* ssp. *vulgaris sl* to either *S. cambrensis* or *S. vernalis*.

The data does however suggest that the present Sectional classification of *Senecio* is not satisfactory.

Chater and Walters (1976) placed all of the taxa used in this study, except *Senecio vulgaris sl* (Section Senecio), *S. vernalis* (Section Senecio) and *S. paludosus*

(Section Doria) into Section Jacobaea on the basis of life-history and capitulum characters. Alexander (1979) placed all the species, except *S. paludosus*, into Section Senecio. On the basis of the data presented here both of these Sectional classifications would appear to be unsatisfactory. In the Chater and Walters (1976) classification, *S. jacobaea* might be expected to have a similar cpDNA to the other taxa of Section *Jacobaea*, it is however much more similar to that of *S. paludosus*. A similar problem is encountered with Section Senecio (*sensu* Alexander) but in this case virtually the entire range of cpDNA divergence values (Table 3.11 and Table 3.12) are found.

Y

Figure 3.3. UPGMA phenogram of *Senecio* chloroplast DNAs probed with total *Lactuca sativa* cpDNA. Constructed from the data in Appendix E, Table E2. Numbers refer to the following taxa:-

1: *S. cambrensis* (cBr)
2: var. *vulgaris* (vMi)
3: *S. squalidus* (sSa)
4: *S. squalidus* (sBr)
5: *S. squalidus* (sSt)
7: var. *hibernicus* (hMo)
8: var. *hibernicus* (hBr)
9: var. *vulgaris* (vYo)
10: *S. squalidus* (sYo)
11: var. *vulgaris* (vBr)
12: var. *hibernicus* (hSa)
13: *S. cambrensis* (cSa)
14: *S. paludosus* (pCa)
15: *S. vernalis* (veGe)
16: ssp. *denticulatus* (dAi)
17: *S. squalidus* (sSh)
18: var. *hibernicus* (hYo)
19: *S. jacobaea* (jTe)

1
17
3
2
9
11
12
13
8
18
5
7
4
10
15
16
19
14
d x 100
6·0
4·8
3·6
2·4
1·2
0·0

Table 3.13. Character state matrix used in the reconstruction of *Senecio* phylogenies from chloroplast DNA data. Character states were determined relative to *S. paludosus* (state 0).

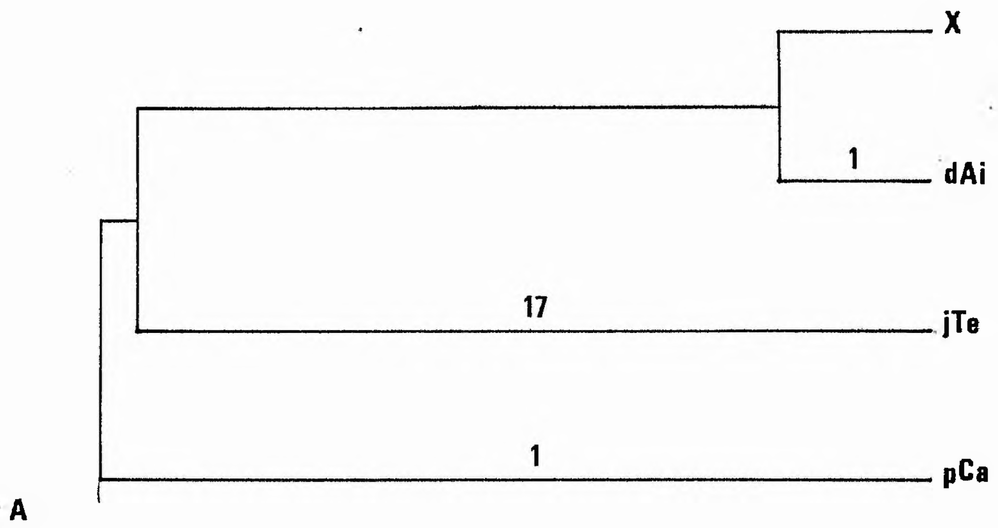
Taxon.	Character states※.
S. vulgaris spp. *vulgaris sensu lato*.	11111 11111 11111 111
S. vulgaris spp. *denticulatus*.	11111 11111 01111 111
S. squalidus.	11111 11111 11111 111
S. cambrensis.	11111 11111 11111 111
S. vernalis.	11111 11111 11111 111
S. jacobaea.	00000 00000 10000 000
S. paludosus.	00000 00000 00000 000

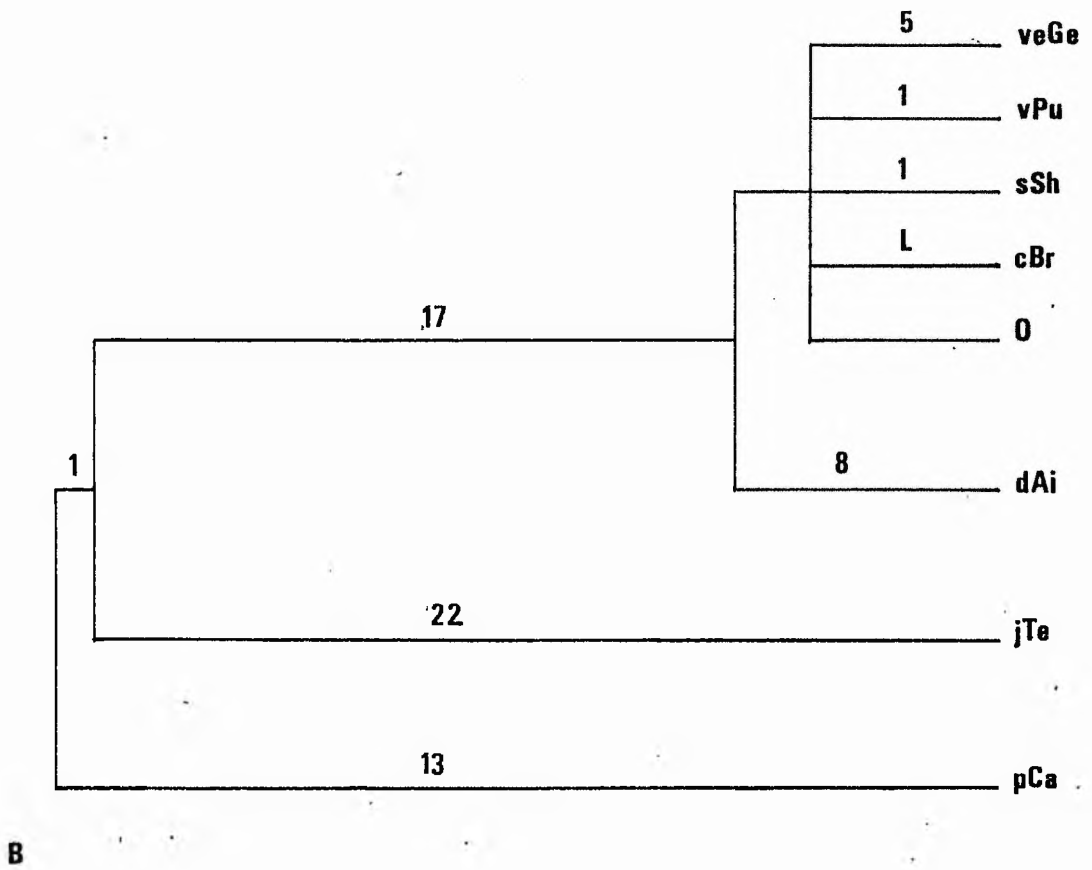
※ PEC's (characters) for particular restriction site mutations used in the phylogeny reconstruction are:

1. pLsC9-BglII.	2. pLsC2-BglII.	3. pLsC4-BglII.
4. pLsC2-BglII.	5. pLsC5ac-BglII.	6. pLsC5ac-BglII.
7. pLsC6-EcoRI.	8. pLsC13/14-EcoRI.	9. pLsC2-BamHI.
10. pLsC4-BamHI.	11. pLsC9-BamHI.	12. pLsC9-HaeIII.
13. pLsC9-HaeIII.	14. pLsC13/14-HaeIII.	15. pLsC13/14-HaeIII.
16. pLsC15-HaeIII.	17. pLsC15-HaeIII.	18. pLsC5ac-HaeIII.

Character states are read (in ascending order) from left to right in the matrix.

Figure 3.4. Phylogenetic relationships among the *Senecio* taxa studied. **A.** Most parsimonious tree using Dollo or Wagner parsimony. Rooted so as to separate *S. jacobaea* and *S. paludosus*. 'X' is a compostite taxon comprised of *S. vulgaris* spp. *vulgaris sl*, *S. squalidus*, *S. vernalis* and *S. cambrensis*. The figures above each branch are the number of site mutations which identify that branch. **B.** Consensus tree containing all the autapomorphic site and length (L) mutations. The figures above each branch are the number of site mutations which identify that branch. 'O' is a composite taxon composed of all other *Senecio* taxa studied.


X
1
dAi
17
jTe
1
pCa
A


5
veGe
1
vPu
1
sSh
L
cBr
O
17
1
8
dAi
22
jTe
13
pCa
B

Discussion.

3.4.1 Chloroplast DNA inheritance.

An important prerequisite for the biosystematic use of cpDNA is to determine its mode of inheritance (Palmer *et al* 1988), since this may have important implications for subsequent data interpretation (Chapter 4). This study has shown that *Senecio* cpDNA is inherited along the maternal line, a result that was not altogether unexpected since two-thirds of all Angiosperms show this mode of plastid transmission (Tilney-Bassett 1978, Sears 1980, Corriveau and Coleman 1988). In ten species of the family Asteraceae, that have been studied all show maternal plastid transmission. Bleyden (1988) showed that cpDNA was transmitted along the female line in inter-varietal crosses of *Senecio vulgaris* ssp. *vulgaris*, which differed in their resistance to the herbicide triazine.

3.4.2 *Senecio* chloroplast DNA organisation.

3.4.2.1 Chloroplast DNA size.

If the size of the average *Senecio* chloroplast genome is assumed to be 93kb then approximately 1.9% of the genome was sampled in this study. This is likely to be an over-

estimate of the proportion of the cpDNA sampled. A value of 110kb was obtained by Bleyden (1988) for the size of the *Senecio vulgaris* ssp. *vulgaris* cpDNA using four enzymes, while Jansen and Palmer (1988), Smith and Ma (1985), Heyraud *et al* (1987), Ma and Smith (1985) and Kolodner and Tewai (1979) give sizes of approximately 150kb for other cpDNAs of the Asteraceae. If this latter value for the size of the cpDNA is taken, then approximately 1.2% of the *Senecio* chloroplast genome has been sampled.

To determine the size of the cpDNA more accurately it would be necessary to map the entire chloroplast genome (Hasebe and Iwatsuki 1990, Palmer 1982, Salts *et al* 1984, Gounaris *et al* 1986, Gordon *et al* 1981, Terauchi *et al* 1989, Fluhr and Edelman 1981) for a number of restriction enzymes and account for every fragment. In this way it may be possible to overcome the problem of identifying comigrating fragments and promiscuous cpDNA sequences.

3.4.2.2 Chloroplast DNA mutations.

The two types of cpDNA data generated in this study, using total cpDNA and cloned cpDNA probes, illustrate the two most commonly used approaches to cpDNA analysis. The use of cloned cpDNA probes, and its associated mutation analysis, is the more accurate method to measure evolutionary distance between taxa, since a single site change is likely to be due to a single base substitution, whereas fragment differences can be interpreted in a number of different ways (Palmer *et al* 1988). For example, multiple insertions and deletions may occur along the

length of a DNA fragment without changing its size, and rearrangement events such as inversions are likely to go undetected unless they span two restriction sites. These problems become more serious as the length of the DNA fragments involved increase.

The absence of apparent inversions in *Senecio* cpDNA is not a suprise as these are rare events (Palmer 1987). However when they do occur they can be extremely informative as phylogenetic markers as they are virtually free of all homoplasy (eg. Jansen and Palmer 1988).

The number of site mutations may be an overestimate, particularly in the light of evidence that substitutions are relatively uncommon in cpDNA (Curtis and Clegg 1984, Zurawski *et al* 1984, Palmer 1985b). Hence, some of the hypothesised site mutations may be length mutations, but could not be identified with other enzymes as a result of the changes involving fragments which were either too large or too small to be resolved under the experimental conditions. This is particularly the case for those fragments that were too small to be resolved (Table 3.7).

Those PECs for which no decision was reached (Table 3.8) are probably the result of multiple length and site mutations which confound interpretation of these patterns. To resolve these difficulties the use of smaller, homologous probes would be desirable.

3.4.2.3 Distribution of chloroplast DNA mutations in *Senecio*.

The distribution of the mutations that were detected with particular cloned probes (Figure 3.2) reveal that a

number of apparent 'hotspots' for site mutations exist within the *Senecio* cpDNA. Regions that are particularly prominent are those covered by probes pLsC6-pLsC7 (IR-LSC region border), pLsC9 (LSC region) and pLsC4-pLsC5ac (IR-SSC region border). The presence of apparent mutation 'hotspots' within the cpDNA has been noted in a number of studies, for example, *Pisum* (Palmer *et al* 1985), *Linum* (Coates and Cullis 1987), *Papaver* (Milo *et al* 1988) and *Cucumis* (Perl-Treves *et al* 1985). 38% of the identified mutations in *Senecio* cpDNA occur in the region that is covered by the 22Kb inversion (pLsC6-pLsC7) in the Asteraceae (Jansen and Palmer 1987a). This distribution of 'hotspots' has not been reported before, although Baldwin *et al* (1990) in a study of the 'Silversword' alliance (*Argyroxiphium*, *Dubautia* and *Wilkesia*) noted a high concentration of restriction site mutations (approximately one mutation per kilobase) in the region of pLsC6. The distribution of mutations in the cpDNA of *Senecio* may reflect some instability in these regions of the cpDNA or be a function of the non-random distribution of restriction sites (see Section 3.4.3).

3.4.3 Chloroplast divergence estimates in *Senecio*.

In Angiosperm cpDNA a relatively narrow range of base composition is found (37-39% GC, Palmer 1985b). Thus the assumption of random site distribution is apparently not satisfied. Adams and Rothman (1982) studied human

mitochondial DNA and viral DNAs from a range of sources and found that restriction site distribution deviated significantly from expectation. In terms of base substitution there is a bias of transitions (AG, TC) over transversions (AT, GC, AC, TC) which is low and apparently constant over evolutionary time in cpDNA (Zurawski *et al* 1984, Zurawski and Clegg 1987, Wolfe *et al* 1987).

It has become apparent that the major events in Angiosperm cpDNA evolution involve large inversions and the accumulation of small insertions/deletions (1-10bp), while base substitutions are apparently rare events (Curtis and Clegg 1984, Gordon *et al* 1982, Palmer and Zamir 1982, Palmer *et al* 1988, Palmer 1987, Zurawski and Clegg 1987). Lehävasilo *et al* (1987) have argued that scoring fragments as the result of base substitutions which are the result of length mutations under-estimates the value of d, but that this is less severe than ignoring the fragments altogether.

The more or less constant size of cpDNA within genera means that the assumption of constant size is not a problem, unless a large deletion is present between taxa being analysed.

3.4.4 Intraspecific cpDNA variation in *Senecio*.

Three polymorphic site mutations and ten polymorphic unidentified mutations (Table 3.8) were identified in *Senecio vulgaris* ssp. *vulgaris sl* and *S. squalidus*. At

least one of these polymorphisms has previously been identified. Bleyden (1988), studying triazine-resistance in *S. vulgaris*, showed that the *Hin*DIII polymorphism (pLsC10/11/12-*Hin*DIII) was often associated with triazine-resistance. The polymorphism was identified as the presence of a 10.2kb fragment (11.3kb in this study) in the triazine-susceptible biotype and the presence of two fragments, 5.6kb and 4.3kb (6.0kb and 4.8kb this study), due to a single site mutation in the triazine resistant biotype (Plate 3.2, Lanes 8, 11-13).

Two polymorphisms were found to be unique for particular *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris* individuals [*Bam*HI-pLsC6 (vMi) and *Eco*RI-pLsC10/11/12 (vPu)]. When total cpDNA was used as a probe a 2.9 kb fragment was found with *Eco*RI digested Puffin Island *S. vulgaris* ssp. *vulgaris* var. *vulgaris* (vPu) cpDNA, as well as when cloned probe pLsC10/11/12 was used. This particular individual was not analysed any further for total cpDNA, because of gel artifacts, and has been excluded from all diversity estimates, so that both sets are comparable.

The other polymorphism, the presence of a 5.7kb fragment in Migvie *S. vulgaris* ssp. *vulgaris* var. *vulgaris* (vMi, *Bgl*II-pLsC6, Plate 3.1b, Lane 2) could not be identified as being due to either a length or site mutation. The only other taxon which had a fragment of similar size with this PEC was Welsh *S. cambrensis* (cBr), which has been shown to be a length mutation (Section 3.3.5). This suggests that although the fragments were identified by the same PEC they were not homologous, since

the occurrence of the 5.7kb fragment in Migvie *S. vulgaris* ssp. *vulgaris* var. *vulgaris* (vMi) was not accompanied by the loss of the 5.3kb fragment. Four hypotheses can be proposed to explain the appearance of this fragment.

1. The 5.7kb fragment may represent a partial digest. However, the absence of potential partial fragments when other probes were used and the reported lack of 5-Methylcytosine in cpDNA (Palmer 1985a) would tend to rule this out.

2. Contamination of the plant material from another source can be ruled out as the only possible source was Welsh *S. cambrensis* (cBr) and in this case a length mutation would have been been identified.

3. Promiscuous DNA may explain the 5.7kb fragment but it was very intense and the lack of putative promiscuous sequences with other probes would rule this out.

4. Two types of cpDNA may have been present in the female parent from which the 'seed' was taken. One of the cpDNAs had the 5.7kb fragment, while the other had a site mutation that resulted in two fragments (5.3kb and 0.4kb). This has three implications:- (i) the maternal parent was a chimera for cpDNA (Tilney-Bassett 1978), (ii) the 'seed' from which that plant developed must have been heteroplasmic for cpDNA and (iii) the mutation must have arisen *de novo*, in the absence of biparental cpDNA inheritance. The individuals within the population would be expected to carry one cpDNA or the other since in an annual plant

vegetative segregation ensures that heteroplasmic egg mother cells are rare (Birky 1988).

These explanations are unsatisfactory in the absence of more cpDNA data on the population from which this material came. If the latter explanation is correct, then it is possible that achenes may have been harvested from more than one individual.

The range of values for 100p within *Senecio squalidus* (0.11%-1.45%, mean 0.68%) and *S. vulgaris* ssp. *vulgaris sl* (0.11%-0.67%, mean 0.36%) are exceptionally high for within species estimates of cpDNA diversity. Other species for which data is available have much lower values (Table 3.14a), indeed these estimates are as high as many cpDNA diversity estimates of congeneric species (Table 3.14b). The modelling studies of Birky *et al* (1989) predict that cpDNA diversity will be greater in taxa where there is extensive population subdivision and a small population size.

3.4.5 Chloroplast DNA in British *Senecio squalidus* and *S. vulgaris* ssp. *vulgaris*.

The chloroplast genomes of *Senecio vulgaris* ssp. *vulgaris* and British *S. squalidus* are identical, based on the PECs used in this study. This result is not unexpected since cpDNA has a conserved sequence and structural evolution. A number of studies have shown that species can have very similar cpDNAs. For example, Hosake (1986) showed that ten wild species of *Solanum* had the W type cytoplasm and Crawford *et al* (1990) has reported that some of the wild species of *Coreopsis* Section *Coreopsis* have identical cytoplasms.

The suprising feature is not that these two taxa have identical cpDNAs but that the putative progenitor of *Senecio vulgaris*, based on cytology and morphology (Kadereit 1984b), ie. *S. vernalis*, has a quite different cpDNA. Two explanations for this observation are suggested; either (i) *S. vernalis* is not the progenitor of *S. vulgaris* or (ii) British *S. squalidus* has gained the *S. vulgaris* ssp. *vulgaris* cytoplasm.

The cpDNA evidence concerning *Senecio vernalis* as the progenitor of *S. vulgaris* is not conclusive since no shared site mutations were found which united *S. vulgaris sl* and *S. vernalis* (Section 3.3.9). Kadereit (1984b) suggests that the origin of *S. vulgaris* ssp. *vulgaris* from *S. vernalis* was via *S. vulgaris* ssp. *denticulatus*, but this interpretation is not supported by the cpDNA evidence, since the cpDNA of ssp. *denticulatus* differs from that of

both *S. vernalis* and *S. vulgaris* ssp. *vulgaris* (Section 3.3.6).

A cytoplasmic exchange may have occurred between *S. vulgaris* ssp. *vulgaris* and *S. squalidus* in either direction. But to explain the similar cpDNAs of these taxa over their range of distribution in the British Isles a single exchange, at the point of introduction of *S. squalidus* would have to be proposed (ie, Oxford Botanic Gardens). It could be envisaged that an introgression event occurred between *S. squalidus* (male parent) and *S. vulgaris* ssp. *vulgaris* (female parent) with the hybrid acting as the female parent in the subsequent backcross to *S. squalidus*. If this were the case the rapid spread of *S. squalidus* in the mid 1800s (Crisp 1972) may have been the result of the introgressant having a competitive advantage. Two further implications of this hypothesis are that:- (i) there has only been one introduction of *S. squalidus* into the British Isles and (ii) *S. vulgaris* ssp. *vulgaris* and *S. squalidus* have accumulated similar cpDNA mutations (Table 3.11 and Table 3.12) over a very short period of time (c. 150 years), ie. *S. squalidus* cpDNA apparently mutates at a much faster rate than *S. vulgaris* ssp. *vulgaris* cpDNA.

These two hypotheses cannot be tested in the absence of knowledge about the situation regarding *S. squalidus* in Continental Europe. Of particular interest would be plants from Southern Europe and the Mediterranean.

3.4.6 Chloroplast DNA and the status of *Senecio vulgaris* ssp. *denticulatus*.

Senecio vulgaris ssp. *denticulatus* is distributed in Europe as an Atlantic-Mediterranean-Montane element (Kadereit 1984a) and is very variable, particularly in characters such as indumentum, leaf shape and generation time (Crisp 1972, Kadereit 1984a). This variation is reflected in its taxonomy. First described by Mueller in 1760 as *S. denticulatus*, over the next 200 years *S. vulgaris* ssp. *denticulatus* was given subspecific and varietal status in a number of species (*S. lividus*, *S. sylvaticus* and *S. vulgaris*) (Allen 1967). Indeed Crisp (1972) states:

> 'Probably, ... , "var. *denticulatus*" refers to a number of maritime races which have some characters in common due to the effects of natural selection.'

In the British Isles, *Senecio vulgaris sl* appears to have two distinct cpDNAs. One is found in *S. vulgaris* ssp. *vulgaris* and Jersey *S. vulgaris* ssp. *denticulatus*, and the other is found in Ainsdale *S. vulgaris* ssp. *denticulatus*. Four hypotheses are proposed to explain this result.

1. Introgression has occurred at the Ainsdale site with a second *Senecio* species that has not been surveyed. It could be envisaged that an initial cross between ssp. *denticulatus* (male parent) and an unidentified species (female parent) may have resulted in a partially fertile hybrid. This then acted as the female parent in a series of backcrosses to ssp. *denticulatus*. The result was a plant that was similar

to ssp. *denticulatus* (Jersey) in both morphology and rDNA (Chapter 2) but had the cytoplasm of the unidentified species. Potential local parents in such a cross are limited to those *Senecio* species which are closely related to *S. vulgaris* and occur in this country (*S. squalidus*, *S. viscosus*, *S. vernalis* and *S. sylvaticus*, Kadereit 1984). Both *S. viscosus* and *S. sylvaticus* can be eliminated as possible parents since they are difficult, if not impossible to artificially cross with *S. vulgaris* (Kadereit 1984b, Gibbs 1971) and natural hybrids have never been confirmed (Benoit *et al* 1975). The cpDNA patterns of *S. squalidus* and *S. vernalis* are both different to Ainsdale ssp. *denticulatus*.

The alternative is that the cross occurred in Europe prior to colonisation of the British Isles by ssp. *denticulatus*. If this is the case then the number of potential parents are increased (*Senecio leucanthemifolius*, *S. lividus*, *S. fructicularis*, *S. chrysanthemifolius*, *S. aethnensis*, *S. gallicus* and *S. glaucus*). Not all of these species have been used in experimental crosses, but of those which have, Kadereit (1984b) found that the cross *S. leucanthemifolius* x *S. vulgaris* ssp. *denticulatus* (Jersey) was successful in 16 out of 20 crosses, he did not however measure pollen fertility of the hybrids.

2. The second possibility is that Ainsdale ssp. *denticulatus* is the progenitor of ssp. *vulgaris sl*

(Kadereit 1984b) and Jersey ssp. *denticulatus* is an introgressed form. This hypothesis would require that an initial cross occurred between ssp. *denticulatus* (male parent) and ssp. *vulgaris* (female parent). The resulting hybrid would then act as the female parent in a series of backcrosses to ssp. *denticulatus*. In this case the result would be a plant with a ssp. *vulgaris* cytoplasm and a morphology and rDNA pattern very similar to ssp. *denticulatus*. This hypothesis still does not, however, address the important problem of the occurrence of two different cpDNAs in *S. vulgaris sl*.

3. The Ainsdale ssp. *denticulatus* cpDNA may represent an ancient wild type *Senecio vulgaris* cpDNA, while the Jersey ssp. *denticulatus* and ssp. *vulgaris* represent types that were inadvertently selected as weeds in the early history of agriculture. The wild type is now only able to survive in a few relict pockets. This could explain the different cpDNAs in the two subspecies, but would require an additional evolutionary event to explain the similarity of ssp. *vulgaris* and *S. squalidus* cpDNAs (Section 3.4.5).

4. Ainsdale ssp. *denticulatus* may be a new taxon that has very close morphological similarity to ssp. *vulgaris* but has a very divergent cpDNA (though the rDNA of both Jersey ssp. *denticulatus* and Ainsdale ssp. *denticulatus* are the same, Chapter 2).

None of these hypotheses are entirely satisfactory, however, in Chapter 7 a hypothesis regarding the origin of *Senecio vulgaris* ssp. *denticulatus* is proposed which may explain both the observed cpDNA and rDNA variation. The variation in *S. vulgaris* ssp. *denticulatus* on the Continent is of vital importance to the interpretation of this data. It may be that the montane and coastal races of ssp. *denticulatus* (Kadereit 1984a) have different cpDNAs and that in the British Isles these taxa have taken up similar habitats.

Lavin *et al* (1990b) have recently published some interesting data which appears to parallel the situation found between the Ainsdale ssp. *denticulatus* and ssp. *vulgaris*. Two legumes (*Gliricidia sepium* and *Astragalus molybdenus*) were surveyed for intraspecific cpDNA variation. In both cases extensive variation was located; in *G. sepium* the taxa at one site differed from the other accessions by at least nine mutations, while in *A. molybdenus* much greater variation was found between individuals from different States than from within a State. In both cases the cpDNA variation occurred in the absence of nuclear and morphological variation.

3.4.7 Chloroplast DNA and *Senecio cambrensis* evolution.

3.4.7.1 *Senecio cambrensis* in Wales.

The length mutation present in Welsh *Senecio cambrensis* (cBr) could have arisen in two ways; either (i)

it was present in the parental material or (ii) it occurred during or shortly after the initial hybridisation event.

Crisp (1972) left the question of the date of origin of *Senecio cambrensis* open. However, Abbott *et al* (1983) have suggested that *S. cambrensis* originated in Wales between 1910 and 1925. If the length mutation occurred during or shortly after the initial hybridisation event then it must have become fixed in the population within 80 years. Fixation of cpDNA mutations is likely to be a very rare event, as a result of the large number of cpDNA molecules per cell and the low mutation rate (Birky *et al* 1983, Klekowski 1988). Whether the mutation occurred in the initial triploid hybrid between *S. squalidus* and *S. vulgaris* ssp. *vulgaris sl* or following chromosome doubling cannot be identified. It could perhaps, be suggested that the presence of a partially foreign nucleus in a native cytoplasm may create some instability in the native cpDNA. Frankel *et al* (1979) have, however, reported changes in the cpDNA during two interspecific cytoplasm introgressions in *Nicotiana*. However, Galau and Wilkins (1990) have conducted a study into the effects on cpDNA of transferring plastids from *Gossypium harknessii* into *G. hirsutum* and *G. barbadense* nuclear backgrounds. After 13 successive backcross generations there were no changes in the 136 restriction fragments examined.

Tragopogon contains two examples of recent allopolyploid speciation, *T. minus* and *T. miscellus*. These species probably originated about 50 years ago in North America. In each case the cytoplasms of the allopolyploid

species were identical to one of the parental species (Soltis and Soltis 1989). Even over much longer time scales the cpDNA of allopolyploids appears to be very stable (*Aegilops triuncialis*, Murai and Tsunewaki 1984). In the case of the amphidiploid *Brassica napus*, the cpDNA was found to be different to the putative maternal parent, *B. oleracea* (Palmer *et al* 1983).

Due to the potential rarity of the initial mutation event and its fixation in a population in a short period of time, the most likely explanation is that one of the parental taxa carries a length mutation which has not been located due to the limited size of the survey. To address this fascinating problem it will be necessary to undertake more extensive sampling of *Senecio cambrensis* and the progenitor taxa in Wales.

3.4.7.2 The origin of *Senecio cambrensis*.

Whatever the cause of the length mutation in the Welsh *Senecio cambrensis* (cBr) it provides supporting evidence for the dual origin of *S. cambrensis* in Wales and Scotland (Ashton 1990), since in *S. cambrensis* from Scotland (cSa) this mutation is absent. If the Scottish *S. cambrensis* (cSa) had been the result of long distance fruit disperal from Wales (Abbott *et al* 1983) then the cpDNA ought to be identical to that of Welsh *S. cambrensis* (cBr). Of interest would be a survey of *S. cambrensis* from Mochdre, Wales since Ashton (1990) has suggested that this may represent a third site of origin of *S. cambrensis*.

The data presented in this Chapter has proved to be unsatisfactory for drawing conclusions regarding phylogeny within the genus as a result of the large number of autapomorphic characters found and the unexpected lack of variation between some taxa (*Senecio vulgaris* ssp. *vulgaris* and *S. squalidus*) and the suprising variation between others (*S. vulgaris* ssp. *vulgaris* and Ainsdale *S. vulgaris* ssp. *denticulatus*). In order to resolve some of the conflicts in this group, particularly those regarding the origin of *S. vulgaris sl* it is suggested that the diploid and tetraploid relatives of *S. vulgaris sl* (Kadereit 1984b) are studied in more detail using a wider range of restriction enzymes and a complete set of cpDNA probes. Similarly because of the high level of variation within *S. vulgaris* ssp. *vulgaris sl* extensive sampling of this and other taxa across Europe would be required.

The broad relationships based on cpDNA support the major morphological and cytological groupings, placing *Senecio vulgaris sl*, *S. squalidus*, *S. vernalis* and *S. cambrensis* as a single group (Figure 3.3 and Figure 3.4).

Chapter 4.

Chloroplast DNA and biosystematics: Some effects of intraspecific diversity and plastid transmission.

" 'You do not know', ... , 'what men have done to win it, and how they have found, too late, that it glitters brightest at a distance, and turns quite dim and dull when handled.' "

Barnaby Rudge.
C. Dickens.

Chloroplast DNA (cpDNA) is increasingly being used by plant taxonomists wishing to answer biosystematic and phylogenetic questions. For example, intrafamilial relationships (Jansen and Palmer 1988, Lavin *et al* 1990a) the evolutionary position of genera (Sytsma and Gottlieb 1986a, French and Kessel 1989), the origin and evolution of species, eg. *Tragopogon miscellus* (Soltis and Soltis 1989) and the degree and partioning of cpDNA variation within species (Neale *et al* 1986, Soltis *et al* 1989a, 1989b, and Soltis and Soltis 1989). The popularity of cpDNA as a marker molecule is based on generalisations about its structure and evolution (Chapter 3, Section 3.1.2.1).

In this review I shall look at two of these assumptions in detail:-

(i) Intraspecific cpDNA variation is low.

(ii) Chloroplast DNA inheritance is predominantly maternal.

Some effects of deviation from these assumptions on the subsequent use of the data in phylogenetic studies will be examined.

4.1 Biosystematic impact of intraspecific chloroplast DNA variation.

One of the assumptions of cpDNA analysis is that there is little or no intraspecific variability in the chloroplast genome (Chapter 3, Section 3.1.2.1). This has led many workers to use single accessions of a taxon from which to draw phylogenetic and more general biosystematic conclusions (eg. Fritzsche *et al* 1981 and Hantula *et al* 1989). However, data that has started to accumulate over the past five years on cpDNA in many different taxa, both wild and cultivated, shows that negligible intraspecific cpDNA variation may not be the rule. In *Brassica napus* up to seven different cytoplasms were identified in 97 individuals examined (Kemble 1987) and in *Lisianthius skinneri* three different cpDNAs were found in three individuals examined (Sytsma and Schaal 1985). A survey of the literature has revealed 60 taxa for which there is evidence of intraspecific cpDNA variation (Table 4.1).

Many of these studies have analysed relatively few individuals (<10), nevertheless, high levels of intraspecific cpDNA variation were found. There have been relatively few studies that have used larger numbers of individuals (>30) to analyse intraspecific cpDNA variation; examples are, *Brassica napus* (Kemble 1987), *Dactylis glomerata sl* (Lumaret *et al* 1989), *Glycine max* (Close *et al* 1989), *Hordeum vulgare sl* (Neale *et al* 1986), *Lupinus texensis* (Banks and Birky 1985), *Pisum sativum* (Teeri *et al* 1985), *Solanum chacoense*, *S. phureja*, *S. sparsipilum*, *S. tuberosum sl* (Hosaka and Hanneman 1988b) and *Tolmiea*

menziesii (Soltis *et al* 1989b). The assumption of low intraspecific cpDNA variation can be traced back to misinterpretations of the work by Banks and Birky (1985). Although three different cpDNA genotypes were found this paper has been used as evidence for the existence of negligible intraspecific cpDNA variation.

Hosaka and Hanneman (1988b) working on the genus *Solanum* and Timothy *et al* (1979) working on *Zea mays sl* have both suggested that intraspecific cpDNA variation may be a common situation. The intraspecific cpDNA diversity found in crop plants may be the result of selection by man for particular cpDNA-encoded characters or the introgression of cpDNA from related species during the early domestication of the taxon (Hosaka and Hanneman 1988b, Dally and Second 1990). An alternative explaination for the occurrence of intraspecific variation may be provided by a model similar to that of Niegel and Avise (1986) for the random survivorship of mitochondrial DNA lineages from a polymorphic progenitor. Mutation fixation is, of course, another possible cause of intraspecific cpDNA variation (Antolin and Strobeck 1986). Although less extensive studies have been conducted with wild taxa, the level of intraspecific variation is apparently still high (eg. *Glycine* Section Glycine, Doyle *et al* 1990b).

Evidence has recently been published to show that <u>within population</u> cpDNA variation may occur. Plants of a single population of *Beta maritima* have been shown to differ in both their chloroplast and mitochondrial genomes (Saumitou-Laprade 1989 in Ecke and Michaelis 1990).

Similarily, intra-populational cpDNA variation has been found in the Hawaiian endemic, *Wilkesia gymnoxiphium* (Baldwin *et al* 1990). One method of generating intra-populational cpDNA variation is via introgression (eg. *Dactylis glomerata*, Lumaret *et al* 1989). In the case of *Beta maritima* refered to above, the possibility of introgression has not, apparently, been ruled out.

The modelling studies of Birky *et al* (1989) predict that cpDNA diversity will be greater in taxa where there is extensive population subdivision and a small population size.

The presence of intraspecific cpDNA variation has two important implications:- (i) When a small sample size is used the probability of not detecting cpDNA variation is high (Baum and Bailey 1989) and (ii) The presence of polymorphism within a taxon is likely to have severe effects on reconstruction of phylogenies since cladistic methods used for analysis are very sensitive to character state changes (Fitch 1984).

Problems of intraspecific cpDNA polymorphism are unlikely to have major effects on phylogenies based on large structural changes in the chloroplast genome, since these are relatively rare [eg. the loss of the invert repeat in Leguminosae subfamily Papilionoideae (Lavin *et al* 1990a) and the distribution of a 22kb inversion in the Asteraceae (Jansen and Palmer 1988)]. However, at the lower levels of the taxonomic hierarchy cpDNA polymorphism is likely to be a problem in phylogeny reconstruction, since

length and site mutations are much more frequent than inversions (Palmer *et al* 1988).

Although, intraspecific cpDNA polymorphism may be a problem in the reconstruction of cpDNA-based phylogenies, it is potentially very useful for analysing other types of evolutionary events, eg. auto-/allo-polyploid speciation (Soltis *et al* 1989a, 1989b, Soltis and Soltis 1989) and introgressive speciation (Lumaret *et al* 1989).

An important point to remember about any phylogeny reconstructed from cpDNA is that it reflects the phylogeny of the chloroplast genome and is essentially a gene phylogeny. It may not be a good representation of the species phylogeny (Nei 1987).

Table 4.1 Taxa for which there is evidence of intraspecific chloroplast DNA variation.

Species	No. Accessions°.	Reference
Aegilops aucheri	3(1-2)	Nakamichi and Tsunewaki (1986)
Aegilops bicornis	4(2-2)	Nakamichi and Tsunewaki (1986)
Aegilops speltiodes	11(1-1-2-7)	Nakamichi and Tsunewaki (1986)
Aegilops speltoides	6(1-5)	Bowman et al (1983)
Aegilops squarrosa	16(1-4-11)	Terachi et al (1985)
Aegilops triuncialis	20(1-6-13)	Ogihara and Tsunewaki (1982)
Aegilops triuncialis	3(1-2)	Murai and Tsunewaki (1984)
Brassica campestris	4(1-3)	Palmer et al (1983)
Brassica campestris	8(1-1-6)	Kemble (1987)
Brassica juncea	7(1-1-5)	Kemble (1987)
Beta macrocarpa	2(1-1)	Kishima et al (1987)
Beta maritima	6(4-2)	Kishima et al (1987)
Brassica napus	3(1-2)	Palmer et al (1983)
Brassica napus	97(1-1-4-7-9-30-45)	Kemble (1987)
Brassica nigra	3(1-1-1)	Palmer et al (1983)
Clarkia biloba	2(1-1)	Sytsma and Gottlieb (1986b)
Cucumis melo	6(1-5)	Perl-Treves and Galun (1985)
Dactylis glomerata sensu lato	38(16-22)	Lumaret et al (1989)
Glycine gracilis	5(1-1-3)	Shoemaker et al (1986)
Glycine latifolia	17(1-1-1-4-10)	Doyle et al (1990b)
Glycine max	26(3-7-16)	Shoemaker et al (1986)
Glycine max	46(1-10-35)	Close et al (1989)
Glycine max forma gracilis	2(1-1)	Close et al (1989)
Glycine microphylla	26(1-1-1-1-2-4-5-11)	Doyle et al (1990b)
Glycine soja	8(1-3-4)	Close et al (1989)
Glycine tabacina	11(1-1-1-1-1-6)	Doyle et al (1990b)
Hedysarum spinosissimum sensu lato	8(7-1)	Baatout et al (1985)
Heuchera grossulariifolia	15(1-1-3-3-7)[2]	Wolf et al (1990)
Heuchera micranthera	28(1-1-1-1-1-2-2-2-3-5-9)	Soltis et al (1989a)
Hordeum glaucum	5(1-4)	Baum and Bailey (1989)
Hordeum leporinum	3(1-2)	Baum and Bailey (1989)
Hordeum murinum	3(1-2)	Baum and Bailey (1989)
Hordeum spontaneum	11(1-1-1-2-3-3)	Holwerda et al (1986)
Hordeum spontaneum	11(3-3-4-1)	Clegg et al (1984)
Hordeum vulgare	11(1-2-2-6)	Holwerda et al (1986)
Hordeum vulgare	9(7-2)	Clegg et al (1984)
Hordeum vulgare ssp. distichum	5(1-4)	Neale et al (1986)
Hordeum vulgare ssp. spontaneum	245(66-80-99)	Neale et al (1986)
Hordeum vulgare ssp. vulgare	46(1-45)	Neale et al (1986)
Lisianthius skinneri	3(1-1-1)	Sytsma and Schaal (1985)
Lupinus texensis	100(88-11-1)	Banks and Birky (1985)
Lycopersicon peruvianum	6(3-2-1)	Palmer and Zamir (1982)
Nicotina debneyi	9(2-7)	Scowcroft (1979)
Oryza latifolia	2(1-1)	Ichikawa et al (1986)
Oryza sativa	22(7-15)	Ishii et al (1986)
Pelargonium x zonale hort.	16(1-2-13)	Metzlaff et al (1981)
Pisum elatius	2(1-1)	Palmer et al (1985)
Pisum humile	12(3-4-5)	Palmer et al (1985)

Table 4.1 Cont.

Species	No. Accessions[a].	Reference
Pisum sativum	13(1-2-3-3-4)	Palmer et al (1985)
Pisum sativum	48(1-3-6-14-24)	Teeri et al (1985)
Solanum chacoense	42(1-2-11-28)	Hosaka and Hanneman (1988a)
Solanum goniocalyx	4(1-3)	Hosaka and Hanneman (1988a)
Solanum phureja	39(6-33)	Hosaka and Hanneman (1988a)
Solanum sparsipilum	37(2-2-33)	Hosaka and Hanneman (1988a)
Solanum stenotomum	15(1-1-13)	Hosaka and Hanneman (1988a)
Solanum tuberosum ssp andigena	113(3-5-5-14-16-70)	Hosaka and Hanneman (1988b)
Solanum tuberosum ssp tuberosum	33(30-2-1)	Hosaka and Hanneman (1988b)
Tolmiea menziesii	37(1-1-3-4-12-16)	Soltis et al (1989b)
Zea mays sensu lato	13(2-4-7)	Doebley et al (1987)
Zea mays sensu lato	7(1-2-4)	Timothy et al (1979)

[a] No. Accessions indicates the total number of individuals/populations examined and in parentheses the number of individuals/populations that fall into each restriction phenotype. The use of cpDNA variant frequencies is not ideal since it is heavily dependent on sample size (Nei 1987), but it does give some indication of the amount of cpDNA variation which occurs.

[2] Unable to locate population 109 given in Table 6 (of Wolf et al 1990) with length mutation L3.

Baldwin et al (1990) reported intraspecific cpDNA variation in Argyroxiphium sandwicense ssp. macrocephalum, Dubautia arborea, D. laxa ssp. hirsuta, D. knudsenii sl, D. ciliolata ssp. glutinosa, D. linearis ssp. linearis, D. plantaginea sl and Wilkesia gymnoxiphium.

Lavin et al (1990b) have reported high levels of intraspecific variation in Gliricidia sepium and Astragalus molybdenus.

Mayer et al (1990) have reported intraspecific variation in Tellima grandiflora.

4.2 Plastid transmission and phylogenetic inference.

The discovery of maternal inheritance of chlorophyll deficiency in *Mirabilis jalapa* (Correns 1909) and biparental, non-Mendelian inheritance of a pigmentation trait in *Pelargonium x zonale hort.* (Baur 1909) at the beginning of this century, stimulated considerable research in the field of plastid transmission (Tilney-Bassett 1978). Three types of plastid transmission have been recognised:- (i) maternal transmission, in which plastids are inherited solely through the female parent, (ii) paternal transmission, in which plastids are inherited solely through the male parent and (iii) biparental transmission, in which plastids are inherited through both the male and female parents. Various aspects of plastid transmission have been reviewed by Birky (1978, 1983, 1988), Sears (1980) and Tilney-Bassett (1978). To study plastid transmission four methods have been used:- (i) Genetic analysis of plastid mutants, (ii) Ultrastructural analysis, (iii) Epifluoresence microscopy and (iv) Chloroplast DNA restriction analysis.

i) Genetic analysis of plastid mutants.The earliest studies used phenotypically recognisable plastid characters, eg. green vs white plastids. This particular methodology has been reviewed by Tilney-Bassett (1978). The approach does however suffer from a number of drawbacks:- (i) Very few plastid characters have been identified and green/white plastid characters may be modified by the nucleus (Tilney-Bassett 1978). (ii) Suitable variation may

not be present in the plants of interest. (iii) Some of the characters used (eg. green vs white plastids) may give a selective disadvantage (eg. high lethality) to an embryo carrying them (Tilney-Bassett 1978).

ii) Ultrastructural analysis. The advent of the electron microscope has allowed ultrastructural studies to be used to try and identify taxa that carry plastids in their male gametophyte. However, Sears (1980) has critised this approach since;- (i) it is very difficult to unequivocally distinguish proplastids from mitochondria and (ii) male gametophytes that carry few proplastids may not be identified unless large numbers of pollen grains are analysed or serial sectioning undertaken.

iii) Epifluoresence microscopy. Staining pollen with the fluorochrome dye DAPI (4′,6-diamidino-2-phenylindole) has been used recently (Corriveau and Coleman 1988) as a rapid way to analyse many plant species for the presence of proplastids in pollen. The confirmation that the bodies stained by DAPI are proplastids has been obtained by comparing DNA extracted from the pollen of a species known to have biparentally inherited plastids (*Medicago sativa*) and a species with maternally inherited plastids (*Antirrhinum majus*). It has been shown that DAPI-stained bodies were present only in the pollen of *Medicago sativa* (Corriveau *et al* 1990). It should, however, be borne in mind that the presence of DAPI-stained bodies does not necessarily indicate that there is paternal plastid input to the embryo, but rather that the potential for this mode of transmission exists. Sears (1980) has suggested that

maternal plastid transmission may occur by:- (i) Exclusion of plastids from the male gamete during spermatogenesis, (ii) Loss of plastids from motile sperm, (iii) Exclusion during fertilisation or (iv) Degradation of plastids or their DNA within the zygote. If one of these mechanisms exist within a given taxon, then the presence of plastids in the pollen is irrelevant.

iv) Chloroplast DNA restriction analysis. This approach has recently been used as a method for the determination of plastid inheritance patterns. The method relies on the ability to identify cpDNA genotypes and perform inter- or intra-specific crosses. The F1 progeny are then used to analyse cpDNA segregation (Hatfield *et al* 1985).

The difficulty of defining the plastid transmission pattern for a taxon is enhanced by the need to examine large numbers of genotypes and follow the fate of any plastids contributed by the paternal parent via crosses. Some of the methods described above do not allow large numbers of genotypes to be feasibly examined, while others that require crosses between taxa may be impractical if these crosses are difficult or impossible. A method is required that will allow the rapid assessment of the plastid type present in an embryo.

These four methods have generated data on many species of Angiosperms (Table 4.2). This Table is by no means comprehensive, but it does serve to illustrate the range of plastid transmission patterns which have been obtained from

different taxa. The majority of these studies have not used large numbers of genotypes per taxon.

Approximately 86 families have been studied to date, 28% of which have at least one taxon that shows the potential for biparental plastid transmission (7% show biparental transmission only, eg. Ericaceae and Geraniaceae). When the 232 genera that have been studied are considered, 21% show the potential for biparental plastid transmission (12% show biparental inheritance only, eg. *Campanula* and *Melilotus*). Within a genus individual species may show different modes of plastid transmission (eg. *Castilleia* and *Lobelia*). Of the 392 species that have been studied 31% show the potential for biparental plastid transmission (27% show biparental inheritance only, eg. *Plumbago auriculata*). Some species show evidence of intraspecific variation in plastid transmission (eg. *Pisum sativum* and *Nepeta cataria*). This data illustrates that biparental transmission, or at least the potential for biparental plastid transmission, is not a rare phenomenon but is both common and widely scattered in the Angiosperms.

In Angiosperms only three published reports show evidence of paternal plastid transmission; *Daucus muricatus* x *D. carota* ssp. *sativus* (Boblenz *et al* 1990), *Medicago sativa* (Schumann and Hancock 1989) and *Nicotiana plumbaginifolia* (Medygesy *et al* 1986). This is in contrast to Conifers where evidence of paternal plastid transmission is much more common (Neale and Sederoff 1989, Neale *et al* 1989, Stine *et al* 1989, Szmidt *et al* 1987, Szmidt *et al* 1988 and Wagner *et al* 1989). Reports of

occasional (or 'leaky') paternal plastid transmission have been published by Schmitz and Kowallik (1986) in *Epilobium*, Dally and Second (1990) in *Oryza* and Galau and Wilkins (1990) in *Gossypium*.

Recent results suggest that it is probably best to consider plastid transmission as a continuum, rather than an 'all-or-nothing' process (Smith 1989, Corriveau and Coleman 1990, Tilney-Bassett and Almouslem 1989). Between the two extremes, of strict maternal plastid transmission and strict paternal transmission, are an array of conditions described as biparental plastid transmission. Studies of *Oenothera* (Chiu *et al* 1988, Corriveau and Coleman 1990) and *Medicago sativa* (Smith 1989) suggest that paternal and maternal genotypes may influence the mode of plastid transmission.

Three consequences of biparental plastid transmission which must be considered, all of which affect the use of cpDNA as a marker molecule, are:- (i) Plastid dynamics within individuals of a population. (ii) Plastid dynamics within and between populations and (iii) the effects of recombination between cpDNA molecules.

4.2.1 Plastid dynamics within individuals.

The number of cpDNA molecules per plastid varies widely with both species and plant maturity (Boffey 1985, Scott and Possingham 1983). These estimates are based mainly on leaves. However, in terms of plastids contributed to later generations, the important figure is the number of plastids that are present in the meristematic initials.

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Tilney-Bassett (1978) suggests that ten plastids per meristematic initial is a reasonable estimate. In the case of biparental inheritance the relative contributions of the maternal and paternal parents to the plastid complement of the zygote are important, since in some cases of biparental inheritance a strong maternal bias may exist (eg. *Pelargonium x zonale hort.*, Tilney-Bassett and Almouslem 1989).

It has been suggested by some workers that the rate of vegatative 'sorting-out' is rapid such that at maturity individual cells are unlikely to be heteroplasmic (Birky 1988, Klekowski 1988), but evidence from *Gossypium hirsutum* suggests that sorting out, at least in some cases, may be a much slower process (Lax *et al* 1987). Although individual cells may not be heteroplasmic, the plant as a whole may possess tissues that have different cpDNA genotypes (ie. a cpDNA chimera). The occurrence of many different variegated cultivars of a range of species is ample evidence of the existence of chloroplast chimeras (Tilney-Bassett 1978). The extent of tissue variegation is influenced by a number of factors including the number of plastids in the meristematic initial (Klekowski 1988).

The appearance of variegated plants is due to differences in plastid colour between tissue sectors. Direct analysis of cpDNA with restriction enzymes may indicate that plants which are not chimeric for plastid colour, may be chimeric for particular restriction fragment patterns. The occurrence of cpDNA chimeras, as a result of biparental inheritance has been documented in relatively

few cases; *Medicago sativa* (Johnson and Palmer 1989, Lee *et al* 1988), *Pelargonium x zonale hort.* (Metzlaff *et al* 1981), *Oryza sativa* (Moon *et al* 1987) and *Pinus banksiana-Pinus contorta* (Govindaraju *et al* 1988). These studies show that although an individual may appear homogeneous in terms of plastid colour, at the DNA level intra-individual variation may occur and in the case of *Pinus banksiana-Pinus contorta*, cpDNA variation within single branches was reported (Govindaraju *et al* 1988). The occurrence of intra-individual cpDNA variation in long-lived perennials and clonally propagated species may be much greater than in a short-lived, sexually reproducing annuals. The other possible source of intra-individual cpDNA variation is somatic mutation (Antolin and Strobeck 1985), but the low probability of fixing a cpDNA mutation that has arisen *de novo* would tend to make this a relatively rare event (Klekowski 1988).

4.2.2 Plastid dynamics within and between populations.

Biparental inheritance of cpDNA affects the dynamics of organelle genes within populations. This problem has not been studied extensively but work by Birky *et al* (1983, 1989), Takahata (1983) and Takahata and Maruyama (1981) have provided useful insights into the population genetics of organelle genomes (mainly animal mitochondria). The study of cpDNA dynamics is made problematic since within any one population of plants there are four levels of cpDNA diversity (between cpDNAs within organelles, between organelles within cells, between cells within individuals

and between individuals within populations). The input of paternal cpDNA via biparental plastid transmission, may influence cpDNA diversity and, therefore, the cpDNA-based genetic structure of the population.

4.2.3 Recombination between chloroplast DNA molecules.

Recombination between cpDNAs and the generation of novel cpDNA is thought to be a rare event in Angiosperms (Palmer *et al* 1988), but recent data from somatic hybridisation studies, suggest that, at least in *Nicotiana*, extensive recombination may occur (Medgyesy *et al* 1985, Thanh and Medgyesy 1989, Fejes *et al* 1990). If recombination between cpDNAs does prove to be more common than originally thought and not just an artifact of somatic hybridisation, then it could have profound effects on the use and interpretation of cpDNA in biosystematic analysis, especially in association with biparental inheritance.

The implicit assumption of only one mode of plastid transmission within a family, genus, or even species could have significant effects on a reconstructed phylogeny because of the sensitivity of cladistic methods to character state change (Fitch 1984). A situation can be envisaged where a shift in plastid transmission pattern has occurred in evolutionary time that has effected the cpDNA diversity. Thus when comparisons are being made between taxa with different modes of plastid transmission, the distribution and evolutionary history of the mode of plastid transmission itself may be critical to a correct phylogenetic reconstruction. The use of cpDNA as a

uniparentally inherited molecular marker makes the implict assumption that the influence of hybridisation can largely be ignored since the chloroplast genome of only one parent is being followed. However, if there is or has been a strong biparental plastid transmission pattern then many of the problems of constructing cladistic phylogenies from morphological data (eg. Funk 1985) will be encountered.

The potential importance of apparently minor events in evolutionary history has been clearly stated by Birky (1978):

> '..., if there are very low levels of paternal gene transmission and recombination, these must be measured for they may become very important over evolutionary time scales even though they are negligible when we look at the results of a single mating.'

Table 4.2 Modes of plastid transmission in Angiosperms. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Acanthaceae	Beleperone guttata			(-)		C
	Eranthemum nervosum			(-)		C
Aceraceae	Acer rubrum			(-)		C
Agavaceae	Yucca filamentosa			(-)		C
Aizoaceae	Fenestraria rhodalophylla			(+)		C
	Mesembryanthemum cordifolium	(M)				T
Aloeaceae	Aloe brevifolia			(-)		C
	Aloe jucunda		(-)			Hs
	Aloe secundiflora		(-)			Hs
	Gasteria verrucosa		(-)			Hs
	Haworthia sp.		(+)			Hs
Apiaceae	Daucus carota			(-)		C
	Daucus muricatus x D. carota ssp. sativus				(P)	Bo
	Foeniculum vulgare			(-)		C
Apocyanaceae	Plumeria rubra			(-)		C
	Vinca major			(-)		C
Araceae	Monstera deliciosa			(-)		C
Araliaceae	Tetrapanax papyriferus			(-)		C
Arecaceae	Phoenix roebelenii			(-)		C
Aristolochiaceae	Aristolochia elegans			(-)		C
Asclepiadaceae	Asclepias syriaca			(-)		C
Asteraceae	Ambrosia psilostachya		(-)			Hs
	Artemisia absinthium			(-)		C
	Cichorium intybus			(-)		C
	Doronicum cordatum			(-)		C
	Grindelia squarrosa			(-)		C
	Helianthus annuus	(M)		(-)		C,S
	Lactuca sativa	(M)		(-)		C,S
	Senecio vulgaris				(M)	B
	Solidago speciosa			(-)		C
	Tragopogon miscellus	(M)				So
Balsaminaceae	Impatiens balsamina		(-)			Hs
	Impatiens capensis	(M)		(-)		C,S
	Impatiens glandulifera		(-)			Hs
	Impatiens walleriana		(-)			Hs
Bignoniaceae	Campsis radicans			(-)		C
Boraginaceae	Borago officinalis	(B)				S
	Echium vulgare			(-)		C
Brassicaceae	Arabidopsis thaliana	(M)		(-)		C,S
	Arabis albida	(M)		(-)		C,S
	Aubrieta graeca	(M)				T
	Aubrieta purpurea	(M)				T
	Brassica campestris	(M)	(-)	(-)		C,D,Hs
	Brassica napus			(-)	(M)	C,E
	Brassica oleraceae			(-)		C
	Capsella bursa-pastoris		(-)			Hs
	Lepidium virginicum			(-)		C
	Raphanus sativus			(-)		C
Bromeliaceae	Tillandsia recurvata			(-)		C
	Tillandsia caput-medusa		(-)			Hs

Table 4.2 Cont. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Cactaceae	Echinocerus engelmanni			(-)		C
	Opuntia basilaris			(-)		C
	Opuntia engelmanni			(-)		C
	Opuntia vulgaris			(-)		C
	Rhipsalidopsis gaertneri			(-)		C
	Zygocactus truncatus			(-)		C
Campanulaceae	Campanula alliariafolia			(+)		C
	Campanula carpatica			(+)		C
	Campanula rapunculoides			(+)		C
	Lobelia erinus		(-,+)			S,Hs
	Lobelia syphilitica			(+)		C
	Platycodon grandiflorum			(+)		C
Cannabaceae	Humulus japonica	(M)				T
Capparidaceae	Cleoma spinosa			(-)		C
Caprifoliaceae	Linnaea borealis			(+)		C
	Lonicera japonica			(+)		C
	Sambucus sp.			(+)		C
Caryophyllaceae	Lychnis alba			(-)		C
	Saponaria offinicalis			(-)		C
	Silene otites	(B)				T
	Silene psuedonites	(B)				S
	Stellaria media	(M)				S
Chenopodiaceae	Beta vulgaris	(M)	(-)	(-)		C,S,Hs
	Chenopodium album	(M)	(-)	(-)		C,W
	Spinacia oleracea		(-)			Hs
Clusiaceae	Hypericum acutum	(B)				T
	Hypericum montanum	(B)				T
	Hypericum pulchrum	(B)				T
	Hypericum quadrangulum	(B)				T
	Hypericum perforatum	(B)		(-)		C,T
Commelinaceae	Tradescantia virginiana			(-)		C
	Tradescantia paludosa		(-)			Hs
Convolvulaceae	Ipomeae nil	(M)		(+)		C,T
Cornaceae	Cornus florida			(-)		C
Crassulaceae	Kalanchoe daigremontiana			(-)		C
Cucurbitaceae	Cucumis sativus			(-)		C
	Cucurbita maxima	(M)				S
Cuscutaceae	Cuscuta sp.			(-)		C
Cyperaceae	Eleocharis palustris		(-)			Hs
Droseraceae	Dionaea muscipula			(+)		C
	Drosera capillaris			(+)		C
Ericaceae	Rhododendron sp.		(+)			Hs
	Rhododendron maximum	(B)		(+)		C,T
	Rhododendron hortense	(B)				T
	Rhododendron japonicum	(B)				T
	Rhododendron kaempferi	(B)				T
	Rhododendron mucronatum	(B)				T
	Rhododendron obtusum	(B)				T
	Rhododendron pulchrum	(B)				T
	Rhododendron ripense	(B)				T

Table 4.2 Cont. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Ericaceae	Rhododendron serpyllifolium	(B)				T
	Rhododendron sublanceolatum	(B)				T
	Rhododendron transiens	(B)				T
	Rhododendron yedoense	(B)				T
Euphorbiaceae	Euphorbia dulcis		(-)			Hs
Fagaceae	Quercus virginiana		(-)			Hs
Gentianaceae	Gentiana punctata			(-)		C
Geraniaceae	Geranium bohemicum	(B)				S
	Geranium bohemicum ssp. deprehensum	(B)				S
	Geranium maculatum	(B)		(+)		C,S
	Geranium pratense		(+)	(+)		C,Hs
	Geranium sanguinium			(+)		C
	Pelargonium denticulatum	(B)				S
	Pelargonium filiciforme	(B)				S
	Pelargonium peltatum			(+)		C
	Pelargonium x zonale hort	(B)	(+)	(+)	(B)	C,T,M,Hs
Gesneriaceae	Saintpaulia hybrida			(-)		C
	Saintpaulia ionantha		(-)			Hs
	Streptocarpus sp.			(-)		C
Hamamelidaceae	Hamamelis virginiana			(-)		C
Hydrangeaceae	Hydrangea arborescens	(M)		(-)		C,T
	Hydrangea hortensis	(M)				S
	Philadelphus sp.			(-)		C
Hydrophyllaceae	Hydrophyllum virginianum			(-)		C
Iridaceae	Iris versicolor			(-)		C
Juglandaceae	Carya pecan		(-)			Hs
Lamiaceae	Coleus blumei			(-)		C
	Mentha verticilliata			(-)		C
	Monarda fistulosa			(-)		C
	Nepeta cataria	(B)		(-)		C,T
	Origanum vulgare			(-)		C
Leguminosae	Acacia decurrens	(B)				T
	Acacia mearnsii	(B)				T
	Apios americana			(-)		C
	Arachis hypogaea			(-)		C
	Astragalus cicer			(-)		C
	Calliandra eriophylla			(-)		C
	Cassia marylandica			(-)		C
	Cercis canadensis			(-)		C
	Cicer aritinum			(+)		C
	Coronilla varia			(-)		C
	Glycine canescens			(-)		C
	Glycine clandestina			(-)		C
	Glycine cryptoloba			(-)		C
	Glycine falcata			(-)		C
	Glycine latifolia			(-)		C
	Glycine max	(M)		(-)		C,P
	Glycine microphylla			(-)		C
	Glycine soja			(-)	(M)	C
	Glycine tabacina			(-)		C

Table 4.2 Cont. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Leguminosae	Glycine tabacina x G. canescens				(M)	Ha,Do
	Glycine tomentella			(-)		C
	Lathyrus japonicus			(+)		C
	Lathyrus odoratus			(+)		C
	Lens culinaris			(-)		C
	Lotus corniculatus			(-)		C
	Lupinus luteus		(+)			Hs
	Lupinus nootkarensis		(+)			Hs
	Lupinus perennis			(-)		C
	Medicago sativa	(B)		(+)	(B/P)	C,Sm,Sc,Ma
	Medicago truncatula	(B)				T
	Melilotus alba			(+)		C
	Melilotus indica			(+)		C
	Melilotus officinalis			(+)		C
	Parkinsonia aculeata		(-)	(-)		C,S,Hs
	Phaseolus aureus			(-)		C
	Phaseolus vulgaris	(B)		(-)		C,S
	Pisum sativum	(M)	(-)	(-/+)		C,S,Hs
	Trifolium arvensis			(-)		C
	Trifolium hybridum			(-)		C
	Trifolium pratense	(M)		(-)		C,S
	Trifolium repens			(-)		C
	Vigna sinensis			(-)		C
	Vicia faba			(-)		C
	Vicia villosa			(-)		C
	Wisteria sinensis			(+)		C
Liliaceae	Allium cepa	(M)	(-)	(-)		C,S
	Allium fistulosum	(M)				T
	Bellevallia lipskyi		(-)			Hs
	Chlorophytum comosum	(M/B)	(-)	(+)		C,T,Hs
	Chlorophytum comosum x C. elatum	(M/B)				T
	Chlorophytum elatum	(M/B)		(+)		C,S
	Convallaria majalis		(-)			Hs
	Endymion puruviana		(-)			S
	Fritillaria imperialis		(-)			Hs
	Fritillaria meleagris		(-)			Hs
	Fritillaria thunbergii		(-)			Hs
	Haemanthus katherinae		(+,-)			S,Hs
	Hemerocalis fulva			(-)		C
	Hippeastrum belladonna		(+)			S
	Hippeastrum vitatum		(-)			Hs
	Hosta japonica		(-)			S,Hs
	Hosta ventricosa		(-)			Hs
	Hyacinthoides non-scriptus		(-)			S
	Lilium candidum		(+)			Hs
	Lilium martagon		(+)			Hs
	Lilium regale		(+)			Hs
	Lilium superbum			(-)		C
	Maianthemum bifolium		(+)			Hs
	Muscari racemosum		(-)			Hs

Table 4.2 Cont. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Liliaceae	Nerine curvifolia			(-)		C
	Ornithogalum nutans		(+)			Hs
	Polygonatum multiflorum		(+)			Hs
	Tulbaghia violacea		(-)			Hs
	Tulipa kolpakowskiana		(-)			Hs
	Tulipa turkestanica		(-)			Hs
Linaceae	Linum usitatissimum		(+)	(-)		C,S,Hs
Magnoliaceae	Liriodendron tulipifera			(-)		C
	Magnolia sp.			(-)		C
Malvaceae	Althaea officinalis			(-)		C
	Hibiscus syriacus			(-)		C
	Gossypium hirsutum	(M)	(-)	(-)		C,K,Hs
	Gossypium trilobum x G. gossypioides				(M)	We
	Gossypium berbadense x G. tomentosum				(M)	We
	Gossypium arboreum x G davidsonii				(M)	We
	Gossypium harknesii x G. hirsutum				(M)	Ga
	Gossypium herbaceum x G. harknesii				(M)	Ga
Menispermaceae	Cocculus laurifolius			(-)		C
Moraceae	Morus alba			(-)		C
Nyctaginaceae	Mirabilis jalapa	(M)	(-)	(-)		C,S,Hs
Nymphaeaceae	Nymphaea colorata			(-)		C
Oleaceae	Olea europea		(-)			Hs
	Syringa vulgaris			(-)		C
Onagraceae	Epilobium sp.		(-)			Hs
	Epilobium angustifolium	(M/B)		(-)		C,S
	Epilobium hirsutum x E. montanum				(M)	Sh
	Epilobium lanceolata x E. watsonii				(M)	Sh
	Epilobium lanceolata x E. montanum				(M)	Sh
	Epilobium parviflorum x E. montanum				(M)	Sh
	Epilobium parviflorum x E. watsonii				(M)	Sh
	Epilobium watsonii x E. montanum				(M/P)	Sh
	Epilobium hirsutum	(M)				T
	Oenothera spp. (Euoenothera, 28 spp.)	(B)	(+)			S
	Oenothera ammophila	(B)		(+)		C,T
	Oenothera berteriona x O. odorata				(M)	H
	Oenothera biennis	(B)		(+)		C,T
	Oenothera erythrosepela		(+)			Hs
	Oenothera grandiflora	(B)		(+)		C,T
	Oenothera hookeri	(B)	(+)	(+)		C,T,Hs
	Oenothera macrosceles			(+)		C
	Oenothera organensis	(B)				Su
	Oenothera perennis			(+)		C
Orchidaceae	Broughtonia sanguinea			(-)		C
	Cattasetum discolor		(-)			S
	Cattleya hyb.			(-)		C
	Cymbidium hyb.			(-)		C
	Cyripedium acaule			(-)		C
	Dendrobium acinaciforme			(-)		C
	Epidendrum cochleatum			(-)		C
	Epidendrum scutella		(-)			S,Hs

Table 4.2 Cont. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Orchidaceae	Epidendrum tampense			(-)		C
	Ornithophora radicans			(-)		C
	Paphiopedilum concolor			(-)		C
	Paphiopedilum micranthum			(-)		C
	Phaius tankervillae		(-)			S
	Phragmeipedium longifolium			(-)		C
	Phragmeipedium warscewiczianum			(-)		C
	Restrepia filamentosa			(-)		C
Oxalidaceae	Oxalis europeae			(-)		C
Papaveraceae	Chelidonium majus			(-)		C
	Eschscholzia californica			(-)		C
Passifloraceae	Passiflora edulis			(+)		C
Pedaliaceae	Sesamum indicum			(-)		C
Phytolaccaceae	Phytolacca americana			(-)		C
Piperaceae	Peperomia griseo-argentea			(-)		C
Pittosporaceae	Hymenosporum flavum			(-)		C
Plantaginaceae	Plantago lanceolata			(-)		C
	Plantago major	(M)		(-)		C,T
	Plantago psyllium			(-)		C
Plumbaginaceae	Limonium carolinianum			(-)		C
	Plumbago auriculata			(+)		C
	Plumbago capensis			(+)		C
	Plumbago larpentae			(+)		C
	Plumbago zeylandica		(+)	(+)		C,Hs
Poaceae	Alopecurus pratensis			(-)		C
	Anthoxanthum oderatum			(-)		C
	Avena sativa	(M)		(-)		C,T
	Avena sativa x A. steralis	(M)				T
	Coix lacryma-jobi	(M)		(-)		C,T
	Digitaria ischaemum			(-)		C
	Echinochloa pungens			(-)		C
	Elymus repens		(-)			Hs
	Hordeum vulgare	(M)	(-)	(-)		C,T,Hs
	Lolium perenne			(-)		C
	Oryza rufipogon				(M)	Da
	Oryza sativa		(-)	(-)		C,S
	Oryza glaberrima x O. rifipogon				(M)	Da
	Oryza longistaminata x O. sativa				(M)	Da
	Panicum miliaceum			(-)		C
	Phleum pratense			(-)		C
	Poa pratensis			(-)		C
	Secale cereale	(B)	(+)	(-)		C,S,Hs
	Sorghum vulgare	(M)		(-)		C,S
	Triticale		(+)	(-)		C,Hs
	Triticum aestivum	(M)	(-)	(-)		C,Pa,Hs
	Triticum aestivum x Secale cereale				(M)	V
	Triticum durum		(+)			Hs
	Triticum durum x Secale cereale				(M)	V
	Triticum timopheevi x Secale cereale				(M)	V
	Triticum vulgare	(M)				T

Table 4.2 Cont. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Poaceae	Zea mays	(M)	(-)	(-)		C,T,Hs
	Zea mays x Z. perennis				(M)	Co
	Zizania aquatica			(-)		C
Polemoniaceae	Phlox divaricata			(-)		C
Polygonaceae	Fagopyrum esculentum	(M/B)		(-)		C,T
	Rumex acetosella			(-)		C
Polygonaceae	Rumex crispus			(-)		C
Primulaceae	Lysimachia clethroides			(-)		C
	Primula sinensis	(M)				T
	Primula vulgaris	(M)				T
Ranunculaceae	Aconitum arendsii			(-)		C
	Aquilegia canadensis			(-)		C
	Clematis sp.			(-)		C
	Nigella damescens			(-)		C
	Ranunculus acris			(-)		C
Rosaceae	Prunus avium		(-)			Hs
	Pyracantha coccinea			(-)		C
	Rosa rugosa			(-)		C
Rubiaceae	Coffea arabica			(-)		C
	Coffea canephora x C. arabica				(M)	Be
Rutaceae	Citrus aurantium			(-)		C
Saxifragaceae	Heuchera micranthera				(M)	Sn
	Tolmiea menziesii				(M)	Sn
Scrophulariaceae	Antirrhinum majus	(M/B)	(-)	(-)		C,T,Hs
	Castilleia foliosa		(-)			S,Hs
	Castilleia wrightii		(+)			S,Hs
	Cordylanthus spp. (3 spp.)	(M)				S
	Cymbalaria muralis			(-)		C
	Linaria vulgaris			(-)		C
	Mimulus cardinalis		(-)			T
	Mimulus quinquevulnerus		(-)			T
	Ophiocephalus angustifolius		(-)			S
	Orthocarpus spp. (2 spp)		(-)			S
	Scrophularia marilandica			(-)		C
	Verbascum thapsus			(-)		C
Solanaceae	Atropa belladonna			(-)		C
	Browallia speciosa	(M/B)				S
	Capsicum annuum	(M)		(-)		C,S
	Datura metel		(+)			Hs
	Datura stramonium			(-)		C
	Hyoscyamus niger		(-)	(-)		C,Hs
	Lycopersicon esculentum	(M)	(-)	(-)		C,S,Hs
	Lycopersicon peruvianum		(-)			Hs
	Nicotiana spp. (7 spp.)	(M)				S
	Nicotiana alata		(-)			Hs
	Nicotiana colossea	(M)				T
	Nicotiana glauca			(-)		C
	Nicotiana glutinosa			(-)		C
	Nicotiana plumbaginifolia				(P)	Me
	Nicotiana plumbaginifolia x N. tabacum				(P)	Me

Table 4.2 Cont. For key see bottom of the table.

FAMILY	SPECIES	Methods 1	2	3	4	REFERENCE
Solanaceae	Nicotiana trigonophylla			(-)		C
	Nicotiana rustuca			(-)		C
	Nicotiana tabacum	(M)		(-)		C,T
	Petunia alkekengi	(M)				S
	Petunia hybrida	(M)	(-)	(-)		C,Po,Hs
	Petunia violacea	(M)				S
	Solanum carolinense			(-)		C
	Solanum chacoense		(-)			Hs
	Solanum dulcamara			(-)		C
	Solanum tuberosum	(M/B)	(-)	(-)		C,T,Hs
	Solanum tuberosum ssp. tuberosum				(M)	Ho
Strelitziaceae	Strelitzia reginea			(+)		C
Theaceae	Camellia sinensis			(-)		C
Tropaeolaceae	Tropaeolum majus			(-)		C
Typhaceae	Typhus latifolia			(-)		C
Ulmaceae	Ulmus americana			(-)		C
Verbanaceae	Verbana hastata			(-)		C
Violaceae	Viola tricolor	(M)		(-)		C,S
Vitaceae	Vitis vinifera			(-)		C
Zingiberaceae	Zingiber officinale			(-)		C

Method 1. Genetic markers (eg. Green vs white plastids, herbicide restistance).
(M) - Maternal inheritance. (B) - Biparental inheritance. (M/B) - Predominantly maternal inheritance but some biparental inheritance.

Method 2. Ultrastructural studies.
(-) - Plastid absent in generative/sperm cell. (+) - Plastid present in generative/sperm cell.

Method 3. Epifluoresence microscopy.
(-) - No fluorescent bodies in pollen. (+) - Fluorescent bodies in pollen. (-/+) - Some pollen with fluorescent bodies.

Method 4. Restriction enzyme markers.
(M) - Maternal inheritance. (B) - Biparental inheritance. (B/P) - Biparental and paternal inheritance. (P) - Paternal inheritance. (M/B) - Maternal inheritance with some paternal inheritance.

References. B - Bleyden (1988), Be - Berthou et al (1983), Bo - Boblenz et al (1990), C - Corriveau and Coleman (1988), Co - Conde et al (1979), D - Darr et al(1981), Da - Dally and Second (1990), Do - Doyle et al (1990b), E - Erickson et al(1983), Ga - Galau and Wilkins (1990), H - Hachtel (1980), Ha - Hatfield et al (1985), Hs - Hageman and Schroder (1989), K -Krishnaswami (1948), M - Metzlaff et al(1981), Ma - Masoude et al(1990), Me - Medgyesy et al(1986) P - Palmer and Mascia (1980), Pa - Pao and Li (1946), Po - Potrykus (1970), S - Sears (1980), Sc - Schumann and Hancock (1989), Sh - Schmitz and Kowallik (1986), Sm - Smith et al (1986), Sn - Soltis et al (1990), So - Soltis and Soltis (1989), Su - Stubbe et al (1984),T - Tilney-Bassett (1978), V - Vedel et al (1981), W - Warwick and Black (1980), We - Wendel (1989).

NB. Galau and Wilkins (1990) report that very rare paternal cpDNA transmission may be inferred from the restoration of fertility in cms x maintainer lines of Gossypium harknessii.

4.3 The generation of biosystematically useful cpDNA data.

The explosion of interest in cpDNA as a biosystematically useful marker has not always resulted in ideal studies. This is apparently the result of either a lack of resources or material. A general criticism is of inadequate sampling, either as a result of using too few enzymes (eg. Hantula *et al* 1989) or too few individuals per taxon (eg. Coates and Cullis 1987). Allied to the problem of sampling is the lack of within individual sampling, since it has been shown that extensive cpDNA variation within individuals may occur (Govindaraju *et al* 1988, Lee *et al* 1988). The methods used to generate data and some of the problems of data analysis have been considered in Chapter 3.

In order to generate more cpDNA data of greater use it is necessary to have more rigorous sampling strategies which take into account the possible non-random distribution of restriction sites (Adams and Rothman 1982) and the increasing evidence of intraspecific cpDNA variation (Section 4.1). It is important to establish the mode of cpDNA inheritance, especially in studies of introgressive and polyploid speciation. This need not be a laborious process since with the advent of epifluoresence microscopy (Corriveau and Coleman 1988) large numbers of genotypes can be quickly surveyed for, at least, their potential to transfer plastids via their pollen. When perennial taxa, taxa with biparental inheritance, and taxa which reproduce extensively by clonal propagation are

studied then intra-individual sampling must be considered since situations can be envisaged where extensive cpDNA variation occurs within such an individual in the absence of extensive morphological variation.

Of all of the readily available methods to analyse cpDNA, site-for-site mapping of mutations is the most useful since mutations can be positively identified (either as site, length or inversion mutations) and the site mutations that are being scored will be evolutionarily homologous. One problem in the use of cpDNA fragment comparison approaches to phylogenetic analysis is the over-estimation of the number of mutations present between two taxa. For example, a single site gain in one taxon may be scored as three separate characters if fragments were compared between a taxon with the site mutation and a taxon lacking this mutation. Although site-for-site comparison is time consuming and expensive, the quality of the data generated and the inferences that can be made make up for the disadvantages (Palmer *et al* 1988).

4.4 Prospects and conclusions.

Chloroplast DNA is undoubtedly an important source of biosystematic information over a wide range of taxonomic levels. Its use must, however, be tempered by a wider consideration of the degree of intra-individual and intraspecific variation and the mode of plastid transmission. Important questions remain to be answered

regarding:- (i) Plastid dynamics within and between populations and the effects on the cpDNA variability. (ii) The influence and effect that a slow 'leakage' of paternal plastid DNA has on an essentially maternal cpDNA phylogeny. (iii) The extent of recombination between cpDNAs in wild plants. (iv) The degree of intra-individual and intraspecific cpDNA variation.

The use of cpDNA is, *a priori*, no better or no worse than any other data source used in biosystematic studies, but in common with other data sources (eg isozymes and secondary metabolites), methods are required which will reconcile apparently divergent data sets. This is but one of the challenges for the continued success and use of cpDNA in biosystematics.

Chapter 5.

Biosystematics of some British and European *Senecio* species: - Random nuclear DNA evidence.

" '... B-o-t, bot, t-i-n, tin, bottin, n-e-y, ney, bottinney, noun substantive, a knowledge of plants. When he has learned that bottinney means a knowledge of plants, he goes and knows 'em. ... ' "

Nicholas Nickleby.
C. Dickens.

Introduction.

Many reviews on aspects of plant genome organisation have been published recently, for example, Flavell *et al* (1980) and Tanksley and Pichersky (1987).

The plant nuclear genome is known to be composed of at least three major reassociation classes of DNA:- (i) Highly repetitive, satellite sequences, (ii) moderately repetitive sequences and (iii) low repetitive or unique copy number sequences (Britten and Koehn 1968, Tanksley and Pichersky 1987). These three genome components can all be used to provide biosystematic information. This Chapter is concerned with the use of highly and moderately repetitive sequences. Ribosomal DNA, a tandem repetitive component of the nuclear genome, is the subject of Chapter 2.

Two classes of moderately repetitive sequences occur, interspersed repeats and tandem repeats. Interspersed repeats occur in short blocks with unique sequence DNA (Flavell 1982). Tandem repeat sequences are localised in particular areas of the genome, eg. ribosomal DNA (Chapter 2) and heterochromatin (Peacock *et al* 1981). A large proportion of the plant genome is composed of repetitive DNA. In the Asteraceae, Tribe Cichorieae, 22% to 55% of the nuclear genome is moderately repetitive DNA (Bachmann and Price 1977). In *Senecio vulgaris*, 74% of the genome has been reported to be repetitive DNA (Flavell *et al* 1974).

In this Introduction I shall outline the use of nuclear restriction fragment length polymorphisms in biosystematics.

5.1.1 Restriction fragment length polymorphisms and biosystematics.

In Chapters 2 and 3 restriction fragment length polymorphisms (RFLPs) have been generated with ribosomal DNA and chloroplast DNA sequences. However, RFLPs can be generated by any DNA sequence for which a probe is available. One definition of a RFLP is the difference in restriction fragment length produced by a particular probe-enzyme combination. This difference is the result of a change in distance between two adjacent restriction enzyme cleavage sites. RFLPs are generated by three main processes:- (i) The loss (or gain) of a single restriction site. (ii) The insertion (or deletion) of a segment of DNA between the two sites of interest. (iii) The inversion of a DNA segment including one (or both) of the restriction sites of interest (see Chapter 3). In addition to the three processes mentioned above, overall differences in RFLP patterns can be the result of differences in sequence copy number and the degree of shared sequence similarity (Figdore *et al* 1988).

Any restriction enzyme analysis will generate RFLPs, whether the probe is 'defined' (eg. cpDNA or rDNA) or 'anonymous' (eg. random genomic). I shall, however, use the term in the slightly stricter sense of random 'anonymous'

probes. These probes may be genomic or complementary DNA (cDNA, Apuya *et al* 1988).

RFLPs are potentially very useful for the identification of hybrids and introgressants (Beckmann and Soller 1983), particularly if a group of RFLPs are identified which are scattered across the genome at a number of loci rather than limited to one or few loci (eg. rDNA).

As might be expected RFLPs have been applied mainly to crops and their close wild relatives. Three major areas have been studied:- (i) the level of RFLP variation present within and between taxa (Apuya *et al* 1988, Keim *et al* 1989a, Nagamine *et al* 1989, Havey and Muehlbauer 1989, Helentjaris *et al* 1985, Figdore *et al* 1988), (ii) the reconstruction of phylogenies (Song *et al* 1988a, 1988b, 1990, Pental and Barnes 1985, Debener et al 1990, Menancio et al 1990) and (iii) the identification of introgression (Keim *et al* 1989b).

The aims of the experiments reported in this Chapter were to establish if RFLPs could be useful in the identification of hybridisation and introgression in *Senecio*. The construction of a small number of random nuclear genomic clones from *Senecio squalidus* was used to test this idea and assess the possible introgressive origin of S. *vulgaris* ssp. *vulgaris* var. *hibernicus*.

Materials and Methods.

5.2.1 Plant material.

The taxa and accessions which were analysed are shown in Table 2.2 (Chapter 2). These represent 6 *Senecio* taxa (*S. cambrensis*, *S. squalidus*, *S. vernalis*, *S. vulgaris* ssp. *vulgaris* var. *hibernicus*, *S. vulgaris* ssp. *vulgaris* var. *vulgaris*, and *S. vulgaris* ssp. *denticulatus*).

5.2.2 Generation of random genomic clones.

Total DNA from a sample of ten individuals of *Senecio squalidus* from Chesterfield (grid ref. SK380710) was prepared and purified as detailed in Appendix A (Sections A2.2 and A2.3.2). The DNA was digested, ligated into the plasmid vector pUC18 and *Escherichia coli* transformed according to the procedures in Appendix A, Section A2.12. Potential recombinant colonies were selected for the presence of *S. squalidus* inserts using a modification of the replica plating technique (Grunstein & Hogness 1975), since relatively few putative recombinant colonies were isolated. These filters were probed with total *S. squalidus* DNA (Appendix A, Sections A2.8 and A2.9) to identify those colonies containing plasmids with *S. squalidus* inserts.

Colonies providing a clear signal were further checked for the presence of a *S. squalidus*-containing plasmids by isolating the plasmid using miniprep procedures (Appendix A, Section A2.12.3), then digesting the isolated plasmid with the cloning enzyme and separating the vector and insert fragments (Appendix A, Section A2.5 and A2.6).

5.2.3 Nuclear restriction fragment length polymorphism study.

Five plasmids isolated by the cloning procedure (Section 5.3.1, Table 5.1) were used as probes to challenge the stripped filters used in the rDNA study (Chapter 2), ie. three enzymes (*Bam*HI, *Eco*RI and *Eco*RV) were used. Between each probing the filters were stripped (Appendix A, Section A10). Filters were washed under the same conditions as for the rDNA probe (Chapter 2, Section 2.2.2).

Results.

5.3.1 *Senecio squalidus* nuclear DNA clones and *Senecio* restriction fragment length polymorphisms.

The initial selection procedure used to identify plasmids with *Senecio squalidus* inserts (white vs blue bacterial colonies) generated 220 clones, however replica plating and miniplasmid preparations (Appendix A, Section A2.11.3) of these clones indicated that only five had *S. squalidus* inserts. The designation and size of the insert, for each of the five isolated plasmids are shown in Table 5.1.

Table 5.1 Designation and size of the *Senecio squalidus* random genomic clones used in this study. All inserts have been cloned in to the vector pUC18 (size = 2.7kb).

Probe designation.	Size (kb).
pSsA19	2.3
pSsA45	2.7
pSsC15	6.2
pSsC35	1.8
pSsC42	9.8

No variation within or between taxa was revealed for the 12 probe-enzyme combinations (PECs) which were used (pSsA19, pSsC15, pSsC35, pSsC42 and *Bam*HI, *Eco*RI, *Eco*RV).

Probe pSsA45 showed a smear of restriction fragments with each of these enzymes, however when this probe was used with partially *Hin*DIII-digested *Senecio squalidus* DNA a ladder pattern with a 320bp periodicity was revealed, indicating that this probe is a tandemly repeated sequence.

Discussion.

The probes which were isolated in this study and the enzymes which were used have not proved useful for the identification of RFLPs within and between the *Senecio* taxa analysed.

An important initial criterion in any biosystematic study is the assessment of the level of variation present within the taxa of interest. In published studies two general trends have been found:- (a) Within species (especially cultivar) RFLP variation is low (Apuya *et al* 1988, Keim *et al* 1989a) and (b) between species RFLP variation is high (Nagamine *et al* 1989). These trends do, however, appear to vary from taxon to taxon.

In *Lens* (Havey and Muehlbauer 1989) and *Glycine* (Apuya *et al* 1988, Keim *et al* 1989a) low levels of intraspecific RFLP variation has been reported. This is in contrast to *Zea* (Helentjaris *et al* 1985) and *Brassica* (Figdore *et al* 1988) where extensive intraspecific RFLP variation has been reported. In *Lycopersicon esculentum* (Helentjaris *et al* 1985), wild races have greater RFLP variation than domesticated lines. The occurrence of high levels of RFLP variation between species has been reported by a number of workers, for example, Nagamine *et al* (1989) in *Beta* species and Helentjaris *et al* (1985) in *Lycopersicon*.

The absence of between species variation in this study is suprising, particularly since *Senecio squalidus* is largely self-incompatible and, therefore, an outcrossing species.

The data reported here does indicate that not all molecular techniques are equally useful in addressing biosystematic questions at all levels. The expectation that DNA will provide a large number of readily scored characters is not borne out here. This is not to say that RFLP data is not useful in the genus *Senecio*, but rather that the PECs which have been examined in <u>this</u> study are not useful.

The failure to reveal any variation within or between the taxa studied may be due to a number of factors:-

(i) The small number of probes isolated. This may have been the result of the low efficiency of obtaining recombinant clones. The use of a total nuclear DNA digest to construct the initial genomic library, rather than a partial digest, may improve the number of clones isolated and hence the number of RFLPs detected.

(ii) Only three enzymes were tried (*Bam*HI, *Eco*RI, *Eco*RV). Thus only a small proportion of the genome was sampled, lowering the probability of detecting a RFLP. An increase in the number of enzymes analysed and using a mixture of tetranucleotide and hexanucleotide cutting enzymes will increase the proportion of the genome analysed and hence, potentially the number of RFLPs detected.

Figdore *et al* (1988) have addressed the question of whether more variation is identified with; (a) fewer probes and more enzymes or (b) more probes and fewer enzymes. In their study of *Brassica* they concluded that the increased RFLP frequency gained by using two as opposed to one enzyme was small (c. 20%) and that it was more economical to use one enzyme and more probes (391 in total) than to reduce probe number and to increase enzyme number. However, the high level of RFLP variation encountered in *Brassica* should be borne in mind.

(iii) The results obtained from the slot blot procedure (Chapter 6) suggests that a lower wash stringency may detect more RFLPs, since variation in signal intensity apparently exists both within and between taxa.

Chapter 6.

Biosystematics of some British and European *Senecio* species: - Taxon-specific probes.

" A hair or two will show where a lion is hidden. A very little key will open a very heavy door. "

Hunted Down.
C. Dickens.

Introduction.

One important application of molecular biology in biosystematics is the delimitation of taxa (Chapter 1), whether families, genera, species or subspecies. This may be accomplished in two ways, either as qualitative differences between taxa based on RFLPs (in their broadest sense) or as quantitative differences in the number of copies of a particular sequence present. The former type of taxon identification has been covered in Chapters 2 (ribosomal DNA), 3 (chloroplast DNA) and 5 (restriction fragment length polymorphisms). It is the latter, quantitative differences, which are the subject of this Chapter.

Two types of quantitative differences can be recognised, those which result in:- (i) A taxon-specific probe which either identifies a taxon or not (all-or-nothing differences, eg. Gupta *et al* 1989). (ii) A probe which shows marked differences in copy number between taxa (abundance differences, eg. Dvorak *et al* 1988).

The term 'taxon-specific' is employed here. However, strictly these sequences should be referred to as genome-specific, since in polyploid taxa apparently taxon-specific sequences may cross hybridise (eg. the C genome probe in *Avena*, Fabijanski *et al* 1990)

Quantitative approaches to taxon identification have a number of advantages over RFLP approaches:- (i) Large numbers of accessions may be quickly and cheaply screened (Schmidt *et al* 1990). (ii) Crude DNA extraction techniques may be employed (Hutchinson *et al* 1985, Metzlaff *et al* 1986, Junghans and Metzlaff 1988).

Taxon-specific sequences have been isolated from:- (i) satellite sequences (eg. *Raphanus*, Grellet *et al* 1986; *Nicotiana tabacum*, Koukalová *et al* 1989) and (ii) moderately repeated sequences (eg. *Secale cereale*, Xin and Appels 1988; *Oryza*, Zhao *et al* 1989). The use of ribosomal DNA as a a source of species-specific sequences has been considered in Chapter 2.

In this Introduction I shall outline the search for taxon-specific probes and their use in tracing introgression events.

6.1. Slot blot analysis: Methodological considerations.

Early work on the identification of taxon-specific sequences employed the techniques of solution DNA-DNA hybridisation (eg. Rimpau *et al* 1978). However, the use of filter-bound DNA-DNA hybridisation technology has simplified the procedure considerably (Britten and Davidson 1985, Meinkoth and Wahl 1984, Rivin 1986). The apparent ease with which taxon-specific sequences can be identified makes them of considerable value.

Slot blot analysis is a method of determining the amount of a particular sequence in a DNA sample (Rivin 1986). The approach relies on two assumptions:- (i) The existence of a linear relationship between sequence quantity and the autoradiographic signal intensity. (ii) The complete saturation of the sequence with the probe under the hybridisation conditions used. These assumptions may not always be met, in particular the signal intensity may rapidly reach satuaration and deviate markedly from a linear response curve (Cullis *et al* 1984).

Signal intensity is not only a function of the quantity of a particular sequence present, but also the stringency at which the filters are washed (Meinkoth and Wahl 1984). Stringency is a measure of the degree of base-pair mismatch which can be tolerated in a DNA-DNA duplex, which is reflected in its melting temperature. Equations derived from experimental studies indicate that T_m is a function of the DNA GC content and the salt concentration in the washing buffer. Hence, as wash temperature is raised the stringency increases and only those sequences with a high similarity to the probe will remain stable.

6.2 Taxon-specific sequences and taxon identification.

Much work on the development of taxon-specific markers has been applied to members of the Poaceae. Reviews of this work have recently been published by Appels (1983), Appels and Moran (1984) and McIntyre *et al* (1988b). From such work

three main types of data have been generated:- (i) Analyses of hybridisation and introgression. (ii) Broad phylogenetic hypotheses. (iii) Analyses of the degree of intraspecific genome variation.

(i) Introgression and hybridisation. One of the most valuable aspects of dispersed, repetitive taxon-specific probes is the potential for following introgression and hybridisation events. Reports on the importance of such probes in introgression studies have been made by Appels and Moran (1984) and McIntyre *et al* (1988b). Zhao *et al* (1989) have obtained sequences specific to the A, C, E and F genomes of the genus *Oryza*. Outside of the Poaceae, Schmidt *et al* (1990) have used *Beta procumbens*-specific sequences to screen addition lines of *B. vulgaris* x *B. procumbens* for beet cyst nematode resistance.

(ii) Phylogenetic applications. The broad phylogenetic application of these sequences has had relatively little use. Those studies which have used this data for phylogenetic purposes (eg. Gupta *et al* 1989, Zhao *et al* 1989, Xin and Appels 1988) have not been as rigorous as some of the studies reported for either ribosomal DNA (Chapter 2) or chloroplast DNA (Chapter 3).

(iii) Intraspecific genome variation. Reviews of extensive intraspecific genome variation and the mechanisms for generation of this variation have recently been published by Walbot and Cullis (1985) and Dover (1988). Strauss and Tsai (1988) showed substantial within population variation for ribosomal RNA gene number in *Pseudotsuga menziesii*, which was correlated with latitude,

elevation and longitude. Similar intraspecific studies of copy number variation have been made by Rivin *et al* (1986) in *Zea mays*, Marazia *et al* (1980) in the Cucurbitaceae and Liang *et al* (1977) in *Triticum*.

Preliminary experiments using the *Senecio squalidus* probes described in Chapter 5 suggested that two of these random genomic clones (pSsA45 and pSsC15) were species-specific. The study was, therefore, extended to included more accessions of *S. squalidus* and *S. vulgaris sl* plus *S. cambrensis*.

Materials and Methods.

6.2.1 Plant material.

A total of 60 accessions were used, which represented 15 accessions each of *Senecio cambrensis*, *S. squalidus*, *S. vulgaris* ssp. *vulgaris* var. *hibernicus* and *S. vulgaris* ssp. *vulgaris* var. *vulgaris*, randomly selected for a detailed slot blot analysis (Table 6.1).

-Y

Table 6.1 Locations of the taxa used in the slot blot study.

Taxon.	Location.	Grid Ref.	No. of Indiv.	Source§
S. vulgaris ssp. vulgaris var. vulgaris.	Monomorphic populations.			
	Brooklands Av., Cambridge.	TL460580	1	SAH
	Grantham St., Lincoln	SK970710	1	SAH
	Migvie, Aberdeenshire.	NJ437068	1	RJA
	Puffin Island, Wales.	SH653824	3	RJA
	Strathkinness Low Rd., St. Andrews.	NO484162	2	SAH
	York.	SE590510	1	PA
	Polymorphic populations.			
	Brymbo, Wales.	SJ296539	1	PA
	Devon Street, Grangemouth.	NS977814	3	SAH
	Leith Docks, Edinburgh.	NT268765	1	PA
	Salamander Street, Edinburgh.	NT276763	1	PA
S. vulgaris ssp. vulgaris var. hibernicus.	Central Station, Lincoln.	SK970710	2	SAH
	Brymbo, Wales	SJ296539	1	PA
	Devon Street, Grangemouth.	NS977814	2	SAH
	Mochdre, Wales.	SH822781	1	PA
	Newcraighall, Edinburgh. (Bank).	NT270730	2	JAI
	Newcraighall, Edinburgh. (Road).	NT270730	1	JAI
	Salamander Street, Edinburgh.	NT276763	1	PA
	York.	SE590510	2	RJA
	York.	SE605508	3	JW

Table 6.1 Cont.

Taxon.	Location.	Grid Ref.	No. of Indiv.	Source§
S. squalidus.	Brymbo, Wales.	SJ296539	2	PA
	Devon Street, Grangemouth.	NS977814	5	SAH
	Leith Docks, Edinburgh.	NT268765	1	PA
	Portland Street, Lincoln.	SK970710	1	SAH
	Sheffield.	SK350870	2	PA
	York.	SE590510	4	PA
S. cambrensis.	Brymbo, Wales.	SJ296539	3	PA
	Ffrith, Wales.	SJ286556	1	PA
	Leith Docks, Edinburgh.	NT268765	1	PA
	Mochdre, Wales.	SH822781	2	PA
	Salamander Street, Edinburgh.	NT276763	8	SAH

§ Initials refer to collectors; JAI - Judith Irwin, JW - John Warren, PA - Paul Ashton, RJA - Richard Abbott, SAH - Stephen Harris.

6.2.2 Determination of DNA concentration.

To provide a more accurate measure of total DNA concentration, than is obtained by visual estimation, densitometry was used. Thirty seven DNA samples (1μl) plus three calf thymus DNA (Pharmacia) concentration standards (62.5ng, 125ng, 250ng) were loaded per 0.8% agarose gel (Appendix A, Section A2.4 and A2.5). The gel was photographed on negative film, developed and scanned with a Shimadzu dual-wavelength flying spot scanner (CS-9000; settings were:- wavelength = 550nm, 1mm zigzag scan, minimum peak area = 1000, all others were the default). A linear DNA concentration vs peak area graph was constructed from the standard data and used to convert sample peak area to concentration (ng/μl).

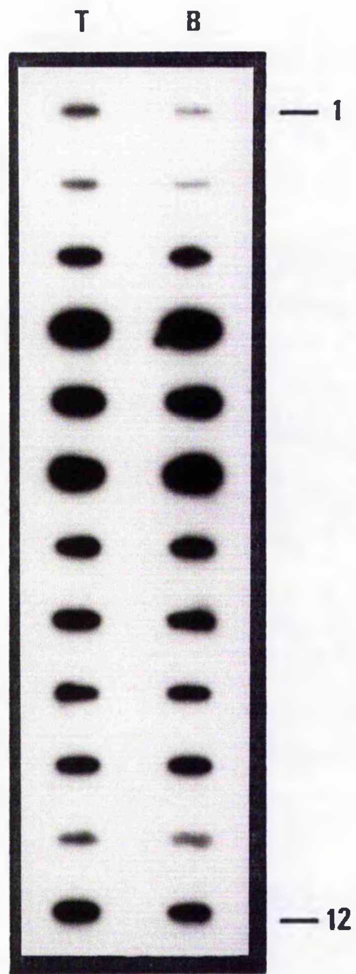
6.2.3 Experimental design.

The slot blot apparatus (Gibco BRL) had two rows of 12 slots available. A total of 96 DNA samples were used, of which 60 were previously defined as the experimental samples. These samples were randomly arranged across eight filters (all filters were prepared from the same batch of Hybond-C Extra). Each filter contained duplicates of each sample in opposite rows (Plate 6.1) and the filters themselves were duplicated (ie. each sample was replicated four times across two filters). In addition to the sample filters, standard filters were prepared in a similar manner (three standards and two controls were replicated four

times across 2 filters). The controls were, buffer alone (to examine background) and buffer plus 100ng calf thymus DNA (to examine non-specific hybridisation). The standards were uncut total plasmid at three concentrations which varied according to the plasmid used.

A total of 18 filters were used, 16 sample filters (2 x 12 samples) and two standard filters (2 x 5 samples). All filters were treated identically following slot blotting of the DNA.

Plate 6.1. Arrangement of DNA samples in a slot blot analysis. Samples probed with the wheat ribosomal DNA probe (pTA71) and washed at 65°C. 'T' indicates top lane and 'B' bottom lane.


T
B
1
12

6.2.4 Slot blot procedure.

The slot blot template was soaked in 1.0M ammonium acetate plus heat-denatured sonicated calf thymus DNA (100μg/ml) for 30 minutes and rinsed in 1.0M ammonium acetate. Prior to use, Hybond-C Extra filters were soaked for 20 minutes in 1.0M ammonium acetate. The apparatus was assembled and used as recommended by the manufacturer.

Samples were prepared according to the method of Rivin (1986). Briefly, DNA samples of known concentration were diluted to 200μl with TE buffer (Appendix A, Section A2.2). The DNA was denatured by adding 20μl 2M sodium hydroxide, vigorously mixing and heating to 95°C for 2 minutes. The sample was neutralised with 55μl 5M ammonium acetate, vigorously mixed and centifuged prior to loading into the wells of the slot blot apparatus. Following loading the tube and slot blot well were rinsed with 400μl 1.0M ammonium acetate, to remove any adhering DNA. After the samples were all loaded the apparatus was disassembled and the filter soaked for 5 minutes in 5xSSC, air dried and baked for 2 hours at 80°C under vacuum. All replicate samples were prepared and loaded independently.

6.2.5 Hybridisation, autoradiography and densitometry.

The filters were probed with only the insert of the probe plasmid, this was prepared as in Appendix A (Section A2.12.4). The three probes used were pSsA45(H5), pSsC15 and pTA71. In the case of pSsA45(H5), the probe was the

smallest fragment (0.63kb) from a *Hin*DIII digest of the psSA45.

All filters were hybridised and washed at the same time. Filters were washed twice in 0.2xSSC + 0.1% SDS (15 minutes each) at four wash temperatures (55°C, 60°C, 65°C, 70°C). Following autoradiography, filters were washed at the next temperature step, until after the 70°C wash they were stripped (Appendix A, Section A2.10) prior to hybridisation with the next probe (at all stages great care was taken to ensure that filters did not dry out). To ensure comparability between sample and standard filters, the standard filters were stripped the same number of times as the sample filters.

Following autoradiography, the area of each signal was determined via densitometry (Shimadzu dual-wavelength flying spot scanner). Each filter autoradiograph was scanned with a 13mm zigzag beam that covered the entire band (slot width = 8mm) and the background determined at the start of each run. The other run parameters were default settings, except for wavelength (550nm) and minimum area (1000 area units). Since the minimum peak area that could be detected in this analysis was 1000 area units, this value was used to indicate a signal from 0 to 1000 area units.

6.2.6 Data analysis.

The DNA samples which were analysed were total genomic extractions (ie. nuclear, chloroplast and mitochondrial

DNAs were present), therefore to determine if chloroplast DNA was a large component of each sample the quantity of chloroplast DNA in each sample was measured by hybridising an invert repeat chloroplast DNA probe (pLsC2, Figure 3.1, Chapter 3) to the sample filters and suitable standards. The quantity of cpDNA loaded per slot was calculated from the peak area data using the formulae derived in Appendix G (Section G1.1).

Area data, which gives a relative indication of the number of sequence copies per genome at a particular stringency was corrected to an equivalent of 100ng of total sample DNA (no account was taken of the very small amount of cpDNA found).

Mean signal intensity readings were calculated for each accession at each stringency and the data was subjected to a series of oneway analyses of variance (ANOVAs). ANOVAs were initially conducted with the VAX-based statistical package GENSTAT. During the course of these calculations the data was tested for deviation from the assumptions of an ANOVA; ie. normality of the data and homogeneity of the variances. Since these assumptions were not fulfilled, all the data points were subjected to a $\log_e$ transformation. The ease of data manipulation provided by the package MINITAB meant that this package was used for all subsequent ANOVAs. Following the oneway ANOVA to test for significant difference between taxa, unplanned pairwise comparisons of the means were conducted to test for significant differences between taxa pairs. The tests used

were the T′-method, the GS2 method and the Tukey-Kramer method (Sokal and Rohlf 1981).

Results.

6.3.1 'Copy number' analysis of *Senecio* species.

Estimation of the quantity of chloroplast DNA present in total DNA extracts of *Senecio* species revealed that between 0.001% and 0.240% (mean 0.031%, SE 0.004%) of the DNA sample showed similarity to the chloroplast DNA under the hybridisation conditions used (0.2xSSC +0.1% SDS at 55°C). No account was taken of this minute quantity of cpDNA when correcting the samples to a standard of 100ng DNA. Similarly, no attempt was made to estimate the quantity of mitochondrial DNA present in each sample.

The graphs shown in Figures 6.1 to 6.3 summarise the variation revealed between *Senecio cambrensis*, *S. squalidus* and *S. vulgaris* ssp. *vulgaris sl* for the probes pTA71, pSsA45(H5) and pSsC15 respectively.

The data derived in this study has been treated simply on the basis of signal intensity corrected to a standard quantity of total DNA (100ng), thus between wash comparisons are not strictly legitimate, due to the absence of suitable standards. This may be seen in the case of pSsA45(H5), where the 55°C wash has a lower signal intensity than the 60°C wash (probably as a result of difference in exposure time for the two sets of autoradiographs). No attempt has been made to derive either

a measure of percentage 'homologous' DNA (Rivin 1986), and hence copy number or equivalent copy number (Strauss and Tsai 1988), since the standards reached signal saturation and were, therefore, non-linear in their response. An additional difficulty was the wide variation between sample signal intensity and the standard signal intensity.

The oneway ANOVA results associated with probes pTA71, pSsA45(H5) and pSsC15 are shown in Tables 6.2 to 6.4 respectively. Following the suggestion of Sokal and Rohlf (1981) the results for the Tukey-Kramer method of unplanned pairwise comparisons are presented since these gave the smallest values for MSD (minimum significant difference).

Table 6.2 Mean, standard errors and oneway ANOVA results for probe pTA71°.

Temperature (°C).		Taxa			
		Sc	NR	RR	Ss
55	n	-	-	-	-
	Mean.	-	-	-	-
	Range.	-	-	-	-
	SE.	-	-	-	-
60	n	14	12	13	13
	Mean.	46338	68859	81112	193263
	Range.	6461-124740	13238-245751	15905-269095	9610-592510
	SE.	8451	21710	20330	50587
65	n	14	15	15	15
	Mean.	12221	29924	51151	78658
	Range.	1727-31905	1833-123575	2975-265881	2897-33925
	SE.	2270	10205	17525	26312
70	n	15	15	15	15
	Mean.	4970	7746	8145	25476
	Range.	657-12961	487-41254	883-23694	300-137962
	SE.	1114	2990	1853	10745

	Wash Temperature (°C)°			
	55	60	65	70
Sc vs NR vs RR vs Ss	-	*	ns	ns
NR vs RR	-	ns	ns	ns
RR vs Ss	-	ns	ns	ns
NR vs Sc	-	ns	ns	ns
NR vs Ss	-	**	ns	ns
RR vs Sc	-	ns	ns	ns
Ss vs Sc	-	**	*	ns

° Sc - Senecio cambrensis, Ss - S. squalidus, NR - S. vulgaris ssp. vulgaris var. vulgaris and RR - S. vulgaris ssp. vulgaris var. hibernicus, * - 0.05 < p < 0.01, ** - 0.01 < p < 0.001, ns - non-significant.

Table 6.3 Mean, standard errors and oneway ANOVA results for probe pSsC45(H5)°.

Temperature (°C).		Taxa			
		Sc	NR	RR	Ss
55	n	12	14	14	14
	Mean.	74165	85539	125697	148619
	Range.	31236-172580	26983-193201	49590-224224	36531-317431
	SE.	12637	13482	14996	23292
60	n	15	14	14	14
	Mean.	86529	104158	162427	236345
	Range.	38961-217854	34701-247972	67764-275214	44419-527549
	SE.	14144	17908	19877	37406
65	n	15	12	12	12
	Mean.	85682	100706	181008	181897
	Range.	22752-260651	20178-288485	48676-355711	32553-443601
	SE.	16901	20191	31356	33925
70	n	15	15	15	15
	Mean.	15965	10675	26922	47928
	Range.	2135-62916	1575-37970	3675-67937	4592-131454
	SE.	4743	2490	7481	11140

	Wash Temperature (°C)			
	55	60	65	70
Sc vs NR vs RR vs Ss	*	*	**	*
NR vs RR	ns	ns	ns	ns
RR vs Ss	ns	ns	ns	ns
NR vs Sc	ns	ns	ns	ns
NR vs Ss	*	**	ns	**
RR vs Sc	ns	ns	*	ns
Ss vs Sc	*	**	*	*

° Sc - Senecio cambrensis, Ss - S. squalidus, NR - S. vulgaris ssp. vulgaris var. vulgaris and RR - S. vulgaris ssp. vulgaris var. hibernicus, * - $0.05 < p < 0.01$, ** - $0.01 < p < 0.001$, ns - non-significant.

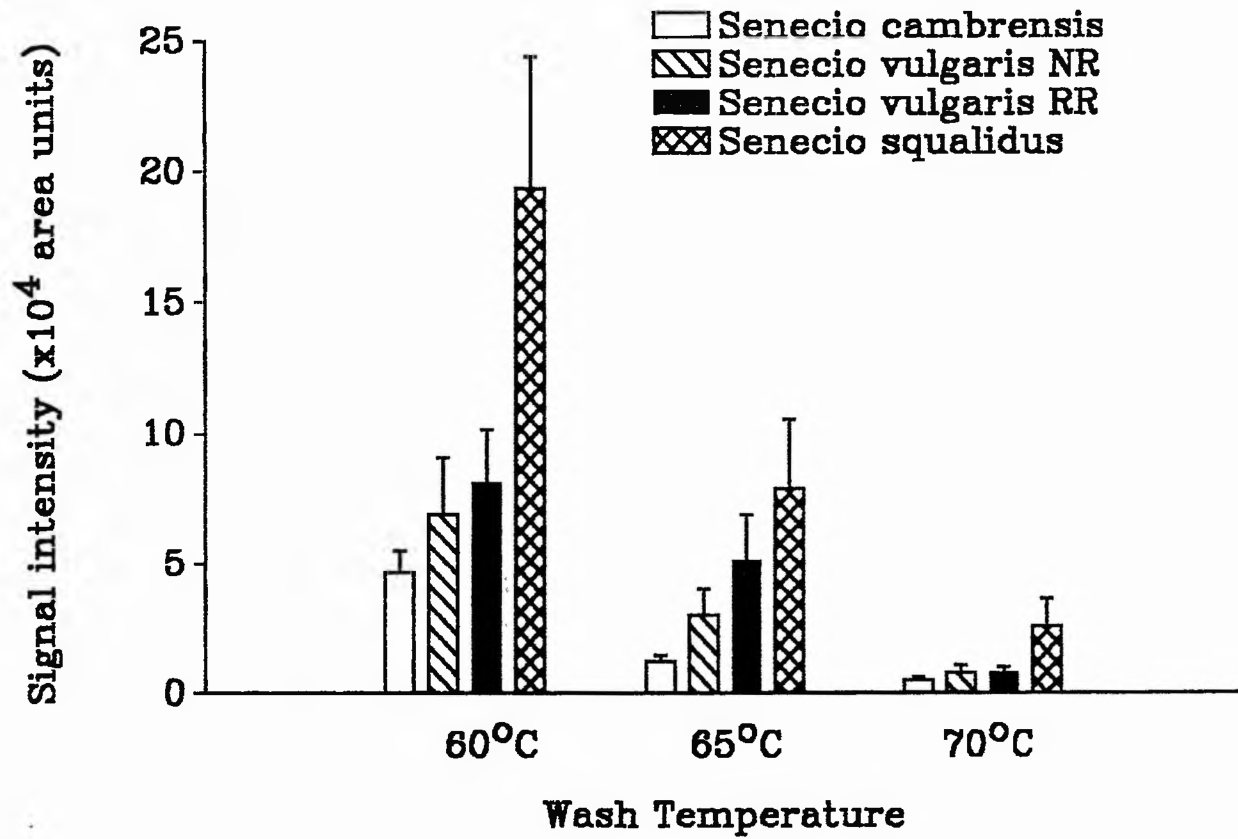
Table 6.4 Mean, standard errors and oneway ANOVA results for probe pSsC15°.

Temperature (°C).		Taxa			
		Sc	NR	RR	Ss
55	n	13	15	15	15
	Mean.	116472	138404	236816	270855
	Range.	27931-598183	32969-415505	47618-560777	34061-666397
	SE.	42782	31937	45469	50875
60	n	15	15	15	15
	Mean.	41560	46701	82363	85766
	Range.	4310-90563	5514-141605	4419-234429	5719-286250
	SE.	15303	13317	18362	20735
65	n	15	15	15	15
	Mean.	21145	31111	38554	45637
	Range.	2034-118879	2051-82433	1791-148592	2772-173868
	SE.	8462	6055	11680	12367
70	n	15	15	15	15
	Mean.	10819	11820	20804	26064
	Range.	1064-70302	1718-47965	1528-86395	1464-112794
	SE.	4751	3444	6742	7887

	Wash Temperature (°C)			
	55	60	65	70
Sc vs NR vs RR vs Ss	**	ns	ns	ns
NR vs RR	ns	ns	ns	ns
RR vs Ss	ns	ns	ns	ns
NR vs Sc	ns	ns	ns	ns
NR vs Ss	ns	ns	ns	ns
RR vs Sc	**	ns	ns	ns
Ss vs Sc	**	ns	ns	ns

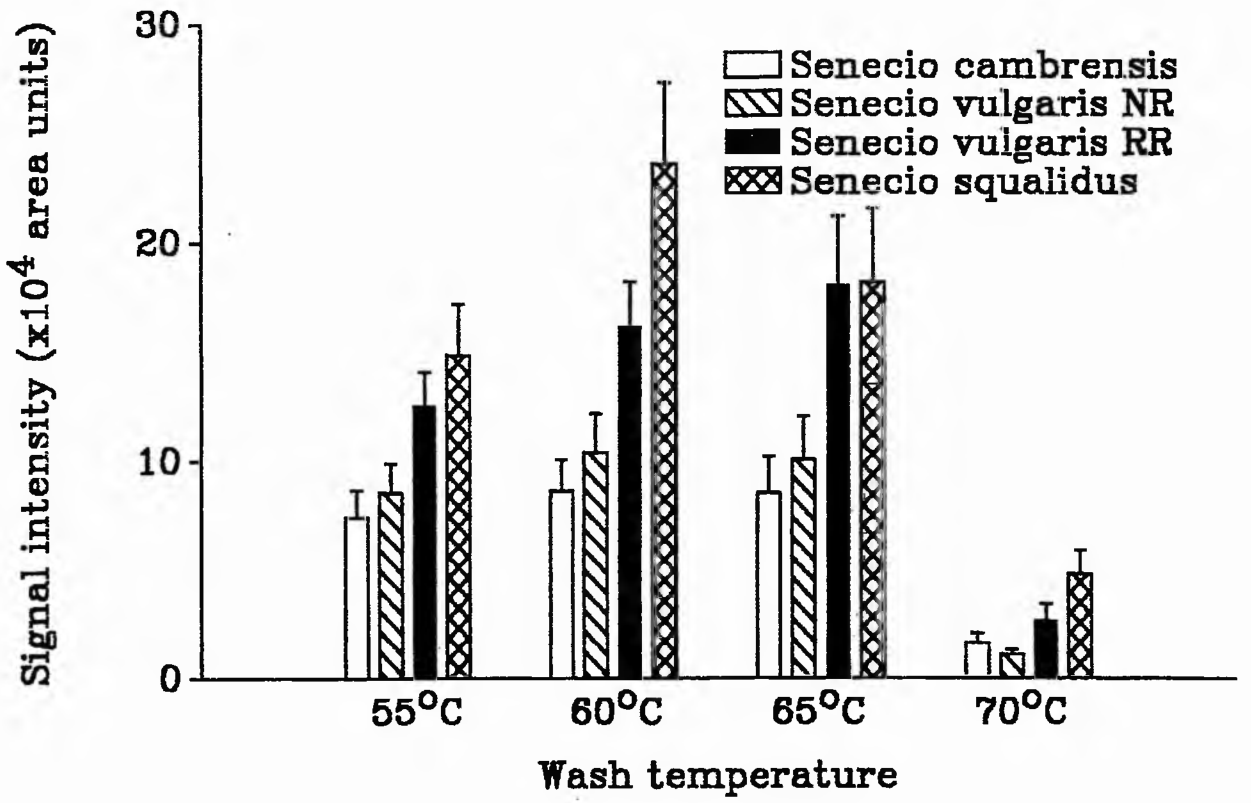
° Sc - Senecio cambrensis, Ss - S. squalidus, NR - S. vulgaris ssp. vulgaris var. vulgaris and RR - S. vulgaris ssp. vulgaris var. hibernicus, ** - $0.01 < p < 0.001$, ns - non-significant.

Figure 6.1. Mean signal intensity of *Senecio cambrensis*, *S. squalidus*, and *S. vulgaris* ssp. *vulgaris sl* when hybridised to the heterologous wheat ribosomal DNA probe pTA71 and washed at different temperatures in a standard buffer (0.2xSSC + 0.1% SDS). Bars indicate standard errors



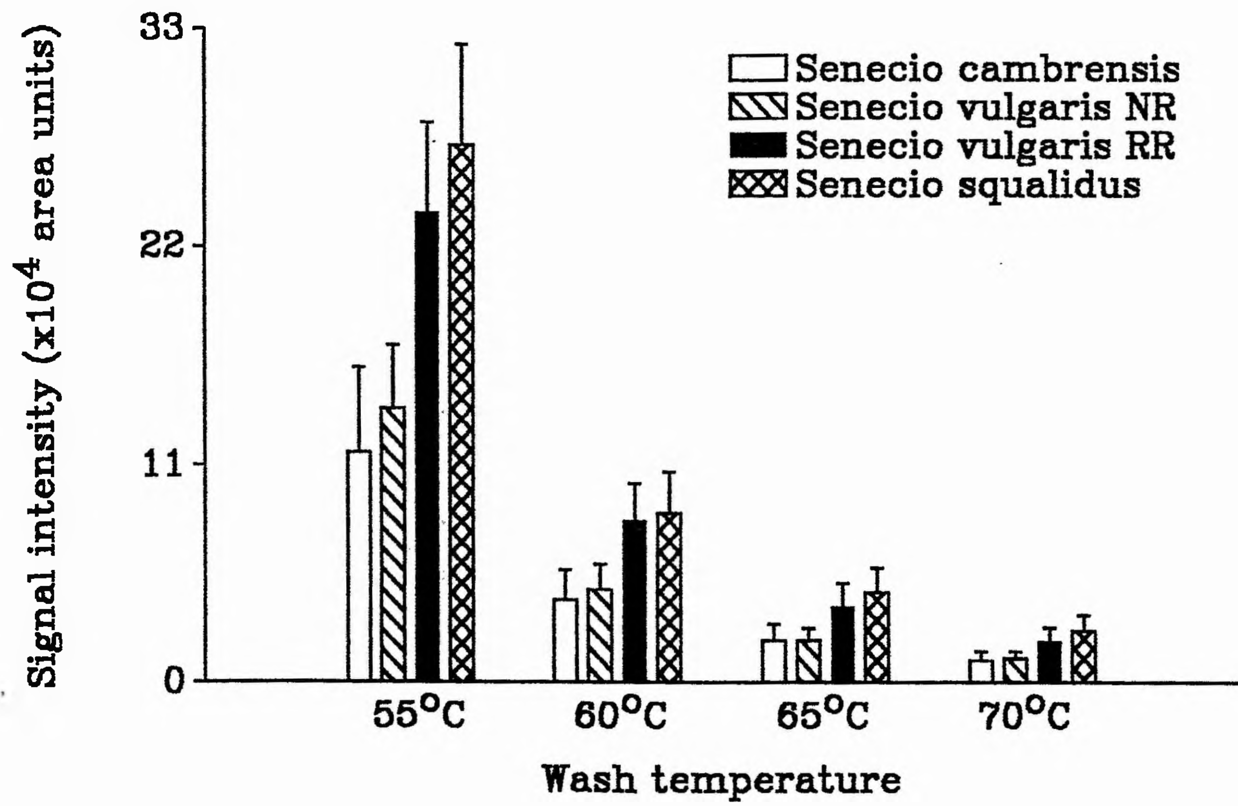
Senecio cambrensis
Senecio vulgaris NR
Senecio vulgaris RR
Senecio squalidus
Signal intensity (x10⁴ area units)
25
20
15
10
5
0
60°C
65°C
70°C
Wash Temperature

Figure 6.2. Mean signal intensity of *Senecio cambrensis*, *S. squalidus*, and *S. vulgaris* ssp. *vulgaris sl* when hybridised to the *S. squalidus* random nuclear probe pSsA45(H5) and washed at different temperatures in a standard buffer (0.2xSSC + 0.1% SDS). Bars indicate standard errors



Signal intensity (x10^4 area units)
30
20
10
0
Senecio cambrensis
Senecio vulgaris NR
Senecio vulgaris RR
Senecio squalidus
55°C
60°C
65°C
70°C
Wash temperature

Figure 6.3. Mean signal intensity of *Senecio cambrensis*, *S. squalidus*, and *S. vulgaris* ssp. *vulgaris sl* when hybridised to the *S. squalidus* random nuclear probe pSsC15 and washed at different temperatures in a standard buffer (0.2xSSC + 0.1% SDS). Bars indicate standard errors



Signal intensity (x10⁴ area units)
33
22
11
0
Senecio cambrensis
Senecio vulgaris NR
Senecio vulgaris RR
Senecio squalidus
55°C
60°C
65°C
70°C
Wash temperature

Discussion.

The experiments which have been reported in this Chapter have shown that the two *Senecio squalidus* random genomic clones do not represent *squalidus*-specific probes. However, further analysis of the data to obtain biosystematically useful data has not proved possible.

The slot blot analysis of sequence similarity measures two variables at any one temperature; the number of copies of a particular sequence which are present (Rivin 1986) and the GC content of the sample DNA relative to that of the probe (Zhao *et al* 1989). Considering the nature of the data, two features of the results presented in Figure 6.1 to Figure 6.3 are of interest:-

(i) For those probe-temperature combinations which show a significant difference between taxa, *Senecio cambrensis* always has a lower mean signal intensity than *S. squalidus*. This was unexpected, almost inexplicable. In a large study conducted with nine repetitive sequences, Rivin *et al* (1986) showed that the copy number of each sequence varied independently of the others, which would be expected. The parallel distribution of signal intensity among the probes may be a result of the small sample of probes analysed.

(ii) *Senecio squalidus* has a greater mean signal intensity at higher temperatures than either *S. cambrensis*

or *S. vulgaris* ssp. *vulgaris* var. *vulgaris* for some of the probe temperature combinations (Tables 6.2 - 6.4). This indicates that *S. squalidus* has a greater sequence similarity to each of the probes compared to the other taxa. This result is not too suprising for the probes isolated from *S. squalidus* [pSsA45(H5) and pSsc15] but is unexpected for the heterologous wheat rDNA probe. In the latter case it would be expected that all *Senecio* taxa would be more-or-less equally divergent from wheat rDNA, rather than the situation suggested here where *S. squalidus* rDNA is more similar to wheat rDNA than are the other *Senecio* taxa studied.

Both of these observations suggest that biosystematic inferences cannot reliably be drawn and that caution should be used in the interpretation of these results, since they may be explained as artifacts based on either saturation of the X-ray film during autoradiography (Rivin 1986) or wide variation in sequence divergence between taxa. The latter point is potentially the case with the rDNA probe since it is known that in DNA-DNA hybridisation thermal stability comparisons sequence divergences of greater than approximately 15% leads to unreliable results (Springer and Krajewski 1989).

An additional consideration is that probes may not represent a homogeneous class of sequence (in terms of evolutionary conservation between taxa). This is certainly the case with the rDNA probe (see Chapter 2) and probably the case with probe pSsC15 (since it represents a 6.2kb sequence). The probe pSsA45(H5) probably represents a

single class of sequence since it is very small (0.630kb). Variation in sequence conservation within a probe can influence the melting properties of DNA-DNA duplexes (Appels and Dvorak 1982a,b).

These problems are unlikely to have occurred if the two probes had proved to be taxon-specific in an 'all-or-nothing' manner. If such probes could be isolated in *Senecio* they would be very valuable in the study of hybridisation and introgression.

Chapter 7.

General discussion.

"He went like one who hath been stunned
And is of sense forlorn,
A sadder and a wiser man
He rose the morrow morn."

Ancient Mariner
S. T. Coleridge.

A considerable body of biosystematic data exists on some British members of the genus *Senecio* and their European relatives. Morphological (Taylor 1984), cytogenetic (eg. Weir and Ingram 1980, Kadereit 1984a,b) and isozyme (Ashton 1990) studies have contributed to this synthesis, which provides an almost unique opportunity to assess the efficiency of molecular biological techniques in analysing evolutionary processes in closely related taxa. It has been demonstrated that molecular evidence can provide new insights into relationships, but can also produce results which are either contradictory to other evidence or inconclusive. Due weight has been accorded to the reasons for such failures in molecular methodology.

Throughout this research, whether analysing the chloroplast or nuclear genomes, three major questions have been addressed:-

(i) What is the degree of intraspecific DNA variation present in *Senecio squalidus*, *S. vulgaris sl* and *S. cambrensis*?

(ii) Did *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus* originate via the introgression of *S. squalidus* genes into *S. vulgaris* ssp. *vulgaris* var. *vulgaris*?

(iii) Is *Senecio cambrensis* the allohexaploid hybrid of *S. squalidus* and *S. vulgaris* ssp. *vulgaris*?

7.1 Intraspecific DNA variation.

7.1.1 *Senecio vulgaris sl*.

Extensive variation has been revealed in both the ribosomal DNA (rDNA) and chloroplast DNA (cpDNA) analyses. At the varietal level no differences in the ranges of rDNA (Chapter 2, Tables 2.2 and 2.3) and cpDNA variation (Chapter 3, Section 3.4.4) have been found, although some evidence was obtained which suggests that some populations of var. *hibernicus* may have different rDNA phenotype frequency distributions (Chapter 2, Section 2.4.1).

At the subspecific level, rDNA revealed distinctive phenotypes between spp. *vulgaris* and ssp. *denticulatus* (Chapter 2, Tables 2.2 and 2.3). These differences were constant between the two populations of ssp. *denticulatus* which were examined. The cpDNA of Ainsdale ssp. *denticulatus* is however, distinct from that of Jersey (which has a ssp. *vulgaris* type cpDNA; Chapter 3, Section 3.3.6).

Both rDNA variation and cpDNA variation have contributed to new insights at the intraspecific level. That is, the distinctive nature of the two subspecies has been confirmed and new questions concerning the origin of

cpDNA variation have been raised. The pattern of cpDNA variation in ssp. *denticulatus* may be explained by one or all of the hypotheses discussed in Chapter 4 (Section 4.4.6).

Hypotheses which invoke introgression of cpDNA into one of the two ssp. *denticulatus* populations would appear unlikely since it would require that introgressive events had no effects on the rDNA of these two populations (Section 6.2). Also, the distinction between these two populations was shown in an isozyme survey of *Senecio* species conducted by Ashton (1990) in which two out of 25 isozyme loci that were surveyed showed Jersey and Ainsdale populations of ssp. *denticulatus* to be distinct and the Jersey population to be identical to ssp. *vulgaris* in its range of variation.

To explain the cpDNA results it is proposed that ssp. *denticulatus* may have had a multiple origin, via reciprocal allopolyploid speciation events between two taxa, one of which had the J (Jersey) type cpDNA and the other which had the A (Ainsdale) type cpDNA. This hybridisation occurred, at least twice, once with the J-type cpDNA donor as the female and once with the A-type cpDNA donor as the female. Following the origin of these two types of ssp. *denticulatus* cpDNA, ssp. *vulgaris* cpDNA may have evolved from the J-type ssp. *denticulatus* cpDNA.

The hybridisation may have occurred at either the diploid (followed by chromosome doubling) or at the tetraploid level. If the hybridisation occurred at the diploid level then chromosome doubling would have to be

proposed in two separate lineages. The more parsimonious solution would be to have the hybridisation occurring at the tetraploid level. Kadereit (1984a) has suggested a similar hypothesis for the origin of *S. vulgaris* ssp. *vulgaris* via ssp. *denticulatus*, but in this case an autopolyploid event with *S. vernalis* as the progenitor was proposed.

A hypothesis which required a single origin of ssp. *denticulatus* would have to explain the large mean cpDNA divergence between ssp. *denticulatus* and ssp. *vulgaris* (1.021% to 1.886%, Tables E1 and E2, Appendix E). Zurawski *et al* (1984) estimated that the synonymous rate of sequence change of the cpDNA-encoded large subunit of the RuBisCO (*rbc*L) gene among grasses is 1 x 10^{-9} nucleotide substitutions per year. If the *Senecio vulgaris sl* cpDNA is evolving at a similar rate (however unrealistic this might be, see Clegg 1990), then the estimated time of divergence between type-A and type-J cpDNAs is 10 to 19 million years!

Multiple species origins, inferred from cpDNA, have been reviewed in Chapter 3, Section 3.1.3 and 3.1.4. Of particular interest here is the observation that racial differences in tetraploid *Aegilops triuncialis* may be the result of reciprocal crosses between the diploids, *A. caudata* and *A. umbellulata* (Murai and Tsunewaki 1984), which seems to parallel the situation in *Senecio*.

A survey of European ssp. *denticulatus* would be expected to reveal populations with either an A-type or a J-type cpDNA. A more extensive study of the taxa related to

S. vulgaris may reveal the progenitor of *S. vulgaris* using a combination of rDNA, cpDNA and isozyme analyses.

7.1.2 *Senecio squalidus*.

Senecio squalidus again shows intraspecific variation in both the rDNA and cpDNA. The rDNA phenotypes could be divided into six phenotypes on the basis of the three enzymes (Chapter 2, Tables 2.2 and 2.3). All of these phenotypes were distinct from those of *S. vulgaris sl*, as was the presence of two repeat lengths in all individuals examined. Thus the two species could be readily distinguished on the basis of rDNA phenotypes.

Although there is extensive cpDNA variation within *Senecio squalidus* the range of restriction profiles are apparently identical to those of *S. vulgaris* ssp. *vulgaris sl*. This is a most suprising result in the face of the rDNA differences between the two species. A phylogenetic or phenetic analysis based on cpDNA data alone would clearly indicate that *S. squalidus* is more closely related to ssp. *vulgaris* (and Jersey ssp. *denticulatus*) than either is to Ainsdale ssp. *denticulatus* (Chapter 3, Figures 3.3 and 3.4). The rDNA analysis gives a quite different picture. This emphasises the necessity to view apparently contradictory results with scepticism and to take into account all lines of evidence.

The apparent similarity of *Senecio squalidus* and *S. vulgaris* ssp. *vulgaris* cpDNAs would suggest that they have closely related chloroplast genomes. The chloroplast DNA

data would appear to be at odds not only with rDNA data, but also with morphological (Taylor 1984), biochemical (Ashton 1990) and some cytological (Kadereit 1984b) evidence.

The similarity could be explained on the basis of a common, slowly diverging chloroplast genome in most European diploid species of *Senecio*, which is shared by *S. squalidus* and progenitor of *S. vulgaris* ssp. *vulgaris sl*. Alternatively it could be explained by hybridisation events following the introduction of *S. squalidus* to Britain (Chapter 3, Section 3.4.5).

The resolution of this enigma lies in an analysis of the European members of the genus *Senecio*. Additional enzymes to analyse both the nuclear and chloroplast genomes would potentially increase resolution and determine whether the similarity of the chloroplast genomes of these two taxa is real or a sampling artifact. Sequencing portions of the chloroplast genome, particularly the region covered by probe pLsC6 (Figure 3.1) may be very instructive.

7.2 The introgressive origin of *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus*.

The ribosomal DNA provides no evidence either for or against the introgressive origin of var. *hibernicus* (Chapter 2, Section 2.4.1.1). The data does, however, suggest that restriction analysis (at least the limited

scope of the present study), using a heterologous wheat rDNA probe, may not be the appropriate approach.

The apparent absence of *Senecio squalidus* rDNA in; (i) one accession (RJA) of var. *hibernicus* from York, which has been investigated by Irwin (1990) who suggested that this may have been the result of fusion between an unreduced *S. squalidus* gamete and a haploid *S. vulgaris* gamete (Chapter 2, Section 2.4.1.1) and (ii) in *S. cambrensis* from Salamander Street would suggest that rDNA may not be an appropiate marker to study introgression in *Senecio*.

Similarly, the absence of apparent variation between the chloroplast genomes of *Senecio vulgaris* and *S. squalidus* did not allow the possible introgressive introduction of *S. squalidus* cytoplasm into *S. vulgaris sl* to be analysed.

In general the mean number of nuclear markers will be halved at each backcross generation and the mean number of chloroplast markers will be one half of this (Rieseberg *et al* 1990, Avise and Saunders 1984). Hence, with a limited sampling of two genomes (as in this this study) the inability to identify introgression is not too suprising.

7.3 The hybrid origin of *Senecio cambrensis*.

Senecio cambrensis produced additive rDNA phenotypes in all the populations studied, except the majority of the individuals from Salamander Street in Edinburgh (Chapter 2, Section 2.4.2). Thus the hybrid nature of *S. cambrensis* has

been confirmed, at least for those populations other than Salamander Street. The apparent non-hybrid nature of the accessions from Salamander Street, conflicts with the nuclear-encoded isozymes which indicate that a hybrid nucleus is present in the material at this site.(Chapter 2, Section 2.4.2).

The occurrence of apparently identical cpDNAs in *Senecio squalidus* and *S. vulgaris* ssp. *vulgaris sl* has meant that the maternal parent of *S. cambrensis* could not be identified. Isozyme studies have revealed that *Senecio cambrensis* has had at least two origins in the British Isles (in Wales and Scotland, Ashton 1990). A dual origin, in Wales and Scotland, is supported by the occurrence of a 350bp insertion in the cpDNA of Welsh *S. cambrensis* which was absent from Scottish *S. cambrensis* and all the other *Senecio* taxa studied (Chapter 3, Section 3.4.7).

7.4 Conclusions.

Both the nuclear and chloroplast genomes have provided some new insights into the relationships and possible origins of members of the genus *Senecio*. As a result of a more extensive data set being available and the fewer problems of interpretation, the chloroplast genome appears to hold considerable promise for analysing phylogenetic relationships, particularly the origin of *S. vulgaris sl*. The species-specific rDNA phenotypes produced by most of the taxa that have been studied (Chapter 2, Section 2.3.2)

may be of use in the identification of material and possibly of hybrids, if the results obtained from Salamander Street *S. cambrensis* are a special case and not the rule.

One facet of this thesis has been the attempt to provide an overview of some of the exciting applications of molecular biology, in the pursuit of answers to biosystematic questions. However, at the same time the problems of these techniques have been highlighted and the view taken that; 'Molecular Techniques' are not a panacea to answer all biosystematic challenges. Rather, molecular biology is only one weapon in an armoury which is available to biosystematists.

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" Quoth the Raven, 'Nevermore.' "

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Appendices.

" '... , we want nothing but Facts, Sir; nothing but Facts!' "

Hard Times
C. Dickens.

Appendix A.

Methods.

A2.1 Growth of plant material.

Achenes were initially sown out on to damp Whatman No.1 filter paper in 5cm tissue culture dishes, and grown until the seedlings reached the first leaf stage. Seedlings were then potted up into 5cm pots containing Arthur Bower universal compost. The plants were subsequently potted-on into 10cm pots. The plants were illuminated for 16 hours per day using 400W halogen or mercury lamps and watered when necessary. Leaf material was harvested when the plants were 10-12 weeks old.

Herbarium vouchers were prepared for representatives of all populations sampled. Specimens were deposited in St. Andrews University herbarium (StA).

A2.2 DNA extraction.

The extraction of intact DNA from the genus *Senecio* was found to be difficult, probably as a result of the number and diversity of secondary plant metabolites that are found in the Tribe Senecioneae (Robins 1977). A number of different methods were tried but only one was found to be consistently successful. Details of the other methods used are given in Appendix B. The successful method was a modification of the protocol of Hattori *et al* (1987).

1. Intact, healthy leaves were excised from the plant and washed in iced water to remove any soil or other adhering debris. Leaves that showed very slight fungal infection were washed in iced 5% sodium hypochlorite (BDH Chemicals) and rinsed in iced water. Before storage at -20°C the leaves were blotted dry and sealed in plastic freezer bags.

2. Approximately 10g of frozen leaves were ground to a very fine powder with liquid nitrogen in an ice-cold mortar. If the material started to thaw more liquid nitrogen was added and the grinding continued. Approximately 30ml of cold extraction buffer (Buffer E) was added to the powder, which was ground again to form a homogeneous paste. The paste was allowed to thaw and more Buffer E was added to give a total of 5-10ml/g fresh weight of material.

3. The homogenate was transfered to a sterile 250ml centrifuge bottle, 20ml of redistilled phenol added and centrifuged at 7000 rpm at 4°C for 10 minutes in a MSE centrifuge (6 x 250ml rotor head). The upper

aqueous layer was removed and sequentially treated with 20ml phenol and 20ml 'wet' chloroform. At each stage the aqueous layer was separated by centrifuging at 7000 rpm at 4°C for 10 minutes. The aqueous extract was treated with 5ml diethyl ether (BDH Chemicals) and the ether chased from the aqueous phase by warming the extract to 65°C, in a water bath, until it was clear (approximately 30 minutes).

4. A bed of polyvinylpolypyrrolidone (PVPP, Sigma P6755) was prepared by washing 2g PVPP per 100ml extraction buffer with 100mM HCl, neutralising with 50mM Tris-HCl (pH 8.0) and filtering through Whatman No. 1 filter paper in a Buchner funnel. The cool extract was filtered through the bed of PVPP by vacuum.

5. Sodium chloride (BDH Chemicals) was added to a final concentration of 0.5M and polyethyleneglycol 8000 (PEG 8000, Sigma P2139) was added to 10% w/v. The extract was gently agitated and then left overnight at 4°C.

6. The nucleic acid-PEG complex was recovered by centrifuging at 8000 rpm at 4°C for 30 minutes. The supernatant was discarded and the pellet resuspended in 5ml of TE buffer at 37°C. DNAase-free RNAase was added to a concentration of 50µg/ml and incubated for 1 hour at 37°C.

7. The extract was deproteinated by adding 2ml phenol and centrifuging at 10000 rpm at room temperature for 10 minutes in a Sorvall SS-34 or SA-600 rotor. The upper aqueous phase was removed and the procedure repeated with 2ml of 'wet' chloroform. The aqueous phase was removed and one-third the volume of 7.5M ammonium acetate (BDH Chemicals) and 2 volumes of ethanol (BDH Chemicals) or propan-2-ol (BDH Chemicals) were added. DNA was precipited overnight at -20°C.

8. The DNA pellet was recovered by centrifugation at 10000 rpm at 4°C for 30 minutes and air-dried at room temperature under vacuum. The pellet was dissolved overnight in approximately 500µl TE.

Buffer-E.

100mM Tris-HCl (pH 8.0) (BDH Chemicals).
50mM disodium-EDTA (BDH Chemicals).
1% sodium dodecyl sulphate (SDS, BDH Chemicals).

Phenol.

Phenol (BDH Chemicals) was redistilled into 50mM Tris-HCl (pH 8.0), 10mM disodium-EDTA.

aqueous layer was removed and sequentially treated with 20ml phenol and 20ml 'wet' chloroform. At each stage the aqueous layer was separated by centrifuging at 7000 rpm at 4°C for 10 minutes. The aqueous extract was treated with 5ml diethyl ether (BDH Chemicals) and the ether chased from the aqueous phase by warming the extract to 65°C, in a water bath, until it was clear (approximately 30 minutes).

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8. The DNA pellet was recovered by centrifugation at 10000 rpm at 4°C for 30 minutes and air-dried at room temperature under vacuum. The pellet was dissolved overnight in approximately 500µl TE.

Buffer-E.

100mM Tris-HCl (pH 8.0) (BDH Chemicals).
50mM disodium-EDTA (BDH Chemicals).
1% sodium dodecayl sulphate (SDS, BDH Chemicals).

Phenol.

Phenol (BDH Chemicals) was redistilled into 50mM Tris-HCl (pH 8.0), 10mM disodium-EDTA.

'Wet' Chloroform.

24 parts chloroform (BDH Chemicals)
1 part isoamyl alcohol (Sigma) or octanol (BDH Chemicals).

TE buffer.

10mM Tris-HCl (pH 8.0).
1mM disodium-EDTA.

A2.3 DNA purification.

Two methods for the further purification of the crude DNA extract were used. Caesium chloride density gradient centrifugation was used occasionally when very high purity DNA was required or when large quantities of DNA were available. However, DNA was routinely purified using DEAE-Sephacel column chromatography.

A2.3.1 Caesium chloride density gradient centrifugation.

When high purity DNA or chloroplast DNA-enriched genomic DNA was required, a CsCl gradient step was incorporated after step 6 of the extraction protocol (Section A2.2).

1. Caesium chloride and ethidium bromide were dissolved in the nucleic acid solution to give a final concentration of 0.75g/ml caesium chloride (BDH Chemicals) and 200µg/ml ethidium bromide (BDH Chemicals). Gradients were centrifuged at 40000 rpm in a fixed angle Sorvall T865.1 rotor at 20°C for 20 hours.

2. The nuclear DNA band was removed from the gradient using a No.19 hypodermic needle and treated with two volumes of TE-saturated butan-1-ol (BDH Chemicals), to remove ethidium bromide. This step was repeated until the organic, upper layer was no longer discoloured.

3. DNA was recovered by precipitation with two volumes of propan-2-ol overnight at room temperature. The DNA pellet was recovered by centrifugation for 30 minutes at 10000 rpm at room temperature in a Sorvall SS-34 or SA-600 rotor. The pellet was air-dried at room temperature under vacuum, then dissolved in 500µl of TE.

A2.3.2 DEAE-Sephacel column chromatography.

Following DNA extraction (Section A2.2) an additional DEAE-Sephacel purification step was routinely incorporated.

1. 1ml DEAE-Sephacel (Sigma I6505) columns were prepared in 5ml syringes using glass fibre filter paper (Whatman GF/A) as the support (NB. 1ml Sephacel will be saturated with approximately 30µg of DNA). The

columns were 'charged' by washing with 3 volumes of 0.6M NaCl in TE, followed by 3 volumes of TE.

2. The DNA solution was diluted to 1ml with TE, added to the column and the run-off collected and reapplied to the column. Three volumes of 0.3M NaCl in TE were added to the column to wash off contaminants, and the run-off discarded. To elute the DNA from the column, 3 volumes of 0.6M NaCl were added and the run-off collected in 500μl aliquots.

3. To each of these aliquots 1ml of propan-2-ol was added and the DNA precipitated overnight at -20°C. The DNA pellet was collected and dried as before, prior to dissolving the DNA sample in a total volume of 100μl of TE. Samples of DNA prepared in this way were stored at 4°C until required.

A2.4 DNA concentration determination.

Three methods of DNA concentration determination were used; UV spectrophotometry, densitometry and visual estimation, but only the latter method was used on a regular basis. For a description of the spectrophotometric method and a discussion of the choice of visual estimation, see Appendix C. The densitometry methodology is given in Chapter 6.

1. To determine the concentration of DNA by visual estimation, a known volume of sample DNA and a standard DNA of known concentration (intact calf thymus DNA at 0.125μg/ml) were run on a 0.8% agarose gel (Section A2.5).

2. The relative concentration of the sample DNA was determined by visual comparison to the fluoresence of the standard. From this the approximate concentration of the sample DNA in terms of ng/μl was determined. This method also allowed a direct assessment of the size of the DNA to be made. The DNA extraction method (Section A2.2) typically gave yields of 5-15μg/g fresh weight of leaf material from *Senecio* species. These DNA yields were apparently influenced by the conditions under which the plants were grown, their age and their species.

A2.5 Agarose gel electrophoresis.

1. To prepare an agarose gel, agarose (Sigma A6013) was dissolved at an appropiate concentration (w/v) in 1xSEB by gentle heating over a bunsen burner, with constant mixing. Once the agarose was dissolved the solution was allowed to boil for a further 5-10 minutes before ethidium bromide was added at a concentration of 0.5μg/ml. The gel solution was allowed to cool to about 50°C before pouring.

2. The gel rigs used were either Gallenkamp Biomed Maxicells (ELE-410) or Biomed Minicells (ELE-400). The open ends of the gel mould, were blocked off with adhesive tape and an appropiate gel comb aligned vertically in the mould. The tape-mould interface was sealed with molten gel solution which was allowed to cool.

3. The gel solution was then poured into the gel mould to a depth of approximately 5mm, by a continuous action to avoid air bubbles in the gel matrix. The gel was allowed to cool for at least 30 minutes, before the ends were untaped and the gel mould arranged in the gel apparatus. Running buffer (1xSEB) was added to just cover the top of the gel.

4. Samples were loaded in the gel wells using a Gilson Pipetteman. The DNA was run towards the positive electrode at an initial current of 100mA. Once the marker had run about 1cm into the gel, the current was reduced to 40mA and the gel run until the marker had run 15-20cm. On each gel size standards were included, which were either Lambda-*Hin*DIII (NBL) or Lambda-*Pst*I (NBL) digests.

5. Once the gel had run, the DNA was visualised on a UV transilluminator (UVP Incorp.) and recorded by photography using Polaroid 667 film.

1x SEB.

0.04M Tris-HCl.
0.02M sodium acetate trihydrate (BDH Chemicals).
1mM disodium-EDTA.
pH to 7.85 with glacial acetic acid.

A2.6 Restriction enzyme digestion.

The restriction enzymes which have been used in this study and their incubation conditions are shown in Table A1. All of the enzymes were purchased from Sigma, Koch-Light or NBL, depending on the cheapest source.

1. Total genomic digestions usually consisted of:

1μg total DNA.
3μl digestion buffer.
5-10 units of enzyme.
distilled water to make up the volume upto 30μl.

2. Following overnight incubation the reactions were stopped by the addition of 1/10 volume of stop buffer.

When digestions were incubated at 65°C, the reactions were overlaid with silicone fluid (Dow Corning 200/50cs), to prevent evapouration.

Stop buffer.

0.25M disodium-EDTA.
50% glycerol (BDH Chemicals).
0.1% SDS.
0.01% bromophenol blue (BDH Chemicals).

A2.7 DNA transfer.

The DNA from agarose gels was transfered to nylon-supported nitrocellulose filters (Hybond-C Extra, Amersham) by the method of Southern (1975).

1. The gel was treated for 30 minutes in denaturation buffer (to denature the DNA), followed by a brief rinse in distilled water.

2. The denaturation buffer was neutralised by putting the gel for in neutralisation buffer for 30 minutes.

3. The Southern blot apparatus was assembled using 20x SSC as the transfer buffer. The filter was aligned with the origin of the gel.

4. Following overnight transfer the top right corner of the filter was removed to identify the origin. The filter was rinsed in 2xSSC (to remove any adhering agarose), air-dried and then baked for 2 hours at 80°C (to fix the DNA).

20x SSC.

3M sodium chloride.
0.3M trisodium citrate (BDH Chemicals).

Denaturation buffer.

1.5M sodium chloride.
0.5M sodium hydroxide (BDH Chemicals).

Neutralisation buffer.

1.5M sodium chloride.
0.5M Tris-HCl (pH7.2).
1mM disodium-EDTA.

A2.8 Probe labelling.

The maintenance of plasmids, their isolation from bacterial cultures and preparation for labelling are given in Section A2.11.

DNA fragments were labelled by random primer extension according to the method of Feinberg & Volgelstein (1983).

1. Each reaction was conducted in a total volume of 25μl, which consisted of:

5μl	HEPES (pH6.6) (Boehringer Mannheim)
5μl	DTM.
1.4μl	OL.
1μl	Bovine serum albumin (BSA, Sigma B2518).
2.5	Units DNA polymerase large fragment (Klenow, NBL).
60ng	probe DNA.
10μCi	Á-32P-dCTP (3000Ci/mM, Amersham).
Distilled water to 25μl.	

The reaction mixture was incubated at room temperature for at least 5 hours, or overnight.

2. Labelled probe DNA was separated from unincorporated nucleotides by Sephadex G-100-120 column chromatography.

A. 1ml columns of Sephadex G-100-120 (Sigma) were prepared immediately prior to use by plugging one end of a narrow bore 1ml syringe with a glass fibre filter paper (Whatman GF/A), wetting the filter with a small volume (approximately 100μl) of TE and adding Sephadex G-100-120 in TE. The column was allowed to settle for approximately 15 minutes before use.

B. The probe mixture was added to the top of the column and TE added to maintain the buffer reservoir. The run-off was discarded until it started to register approximately 10cps on a Geiger-Muller tube. Fractions were removed from the column until the peak activity had passed and the activity was starting to rise again. These fractions were pooled and used as the probe.

3. If necessary the labelled probe was frozen for up to seven days, depending on the activity date of the radioisotope.

DTM.

100μM dATP (Pharmacia).
100μM dGTP (Pharmacia).
100μM dTTP (Pharmacia).
Dissolved in TM.

TM.

250mM Tris-HCl (pH 8.0).
25mM Magnesium chloride (BDH Chemicals).
50mM ß-Mercaptoethanol (Sigma M6250).

OL.

1mM Tris-HCl.
1mM disodium-EDTA.
90 optical density units/ml hexaoligodeoxyribo-nucleotides (Pharmacia Cat. No. 27-2166-01).

Sephadex G100-120 preparation.

2-3g of Sephadex was presoaked in 10ml TE for, at least, 24 hours.

A2.9 Filter prehybridisation and hybridisation.

1. All filters in this study were hybridised in plastic bags, using approximately 10ml of prehybridisation solution per filter. 1 to 12 filters were sealed in each plastic bag with an appropriate volume of Prehybridisation Buffer III [containing 10μg/ml heat denatured sonicated calf thymus DNA (Pharmacia)] and prehybridised at 65°C for 6 hours.

2. Labelled probe was heat denatured for two minutes at 96°C, then injected into a bag with a hypodermic needle (No.25). The bag was resealed and the filter allowed to hybridise for about 16 hours at 65°C.

3. Following hybridisation the filters were washed in varying concentrations of SSC and SDS depending on the stringency required.

Prehybridisation Buffer III.

0.6M sodium chloride.
10mM PIPES (pH6.8, Boehringer Mannheim).
1mM disodium-EDTA (pH 8.5).
10x Denhardt's solution.

100x Denhardt's solution.

0.2% bovine skin gelatine type B (Sigma, G6269).
0.2% Ficoll 400 (Sigma, F9378).
0.2% polyvinylpyrollidone-360 (Sigma, PVP-360).
1% SDS.
0.05% tetrasodium pyrrophosphate (BDH Chemicals).

A2.10 Autoradiography and fragment size determination.

A2.10.1 Autoradiography.

1. Once filters had been washed they were blotted dry on paper towels and wrapped in either cling film or plastic sheeting. It was important that the filters did not dry out, as this made removal of the probe at later stages difficult, if not impossible.

2. Wrapped filters were placed DNA-side up in autoradiography cassettes (Genetic Research Instruments) with two intensifying screens (CAWO Special screens). A sheet of X-ray film (Agfa Curix) was placed on the DNA-side of the filter with one edge of the film aligned with the top (origin) edge. The X-ray films were exposed for varying periods (from 1 hour to 3 weeks) at -70°C.

3. Exposed films were developed in an X-ray processing machine (Fuji, RG II Processor).

4. Following autoradiography the filters were stripped of the probe, to enable them to be reprobed up to four times, by washing in boiling 1xSSC + 0.1% SDS and allowing the solution to cool. This treatment was repeated three times until no activity remained on the filters. Filters were air-dried and stored at room temperature until required.

A2.10.2 Fragment size determination.

In order to determine DNA fragment sizes the distance that the fragments migrated from the origin was determined and the sizes calculated from the standards using a IBM-PC compatible computer programme supplied by M. Krawczak (Krawczak 1988).

A2.11 Microbiological procedures.

All of the microbiological procedures are conducted under category 0 containment.

A2.11.1 Media.

The basic medium used was a nutrient broth, either commercially prepared (Oxoid Nutrient Broth CM1) or L-Broth, to which agar (Oxoid) was added at 1.5% to make plates. Selective plates and cultures were prepared by making additions to nutrient broth or agar just before use. Ampicillin cultures contained ampicillin (sodium salt, Boehringer Mannheim) at 50μg/ml. The selective plates for cloning were composed of 50μg/ml ampicillin, 7.8μg/ml isopropyl-ß-D-thiogalacto-pyranoside (IPTG, NBL) and 32.5μg/ml 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-Gal, NBL).

L-Broth.

0.5% yeast extract (Oxoid).
1% tryptone (Oxoid).
1% sodium chloride.
pH 7.2.

A2.11.2 Plasmid maintenance.

Plasmids were maintained, in their bacterial hosts, for short periods (approximately one month) on selective plates at 4°C. For longer periods, 0.5ml cultures were grown in selective broth overnight at 37°C and then 0.5ml sterile glycerol (BDH Chemicals) was added prior to storage

at -20°C. For indefinite storage, cultures were frozen at -70°C in freezing broth.

Freezing Broth.

1% tryptone.
0.5% yeast extract.
0.5% sodium chloride.
0.63% dipotassium hydrogen phosphate trihydrate (BDH Chemicals).
0.045% trisodium citrate.
0.009% magnesium sulphate heptahydrate (BDH Chemicals).
0.09% ammonium sulphate (BDH Chemicals).
0.18% potassium dihydrogen phosphate (BDH Chemicals)
4.4% glycerol.
pH 7.2.

A2.11.3 Plasmid minipreparations.

1. Single colonies of the plasmid containing bacterium were inoculated into 10ml selective broth cultures and grown in a shaking incubator overnight at 37°C.

2. The cells were pelleted at 2000rpm at room temperature for 10 minutes, the supernatant poured off, and the vial rested in an ice-bucket so that the remaining culture medium drained away from the pellet.

3. The drained pellet was resuspended in 100µl 25% sucrose (BDH Chemicals) in 50mM Tris-HCl (pH 8.0) and transfered to a 0.5ml microfuge tube.

4. 600 µl MSTET was added to the bacterial suspension, followed by 14µl freshly prepared lysozyme (40mg/ml in 25% sucrose, Boehringer Mannheim). The tube contents were mixed and then heated in a boiling water bath for exactly one minute.

5. The tubes were spun at 13000 rpm at 4°C for 30 minutes and the pellet removed with a sterile toothpick.

6. 200µl phenol (containing 0.8% 8-hydroxy-quinoline) was added to the tube, mixed, then centrifuged at 13000 rpm at room temperature for 10 minutes

7. 600µl of the supernatant was transferred to a new tube. 60µl 7.5M ammonium acetate and 840µl propan-2-ol were added to the tube, thoroughly mixed and placed at -70°C for 45 minutes to precipitate nucleic acids.

8. The nucleic acids were pelleted at 10000 rpm at 4°C for 10 minutes, resuspended in 200µl TE and 5µl DNAase-free RNAase (10mg/ml) was added to the tube. The tube was then incubated at 37°C for 30 minutes and the solution deproteinated with phenol as before.

9. 180μl of the aqueous layer was removed. 18μl 7.5M ammonium acetate and 500μl propan-2-ol was added to precipitate the DNA as usual. The DNA was finally pelleted and dissolved in 20μl TE before storage at -20°C.

10. To check on the concentration and size of the isolated insert, the plasmid was digested with the cloning enzyme and run on a 0.8% agarose gel (Section A2.4).

MSTET.

5% Triton-X-100 (Sigma).
50mM Tris-HCl (pH 8.0).
50mM disodium-EDTA.
5% sucrose.

A2.11.4 Probe preparation.

Prior to use all of the plasmids were digested with the cloning enzyme and deproteinated with 'wet' chloroform. Both the insert and the vector were included in the labelling reaction (Section A2.8), except where the vector was larger than the insert, in which case the insert was isolated on filter paper according to the method of Maniatis *et al* (1982).

A2.12 Cloning procedures.

DNA fragments were cloned into the general cloning vector pUC18 at an *Eco*RI site. The vector carries an ampicillin resistance gene and a functional lacZ gene (which codes for ß-Galactosidase) containing a multicloning site. Any bacterium carrying this plasmid will be ampicillin resistant. In the presence of a chromogenic substrate, eg. 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-Gal), non-recombinant plasmids will produce blue colonies due to the presence of a functional ß-galactosidase, but colonies carrying recombinant plasmids will be white due to their inability to produce functional ß-galactosidase.

A2.12.1 Genomic DNA preparation.

Caesium chloride purified total DNA was further purified on a NACS column (BRL, 1525NP) according to the manufacturers instructions. One microgramme of pure DNA was partially digested with 0.01 units/μg *Eco*RI, in a total volume of 30μl, at 37°C for 1 hour, to yield a range of size fragments from greater than 23kb to less than 3kb. The reaction was deproteinated with TE-saturated phenol/ chloroform (50:50 v/v), the DNA precipitated in ethanol at -20°C. The pellet dissolved in 90μl TE.

2.12.2 Vector preparation.

1) 4μg pUC18 was digested with 5 units of *Eco*RI for two hours at 37°C. THe reaction was stopped by the addition of 3μl 0.5M disodium-EDTA, deproteinated with

100μl phenol/chloroform (50:50 v/v) and the DNA precipitated from the aqueous phase with 10μl 7.5M ammonium acetate and 80μl ethanol.

2) The DNA pellet was recovered by centrifugation and dissolved in 100μl TE. 20μl of the solution was kept for control experiments.

3) The remaining 80μl was dephosphorylate with 2.5 units of calf intestinal alkaline phosphatase (NBL) for 30 minutes at 37°C. The reaction was stopped at 55°C for 45 minutes and deproteinated with two treatments of 100μl phenol/chloroform. The aqueous phase was recovered and the DNA precipitated as before. The pellet was dissolve in 31.5μl TE.

2.12.3 Ligation.

Three ligation reactions were set up; two controls (*Eco*RI-digested pUC18 and dephosphatased *Eco*RI-digested pUC18) and one experimental reaction.

The reaction conditions were similar for both control and experimental reactions.

100ng of pUC18 (dephosphatased or otherwise)
1μl 10xT4 ligation buffer (as supplied with enzyme)
1 unit T4 ligase (NBL)
Distilled water upto 10μl
Incubated overnight at 12°C.

In the experimental reaction 200ng genomic DNA was included.

Following incubation, 90μl TMC was added to each 10μl ligation reaction.

TMC.

10mM Tris-HCl (pH7.5)
10mM magnesium chloride (BDH Chemicals)
10mM calcium chloride (BDH Chemicals)

2.12.4 Transformation procedure.

2.12.4.1 Competent cell preparation.

1) Single colonies of *Escherichia coli* strain TG2 were inoculated into 10ml broth cultures and incubated overnight at 37°C in stationery culture.

2) 2ml of the overnight culture was added to a 100ml broth culture (in a 250ml flask), and the culture grown to an OD_{600} of 0.5-0.6 in a shaking incubator at 37°C.

3) 40ml of the culture was kept on ice for 15 minutes prior to centrifugation at 2000 rpm at 4°C for 10 minutes in a Sorvall SS-34 rotor.

4) The pellet was gently resuspended in 20ml 100mM magnesium chloride, centrifuged as above and resuspended in 4ml 100mM calcium chloride. The competent cells were left on ice for 30 minutes prior to use.

5) 200μl of competent cells were added to each ligation mixture and left on ice for 30 minutes. The cells were then heat shocked at 42°C for two minutes.

6) The mixture was cooled at room temperature. 100μl nutrient broth was added and incubated for one hour at 37°C.

7) 200μl of control ligations and 400μl of the experimental ligation were added to dried selective plates (X-Gal/IPTG, see Section A2.11.1). Aliquots were spread and the plates incubated overnight at 37°C.

8) The following day plates were scored for the presence of white (recombinant) colonies.

2.12.4.2 Screening procedures for the identification of recombinant colonies.

1) A dried ampicillin plate was overlaid with a gridded nitrocellulose filter (Schleicher & Schnell, BA 85/21 membrane filter 0.45μm, 82mm diameter). A second (master) plate was placed on to a piece of gridded paper such that relative positions on the two plates could be identified.

2) White colonies were picked from the experimental plates with sterile toothpicks, smeared onto the nitrocellulose plate and stabbed into the master plate at a corresponding position (as an internal control one of the blue colonies was included on each plate).

3) The plates were inverted and incubated at 37°C overnight. The master plate was sealed with 'Nescofilm' and stored at 4°C.

4) The nitrocellulose filter was removed from the other plate and placed sequentially, face-up for five minutes each, on filter papers soaked in the following solutions;

i) Southern denaturation buffer.
ii) Southern neutralisation buffer (2 treatments).

5) The filter was gently washed in 2xSSC + 0.1%SDS to remove any adhering colonies, air-dried and baked at 80°C f two hours.

Table A1. Cutting sequence and incubation conditions for the restriction enzymes used in this study.

ENZYME	CUTTING SEQUENCE	DIGESTION BUFFER	INCUBATION TEMPERATURE(°C)
7bp cutting enzymes.			
*Bst*EII	G/GTNACC	TA	60
6bp cutting enzymes.			
*Bam*HI	G/GATCC	TA	37
*Bgl*II	A/GATCT	TA	37
*Eco*RI	G/AATTC	TA	37
*Eco*RV	GAT/ATC	TA	37
*Hin*DIII	A/AGCTT	TA	37
*Kpn*I	GGTAC/C	S	37
*Pst*I	CTGCA/G	TA	37
*Sac*I	GAGCT/C	TA	37
*Sal*I	G/TCGAC	TA	37
*Xba*I	T/CTAGA	TA	37
*Xho*I	C/TCGAG	TA	37
4bp cutting enzymes.			
*Hae*III	GG/CC	TA	37
*Taq*I	T/CGA	TA	60

TA 33mM Tris-Acetate (pH 7.9).
66mM Potassium acetate.
10mM Magnesium acetate.
4mM Spermidine tetrachloride (Sigma).
0.5mM Dithiothreitol.

S 60mM Tris-HCl (pH 7.5).
60mM sodium chloride.
60mM magnesium chloride.
60mM ß-mercaptoethanol.

Appendix B.

Unsuccessful DNA extraction procedures.

The success of a DNA extraction method was judged by three criteria; the ability to process a large number of individual plants, the yield and the 'intactness' of the DNA. The unsuccessful methods and the modifications which were tried are shown below.

Taxon°	Method	Modifications''
S.vulgaris RR	Britten *et al* (1974, p377)	
S.vulgaris NR	King (1986)	
S.vulgaris RR	Belford (1979)	
S.vulgaris NR	Draper *et al* (1988)	
S.vulgaris NR *S.squalidus*	Zimmer *et al* (1981)	Extraction buffers A, B & C.
S.vulgaris RR	Valejos *et al* (1986)	
S.vulgaris ssp. denticul-atus.	Doyle & Beachy (1985)	S and S + 0.1M NaDETC.
S.vulgaris NR *S.squalidus*		S + 0.5%SDS S + 0.5%SDS + 20μg/ml proteinase K
S.vulgaris NR	Rogers & Bendich (1986)	50-500mg method

° NR - Senecio vulgaris ssp. vulgaris var. vulgaris.
RR - Senecio vulgaris ssp. vulgaris var. hibernicus.

'' S - Standard extraction buffer.
NaDETC - Sodium diethylthiocarbamate (Sigma).

Appendix C.

Rationale for visual estimation of DNA concentration.

Introduction.

One method for determining the concentration and purity of DNA is to measure its absorbance at 230nm, 260nm and 280nm. The value for A_{260} can be used to calculate DNA concentration, knowing that: in a 10mm pathlength, 50µg/ml DNA has an optical density of 1. Similarly, the purity of the DNA sample can be derived from the A_{260}:A_{230} and A_{260}:A_{230} ratios, both of which should approach 1.8 for high purity DNA.

Method.

1. Total DNA was extracted from *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus* (material from crossing programme, Irwin 1990) as in Appendix A, Section A2.2. The concentration and purity of the DNA was then measured on a Unicam spectrophotometer.

2. The DNA was further purified by passage down a 2.7ml hydroxyapatite (HAP) column. The HAP column was prepared by resuspending 5g HAP (Biorad DNA grade Bio-Gel HTP) in 0.24M sodium phosphate (pH 6.8, BDH Chemicals) and adding the suspension to a 5ml syringe plugged with glass fibre filter paper (Whatman GF/A) [bed volume of 2.7ml]. The column was washed with 20ml 0.24M sodium phosphate (pH 6.8).

3. In order to remove any EDTA that may interfere with the HAP, the DNA was precipitated with one-third the volume of 7.5M ammonium acetate and two volumes of ethanol overnight at -20°C. The DNA pellet was recovered by centrifugation and resuspended in 2ml 0.24M sodium phosphate (pH 6.8).

4. The DNA solution was applied to the column, and the column washed with three separate solutions:- 60ml 0.24M sodium phosphate (pH 6.8), until the A_{254} value had fallen below 0.05; 150ml 0.12M sodium phosphate (pH 6.8); 150ml 0.48M sodium phosphate (pH 6.8). The run-off from the column was collected in 10ml aliquots. The values of A_{260}, A_{230} and A_{280} were measured for each of these aliquots and an elution profile constructed.

5. 1ml aliquots from the A_{260} peaks were treated with 7.5M ammonium acetate and 2 volumes of ethanol to precipitate the DNA. The DNA was pelleted, dissolved

in 10μl TE and run on a 0.8% agarose gel (Appendix A, Section A2.5).

Results.

The initial spectrophotometer measurements indicated that 1275μg of DNA had been loaded on the column. The DNA elution profile for the sample of *Senecio vulgaris* DNA is shown in Figure C1. It is clear that two A_{260} peaks are present, the first peak (eluted by 0.24M sodium phosphate) is approximately 3.4 times larger than the second peak (eluted by 0.48M sodium phosphate). Inorder to confirm the presence of DNA in these peaks, samples were run on an agarose gel and the staining intensity measured by visual estimation (Section A2.4). These results are shown in Table C1.

From a comparison of the elution profile (Figure C1) and the relative staining intensities (Table C1) it is clear that the two peaks do not coincide. The largest amount of DNA was found in the second peak, while the greatest A_{260} value was found in the first peak. These results indicate that there is an A_{260}-absorbing material which is contaminanting the DNA extract. The amount of DNA loaded onto the HAP column, calculated from the gel, was approximately 200μg.

Table C1. Relative ethidium bromide staining intensities of *Senecio vulgaris* ssp. *vulgaris* var *hibernicus* DNA purified on a hydroxyapatite column.

HAP Fraction number.°	Relative staining intensity.*
1	1.00
2	0.25
3	0.00
22	10.00
23	1.00
24	0.50

° Fractions 1-3 0.24M sodium phosphate, Fractions 22-24 0.48M sodium phosphate.

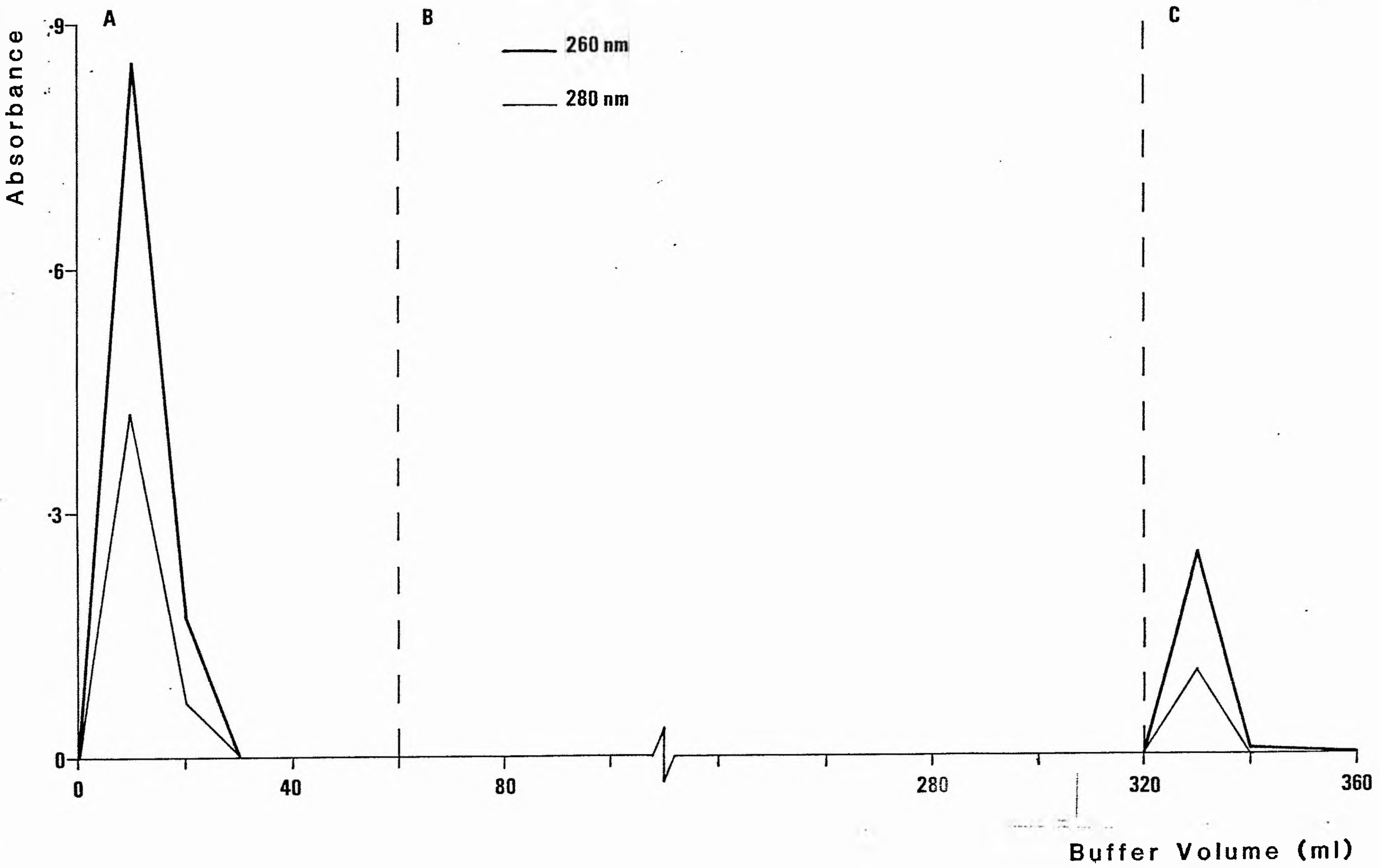
* Compared to Fraction 1.

Discussion.

The results presented in Figure C1 and Table C1 show that although the DNA extraction method used will yield DNA suitable for restriction digestion, the DNA is heavily contaminated with a strongly 260nm-absorbing compound. This

clearly shows that spectrophotometry is an unsuitable method for determining the concentration of *Senecio* DNA, since any estimate of DNA concentration would severly over-estimate the amount actually present. It is, therefore, necessary to have a more robust method of DNA concentration estimation. The visual estimation method is such a solution. Although this method is not ideal it does provide a quick estimate of DNA concentration. It has been found that with practise, the estimate is fairly good when judged in comparison to densitometry.

Figure C1. Elution profile of *Senecio vulgaris* ssp. *vulgaris* var. *hibernicus* total DNA from a HAP column. A. Column washed with 0.24M sodium phosphate buffer. B. Column washed with 0.12M sodium phosphate buffer. C. Column washed with 0.48M sodium phosphate buffer.



Absorbance
·9
·6
·3
0
A
B
C
260 nm
280 nm
0
40
80
280
320
360
Buffer Volume (ml)

Appendix D.

Fragment sizes for the chloroplast DNA data.

The sizes of the fragments produced when different probe enzyme combinations were used on various *Senecio* taxa. The code for each of the accessions is given in Chapter 3, Table 3.2.

Enzyme: BamHI.
Probe: Total cpDNA.

38.00kb	16.90kb	12.50kb	08.57kb	07.55kb	05.38kb	cBr.
03.91kb	03.45kb	03.21kb	02.69kb	02.48kb	02.45kb	
02.31kb	02.09kb	02.04kb	01.77kb	01.50kb	01.27kb	
	[Some fragments >> 40kb]					
38.00kb	16.90kb	12.50kb	07.55kb	05.38kb	03.91kb	cSa, dAi, hBr, hMo, hSa,
03.45kb	03.21kb	02.69kb	02.48kb	02.45kb	02.31kb	sBr, sSa, sSh, sSt, sYo,
02.09kb	02.04kb	01.77kb	01.50kb	01.27kb		veGe, vMi, vYo.
	[Some fragments >> 40kb]					
29.60kb	16.09kb	11.05kb	07.55kb	05.38kb	03.91kb	vPu.
03.45kb	03.21kb	02.69kb	02.48kb	02.45kb	02.31kb	
02.09kb	02.04kb	01.77kb	01.50kb	01.27kb		
	[Some fragments >> 40kb]					
33.30kb	15.90kb	11.50kb	09.35kb	07.55kb	05.38kb	pCa.
03.91kb	03.45kb	03.21kb	02.69kb	02.48kb	02.45kb	
02.31kb	02.09kb	02.04kb	01.77kb	01.50kb	01.27kb	
	[Some fragments >> 40kb]					
38.00kb	16.90kb	12.50kb	07.55kb	05.38kb	03.91kb	hYo.
03.45kb	03.21kb	02.69kb	02.31kb	02.09kb	02.04kb	
01.77kb	01.50kb	01.27kb				
	[Some fragments >> 40kb]					

[Not determined for jTe, vBr]

Enzyme: BamHI.
Probe: pLsC1.

33.30kb	07.38kb	03.35kb	02.06kb	All Accessions.

Enzyme: BamHI.
Probe: pLsC2.

03.21kb	02.97kb	01.76kb	01.25kb	01.00kb	0.448kb	cBr, cSa, dAi, hBr, hMo,
0.503kb						hSa, hYo, sBr, sSa, sSh,
						sSt, sYo, veGe, vBr, vMi,
						vPu, vSa, vYo.
03.21kb	02.37kb	01.76kb	01.25kb	01.00kb	0.651kb	jTe, pCa.
0.448kb						

Enzyme: BamHI.
Probe: pLsC4.

19.10kb	07.03kb	04.31kb	cBr, cSa, dAi, hBr, hMo,
			hSa, hYo, sBr, sSa, sSh,
			sSt, sYo, veGe, vBr, vMi,
			vPu, vSa, vYo.
24.90kb	07.03kb	04.01kb	jTe, pCa.

Enzyme: BamHI.
Probe: pLsC5ac.

25.30kb 07.98kb 04.70kb cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.

33.6kb 07.98kb 04.70kb jTe.

[Not determined for pCa]

Enzyme: BamHI.
Probe: pLsC6.

08.04kb 05.70kb 02.11kb 01.86kb 01.66kb 01.49kb 0.914kb cBr.

08.04kb 05.70kb 05.36kb 02.11kb 01.86kb 01.66kb 01.49kb 0.914kb vMi.

08.04kb 05.36kb 02.11kb 01.86kb 01.66kb 01.49kb 0.914kb cBr, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, vBr, vPu, vSa, vYo.

08.04kb 06.25kb 02.11kb 01.86kb 01.66kb 01.49kb 0.954kb pCa.

08.04kb 05.96kb 02.11kb 01.86kb 01.66kb 01.49kb 0.914kb veGe.

08.04kb 05.16kb 02.11kb 01.86kb 01.66kb 01.49kb 0.914kb dAi.

08.04kb 05.38kb 02.11kb 01.86kb 01.66kb 01.49kb 0.897kb jTe.

Enzyme: BamHI.
Probe: pLsC7.

07.13kb All Accessions

Enzyme: BamHI.
Probe: pLsC9.

07.42kb	06.78kb	02.32kb	01.97kb	cBr, cSa, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.
06.78kb	06.25kb	02.37kb	01.97kb	pCa.
06.78kb	06.25kb	02.32kb	01.97kb	dAi.
06.78kb	04.82kb	02.32kb	01.97kb	jTe.

Enzyme: BamHI.
Probe: pLsC10/11/12.

11.80kb	08.50kb	03.12kb	02.97kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.
19.27kb	03.12kb	02.97kb		pCa.

Enzyme: BamHI.
Probe: pLsC13/14.

[Not determined]

Enzyme: BamHI.
Probe: pLsC15.

[Not determined]

Enzyme: BglII.
Probe: Total cpDNA.

08.77kb	07.87kb	06.90kb	06.03kb	05.66kb	04.80kb	cBr, cSa, dAi, hBr, hMo,
04.25kb	03.92kb	03.10kb	02.85kb	02.43kb	02.24kb	hSa, hYo, sSh, vBr, vMi,
01.87kb	01.71kb	01.49kb	01.38kb	01.10kb	0.658kb	vYo.
12.60kb	08.77kb	07.87kb	06.90kb	06.03kb	05.66kb	sSa, sYo.
04.80kb	04.25kb	03.92kb	03.10kb	02.85kb	02.43kb	
02.24kb	01.87kb	01.71kb	01.49kb	01.38kb	01.10kb	
0.658kb						
13.10kb	12.60kb	08.77kb	07.87kb	06.90kb	06.03kb	sBr.
05.66kb	04.80kb	04.25kb	03.92kb	03.10kb	02.85kb	
02.43kb	02.24kb	01.87kb	01.71kb	01.49kb	01.38kb	
01.10kb	0.658kb					
13.10kb	08.77kb	07.87kb	06.90kb	06.03kb	05.66kb	sSt.
04.80kb	04.25kb	03.92kb	03.10kb	02.85kb	02.43kb	
02.24kb	01.87kb	01.71kb	01.49kb	01.38kb	01.10kb	
0.658kb						
13.60kb	08.77kb	07.87kb	06.90kb	06.03kb	05.66kb	vPu.
04.80kb	04.25kb	03.92kb	03.10kb	02.85kb	02.43kb	
02.24kb	01.87kb	01.71kb	01.49kb	01.38kb	01.10kb	
0.658kb						
08.77kb	08.20kb	06.90kb	06.03kb	05.66kb	04.80kb	pCa.
04.25kb	03.92kb	03.64kb	03.10kb	02.85kb	02.43kb	
02.24kb	01.87kb	01.71kb	01.49kb	01.38kb	01.10kb	
0.658kb						
11.30kb	08.77kb	07.87kb	06.90kb	06.03kb	05.66kb	veGe.
04.80kb	04.25kb	03.92kb	03.10kb	02.85kb	02.43kb	
02.24kb	01.87kb	01.71kb	01.49kb	01.38kb	01.10kb	
0.658kb						
08.77kb	08.20kb	07.87kb	06.90kb	06.03kb	05.84kb	jTe.
04.80kb	04.25kb	03.92kb	03.10kb	02.85kb	02.43kb	
02.24kb	01.87kb	01.71kb	01.49kb	01.38kb	01.10kb	
0.658kb						

Enzyme: BglII.
Probe: pLsC1.

05.48kb	03.68kb	02.66kb	01.39kb	01.29kb	01.02kb	cBr, cSa, dAi, hBr, hMo,
0.503kb						hSa, hYo, jTe, pCa, sBr,
						sSa, sSh, sSt, sYo, veGe,
						vBr, vMi, vYo.
05.48kb	03.68kb	02.66kb	02.27kb	01.39kb		vPu.
01.29kb	01.02kb	0.503kb				

Enzyme: BglII.
Probe: pLsC2.

05.37kb 03.34kb 01.60kb 0.770kb 0.503kb — cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.

03.76kb 03.34kb 01.60kb 01.22kb 0.770kb — jTe, pCa.

Enzyme: BglII.
Probe: pLsC4.

08.66kb 07.59kb — cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.

08.66kb 08.02kb — jTe, pCa.

Enzyme: BglII.
Probe: pLsC5ac.

08.38kb 07.42kb 06.56kb 0.635kb 0.524kb — cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.

08.21kb 08.04kb 06.56kb 0.635kb 0.524kb — jTe, pCa.

Enzyme: BglII.
Probe: pLsC6.

04.56kb 03.31kb 03.14kb 02.46kb 01.30kb 01.19kb — cBr, pCa.

04.56kb 03.14kb 02.95kb 02.46kb 01.30kb 01.19kb — cSa, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, vBr, vMi, vPu, vSa, vYo.

04.56kb 03.45kb 03.31kb 02.46kb 01.30kb 01.19kb — veGe.

04.56kb 03.14kb 02.81kb 02.46kb 01.30kb 01.19kb — dAi.

04.56kb 03.14kb 02.86kb 02.46kb 01.26kb 1.19kb — jTe.

Enzyme: BglII.
Probe: pLsC7.

06.01kb 03.00kb 01.58kb 01.32kb — cBr, cSa, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, vBr, vMi, vPu, vSa, vYo.

06.01kb 3.00kb 02.75kb — pCa.

06.01kb 02.75kb — jTe.

06.01kb 03.38kb 01.58kb 01.32kb — veGe.

06.01kb 03.00kb 01.66kb 01.32kb — dAi.

Enzyme: BglII.
Probe: pLsC9.

03.31kb 03.04kb — cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.

04.36kb 03.04kb — jTe, pCa.

Enzyme: BglII.
Probe: pLsC10/11/12.

04.12kb 03.85kb 02.77kb 02.40kb 02.22kb 02.12kb 'c
r, cSa, dAi, hBr, hMo,
01.68kb 01.44kb 01.13kb 0.837kb 0.652kb — hSa, hYo, pCa, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.

04.12kb 03.85kb 02.77kb 02.40kb 02.22kb 01.81kb — jTe
01.68kb 01.44kb 01.13kb 0.837kb 0.652kb

Enzyme: BglII.
Probe: pLsC13/14.

04.53kb 01.92kb 01.81kb 01.74kb — All Accessions.

Enzyme: BglII.
Probe: pLsC15.

04.20kb 01.95kb 01.35kb — All Accessions.

Enzyme: BstEII.
Probe: Total cpDNA.

Fragments						Accessions
16.20kb	11.00kb	07.90kb	03.45kb	03.27kb	03.19kb	cBr, sBr, sSa, sSh,
02.78kb	02.29kb					sYo, vMi, vYo.
16.20kb	11.00kb,	07.90kb	03.45kb	03.27kb	02.78kb	hBr, hMo, hSa, sSt,
02.29kb						vBr.
16.20kb	11.00kb	07.90kb	03.45kb	03.27kb	02.78kb	cSa, veGe.
16.20kb	11.00kb	06.70kb	03.45kb	03.27kb	02.78kb	dAi.
02.29kb						
15.10kb	10.50kb	07.90kb	03.45kb	03.27kb	03.19kb	hYo.
02.78kb						
16.20kb	11.00kb	03.45kb	03.27kb	02.88kb	02.78kb	jTe.
14.20kb	10.13kb	03.45kb	03.27kb	02.78kb		pCa.

Enzyme: BstEII.
Probe: pLsC1.

Fragments						Accessions
03.63kb	02.92kb	02.10kb	01.98kb	01.62kb	01.32kb	cBr, cSa, dAi, hBr, hMo,
01.12kb	0.610kb	0.178kb	0.072kb			hSa, hYo, sBr, sSt, sYo,
						veGe, vBr, vMi, vYo.
03.63kb	02.92kb	02.10kb	01.98kb	01.32kb	01.12kb	jTe.
0.610kb	0.178kb	0.072kb				

[Not determined for sSa, pCa, sSh]

Enzyme: BstEII.
Probe: pLsC2.

Fragments		Accessions
20.75kb	05.24kb	All Accessions.

Enzyme: BstEII.
Probe: pLsC4.

Fragments			Accessions
17.50kb	04.95kb	03.64kb	cBr, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.
17.50kb	04.95kb		cSa, sSh.
16.20kb	05.45kb	04.95kb	pCa.

Enzyme: BstEII.
Probe: pLsC5ac.

Fragments			Accessions
04.86kb	04.01kb	03.64kb	All Accessions.

Enzyme: BstEII.
Probe: pLsC6.

08.13kb 07.38kb — sSt, hBr, hMo, sYo, vBr, hSa, dAi, hYo, jTe,

[Not determined for cBr, cSa, pCa, sBr, sSa, sSh, veGe, vMi, vYo]

Enzyme: BstEII.
Probe: pLsC7.

28.00kb 04.27kb — All accessions.

Enzyme: BstEII.
Probe: pLsC9.

[Not determined]

Enzyme: BstEII.
Probe: pLsC10/11/12.

[Not determined]

Enzyme: BstEII.
Probe: pLsC13/14.

[Not determined]

Enzyme: BstEII.
Probe: pLsC15.

05.17kb 04.22kb — All Accessions.

Enzyme: EcoRI.
Probe: Total cpDNA.

14.10kb	10.10kb	08.00kb	05.08kb	04.39kb	04.05kb	cBr, sBr, vMi.
03.47kb	03.07kb	02.70kb	02.38kb	02.19kb	01.64kb	
01.44kb	01.29kb	0.932kb	0.767kb	0.670kb	0.561kb	
14.10kb	10.10kb	08.00kb	05.08kb	04.39kb	04.05kb	hBr, hMo, hSa, hYo, sSt,
03.47kb	02.70kb	02.38kb	02.19kb	01.64kb	01.44kb	sYo, vBr, vYo.
01.29kb	0.932kb	0.767kb	0.670kb	0.561kb		
09.84kb	08.00kb	05.08kb	04.39kb	04.05kb	03.47kb	vPu.
03.07kb	02.70kb	02.38kb	02.19kb	01.64kb	01.44kb	
01.29kb	0.932kb	0.767kb	0.670kb	0.561kb		
14.10kb	10.10kb	07.67kb	05.08kb	04.39kb	03.945kb	veGe.
03.47kb	02.70kb	02.38kb	02.19kb	01.64kb	01.44kb	
01.29kb	0.932kb	0.767kb	0.670kb	0.561kb		
14.10kb	10.10kb	08.00kb	05.08kb	04.39kb	04.16kb	dAi.
03.47kb	02.70kb	02.43kb	02.25kb	01.64kb	01.44kb	
01.29kb	0.932kb	0.767kb	0.670kb	0.561kb		
14.10kb	10.10kb	08.00kb	05.35kb	05.08kb	04.39kb	jTe.
03.47kb	03.12kb	02.38kb	02.19kb	01.64kb	01.44kb	
01.29kb	0.932kb	0.767kb	0.670kb	0.561kb		

[Not determined for cSa, pCa, sSa,sSh]

Enzyme: EcoRI.
Probe: PLsC1.

14.6kb	All Accessions.

Enzyme: EcoRI.
Probe: PLsC2.

09.16kb	02.21kb	01.90kb	All Accessions.

Enzyme: EcoRI.
Probe: PLsC4.

02.13kb	01.13kb				cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa.
03.03kb	02.13kb	01.98kb	01.65kb	01.13kb	sSa.
03.03kb	02.13kb	01.65kb	01.13kb		pCa, vYo.
02.23kb	01.13kb				jTe.

Enzyme: EcoRI.
Probe: PLsC5ac.

03.06kb 02.50kb 02.36kb — cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.

[Not determined for pCa]

Enzyme: EcoRI.
Probe: PLsC6.

02.02kb 01.88kb 01.70kb 01.62kb 01.53kb 01.21kb — cBr, dAi, hMo, hSa, hYo, sBr, sSh, sSt, sYo, vBr, vMi, vPu, vSa, vYo.
[Other fragments present that were not resolved]

02.49kb 02.12kb 02.02kb 01.88kb 01.70kb 01.62kb 01.53kb 01.21kb — sSa
[Other fragments present that were not resolved]

02.02kb 01.88kb 01.70kb 01.62kb 01.53kb — cSa, hBr, veGe.
[Other fragments present that were not resolved]

02.49kb 02.12kb 01.88kb 01.62kb 01.53kb 01.21kb — pCa.
[Other fragments present that were not resolved]

02.49kb 01.88kb 01.62kb 01.53kb — jTe.
[Other fragments present that were not resolved]

Enzyme: EcoRI.
Probe: PLsC7.

08.28kb 04.63kb 01.66kb 01.25kb 0.346kb — cBr, dAi, hBr, hSa, hYo, pCa, sBr, sSa, sSh, sYo, veGe, vBr, vMi, vPu, vSa, vYo.

08.28kb 04.63kb 01.66kb 01.41kb 01.25kb 0.765kb 0.346kb — sSt, vBr.

08.28kb 04.63kb 01.66kb 01.36kb 01.25kb 0.346kb — hMo.

08.28kb 04.63kb 01.66kb 01.25kb 0.410kb — jTe.

Enzyme: EcoRI.
Probe: PLsC9.

12.43kb 05.40kb — cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.

12.40kb 05.65kb — pCa.

Enzyme: EcoRI.
Probe: PLsC10/11/12.

11.50kb	04.17kb	03.61kb	02.95kb	02.61kb	02.37kb	cBr, cSa, hBr, hMo, hSa,
02.04kb	01.96kb					hYo, sBr, sSa, sSh, sSt,
						sYo, veGe, vBr, vMi, vSa,
						vYo.

[Other fragments present that were not resolved]

11.50kb	04.17kb	03.61kb	02.87kb	02.61kb	02.37kb	vPu.
02.04kb	01.96kb					

[Other fragments present that were not resolved]

11.50kb	04.17kb	03.61kb	03.04kb	02.61kb	02.37kb	dAi.
02.04kb	01.96kb					

[Other fragments present that were not resolved]

11.50kb	03.87kb	03.61kb	02.95kb	02.61kb	02.37kb	jTe.
02.04kb	01.93kb					

[Other fragments present that were not resolved]

[Not determined for pCa]

Enzyme: EcoRI.
Probe: PLsC13/14.

03.97kb	01.86kb	01.77kb	01.53kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.
03.04kb	01.86kb	01.77kb	01.53kb	jTe, pCa.

Enzyme: EcoRI.
Probe: PLsC15.

02.61kb	02.00kb	0.952kb	0.807kb	0.597kb	0.410kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, pCa, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.
03.15kb	01.39kb	0.952kb	0.807kb	0.597kb	0.367kb	jTe.

Enzyme: EcoRV.
Probe: Total cpDNA.

17.90kb	14.30kb	11.80kb	10.80kb	09.00kb	08.25kb	cBr.
06.68kb	06.20kb	05.18kb	04.67kb	04.50kb	03.94kb	
03.09kb	02.84kb	02.60kb	01.94kb	01.59kb		
	[2 fragments << 1.5kb outside marker range]					
17.90kb	14.30kb	11.80kb	10.80kb	09.00kb	07.97kb	cSa, hBr, hMo, hSa,
06.68kb	06.20kb	05.18kb	04.67kb	04.50kb	03.94kb	hYo, sBr, sSa, sSh,
03.09kb	02.84kb	02.60kb	01.94kb	01.59kb		sSt, sYo, vBr, vMi, vYo.
	[2 fragments << 1.5kb outside marker range]					
13.60kb	11.00kb	10.30kb	09.94kb	09.00kb	08.40kb	pCa.
06.58kb	06.11kb	05.18kb	04.67kb	04.50kb	03.94kb	
03.09kb	02.84kb	02.60kb	01.94kb	01.59kb		
	[2 fragments << 1.5kb outside marker range]					
17.90kb	14.30kb	11.80kb	10.80kb	09.00kb	08.70kb	veGe.
06.68kb	06.20kb	05.32kb	04.67kb	04.39kb	03.94kb	
03.09kb	02.84kb	02.60kb	01.94kb	01.59kb		
	[2 fragments << 1.5kb outside marker range]					
17.90kb	14.30kb	11.80kb	10.80kb	09.00kb	07.84kb	dAi.
06.68kb	06.20kb	05.18kb	04.80kb	04.50kb	03.94kb	
03.09kb	02.84kb	02.60kb	01.94kb	01.59kb		
	[2 fragments << 1,5kb outside marker range]					
17.90kb	14.30kb	11.80kb	10.80kb	09.00kb	07.97kb	jTe
06.68kb	06.20kb	05.18kb	04.67kb	03.09kb	02.84kb	
02.58kb	01.94kb	01.59kb				

Enzyme: EcoRV.
Probe: pLsC1

03.96kb	03.79kb	03.76kb	03.75kb	All Accessions.
	[More fragments apparent but not resolved]			

Enzyme: EcoRV.
Probe: pLsC2.

14.20kb	10.80kb	03.84kb	01.01kb	cBr, dAi, hBr, hSa, hYo, sBr, sSa, sSt, sYo, veGe, vBr, vMi, vSa, vYo.
14.20kb	10.80kb	04.10kb	01.01kb	jTe.

[Not determined for cSa, hMo, pCa, sSh, vPu]

Enzyme: EcoRV.
Probe: pLsC4.

16.68kb	12.16kb					cBr, cSa, hBr, hSa, hYo, jTe, sBr, sSa, sSt, sYo, vBr, vMi, vYo.

[Not determined for dAi, hMo, pCa, sSh, veGe, vPu.]

Enzyme: EcoRV.
Probe: pLsC5ac.

22.40kb	04.86kb					cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.
19.00kb	04.86kb					pCa.

Enzyme: EcoRV.
Probe: pLsC6.

07.79kb	05.11kb	04.71kb	03.29kb	03.12kb	02.03kb	cBr, hSa, sSa, vMi, vPu,
01.55kb	01.30kb					vYo.
07.79kb	05.11kb	04.71kb	03.12kb	02.03kb	01.55kb	dAi, hBr, hMo, hYo, sBr,
01.30kb						sSt, vBr, vSa.
08.73kb	05.11kb	04.71kb	03.29kb	03.12kb	02.03kb	veGe.
01.55kb	01.30kb					
07.79kb	05.11kb	04.71kb	03.29kb	03.12kb	02.03kb	jTe.
01.41kb	01.30kb					

[Not determined for cSa, pCa, sSh, vPu]

Enzyme: EcoRV.
Probe: pLsC7.

18.70kb	04.56kb	01.52kb				cBr, hBr, hMo, hSa, hYo, sBr, sSa, sSt, sYo, veGe, vBr, vMi, vYo.
18.70kb	04.72kb	01.52kb				dAi.
18.70kb	04.56kb	01.40kb				jTe.

[Not determined for cSa, pCa, sSh, vPu]

Enzyme: EcoRV.
Probe: pLsC9.

04.97kb	dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSt, sYo, veGe, vBr, vYo.

[Not determined for cBr, cSa, pCa, sSa, sSh, vMi, vPu vSa]

Enzyme: EcoRV.
Probe: pLsC10/11/12.

24.8kb	11.9kb	10.3kb	04.91kb	02.65kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.
24.8kb	11.9kb	09.29kb	04.91kb	02.65kb	pCa.

Enzyme: EcoRV.
Probe: pLsC13/14.

07.79kb	05.08kb	cBr, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSt, sYo, veGe, vBr, vMi, vSa, vYo.

[Not determined for cSa, pCa, sSa, sSh, vPu]

Enzyme: EcoRV.
Probe: pLsC15.

06.56kb	06.02kb	cBr, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSt, sYo, veGe, vBr, vMi, vSa, vYo.

[Not determined for cSa, pCa, sSh, vPu]

Enzyme: HaeIII.
Probe: Total cpDNA.

[Not determined, due to the difficulty of band resolution]

Enzyme: HaeIII.
Probe: pLsC1.

11.30kb	01.46kb	01.17kb			cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, vBr, vMi, vSa, vYo.
10.90kb	01.46kb	01.17kb			cPa.

[Not determined for veGe]

Enzyme: HaeIII.
Probe: pLsC2.

01.81kb	01.69kb	01.56kb	01.06kb	0.685kb	All Accessions.

Enzyme: HaeIII.
Probe: pLsC4.

03.00kb	01.42kb	0.848kb		cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vSa, vYo.
03.23kb	01.66kb	01.33kb	0.848kb	cPa.
03.40kb	03.13kb	0.848kb		jTe.

Enzyme: HaeIII.
Probe: pLsC5ac.

03.00kb	02.05kb	0.910kb		cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vYo.
02.90kb	02.38kb	0.910kb		pCa.
03.00kb	02.57kb	02.38kb	0.910kb	jTe.

Enzyme: HaeIII.
Probe: pLsC6.

03.51kb	02.56kb	02.25kb	01.84kb	01.31kb		cBr, cSa, hBr, hMo, hSa,
01.10kb	0.956kb	0.872kb	0.758kb			hYo, sBr, sSa, sSt, sYo,
	[Other fragments present that were not resolved]					veGe, vBr, vMi, vSa, vYo.
03.51kb	02.56kb	02.11kb	01.84kb	01.48kb		cPa.
01.31kb	01.10kb	0.956kb	0.872kb	0.758kb		
	[Other fragments present that were not resolved]					
03.51kb	02.39kb	02.25kb	01.84kb	01.31kb		dAi.
01.10kb	0.956kb	0.872kb	0.758kb			
	[Other fragments present that were not resolved]					
03.51kb	02.56kb	02.11kb	01.84kb	01.31kb		sSh.
01.10kb	0.956kb	0.872kb	0.758kb			
	[Other fragments present that were not resolved]					
03.51kb	02.25kb	02.11kb	01.84kb	01.10kb	0.956kb	jTe.
0.872kb	0.758kb					
	[Other fragments present that were not resolved]					

Enzyme: HaeIII.
Probe: pLsC7.

0.908kb	0.823kb	0.769kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, pCa, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.
0.908kb	0.823kb	0.605kb	jTe

Enzyme: HaeIII.
Probe: pLsC9.

01.48kb	0.895kb	0.802kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.
01.36kb	0.802kb	0.631kb	cPa, jTe.

Enzyme: HaeIII.
Probe: pLsC10/11/12.

02.66kb 02.54kb 01.47kb 01.18kb 0.979kb 0.910kb 0.631kb 0.513kb
cBr, cSa, hBr, hMo, hSa, hYo, jTe, pCa, sBr, sSa, sSh, sSt, sYo, vBr, vMi, vSa, vYo.

02.54kb 01.47kb 01.18kb 0.979kb 0.910kb 0.631kb 0.513kb
dAi, veGe.

[Not determined for vPu]

Enzyme: HaeIII.
Probe: pLsC13/14.

03.10kb 02.19kb 01.58kb 01.13kb 0.545kb
cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.

02.19kb 01.99kb 01.06kb 0.545kb
pCa, jTe.

Enzyme: HaeIII.
Probe: pLsC15.

01.88kb 01.67kb 01.42kb 0.860kb 0.685kb
cBr, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSt, sYo, veGe, vBr, vMi, vSa, vYo.

01.88kb 01.67kb 0.860kb 0.685kb
cSa, sSh, vPu.

01.88kb 01.63kb 0.898kb 0.685kb
jTe, pCa.

Enzyme: HinDIII.
Probe: Total cpDNA.

18.70kb 12.70kb 11.20kb 09.92kb 07.79kb 07.31kb — cBr, dAi, hMo, hYo, sBr,
06.90kb 04.94kb 04.55kb 04.29kb 04.18kb 03.92kb — sSa, sSh, sSt, sYo, veGe,
03.38kb 02.70kb 02.49kb 02.07kb 0.605kb — vMi, vYo.
[Other fragments present that were not resolved]

27.20kb 17.80kb 12.30kb 11.10kb 09.65kb 07.79kb — vPu.
07.31kb 06.90kb 04.94kb 04.55kb 04.29kb 04.18kb
03.92kb 03.38kb 02.70kb 02.49kb 02.07kb 0.605kb
[Other fragments present that were not resolved]

18.70kb 12.70kb 09.92kb 07.79kb 07.31kb 06.90kb — cSa, hBr, hSa, vBr.
06.09kb 04.94kb 04.55kb 04.29kb 04.18kb 03.92kb
03.38kb 02.70kb 02.49kb 02.07kb 0.605kb
[Other fragments present that were not resolved]

16.90kb 11.90kb 09.40kb 07.62kb 07.03kb 06.30kb — pCa.
05.00kb 04.55kb 04.29kb 03.38kb 02.70kb 02.49kb
02.07kb 01.74kb 0.691kb
[Other fragments present that were not resolved]

18.70kb 12.70kb 09.92kb 07.76kb 07.31kb 06.90kb — jTe.
05.00kb 04.55kb 04.29kb 03.38kb 02.77kb 02.56kb
02.07kb 0.605kb
[Other fragments present that were not resolved]

Enzyme: HinDIII.
Probe: pLsC1.

11.50kb 0.475kb — All Accessions.
[One fragment << 0.4kb present]

Enzyme: HinDIII.
Probe: pLsC2.

08.15kb 07.11kb — All Accessions.

Enzyme: HinDIII.
Probe: pLsC4.

02.27kb — cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.

03.33kb 02.27kb — pCa.

Enzyme: HinDIII.
Probe: pLsC5ac.

10.3kb	04.80kb	03.67kb				cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.
06.79kb	03.27kb					pCa.
10.30kb	06.69kb	04.80kb	03.67kb			jTe.

Enzyme: HinDIII.
Probe: pLsC6.

17.1kb	07.55kb			cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.
18.30kb	16.10kb	08.08kb	07.39kb	pCa.

Enzyme: HinDIII.
Probe: pLsC7.

07.19kb	06.11kb	02.49kb	02.32kb	All Accessions.

Enzyme: HinDIII.
Probe: pLsC9.

04.30kb	03.21kb	02.37kb			cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.
09.16kb	08.48kb	04.30kb	03.33kb	02.37kb	pCa.
04.30kb	03.54kb	02.37kb			jTe.

Enzyme: HinDIII.
Probe: pLsC10/11/12.

11.30kb	07.44kb	07.05kb	04.80kb			cBr, dAi, hMo, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vMi, vPu, vYo.
07.44kb	07.05kb	06.02kb	04.80kb			cSa, hBr, hSa, vBr.
11.90kb	09.23kb	07.44kb	05.41kb	04.80kb	03.24kb	pCa.
12.60kb	07.44kb	07.05kb	04.80kb			jTe.

Enzyme: HinDIII.
Probe: pLsC13/14.

08.15kb 05.82kb 02.89kb All Accessions.

Enzyme: HinDIII.
Probe: pLsC15.

08.35 All Accessions.

Enzyme: KpnI.
Probe: Total cpDNA.

20.60kb	14.30kb	12.00kb	06.26kb	05.01kb	04.75kb	cSa, hBr, hMo, hSa, sSt,
03.34kb	02.89kb					sYo, veGe, vBr, vYo.
20.70kb	14.30kb	12.00kb	06.26kb	05.01kb	03.34kb	dAi.
02.89kb						

[Not determined for cBr, hYo, jTe, pCa, sBr, sSa, sSh, vMi, vPu]

Enzyme: KpnI.
Probe: pLsC1.

[Not determined]

Enzyme: KpnI.
Probe: pLsC2.

[Not determined]

Enzyme: KpnI.
Probe: pLsC4.

21.8kb 12.4kb All Accessions.

Enzyme: KpnI.
Probe: pLsC5ac.

10.6 cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSt, sYo, veGe, vBr, vYo.

[Not determined for pCa, sSh, vMi, vPu]

Enzyme: KpnI.
Probe: pLsC6.

05.46kb	cBr, cSa, hSa, hYo, jTe, pCa, sBr, sSa, sSh, sYo, veGe, vMi, vPu, vYo.
05.69kb	dAi.

[Not determined for hBr, hMo, sSt, vBr]

Enzyme: KpnI.
Probe: pLsC7

04.51kb 04.30kb	All Acessions.

Enzyme: KpnI.
Probe: pLsC9

11.40kb	cBr, cSa, dAi, hSa, hYo, jTe, pCa, sBr, sSa, sSh, sYo, veGe, vMi, vPu, vYo.

[Not determined for hBr, hMo, sSt, vBr]

Enzyme: KpnI.
Probe: pLsC10/11/12.

11.8kb 06.17kb 05.42kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSt, sYo, veGe, vBr, vPu, vYo.

[Not determined for pCa, sSh,vMi]

Enzyme: KpnI.
Probe: pLsC13/14.

13.40kb 03.37kb	All Accessions.

Enzyme: KpnI.
Probe: pLsC15.

03.30	All Accessions.

Enzyme: PstI.
Probe: Total cpDNA.

38.80kb	25.10kb	17.60kb	11.10kb	10.00kb	04.79kb	cBr, cSa, dAi, hBr, hMo,
04.53kb	02.95kb					hSa, hYo, sBr, sSa, sSh,
						sSt, sYo, veGe, vBr, vYo.
34.10kb	23.10kb	16.60kb	11.10kb	10.00kb	04.79kb	vPu.
04.53kb	02.95kb					
11.10kb	10.00kb	04.79kb	04.53kb	02.95kb		jTe.

[Not determined for pCa, vMi]

Enzyme: PstI.
Probe: pLsC1.

10.00kb	9.14kb	02.81kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.
10.00kb	9.14kb	02.88kb	pCa.

Enzyme: PstI.
Probe: pLsC2.

24.00 — All Accessions.

Enzyme: PstI.
Probe: pLsC4.

[Not determined]

Enzyme: PstI.
Probe: pLsC5ac.

17.50kb 04.71kb — All Accessions.

Enzyme: PstI.
Probe: pLsC6.

41.80kb 12.50kb — All Accessions.

Enzyme: PstI.
Probe: pLsC7.

10.20kb — All Accessions.

Enzyme: PstI.
Probe: pLsC9.

19.00kb	All Accessions.

Enzyme: PstI.
Probe: pLsC10/11/12.

18.60kb 04.65kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, pCa, sBr, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vYo.

[Not determined for sSa]

Enzyme: PstI.
Probe: pLsC13/14.

[Not determined]

Enzyme: PstI.
Probe: pLsC15.

18.80kb 02.92kb	All Accessions.

Enzyme: SacI.
Probe: Total cpDNA.

15.10kb	12.40kb	11.70kb	10.50kb	09.62kb	09.27kb	cBr, sSa sYo, vMi.
07.54kb	06.68kb	04.03kb	03.73kb	03.53kb	02.98kb	
02.16kb	01.74kb					
18.70kb	15.70kb	12.00kb	10.50kb	09.62kb	09.27kb	cSa, hBr, hSa, jTe, sBr,
07.54kb	06.68kb	03.73kb	03.53kb	02.98kb	01.74kb	sSh, sSt, vBr, vPu, vYo.

[Not determined for dAi, hMo, hYo, pCa, veGe]

Enzyme: SacI.
Probe: pLsC1.

14.10kb 11.40kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSt, sYo, veGe, vBr, vMi, vYo.

[Not determined for pCa, sSh, vPu]

Enzyme: SacI.
Probe: pLsC2.

13.50kb 08.49kb 02.65kb 02.58kb	All Accessions.

Enzyme: SacI.
Probe: pLsC4.

01.85kb						cBr, cSa, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, vBr, vMi, vPu, vSa, vYo.
01.85kb	01.44kb					dAi, pCa, veGe.

[NB. some of these digests have a number of large fragments that are probably the result of partial digestion]

Enzyme: SacI.
Probe: pLsC5ac.

20.30	cBr, cSa, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSt, sYo, veGe, vBr, vMi, vYo.

[Not determined for sSa, sSh, pCa, vPu]

Enzyme: SacI.
Probe: pLsC6.

04.82kb	03.49kb	03.35kb	02.05kb	01.50kb	01.37kb	cBr.
01.28kb						
04.82kb	03.35kb	03.11kb	02.05kb	01.50kb	01.37kb	sSa, vMi.
01.28kb						
03.35kb	03.11kb	02.05kb	01.50kb	01.37kb	01.28kb	hBr, hMo, hSa, hYo, sBr, sSt, sYo, vBr, vSa, vYo.
04.82kb	03.49kb	03.35kb	02.58kb	02.05kb	01.50kb	sSh, vPu.
01.37kb	01.28kb					
05.50kb	04.82kb	03.28kb	03.49kb	02.05kb	01.89kb	pCa.
01.79kb	01.37kb	01.56kb				
04.82kb	03.35kb	03.05kb	02.58kb	02.05kb	01.50kb	jTe.
01.28kb						

[Not determined for cSa, dAi, veGe]

Enzyme: SacI.
Probe: pLsC7.

06.46	All Accessions.

Enzyme: SacI.
Probe: pLsC9.

03.57kb	03.31kb	02.01kb	01.82kb	cBr, hBr, hMo, hSa, hYo, sBr, sSa, sSh, sSt, sYo, vBr, vMi, vPu, vSa, vYo.
03.98kb	03.31kb	02.01kb	01.82kb	pCa.
04.58kb	03.31kb	02.01kb	01.82kb	jTe.

[Not determined for cSa, dAi, veGe]

Enzyme: SacI.
Probe: pLsC10/11/12.

08.67kb	06.65kb	06.06kb	cBr, cSa, dAi, hBr, hMo, hSa, hYo, sBr, sSt, sYo, veGe, vBr, vMi, vYo.
06.85kb	06.65kb	06.06kb	jTe.

[Not determined for pCa, sSa, sSh, vPu]

Enzyme: SacI.
Probe: pLsC13/14.

09.42kb 04.05kb 02.30kb 01.76kb — All Accessions.

Enzyme: SacI.
Probe: pLsC15.

13.00kb 06.87kb — All Accessions.

Enzyme: XhoI.
Probe: Total cpDNA.

[Not determined]

Enzyme: XhoI.
Probe: pLsC1.

08.25kb — All Accessions.

Enzyme: XhoI.
Probe: pLsC2.

01.70kb 09.42kb 08.05kb 07.16kb 03.22kb — All Accessions.

Enzyme: XhoI.
Probe: pLsC4.

[Not determined]

Enzyme: XhoI.
Probe: pLsC5ac.

[Not determined]

Enzyme: XhoI.
Probe: pLsC6.

03.38kb	03.12kb	02.11kb	01.92kb	01.73kb	cBr, dAi, hBr, hMo, hSa, hYo, jTe, sBr, sSa, sSh, sSt, sYo, veGe, vBr, vMi, vPu, vSa, vYo.

[Not determined for cSa, pCa]

Enzyme: XhoI.
Probe: pLsC7.

16.60kb	03.19kb	02.22kb		cBr, cSa, hBr, hMo, hYo, sBr, sSa, sSh, sSt, sYo, veGe, vMi, vPu, vSa.
16.60kb	03.19kb	02.32kb	02.22kb	hSa, vBr, vYo.
16.60kb	05.62kb	03.19kb	02.22kb	pCa.
16.60kb	03.33kb	02.22kb		dAi.
16.60kb	03.26kb	02.22kb		jTe.

Enzyme: XhoI.
Probe: pLsC9.

16.30kb	All Accessions.

Enzyme: XhoI.
Probe: pLsC10/11/12.

10.30kb	All Accessions.

Enzyme: XhoI.
Probe: pLsC13/14.

[Not determined]

Enzyme: XhoI.
Probe: pLsC15.

03.57kb 01.40kb 0.626kb All Accessions.

Appendix E.

Chloroplast DNA divergence matrices.

Table E1. Maximum liklihood estimates of the proportion of nucleotide substitutions per restriction site, given as values of 100p, for the Senecio taxa used in this study.

	vMi	vYo	vBr	hMo	hBr	hSa	hYo	dAi	veGe
vMi	-	0.275	0.577	0.667	0.609	0.330	0.276	1.061	1.521
vYo		-	0.462	0.549	0.492	0.217	0.163	0.934	1.385
vBr			-	0.299	0.135	0.242	0.354	1.016	1.707
hMo				-	0.218	0.494	0.440	1.448	1.813
hBr					-	0.327	0.383	1.050	1.685
hSa						-	0.109	0.876	1.445
hYo							-	0.764	1.390
dAi								-	1.452
veGe									-
sSa									
sBr									
sSt									
sYo									
sSh									
cBr									
cSa									
jTe									
pCa									

-Y

Table E1. Cont.

	sSa	sBr	sSt	sYo	sSh	cBr	cSa	jTe	pCa
vMi	0.362	0.221	0.578	0.247	0.977	0.193	1.396	3.618	5.549
vYo	0.247	0.109	0.462	0.136	1.208	0.247	1.319	3.449	5.636
vBr	0.663	0.335	0.107	0.326	1.474	0.549	1.466	3.593	6.027
hMo	0.754	0.441	0.190	0.411	1.334	0.638	1.448	3.724	5.970
hBr	0.694	0.384	0.135	0.355	1.513	0.580	1.443	3.579	6.103
hSa	0.414	0.164	0.407	0.108	1.269	0.302	1.257	3.319	5.791
hYo	0.360	0.109	0.354	0.027	1.152	0.247	1.262	3.331	7.274
dAi	1.151	0.824	1.016	0.791	1.904	0.850	1.455	3.776	7.724
veGe	1.614	1.355	1.707	1.358	2.549	1.248	1.955	4.398	8.557
sSa	-	0.305	0.663	0.331	0.949	0.333	1.367	3.658	5.368
sBr		-	0.355	0.082	1.095	0.193	1.204	3.408	5.682
sSt			-	0.326	1.475	0.549	1.591	3.658	6.105
sYo				-	1.180	0.219	1.291	3.293	5.763
sSh					-	1.188	0.982	1.191	6.295
cBr						-	1.300	3.382	5.500
cSa							-	4.387	5.289
jTe								-	7.313
pCa									-

Y

Table E2. Estimates of sequence divergence, d, when total Lactuca sativa cpDNA was used as a probe for the Senecio taxa used in this study. Values of d are shown in the top right of the Table, while in the bottom left values for the standard errors are given.

	vMi	vYo	vBr	hMo	hBr	hSa	hYo	dAi	veGe
vMi	-	0.0008	0.0092	0.0076	0.0098	0.0083	0.0097	0.0234	0.0176
vYo	0.0008	-	0.0090	0.0057	0.0083	0.0070	0.0079	0.0215	0.0124
vBr	0.0029	0.0027	-	0.0015	0.0015	0.0000	0.0055	0.0200	0.0093
hMo	0.0023	0.0019	0.0011	-	0.0025	0.0013	0.0033	0.0154	0.0203
hBr	0.0027	0.0024	0.0011	0.0013	-	0.0013	0.0059	0.0182	0.0092
hSa	0.0025	0.0022	0.0000	0.0009	0.0009	-	0.0046	0.0168	0.0078
hYo	0.0027	0.0023	0.0021	0.0015	0.0020	0.0018	-	0.0167	0.0088
dAi	0.0044	0.0041	0.0042	0.0034	0.0037	0.0035	0.0035	-	0.0143
veGe	0.0037	0.0030	0.0028	0.0039	0.0025	0.0023	0.0025	0.0033	-
sSa	0.0008	0.0017	0.0040	0.0033	0.0031	0.0032	0.0025	0.0043	0.0038
sBr	0.0022	0.0022	0.0019	0.0013	0.0019	0.0016	0.0015	0.0033	0.0026
sSt	0.0023	0.0020	0.0013	0.0006	0.0052	0.0011	0.0013	0.0034	0.0024
sYo	0.0022	0.0019	0.0015	0.0009	0.0016	0.0013	0.0015	0.0186	0.0023
sSh	0.0022	0.0016	0.0023	0.0013	0.0022	0.0018	0.0018	0.0031	0.0091
cBr	0.0014	0.0015	0.0033	0.0009	0.0030	0.0028	0.0027	0.0045	0.0034
cSa	0.0027	0.0026	0.0009	0.0013	0.0013	0.0007	0.0017	0.0031	0.0022
jTe	0.0059	0.0060	0.0076	0.0059	0.0062	0.0062	0.0066	0.0076	0.0113
pCa	0.0092	0.0092	0.0111	0.0094	0.0093	0.0091	0.0088	0.0093	0.0096

Y

Table E2. Cont.

	sSa	sBr	sSt	sYo	sSh	cBr	cSa	jTe	pCa
vMi	0.0008	0.0068	0.0076	0.0069	0.0054	0.0027	0.0082	0.0329	0.0568
vYo	0.0037	0.0072	0.0063	0.0057	0.0070	0.0033	0.0089	0.0345	0.0568
vBr	0.0124	0.0039	0.0019	0.0025	0.0046	0.0123	0.0009	0.0420	0.0617
hMo	0.0130	0.0026	0.0006	0.0012	0.0018	0.0109	0.0022	0.0377	0.0585
hBr	0.0117	0.0052	0.0032	0.0038	0.0054	0.0124	0.0022	0.0356	0.0587
hSa	0.0124	0.0039	0.0019	0.0025	0.0036	0.0109	0.0007	0.0356	0.0559
hYo	0.0077	0.0033	0.0026	0.0033	0.0035	0.0103	0.0038	0.0392	0.0521
dAi	0.0208	0.0143	0.0160	0.0166	0.0101	0.0224	0.0115	0.0482	0.0576
veGe	0.0165	0.0094	0.0084	0.0078	0.0064	0.0154	0.0060	0.0490	0.0603
sSa	-	0.0084	0.0093	0.0107	0.0018	0.0038	0.0116	0.0386	0.0605
sBr	0.0026	-	0.0032	0.0013	0.0026	0.0094	0.0031	0.0368	0.0735
sSt	0.0028	0.0015	-	0.0019	0.0027	0.0088	0.0015	0.0345	0.0568
sYo	0.0030	0.0009	0.0011	-	0.0035	0.0094	0.0037	0.0353	0.0577
sSh	0.0013	0.0015	0.0016	0.0018	-	0.0103	0.0046	0.0199	0.0559
cBr	0.0017	0.0026	0.0025	0.0026	0.0020	-	0.0113	0.0168	0.0622
cSa	0.0031	0.0016	0.0011	0.0017	0.0016	0.0031	-	0.0381	0.0577
jTe	0.0071	0.0062	0.0060	0.0061	0.0050	0.0042	0.0071	-	0.0797
pCa	0.0095	0.0105	0.0092	0.0092	0.0091	0.0097	0.0092	0.0113	-

Appendix F.

Ribosomal DNA data.

Table F1a Phenotypes of each of single and double digests used in the construction of the *Senecio vulgaris* ssp. *vulgaris* var. *vulgaris* restriction maps shown in Chapter 2, Figure 2.2. All fragment sizes are in kilobases (kb). Figures in parentheses refer to fragments which were faint (partial digests?), but useful in the construction of the maps.

Enzyme: EcoRI.
Sizes: 7.8, 7.5, 3.1.
Enzyme: BamHI.
Sizes: 7.2, 5.6, 3.1, 2.8, 1.8, (1.5), 1.28, 1.21.
Enzyme: EcoRV.
Sizes: 6.8, 6.6, 4.0.
Enzyme: XbaI.
Sizes: 15.
Enzyme: XhoI.
Sizes: 15.
Enzyme: BstEII.
Sizes: 12.9.
Enzyme: EcoRI + BamHI.
Sizes: (8.3), (6.9), 2.3, 1.44, 1.28, 1.21, 0.99.
Enzyme: EcoRI + EcoRV.
Sizes: 6.3, 6.1, 3.5.
Enzyme: EcoRI + XbaI.
Sizes: 3.1, 1.4.
Enzyme: EcoRI + XhoI.
Sizes: 4.7, 4.4, 3.1, 2.9.
Enzyme: EcoRI + BstEII.
Sizes: 3.8, 3.4.
Enzyme: BamHI + EcoRV.
Sizes: 2.84, 1.53, 1.28, 1.21, 1.08.
Enzyme: BamHI + XbaI.
Sizes: 3.1, 2.8, 1.8, 1.2.
Enzyme: BamHI + XhoI
Sizes: 3.1, 2.8, 1.8, (1.5), 1.28, 1.21.
Enzyme: BamHI + BstEII.
Sizes: 3.1, 2.8, 1.8, (1.5), 1.28, 1.21.
Enzyme: EcoRV + XbaI..
Sizes: 6.8, 6.6, 4.0, 2.0.
Enzyme: EcoRV + XhoI.
Sizes: 6.8, 6.6, 4.0.
Enzyme: EcoRV + BstEII.
Sizes: 4.7, 4.0.
Enzyme: XbaI + XhoI.
Sizes: 15.
Enzyme: XbaI + BstEII.
Sizes: Not digested.
Enzyme: XhoI + BstEII
Sizes: Not digested.

Table F1b Phenotypes of each of single and double digests used in the construction of the *Senecio squalidus* restriction maps shown in Chapter 2, Figure 2.2. All fragment sizes are in kilobases (kb). Figures in parentheses refer to fragments which were faint (partial digests?), but useful in the construction of the maps.

Enzyme: EcoRI.
Sizes: 8.6, 7.5, 5.5.
Enzyme: BamHI.
Sizes: 8.5, 7.1, 2.8, 1.2.
Enzyme: EcoRV.
Sizes: 7.7, 6.6, 4.4.
Enzyme: XbaI.
Sizes: 14.1, 12.8.
Enzyme: EcoRI + BamHI.
Sizes: 7.9, 6.4, 1.2, 1.0, 0.6.
Enzyme: EcoRI + EcoRV.
Sizes: 7.7, 6.1, 5.5.
Enzyme: EcoRI + XbaI.
Sizes: 7.9, 6.4, 5.5, 1.4.
Enzyme: BamHI + EcoRV.
Sizes: 6.3, 5.0, 1.5, 1.4, 1.2, 1.0.
Enzyme: BamHI + XbaI.
Sizes: 8.4, 7.3, 2.8, 1.2.
Enzyme: EcoRV + XbaI.
Sizes: 4.4, 1.9.

Table F2 Phenotypes of each of the accessions used in the rDNA study. Numbers refer to the phenotypes shown in Chapter 2, Figure 2.3. '-' indicates that data is not available for this particular enzyme.

Location.	Individual No.	Enzyme. EcoRI	EcoRV	BamHI
Senecio cambrensis.				
Brymbo.	3	4	7	8
Brymbo.	1	4	7	8
Brymbo.	7	4	7	8
Edinburgh, Leith Docks.	1	4	7	7
Mochdre.	2/86	4	7	8
Mochdre.	7/86	4	7	8
Mochdre.	6/86	4	7	-
Edinburgh, Salamander St.	1	4	7	1
Edinburgh, Salamander St.	21	-	-	1
Edinburgh, Salamander St.	6/86	1	1	-
Edinburgh, Salamander St.	7/86	1	1	1
Edinburgh, Salamander St.	6	1	1	1
Edinburgh, Salamander St.	9	1	1	1
Edinburgh, Salamander St.	19	1	1	1
Senecio squalidus.				
Brymbo.	8	4	-	-
Cardiff.	10	-	2	-
Cardiff.	6	3	2	4
Cardiff.	8	3	2	4
Devon St.	6	4	2	4
Devon St.	7	4	2	4
Devon St.	8	4	2	4
Edinburgh, Leith Docks.	88/1	3	-	4
Edinburgh, Leith Docks.	10	5	-	4
Edinburgh, Leith Docks.	14	3	-	4
Edinburgh, Leith Docks.	11	3	3	4
Edinburgh, Leith Docks.	13	5	-	4
Edinburgh, Leith Docks.	88/4	3	3	4
Edinburgh, Leith Docks.	3	-	2	4
Edinburgh, Leith Docks.	1	-	2	4
Edinburgh, Leith Docks.	6	5	-	4
Edinburgh, Leith Docks.	7	3	3	4
Edinburgh, Leith Docks.	8	4	-	4
Lincoln.	3	4	2	4
Lincoln.	4	-	2	4
Lincoln.	1	3	2	4
Lincoln.	3	3	3	4
Mochdre.	102	3	3	4
Mochdre.	101	4	3	4
Sheffield.	3	4	2	4
Sheffield.	6	3	2	4
York.	2a	-	-	4
York.	3	-	-	4
York.	4	-	-	4

Location.	Individual No.	Enzyme. EcoRI	EcoRV	BamHI
Senecio squalidus.				
York.	14	4	2	4
York.	10	4	2	-
York.	2	3	3	4
York.	8	3	-	4
Senecio vulgaris ssp. denticulatus.				
Ainsdale, Lancashire.		2	1	6
Ainsdale, Lancashire.		2	1	6
Jersey.	4	2	1	6
Jersey.	2	2	1	6
Jersey.	1	-	1	6
Senecio vernalis.				
Germany.	2	1	4	5
Germany.	5	1	4	5
Germany.	6	-	5	5
Senecio vulgaris ssp. vulgaris var. vulgaris.				
Boness.	2	-	1	-
Brymbo.	4	1	1	1
Brymbo.	5	1	1	1
Brymbo.	6	1	1	2
Brooklands Avenue, Cambridge.	2	1	1	1
Brooklands Avenue, Cambridge.	2a	-	1	-
Brooklands Avenue, Cambridge.	3	1	1	-
Brooklands Avenue, Cambridge.	4	1	1	1
Devon St.	1	1	1	1
Devon St.	2	-	1	1
Devon St.	4	-	6	1
Devon St.	6	1	-	1
Devon St.	7	1	1	1
Edinburgh, Leith Docks.	2	-	1	3
Edinburgh, Leith Docks.	3	1	1	1
Edinburgh, Leith Docks.	5	1	1	1
Lincoln, Central.	4	1	1	1
Lincoln, Grantham St.	5	1	1	1
Migvie, Aberdeenshire.	A	1	1	1
Mochdre.	8	1	-	1
Mochdre.	9	-	1	1
Puffin Is.	N21	1	1	3
Puffin Is.	N22	-	1	3
Edinburgh, Salamander St.	7	1	1	2
Edinburgh, Salamander St.	9	1	1	2
Edinburgh, Salamander St.	6n	1	1	1
Edinburgh, Salamander St.	7n	1	1	1
Strathkinness Low Road, St. Andrews.	1	-	1	1
Strathkinness Low Road, St. Andrews.	2	-	6	1
Strathkinness Low Road, St. Andrews.	5	-	1	1

Location.	Individual No.	Enzyme. EcoRI	EcoRV	BamHI
<u>Senecio vulgaris ssp. vulgaris var. vulgaris</u>.				
Strathkinness Low Road, St. Andrews.	14	-	1	1
Strathkinness Low Road, St. Andrews.	15	1	6	1
Strathkinness Low Road, St. Andrews.	17	1	1	1
Strathkinness Low Road, St. Andrews.	18	-	1	1
Strathkinness Low Road, St. Andrews.	29	-	6	1
York.	3	1	-	2
York.	4	-	1	-
York.	6	1	1	1
York.	9	1	1	2
<u>Senecio vulgaris ssp. vulgaris var. hibernicus</u>.				
Devon St.	1	1	1	1
Devon St.	2	1	1	1
Devon St.	3	1	1	1
Devon St.	7	1	6	1
Devon St.	9	-	6	-
Edinburgh, Leith Docks.	16	-	1	2
Edinburgh, Leith Docks.	17	-	1	1
Edinburgh, Leith Docks.	18	1	1	1
Lincoln, Central.	1	1	1	1
Mochdre.	28/27	1	1	4
Edinburgh, Newcraighall, Bank..	7(2)	-	6	-
Edinburgh, Newcraighall, Bank.	9(2)	-	1	1
Edinburgh, Newcraighall, Bank.	10	-	6	1
Edinburgh, Newcraighall, Bank.	13	1	6	1
Edinburgh, Newcraighall, Bank.	25	-	1	-
Edinburgh, Newcraighall, Road.	15	1	6	2
Edinburgh, Salamander St.	3	1	-	1
Edinburgh, Salamander St.	8	1	-	1
Edinburgh, Salamander St.	20	1	-	1
Edinburgh, Salamander St.	30	1	-	2
Edinburgh, Salamander St.	31	1	-	2
Edinburgh, Salamander St.	43	1	1	3
Edinburgh, Salamander St.	44	-	1	1
York RJA	2	1	1	2
York RJA	3	1	1	1
York RJA	4	1	1	2
York RJA	11	1	1	2
York RJA	15	1	1	2
York RJA	17	1	1	2
York RJA	18	1	-	2
York RJA	19	1	1	2
York RJA	25	1	1	2
York RJA	25a	1	1	2
York RJA	26	1	1	2
York Warr.	3	1	1	1
York Warr.	8	1	1	-
York Warr.	20	1	1	4

Appendix G.

Determination of the quantity of chloroplast DNA per total DNA sample.

G1.1 Calculation of the amount of chloroplast DNA in each total DNA sample.

1pmol of 1kb DNA = 0.66μg.

ie. 1 pmol of xkb DNA = 0.66xμg.

$$\text{ie.}\quad \frac{0.001\ \mu g/ng}{0.66x\ \mu g/pmol} = \frac{1.52 \times 10^{-3}}{x}\ pmol/ng$$

$$\frac{1.52 \times 10^{-15}}{x}\ mol/ng \times 6.02 \times 10^{23}$$

$$\text{ie}\quad \frac{9.15 \times 10^{8}}{x}\ \text{DNA molecules/ng} \qquad [1]$$

Let A be the area of the standard amount, y ng

$$\text{ie.}\quad \frac{9.15 \times 10^{8} \times y}{x}\ \text{DNA molecules} = \text{A area units}$$

Therefore:

$$\frac{9.15 \times 10^{8} \times y}{xA}\ \text{DNA molecules/unit area} \qquad [2]$$

However, to determine the number of insert molecules/unit area it is necessary to multiply Eq. [2] by the correction factor 9.9/12.6. Where 9.9kb is the size of the insert and 12.6kb is the total plasmid size (vector plus insert).

If sample area, k, produced by z ng of DNA, then

1 unit area = z/k ng DNA.

$$\text{ie.}\quad \text{Sample Area} = \frac{7.19 \times 10^{8} \times yk}{zxA}\ \text{molecules} \qquad [3]$$

1pg DNA = 0.965×10^{9} bp.

Since pLsC2 is an invert repeat sequence probe then Eq. [3] must be divided by 2.

Let S be the size of the cpDNA molecule. Assume that this is constant across taxa and is approximately 150kb.

Where x = 12.6kb.

Therefore, the total mass of cpDNA in the sample is:

$$\frac{4433.83yk}{zA} \text{ pg} \qquad [4]$$