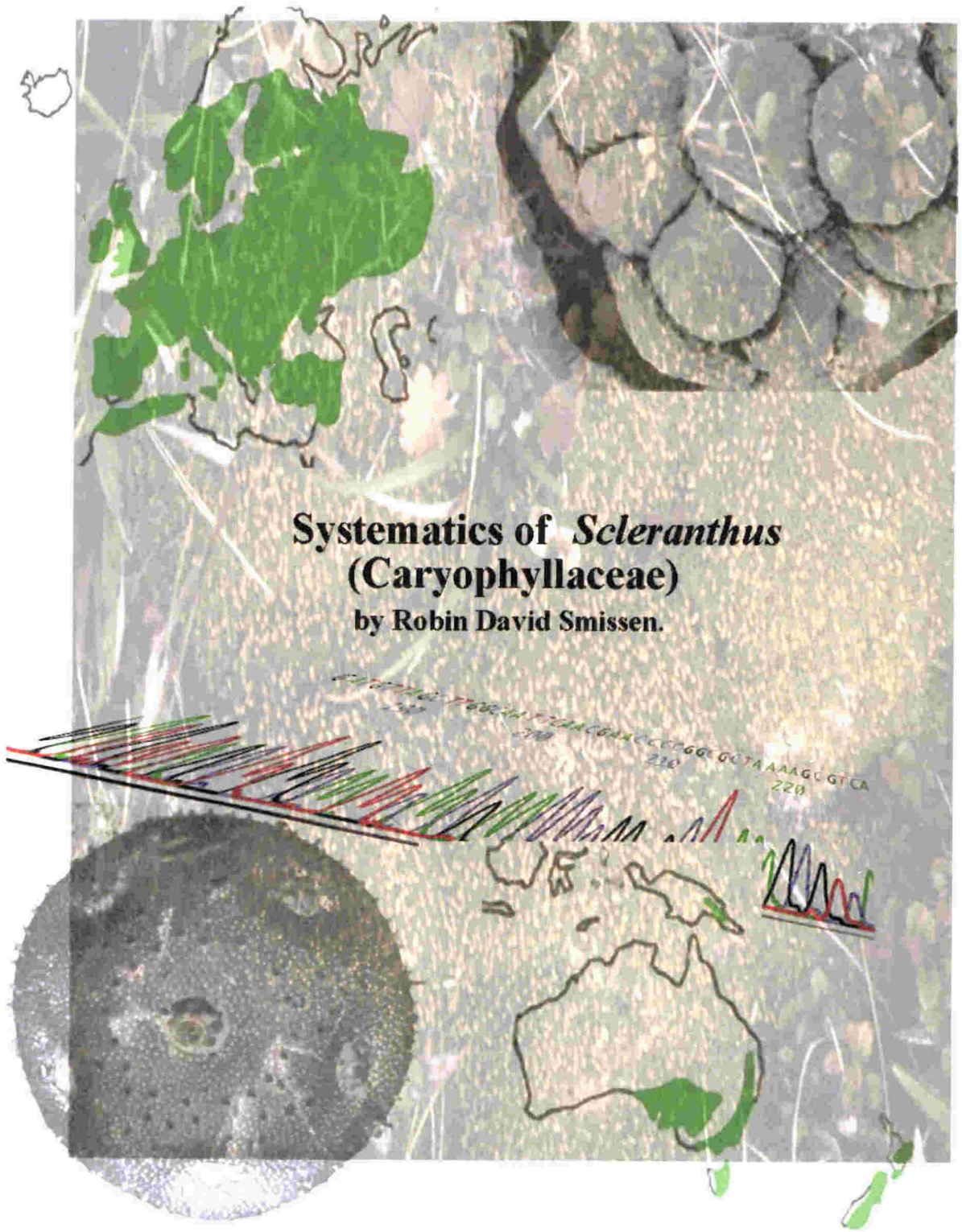

Systematics of *Scleranthus* (Caryophyllaceae)

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Abstract

Scleranthus is a genus of about 12 species of herbaceous flowering plants or small shrubs with a disjunct Eurasian/Australasian distribution. Monophyly of the genus is supported by the close similarity of gynoecial development of all species and consistent with nuclear ITS DNA sequence analysis. Traditionally the genus had been divided into two sections, section *Scleranthus* and section *Mniarum*. Section *Mniarum* is exclusively Australasian while section *Scleranthus* has been circumscribed to contain exclusively European species or a combination of European and Australasian species. Pollen and floral characters align the species into Australasian and Eurasian groups also supported by nuclear ITS DNA sequence analysis. Section *Scleranthus* as more broadly defined (i.e., *sensu* West and Garnock-Jones, 1986) is therefore at least paraphyletic or at worst polypyhyletic. Phylogenetic reconstructions based on morphological characters differ from those based on ITS sequences in supporting different relationships within the Australasian species of *Scleranthus*. Hybridisation and introgression within the genus are discussed and suggested as the cause of discordance between morphology and DNA sequence based trees. Low sequence divergence among *Scleranthus* ITS sequences suggests that the European and Australasian clades within the genus diverged within the last 10 million years. Biogeographic implications of these dating and competing hypotheses explaining the disjunct North-South distribution of the genus are discussed.

Nuclear ITS and chloroplast *ndh*F DNA sequences both suggest that *Scleranthus* belongs to a clade within the family Caryophyllaceae consisting of members of subfamilies Alsinoideae and Caryophylloideae. Phylogenetic relationships between genera belonging to the three subfamilies of Caryophyllaceae (Alsinoideae, Caryophyloideae, and Paronychioideae) are addressed in this thesis through *ndh*F sequence analysis, which provides no support for the monophyly of traditionally recognised groups.

Morphological character data sets are likely to always encompass multiple incongruent data partitions (*sensu* Bull *et al.* 1993). It may therefore be appropriate to combine data from DNA sequence and morphology for parsimony analysis even where the two are significantly incongruent.

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Chapter 1: Introduction to the thesis

"...in all probability we are just about at the beginning of human history, and the generations which will put us right are likely to be far more numerous than those whose knowledge we - often with a considerable degree of contempt - have the opportunity to correct."

Friedrich Engels (1894).

This thesis centers on the flowering plant genus *Scleranthus* (Caryophyllaceae), known commonly in Europe as knawels. To aid the reader an informal description of the plants is provided below (section 1.2). However, to ensure clarity in discussion of subgeneric groupings, taxonomic issues in the genus are discussed first.

1.1 Taxonomy

The genus consists of 11 accepted named species plus one as yet unnamed species (West and Garnock-Jones, 1986). Although relatively little has been written on the subgeneric taxonomy of *Scleranthus,* classification of these plants has had a somewhat confused history. The historical outline provided here is not intended as a comprehensive listing of names, merely a summary of the differing usage of the names *Scleranthus* and *Mniarum.*

Two European species of *Scleranthus* were named by Linnaeus in 1753, *S. perennis* and *S. annuus* (the perennial and annual knawel respectively). A plant of New Zealand origin was subsequently named by J. R. and G.

Forster in 1776 as *Mniarum biflorum* (*Ditoca muscosa* Banks and Solander ex Gaertn. is a later synonom). Robert Brown added new species from Australia to both the above genera in his *Prodromus Florae Novae Hollandiae* (1810): *M. fasciculatum, S. pungens* and *S. diander.* Brown (1810) noted that the two genera differed in that *Mniarum* plants bore flowers with only one stamen, had different (presumably smaller) inflorescences and four- rather than five- parted perianth (although in *M. fasciculatum* sepal number is actually variable). These differences were not deemed sufficient to merit separate genera by J. D. Hooker (1853) who reduced *Mniarum* to a synonym of *Scleranthus* in his *Flora Novae-Zelandiae* stating:

"I see no grounds on which to separate this genus [Mniarum] *from* Scleranthus, *to two Tasmanian species of which the present is very closely allied."*

Pax (1889) follows Hooker (1853) in recognizing only one genus, *Scleranthus*, for these plants, but recognized two subgenera: subgenus *Euscleranthus* growing in Europe, Asia and Africa (and including *S. perennis* and *S. annuus*), and subgenus *Mniarum* growing in Australia (he lists only *S. biflorus* by name). Later, Pax and Hoffman (1934) dealt with *Scleranthus* classification somewhat more thoroughly, recognizing two subgeneric groups at sectional rank: section *Euscleranthus* (including the Eurasian/Mediterranean species *S. perennis* and *S. annuus,* along with three other similarly distributed species, of which only *S. uncinatus* is currently recognized: Sell, 1964); and section *Mniarum* (now explicitly including all the Australian species previously ascribed to either *Mniarum* or *Scleranthus*).

West and Garnock-Jones (1986) present what they refer to as a "traditional" classification recognizing the distinction between the single stamened plants of Australia (their section *Mniarum*, in which they recognized, 5 named and one as yet unnamed species); and the larger flowered plants of Australia, Eurasia and North Africa (their section *Scleranthus,* 6 species: see Table 1.1). These authors argued that the three Australian species they classified in section *Scleranthus* might be phylogenetically closer to members of their section *Mniarum,* and if this was so, that they should be transferred to section *Mniarum*. Since they cast doubt on the monophyly of the sections they recognized this appears to be something of a 'straw-man' argument. This classification (i.e., West and Garnock-Jones 1986) appears to have no formal history prior to their paper (other than Brown, 1810). However, their narrow circumscription of section *Mniarum* is useful in addressing *Scleranthus* phylogeny as it is a morphologically distinct and apparently strictly monophyletic grouping. Moreover, earlier classifications are not accompanied by explicit discussion of phylogenetic relationships and should not be interpreted as being phylogenetically based. For these reasons, and as it is the most recent taxonomic treatment of the genus, the classification of West and Garnock-Jones (1986), as described in Table 1.1, is followed in this thesis.

Table 1.1. *Scleranthus* species names by section according to West and Garnock-Jones (1986). [a] Australian species included in section *Mniarum* by Pax and Hoffman (1934) but treated as section *Scleranthus* in West and Garnock-Jones (1986) and this thesis.

Section *Scleranthus*	Section *Mniarum*
S. annuus L. (1753)	*S. biflorus* (J. R. Forst & F. Forst.) Hook. f. (1852)
S. diander R. Br. (1810)[a]	*S. brockiei* P. A. Will. (1956)
S. minusculus F. Muell. (1890)[a]	*S. fasciculatus* (R. Br.) Hook. f. (1855)
S. perennis L. (1753)	*S. singuliflorus* (F. Muell.) Mattf. (1938)
S. pungens R. Br. (1810)[a]	*S. uniflorus* P. A Will. (1956)
S. uncinatus Schur (1850)	

1.2 *Scleranthus* biology

All species of the genus *Scleranthus* are low growing herbs or dwarf shrubs with short, single-veined linear leaves and very small green flowers with no petals. In some species, internodes are short and the leaves densely packed forming a cushion (e.g., *S. uniflorus*: Figure 1.1) whereas in other species internodes are longer producing a more spreading habit (e.g., *S. perennis*: Figure 1.2). In all species flowers produce a single ovule that usually develops into a fertile seed. Perigyny is well developed in all species and the sepals and floral cup are persistent so that fruits are shed surrounded by the dry dead tissue of these organs.

Stamen number is variable from 1 to 10 in the genus, presumably reflecting differing pollination biology among species. Pollination biology has been examined in *S. perennis* and *S. annuus*. In the 10-stamened *S. perennis,* cross-pollination is apparently mediated by ants attracted to flowers by

nectar produced by glands at the base of stamens (Svensson, 1985). In 2-stamened *S. annuus,* cross-pollination is apparently rare (Svensson, 1990).

1.2.1 Section *Scleranthus*

Section *Scleranthus*, as defined in West and Garnock-Jones (1986), contains three species found in Europe, West Asia and North Africa and three species endemic to Australia. One of the Eurasian species, *S. annuus,* is naturalized in many parts of the world. The three Eurasian species are morphologically similar to one another, all being small spreading herbs. Of these three species, *S. annuus* and *S. uncinatus* are annuals and *S. perennis* a semi-annual or "biennial" (Svensson, 1985). All species occupy disturbed, dry and often sandy habitats such as pasture and roadsides. Flowers of these species, although small, are often numerous and borne in dense inflorescences (Figure 1.3). In *S. perennis* the sepals are rounded and spreading with a white margin while in the other two species they are narrow and have little or no white margin (Figure 1.4). *Scleranthus* is morphologically variable through Europe with many species names in synonomy. A number of subspecies of *S. perennis* and *S. annuus* are accepted in *Flora Europaea* (Sell, 1964). The three Australian species of section *Scleranthus* are more morphologically diverse than their European counterparts and include two small perennials (*S. diander* and *S. pungens*) and a semi-herbaceous annual (*S. minusculus*). Like their European counterparts, the Australian members of section *Scleranthus* have inflorescences of many flowers, which in the case of *S. pungens* are comparatively large and have sepals with white margins. The lanceolate sepals of *S. minusculus* become several millimetres long at the fruiting stage although they are shorter and connivent at anthesis.

A specimen of *S. pungens* is illustrated (Figure 1.5). The habitats of Australian members of section *Scleranthus* include montane grassland (*S. diander*), rocky out-crops (*S. pungens*), and sand hills and open woodland (*S. minusculus*).

1.2.2 Section *Mniarum*

The species included by West and Garnock-Jones (1986) in section *Mniarum* (*S. biflorus, S. uniflorus, S. singuliflorus, S. fasciculatus,* and *S. brockiei*) occur only in Australasia and Papua New Guinea and are morphologically very similar to each other. All have very small inconspicuous flowers without any trace of visual attractant (figure 1.6). Their inflorescences are reduced to pairs or solitary flowers that are most obvious at fruiting when they are borne on elongated peduncles (Figure 1.7). The reduction in size of flowers has been accompanied by reduction of the androecium to a single stamen (in most flowers) which usually dehisces in contact with the stigmas. Some species of this group (*S. biflorus, S. uniflorus* and *S. brockiei*) form dense cushions that are often compared with (and sometimes mistaken for) mosses (Figure 1.1). These types are commonly grown as ornamentals. *S. fasciculatus* and *S. biflorus* are illustrated below. Section *Mniarum* species are often found in montane, subalpine, coastal or dry riverbed habitats. Differences between the two sections of *Scleranthus* are summarized in Table 1.2.

Table 1.2. Comparison of *Scleranthus* sections. Adapted from West and Garnock-Jones 1986). [a] *sensu* West and Garnock-Jones 1986.

	Section *Scleranthus*[a]	Section *Mniarum*[a]
Flowers	clustered, showy	insignificant, paired or solitary
Stamens	2 – 10	1
Sepals	spreading with cream or white scarious margins	connivent, without scarious margins
Pollen/ovule ratio	high	low
Biogeography	Australia, Eurasia, North Africa	Australia, Papua New Guinea, New Zealand

Figure 1.1 ***Scleranthus uniflorus***

Figure 1.2 ***Scleranthus perennis*** **vegetative habit. Scale bar 10 mm.**

Figure 1.3 ***Scleranthus annuus*** **several flowering plants. Scale bar 100mm.**

Figure 1.4 ***Scleranthus annuus*** **inflorescence. Scale bar 10mm.**

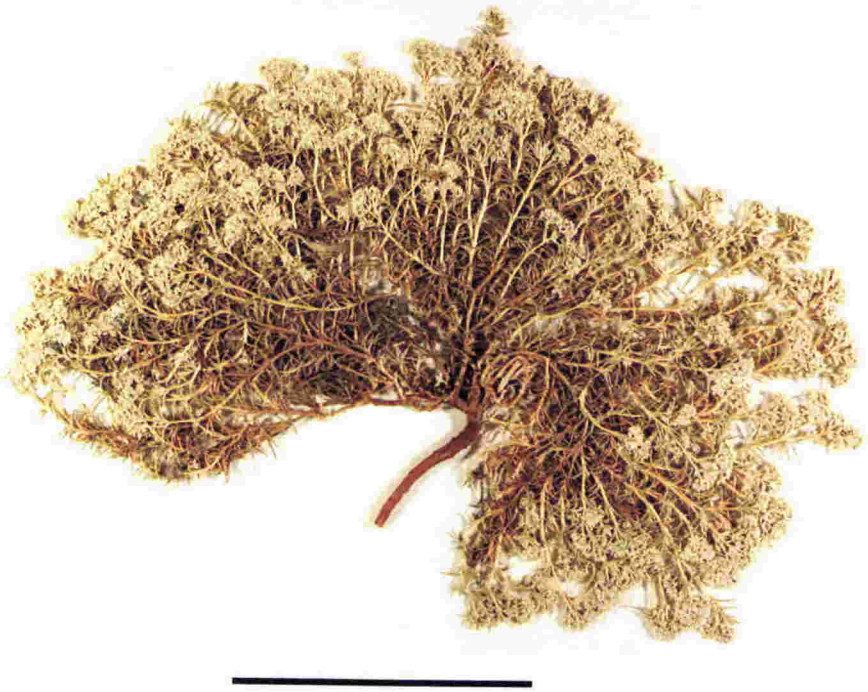

Figure 1.5 ***Scleranthus pungens*** **(herbarium specimen). Scale bar 100 mm.**

Figure 1.6 ***Scleranthus uniflorus*** **flower. Scale bar 1 mm.**

Figure 1.7 ***Scleranthus fasciculatus*** **fruiting plant. Scale bar 10 mm.**

1.3 Why study *Scleranthus*?

1.3.1 Biogeography

Scleranthus is a small genus that exemplifies some of the biological and biogeographic puzzles often presented by Southern Hemisphere plants. Perhaps the most striking feature of *Scleranthus* is its biogeography, with widely separated native ranges centered in Europe and Australasia (Figure 1.8.). Many components of Australasian floras, especially many New Zealand alpine and subalpine plants, are presumed to be derived from Northern Hemisphere stocks dispersing into the area through South East Asia (Raven, 1973). Such a simple explanation of *Scleranthus* origins in Australasia is complicated by the absence of the genus from East and South East Asia and the greater morphological diversity represented by Australasian species. The most northerly occurrence of the southern species of *Scleranthus* (*S. singuliflorus*, found in Papua New Guinea) is part of section *Mniarum,* whose species share several derived character states. This implies its presence in PNG is the result of a secondary colonization and is not a relict of island hopping into Australasia. A northern origin hypothesis therefore requires either extinction of intermediary *Scleranthus* populations throughout Asia, or direct dispersal across most of the globe. It should be noted that *Scleranthus* propagules are apparently not adapted for long distance dispersal, being of small but not minute size, possessing no wings or other structures encouraging wind dispersal and having no obvious attraction for animal dispersers nor any hooks or other physical structures to promote adherence to animals. Alternative explanations of *Scleranthus* distribution were presented by West and Garnock-Jones (1986) and include

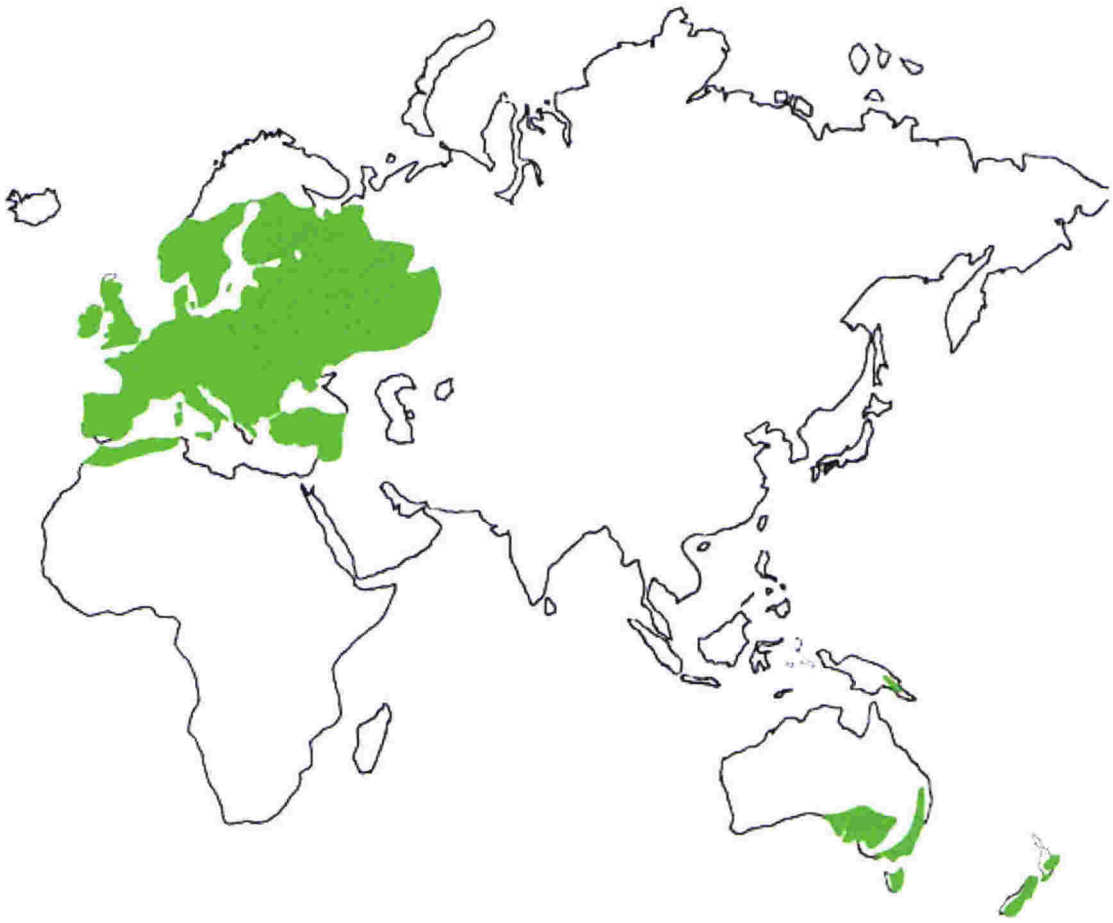

Figure 1.8 Worldwide native distribution of *Scleranthus*.

separate progenitors for northern and southern *Scleranthus* species (non-monophyly of *Scleranthus*) and derivation of European species from Australasian ancestors. These are more fully elaborated and discussed in chapter 3.

Another interesting element to *Scleranthus* biogeography is the trans-Tasman distribution of two species of section *Mniarum*, *S. biflorus* and *S. brockiei*, which occur in Australia and New Zealand. One explanation is that the species are ancient (or have evolved along parallel lines from ancestral stock) and that the disjunction is the result of the separation and drift of New Zealand and Australian landmasses. Alternatively, these distributions might result from multiple long-distance dispersal across the Tasman Sea.

Evaluation of alternative hypotheses explaining either of these aspects of *Scleranthus* biogeography requires improved knowledge of *Scleranthus* phylogeny, providing much of the impetus for this thesis. *Scleranthus* sub-generic classification is also of some interest. If the genus as a whole is monophyletic, then it is possible that the morphologically uniform species of section *Mniarum* are derived from within the more variable section *Scleranthus*, making the latter paraphyletic (West and Garnock-Jones, 1986). This group is, therefore, one in which application of cladistic methodology has potential to provide a more objectively based taxonomy better reflecting the relationships between species.

Intrinsic to any cladistic approach (and indeed, all modern taxonomic method) is the evaluation of variable characters. The reduced vegetative habit and flowers of *Scleranthus* render the number of easily obtainable, traditional morphological characters insufficient to reliably reconstruct

phylogenetic relationships between species. Two approaches have been employed to provide additional character information in this thesis, DNA sequencing and microscopic examination of flowers.

1.3.2 Phylogeny of the family Caryophyllaceae

Scleranthus is a member of the family Caryophyllaceae which includes ornamentals such as *Gypsophila* and *Dianthus*, and weedy herbs such as *Stellaria* (chickweeds) and *Sagina* (pearlworts). The family is traditionally divided into three subfamilies, Caryophylloideae, Alsinoideae and Paronychioideae. Paronychioideae, or part thereof, is sometimes recognized as a separate family, Illecebraceae (e.g., Hutchinson, 1974; Cheeseman, 1906)). Phylogenetic relationships within and between these subfamilies have received little recent attention except in the tribe Sileneae of Caryophylloideae (Oxelman and Liden, 1995; Oxelman, 1997). The monophyly of the Caryophylloideae is supported by a number of morphological and chromosomal apomorphies, but the monophyly of Alsinoideae and that of the Paronychioideae are doubtful (Bittrich, 1993). Several lineages in the family have apparently evolved a reduced floral morphology (associated with self-pollination) independently and a number of genera are poorly known. Several genera, including *Scleranthus*, have previously been included in both the subfamilies Alsinoideae (Pax and Hoffman, 1934; Bittrich 1993) and Paronychioideae (or family Illecebraceae: Cheeseman, 1906; Hutchinson, 1974). The poor level of understanding of (or at least the lack of consensus on) subfamilial relationships not only leads to unstable classifications but also causes problems in selecting outgroup taxa for cladistic studies in groups such as

Scleranthus. Consequently this thesis also explores the relationship between *Scleranthus* and the other genera of the Caryophyllaceae and makes a contribution toward molecular phylogenetic study of the family.

To summarize, the aims of the thesis at the outset were:

1. **To test the monophyly of *Scleranthus* considering the possibility of bi-hemispheric (polyphyletic) origin of the genus**

2. **To test alternative phylogenies for the species of *Scleranthus* using nrDNA ITS sequences and novel morphological/anatomical data.**

3. **To evaluate alternative explanations of *Scleranthus* biogeography in the light of improved phylogenetic information and assess current classification.**

4. **To explore subfamilial phylogeny in Caryophyllaceae with a view to finding the sister group (or groups) of *Scleranthus* from amongst candidate genera of Alsinoideae and Paronychioideae.**

1.4 Thesis structure and content

This thesis contains three results chapters (Chapter 2, 3, and 4) written in the form of journal articles (it is anticipated that these will be submitted for publication in only slightly modified forms) followed by an overall summary and discussion. Each results chapter deals with different aspects of the study but as some overlap is in places inevitable this has been dealt with by

reference between chapters. A description of the contents and conclusions of each chapter follows.

1.4.1 Chapter 2: Morphology and Phylogeny of *Scleranthus* (Caryophyllaceae)

Chapter 2 reports the results of primarily descriptive work dealing with *Scleranthus* flower anatomy, morphology, embryology and palynology along with a cladistic analysis of characters derived from this work and from previous study (West and Garnock-Jones, 1986 and unpublished). Although floral anatomy and embryology of the genus are largely uniform, variation in pollen morphology, external gynoecial morphology and nectary shape provided cladistic characters. Monophyly of the genus as a whole is not addressed by cladistic analysis in this chapter, but is best supported by the uniformity of gynoecial development and anatomy in the genus, and by the lack of variation in embryology. Pollen grains of the genus fall into two groups, larger grains with thicker exine in European species and smaller, thinner-walled grains in the Australasian species. Cladistic analysis is conducted without reference to outgroups but European and Australasian species are separated by the longest branch of the shortest tree obtained for the character matrix used. Moreover, some of the characters shared within each group are likely to be apomorphic based on informal comparisons with likely out-groups. On this basis it is concluded that Australasian native species of *Scleranthus* are a monophyletic group and that section *Scleranthus* (*sensu* West and Garnock-Jones, 1986) is paraphyletic. Monophyly of section *Mniarum* is upheld, but it is not clear from morphological evidence whether it is nested within or is a sister group to the

Australian species of section *Scleranthus*. No firm conclusions are drawn as to subfamilial placement or sister group relations with other Caryophyllaceae.

Interesting variation in anther number (and hence pollen/ovule ratios) was found between species. The species with the highest pollen/ovule ratio, *S. pungens*, was also found to have elaborated nectaries between stamens that were previously interpreted as staminodes. Biological implications of these data are discussed, but field and genetic data required to test emergent hypotheses about breeding strategies and out-crossing rates are currently unavailable.

1.4.2 Chapter 3: Phylogeny and biogeographical history of *Scleranthus* (Caryophyllaceae) inferred from ITS nucleotide sequence data.

Chapter 3 reports the results of nuclear ribosomal DNA Internal Transcribed Spacer (ITS) sequence analysis with bearing on *Scleranthus* phylogeny and its relationships to other Caryophyllaceae. Sequences were gathered from collections of all but one species of *Scleranthus* and a selection of other Caryophyllaceae. ITS sequences were found to vary little within *Scleranthus,* but were highly divergent among most genera belonging to the Caryophyllaceae. Numerous insertions/deletions (indels) and possibly other rearrangements prevented reliable alignment of sequences of different genera for phylogenetic analysis. The ITS2 region was found to be more readily aligned than ITS1 in the family as it had more regions of conserved sequence. ITS2 sequences for a selection of Caryophyllaceae species

covering all three subfamilies were compared phenetically by constructing a dendrogram from pairwise comparisons of sequences using the Guidetree function of the ClustalW computer package. The close similarity of all *Scleranthus* ITS sequences quantified by this approach is consistent with monophyly of the genus. Other close relationships consistent with pairwise comparison of ITS2 sequences are between *Sagina* and *Colobanthus* (two morphologically similar genera of subfamily Alsinoideae) and between *Drymaria* (Paronychioideae) and *Pycnophyllum* (Alsinoideae). However, little hierarchical structure was found in the variation of ITS2 sequence similarity suggesting either that the informative variable regions of sequence had become saturated with substitutions (or indels), or that extant lineages diverged in a rapid ancient radiation resulting in an apparent "star phylogeny". These ideas are further explored in chapter 4.

Relationships within *Scleranthus* were assessed by cladistic analysis of combined ITS1 and ITS2 sequence using *Sagina* as outgroup. *Sagina* and *Colobanthus* sequences were the most readily aligned with those of *Scleranthus* and were most similar in the Guidetree analysis. An incomplete and partly arbitrary alignment was used because even these most similar sequences were still highly divergent. All shortest trees found for this data matrix had European and Australasian species grouped as sister clades. One Australian species, *S. diander* (section *Scleranthus*), had an ITS sequence interpretable as an additive combination of *S. minusculus* and either *S. uniflorus* or *S. biflorus* sequences. Despite having sequence for only one specimen of the species it is suggested that a hybrid origin of *S. diander* deserves serious examination given that it is also intermediate in some aspects of its morphology and ecology.

Trees produced from ITS sequences disagree with morphology-derived trees with regard to relationships among Australian species. Whereas cladistic analysis of morphological characters reported in chapter 1 supports monophyly of section *Mniarum*, the ITS sequences give parsimony and Neighbor-Joining trees in which the Australasian species of section *Scleranthus* are nested within section *Mniarum*. A number of possible explanations for the discordance between morphological and ITS data are discussed. A combination of hybridization and introgression is advanced as the best explanation. A combined analysis of morphological and ITS characters is presented despite some reservations over combining discordant data. The trees produced combine elements of trees from separate analysis of each data partition. Parsimony analysis of combined data results in a fully resolved single shortest tree consistent with the consensus of shortest morphology trees.

Samples from Australian and New Zealand *S. biflorus* and *S. brockiei* differ by 1 substitution in each species, indicating that dispersal across the Tasman Sea was recent in both cases.

Date estimates are made for the divergence of European and Australasian *Scleranthus* lineages and for the earliest divergence within the Australasian clade. In addition to the usual problems of assuming that nucleotide substitution rates are constant in divergent lineages (the "molecular clock" hypothesis), serious problems confound date estimation for *Scleranthus* evolution. The pollen grains of many genera of Caryophyllaceae are difficult to distinguish and it is difficult to assign fossil pollen to extant lineages on the basis of apomorphic characters. For this reason I have assumed that the divergence of the lineage ancestral to members of subfamily

Paronychioideae from the lineage ancestral to Alsinoideae and Caryophylloideae (including *Scleranthus*) took place close to the earliest appearance of Caryophyllaceae pollen in the fossil record (i.e., the most conservative position possible). Relationships between subfamilies of Caryophyllaceae were drawn from chapter 4 (see below). Also, the high levels of ITS sequence divergence between Paronychioid and Alsinoid/Caryophylloid sequences mean that distance estimation must of necessity be inaccurate. Despite the limitations of molecular clock date estimates in general, and the particular problems encountered in this case, it is clear that all extant species of *Scleranthus* are the result of recent (uppermost Tertiary or Quaternary) radiations. Vicariance explanations of disjunct Eurasian-Australasian and trans-Tasman distributions are rejected on these grounds.

1.4.3 Chapter 4: Subfamilial relationships within Caryophyllaceae as determined from Parsimony and Neighbor-Joining analyses of 5' *ndh*F sequences.

Chapter 4 reports the results of 5' *ndh*F gene sequence analysis of 15 Caryophyllaceae sequences and two outgroups. The *ndh*F gene is one of a number of alternative (to *rbc*L) chloroplast genes used in recent years for flowering plant phylogenetics (e.g., Bohs and Olmstead, 1997; Terry, Brown and Olmstead, 1997). Published work on phylogeny of the Caryophyllales suggests that *rbc*L evolves at too slow a rate to provide enough variable characters to be applicable at subfamilial level in the Caryophyllaceae. Work by John Clement at the University of Texas, Austin indicated that the 5'

region of *ndh*F could be amplified from Caryophyllaceae and that it had a higher substitution rate than *rbc*L in the group.

Three main lineages within the Caryophyllaceae were supported by parsimony and Neighbor-Joining analysis of 5' *ndh*F sequences. Two of these lineages were composed of genera usually ascribed to subfamily Paronychioideae and it was not determined whether they were sister to each other (thereby making Paronychioideae monophyletic) or whether one or the other was sister to the remaining taxa. Paronychioideae has been divided into tribes Polycarpeae and Paronychieae (e.g., Bittrich, 1993). One of the lineages supported in the *ndh*F sequence analysis included only members of Paronychieae but another included plants from both tribes (at high bootstrap and decay values). Paronychieae is concluded to be a polyphyletic grouping of plants sharing independently-evolved reduced flowers. The third major lineage supported by analysis of *ndh*F sequences included members of subfamilies Alsinoideae and Caryophylloideae, which have previously been suggested to constitute a monophyletic group (Bittrich, 1993). Phylogenetic resolution within this group was very limited with only one clade (comprising species of the Alsinoideae genera *Stellaria* and *Cerastium*) well supported, despite considerable divergence among sequences. Ancestral Alsinoideae/Caryophylloideae appear to have undergone a rapid period of radiation, generating lineages ancestral to most extant genera sometime in the distant past. *Scleranthus* is well supported as a member of the Alsinoideae/Caryophylloideae clade.

1.4.4 Synthesis

Following the three results chapters a synthesis of findings and general conclusions is provided. Included in this section of the thesis is a brief discussion of the validity of combining data from morphological and DNA sequence studies in phylogenetic analyses and the implications of incongruence between data sets.

1.5 References

Bittrich, V. (1993): Caryophyllaceae. In K. Kubitzki, J. Rohwer, and V. Bittrich (Eds.) "The families and genera of vascular plants". Volume 2. Magnoliid, Hamamelid, and Caryophyllid families. Springer Verlag, Berlin.

Bohs, L., and Olmstead, R. G. (1997): Phylogenetic relationships in *Solanum* (Solanaceae) based on *ndh*F sequences. Systematic Botany 22(1): 5-17.

Brown, R. (1810): *Prodromus Florae Novae Hollandiae*. Hafner Publishing Co., New York.

Cheesman, T. F. (1906): Manual of the New Zealand Flora. Government Printer, Wellington.

Engels, F. (1894): Anti-Duhring: Herr Duhring's Revolution in Science. Third edition. Moscow Foreign Languages Publishing House.

Hooker, J. D. (1853): The Botany of the Antarctic voyage of H.M. Discovery ships Erebus and Terror in the years 1839-1843 under the command of Captain Sir James Clark Ross. Pt. 2. Flora Novae Zelandiae. Lovell Reeve and Co. London.

Hutchinson, J. (1974): The families of flowering plants. Oxford University Press.

Oxelman, B. (1996): RAPD patterns, nrDNA ITS sequences and morphological patterns in *Silene* section *Sedoideae* (Caryophyllaceae). Plant Systematics and Evolution 201: 93-116.

Oxelman, B., and Liden, M. (1995): Generic boundaries in the tribe Sileneae (Caryophyllaceae) as inferred from nuclear ribosomal DNA sequences. Taxon 44: 525-542.

Pax, F. (1889): Caryophyllaceae. In A. Engler and K. Prantl (Eds.) "Die natürlichen Pflanzenfamilien, III. Teil, 1b. Halfte". Engelmann: Leipzig.

Pax, F., and Hoffman, K. (1934): Caryophyllaceae. In A. Engler and K. Prantl (Eds.) "Die natürlichen Pflanzenfamilien, Bd 16c". Engelmann: Leipzig.

Raven, P. H. (1973): Evolution of subalpine and alpine plant groups in New Zealand. New Zealand Journal of Botany 11: 177-200.

Sell, P. D. (1964): *Scleranthus* (Caryophyllaceae). Flora Europaea 1: 148-149.

Svensson, L. (1985): An estimate of pollen carry over by ants in a natural population of *Scleranthus perennis* L. (Caryophyllaceae). Oecologia (Berlin) 66:273-377.

Svensson, L. (1990): Floral morphology and the effects of crossing distance in *Scleranthus*. PhD dissertation. University of Lund. Sweden.

Terry, R. G., Brown, G. R., Olmstead, R. G. (1997): Examination of subfamilial phylogeny in Bromeliaceae using comparative sequencing of the plastid locus *ndh*F. American Journal of Botany 54(5): 664-670.

West, J. G. and Garnock-Jones, P. J. (1986): Evolution and biogeography of *Scleranthus* (Caryophyllaceae) in Australasia. In B. G. Barlow (Ed.) "Flora and Fauna of Alpine Australasia". CSIRO 1986.

Chapter 2: Morphology and phylogeny of *Scleranthus* (Caryophyllaceae)

Abstract

Floral anatomy, ontogeny, embryology, and pollen morphology is described for *Scleranthus* (Caryophyllaceae), a small genus of flowering plants with disjunct Eurasian/Australasian distribution. Monophyly of the genus is supported by the close similarity of gynoecial development of all species. Pollen and floral characters divide the species into Australasian and Eurasian groups. Cladistic analysis of morphological characters suggests that section *Scleranthus* (*sensu* West and Garnock-Jones, 1986) is paraphyletic with respect to the single-stamened species of section *Mniarum*. Variation in pollen ovule ratios among genera is discussed.

2.1 Introduction

The genus *Scleranthus* comprises 11 accepted species (West and Garnock-Jones, 1986) of semi-herbaceous annuals or perennials, and dwarf woody perennial shrubs in the family Caryophyllaceae. The genus is usually divided into section *Scleranthus* and section *Mniarum*. As defined by West and Garnock-Jones (1986), section *Scleranthus* contains 3 European native species (with ranges extending into western Asia and northern Africa); *S. perennis, S. uncinatus*, and *S. annuus* (Sell, 1964); and 3 species endemic to Australia; *S. diander, S. pungens* and S. *minusculus*. Eurasian native *S. annuus* is naturalized in many parts of the world including Australia and New Zealand (Garnock-Jones, 1981). Section *Mniarum* is restricted to Australasia and New

Guinea with *S. biflorus* and *S. brockiei* occurring in both New Zealand and Australia, *S. singuliflorus* found in Australia and New Guinea, *S. fasciculatus* endemic to Australia (naturalized in New Zealand), and *S. uniflorus* endemic to New Zealand. An earlier classification (Pax and Hoffman, 1934) groups all Australasian species together in section *Mniarum*.

Scleranthus plants bear small, mostly inconspicuous flowers without petals. The sepals are green, sometimes with white margins. Perigyny is well developed in all species and the sepals and perigynum are persistent, forming part of the dispersal unit along with the fruit and single seed. Flowers of section *Scleranthus* plants are comparatively large, multistaminate and borne in compact dichasia. Flowers of section *Mniarum* (*sensu* West and Garnock-Jones, 1986) plants are smaller, usually single stamened, borne either in pairs or solitary. The fruits of section *Mniarum* plants are presented on elongated peduncles.

Ants are implicated in pollination of *S. perennis* (Svensson, 1985) which is a comparatively large-flowered species of *Scleranthus* that exhibits strong protandry and has a high pollen/ovule ratio. Ants or other pollinators may also be important in the pollination of other multi-staminate species. Other species of the genus, especially the members of section *Mniarum,* with reduced stamen number and flower size, are generally assumed to be highly selfing.

The genus has been included in subfamily Alsinoideae (e.g., Pax and Hoffman, 1934; Bittrich, 1993) and subfamily Paronychioideae [=family Illecebraceae] (e.g. Hutchinson, 1974) by different authors. Also, the unusual disjunct distribution of the genus worldwide has led some to question its monophyly (West and Garnock-Jones, 1986) and at least raises interesting

biogeographic questions which warrant examination of the systematics of the group. West and Garnock-Jones (1986) also suggested that the Australasian species ascribed to section *Scleranthus* are more closely related to the species of section *Mniarum* than to the European species (i.e., section *Scleranthus* as circumscribed by them is paraphyletic).

Scleranthus, due to its reduced habit and tiny flowers, is relatively bereft of traditional taxonomic characters for use in phylogeny reconstruction. Here I report on a variety of aspects of the floral morphology and anatomy of the genus, including vasculature, embryology, ontogeny and pollen morphology, with the aim of evaluating its monophyly and subfamilial placement. Cladistic analysis of these characters and others discussed previously (West and Garnock-Jones, 1986) is undertaken and the taxonomic implications discussed.

Hofmann (1994) described floral morphology and ontogeny of the Caryophyllales using Scanning Electron Microscopy (SEM) and serial sections. Included in her study were numerous Caryophyllaceae including *Scleranthus annuus*. She found that the sepals were initiated successively, in a 2/5 spiral in flowers with five sepals. After the appearance of the sepals, ten (or fewer) primordia are usually initiated at about equal distance from the floral apex. The five antisepalous members of this ring of primordia develop directly into stamens. In species with petals, the alternisepalous primordia are described as subdividing to each produce an inner stamen primordium and an outer petal primordium. While this basic pattern varies with numbers of floral parts it appears characteristic for the family. Sattler (1973) described the floral ontogeny of *Silene cucubalus*. The sequence of events he describes is essentially similar to that outlined by Hofmann for the family as a whole.

These works provide a good background on which to base an ontogenetic study of floral variation in *Scleranthus.*

Embryological characters have been surveyed in a number of genera of Caryophyllaceae (Davis, 1966; Johri *et al.*, 1992). They provide a generally conservative set of characters potentially useful to assess the subfamilial placement of *Scleranthus* and to investigate whether the genus is monophyletic. Davis (1966) treats *Scleranthus* in the Illecebraceae which are embryologically very similar to Caryophyllaceae as reported by her. The principal embryological difference between the two groups Davis describes is that Illecebraceae has members with both Solanad (*Herniaria* and *Polycarpon*) embryogeny and Caryophyllad embryogeny (*Scleranthus*) while the Caryophyllaceae have uniformly Caryophyllad embryogeny. Johri *et al.* (1992) apparently include the Illecebraceae within the Caryophyllaceae but make no mention of any other than the Caryophyllad type of embryogeny.

Thomson (1942) studied the floral anatomy of a range of species of Caryophyllaceae, paying specific attention to vascularization. She found that:

"The entire family is characterized by sepal lateral veins which arise commissurally - that is, as single strands (representing two laterals of adjacent sepals) on the petal radii which show no indication of doubleness until they fork into pairs of marginal veins".

She found the fusion of floral whorls did not affect the carpels, which were separated from the "lower whorls" by a stelar internode. In the subfamily Alsinoideae she found perigyny by fusion of sepal lateral, petal and stamen vascular bundles and associated soft tissues and fusion between sepal midrib

and antisepalous stamen traces in smaller forms, with the result that only two whorls of traces depart from the stele to supply all floral members. As perigyny is one of the characters uniting the species of *Scleranthus* and as a body of research into vasculature of Caryophyllaceae was available these aspects were considered in *Scleranthus.*

Pollen characters have been surveyed in most large genera of Caryophyllaceae (Nowicke, 1975; Nowicke, 1994). Pantoporate grains with punctate/perforate exine and spinules are the most common type. *Scleranthus perennis* has this type of grain (Nowicke, 1974) but spinules are reported to be absent in *S. biflorus* (Moar, 1993). Comparison of these published descriptions indicates additional interspecific variation in characters such as pollen grain size, possession of concave mesoporal exine and pore number.

2.2 Materials and methods

Field collections (**WELTU 16766, WELTU 19658, WELTU 19659, WELTU 19660, WELTU 19661, WELTU 19662, WELTU 19663, *West* 5076, *West* 5096,**) were fixed in FAA (Formalin-aceto-alcohol: Johansen, 1940) and preserved in 70% ethanol. Material for serial sectioning was dehydrated in a *t*-butyl alcohol series and embedded in paraplast™ embedding medium. Sections were cut on a rotary microtome at 10 μm thickness. Sections were stained with safranin and fast green or safranin and astra blue and mounted in DPX™ or Eukit™ mountant.

Material for examination under SEM was dehydrated in ethanol with an increasing proportion of acetone and then critical point dried with liquid CO_2. Samples were sputter coated with 4 - 10 nm of gold and observed with a

Philips 505 SEM.

Pollen for light microscope examination was acetolysed by heating samples in a solution of 9:1 acetic anhydride:conc. sulphuric acid at 70 °C for 10 minutes. This was conducted in Eppendorf tubes in a water bath with a small plug of glass wool placedin the bottom of each tube. After acetolysis samples were briefly centrifuged then washed in 25% ethanol and centrifuged again. Glass wool was used to help retain the small numbers of pollen grains in each sample. Acetolysed pollen was observed mounted in glycerine jelly.

To estimate the number of pollen grains contained in anthers of *S. biflorus*, *S. minusculus*, and *S. pungens,* anthers were crushed in known volumes of water. Pollen grains were counted by applying 5 µL samples to microscope slides and observing under 40 × magnification with a Zeiss stereo-microscope. For *S. biflorus* and *S. minusculus,* anthers were crushed in 25 µL of water. Of this suspension, grains were counted in a total of 20 µL (i.e., 4 spots of 5 µL). The remaining 5 µL was not counted, instead the number of grains counted in 20 µL was multiplied by 1.25 to give the final estimate of total pollen grains. *S. pungens* anthers were crushed in 25 µL of water and then a 5 µL aliquot of this suspension was diluted in a total volume of 50 µL. Five 5 µL aliquots of the 50 µL suspension were then spotted on to microscope slides and the number of pollen grains counted. The final estimate of number of pollen grains per anther in *S. pungens* was obtained by summing the counts and then multiplying by 10.

Pollen stainability was assessed according to Alexander (1969).

Fluid was removed from flowers by capillary tube and tested for sugar content by applying it to wetted Glucostix® reagent strips.

Phylogenetic analysis was conducted using PAUP* 4.0b2 (Swofford, 1999) on test release. Exhaustive searches were conducted with no character weighting. Character mapping was conducted using MacClade (Maddison and Maddison, 1992).

2.3 Results and observations

2.3.1 Mature flower morphology

Detailed examination of flowers of the named species accepted in West and Garnock-Jones (1986), except *S. uncinatus, S. singuliflorus* and *S. diander* was carried out by dissection under the stereo-microscope and examination with scanning electron microscope. Some observations were made of herbarium specimens of *S. singuliflorus* and *S. diander.*

The sepals of *S. minusculus* display a unique aspect of development. At anthesis, the sepals of this species are more or less connivent and not notable for their size (Figure 2.1). However, as the fruit develops the sepals open (Figure 2.2), and grow considerably to a final size of about 3mm long. Sepals of other species do not grow significantly after anthesis.

2.3.1.1 Androecium and nectary

Stamen number is variable in *Scleranthus. S. perennis* has up to 10 stamens (Figure 2.3 and Figure 2.4). The flowers of *S. perennis* are strongly protandrous. Protandry was also observed in *S. annuus* and *S. biflorus* (weakly). *S. annuus* typically has 2 fertile stamens but can have up to 10 (Svensson 1990b). All the material examined in this study consisted of two-stamened flowers with a variable number of staminodes (Figure 2.5 and Figure 2.6). *S. minusculus* and *S. diander* have 2 stamens, and the remaining species usually one. Occasional flowers of *S. biflorus* and *S. brockiei* were found with two stamens rather than the usual one. Occasional female flowers were also found in *S. biflorus* and *S. brockiei,* which like the flowers of other species of *Scleranthus* are usually hermaphroditic.

At the base of the stamens is a disk of glandular nectary (except in *S. pungens*, where the stamens depart the perigynum below the sepals and there is no disk). Instead *S. pungens* flowers possess a whorl of organs in alternisepalous position apparently the same distance, or perhaps marginally further, from the center of the flower than the stamens (of which there are five in episepalous position). These organs are shorter than the stamens, not vascularized and often divide dichotomously one or two times to form a branching structure (Figure 2.7 and Figure 2.8). Sections of flowers reveal the cells of these organs have densely staining cytoplasm, spherical nuclei and vacuoles similar to nectary cells in other species (Figure 2.9 and Figure 2.10). Unfortunately young flower buds were not available, preventing proper examination of the initiation of these organs. However their dichotomous growth form and lack of vascularization suggest that, despite their positioning, they are outgrowths of nectary tissue and do not represent either staminodes (as described previously, e.g. Chorney, 1986) or petals. *S. pungens* is also notable in that the anthers (Figure 2.11) are larger than in other species (measuring approximately 600 µm long compared to 250 - 400 µm in other species).

Cells flanking the stamens on the nectary disk have short finger like projections in *S. perennis* (Figure 2.12 and Figure 2.13) and occasionally in *S. annuus*. These are presumably associated with secretion of nectar. Sugar-containing secretions were removed from floral disks of *S. annuus, S. perennis, S. brockiei* and *S. biflorus*. Stomata are common on sepals and also occur on the nectary disk, sometimes in groups. Crystals were commonly observed in the soft tissue of the flower cup (as rectangular prisms, Figure 2.15) of all species and in the connective of stamens of *S. perennis* (as druses, Figure 2.14). Crystals were also occasionally observed in the sepals.

Figure 2.1. *Scleranthus minusculus* opened bud, just before anthesis, with one stamen removed, showing remaining stamen, styles, disk and top of ovary.

Figure 2.2. *Scleranthus minusculus* persistent sepals and perigynum surrounding fruit during expansion and opening of sepals. Scale bar 1mm.

Figure 2.1

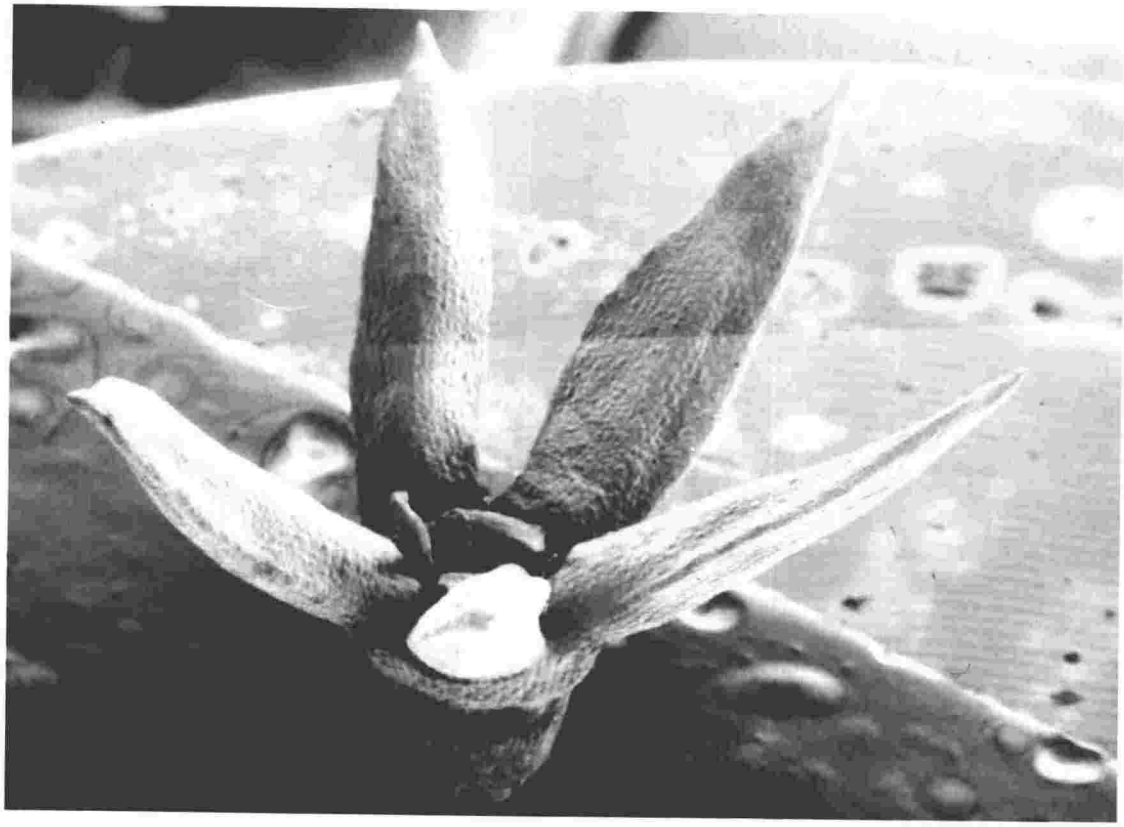

Figure 2.2

Figure 2.3. *Scleranthus perennis* flower during male phase. Scale bar 1mm.

Figure 2.4. *Scleranthus perennis* flower during female phase.

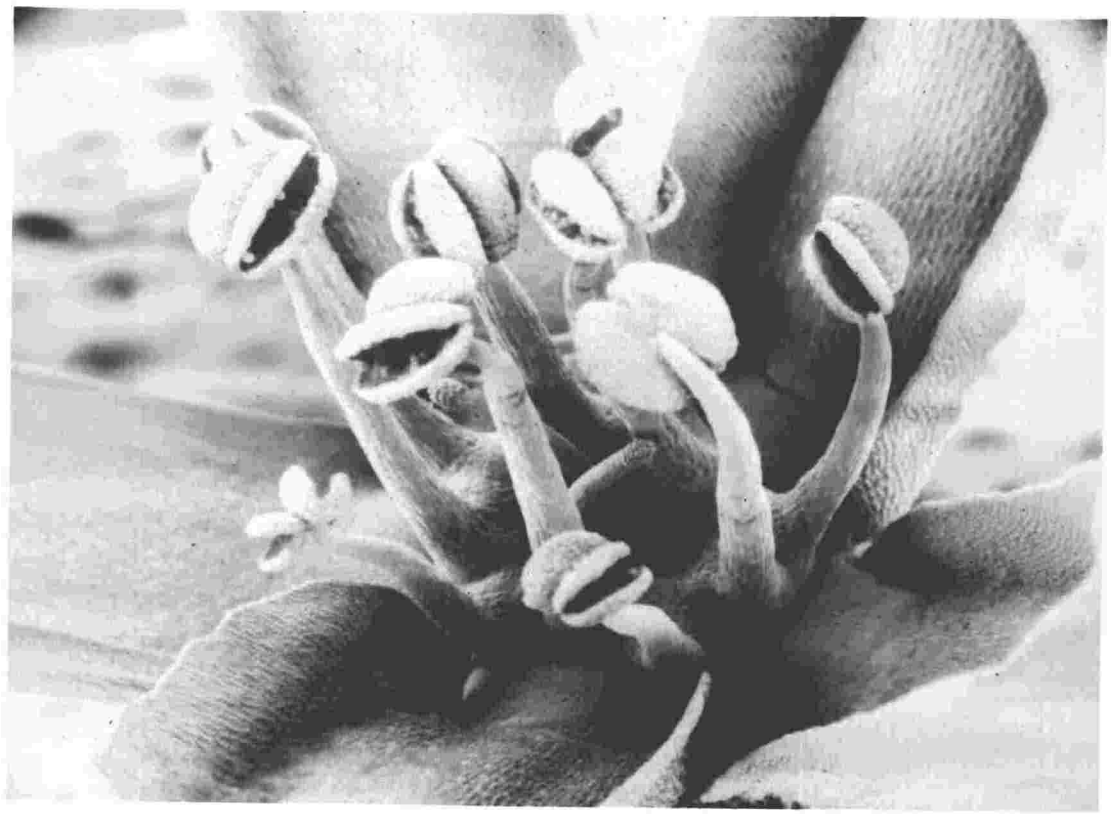

Figure 2.3

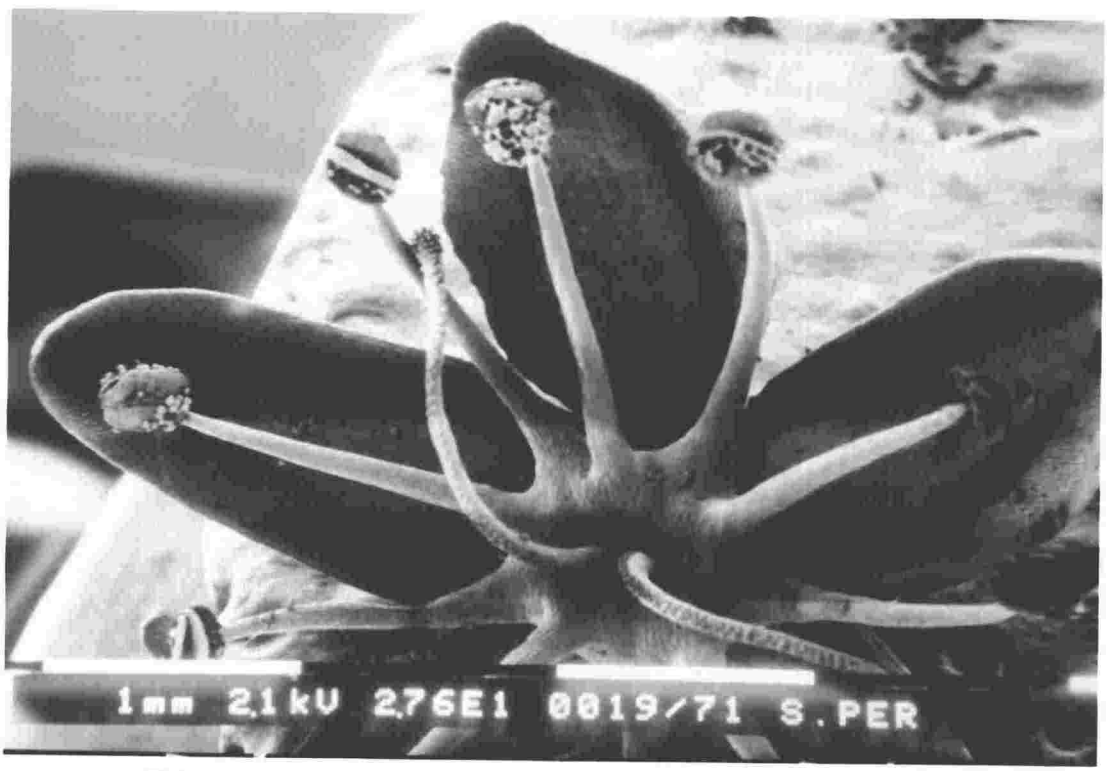

Figure 2.4

Figure 2.5. *Scleranthus annuus* flower. Scale bar 1mm.

Figure 2.6. *Scleranthus annuus* flower, close up showing stamens and staminodes, and stigmas (arrow). Scale bar 100 μm.

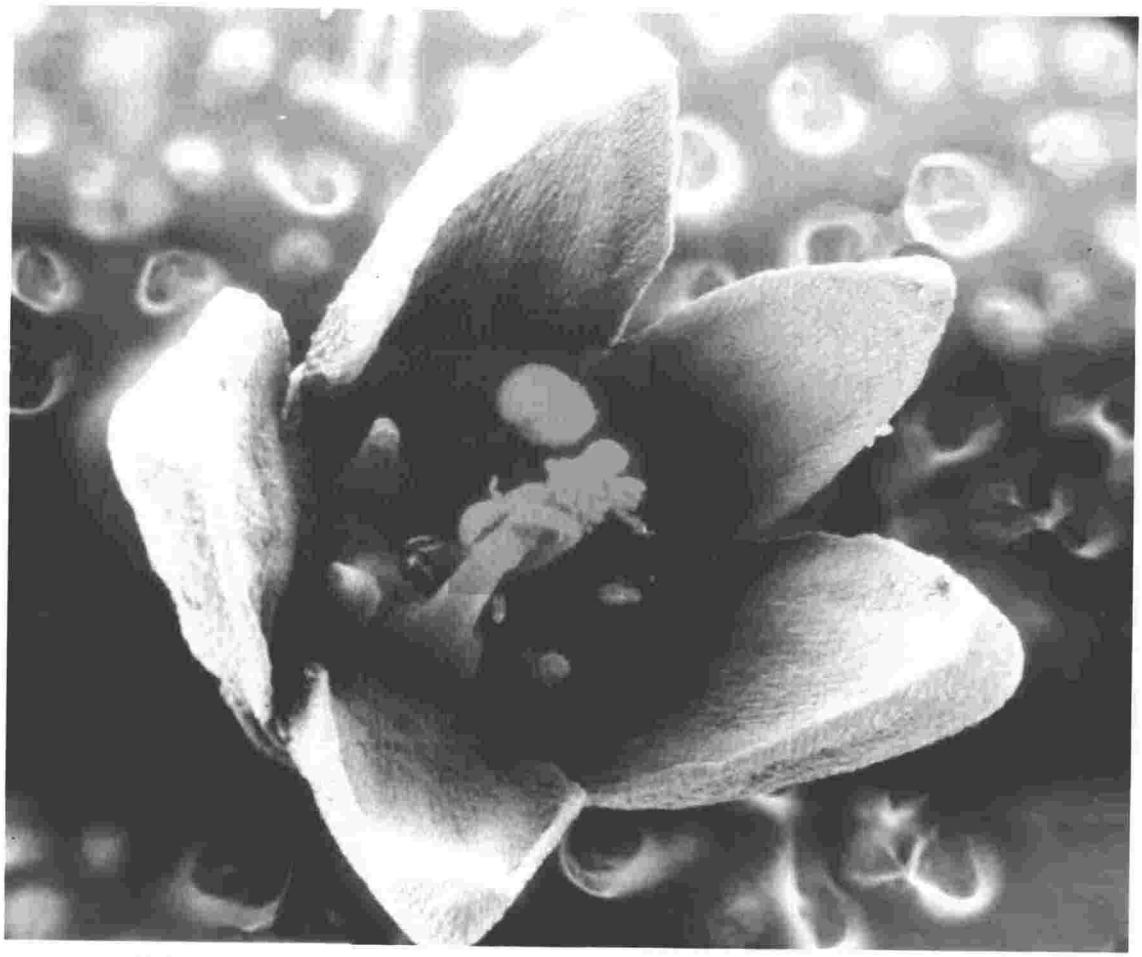

Figure 2.5

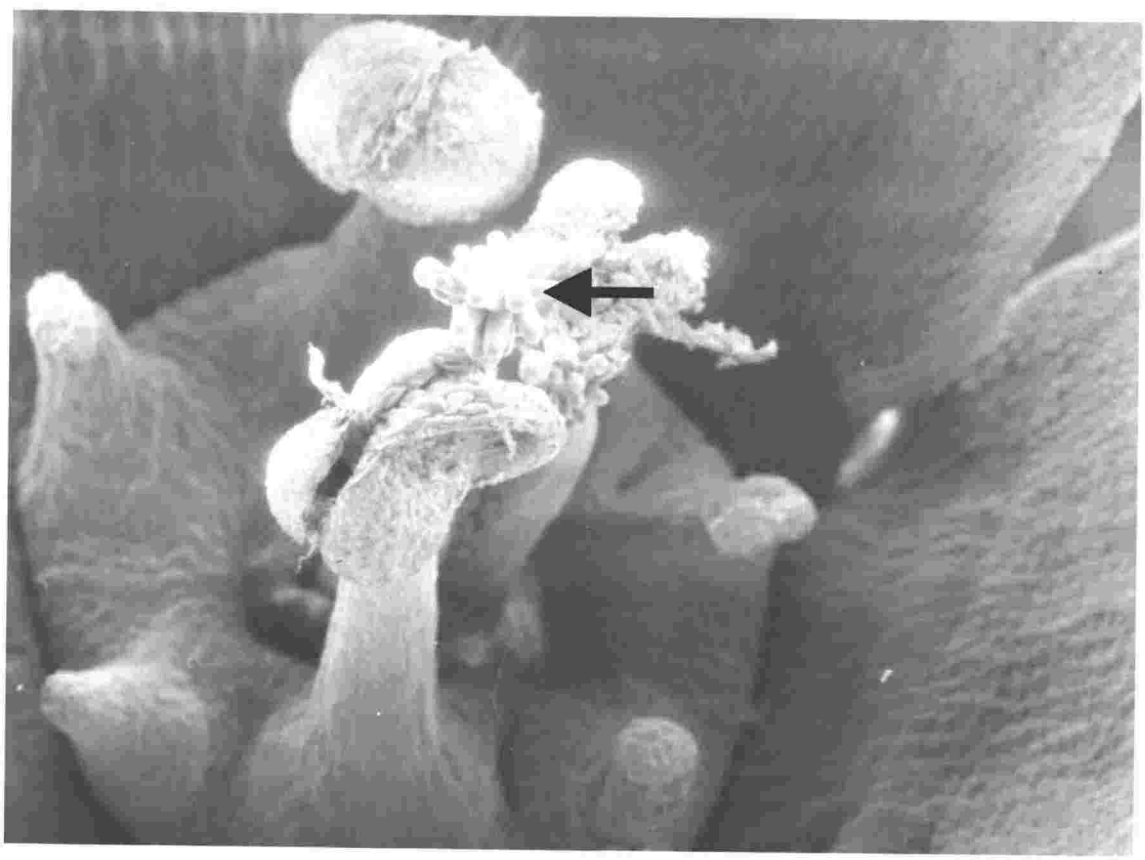

Figure 2.6

Figure 2.7. *Scleranthus pungens* dissected flower showing dichotomising nectary filaments (small arrow) and stamen filaments (large arrow).

Figure 2.8. *Scleranthus pungens* dissected young bud showing stamens (large arrow) and nectary filaments (small arrow). Scale bar 100 μm.

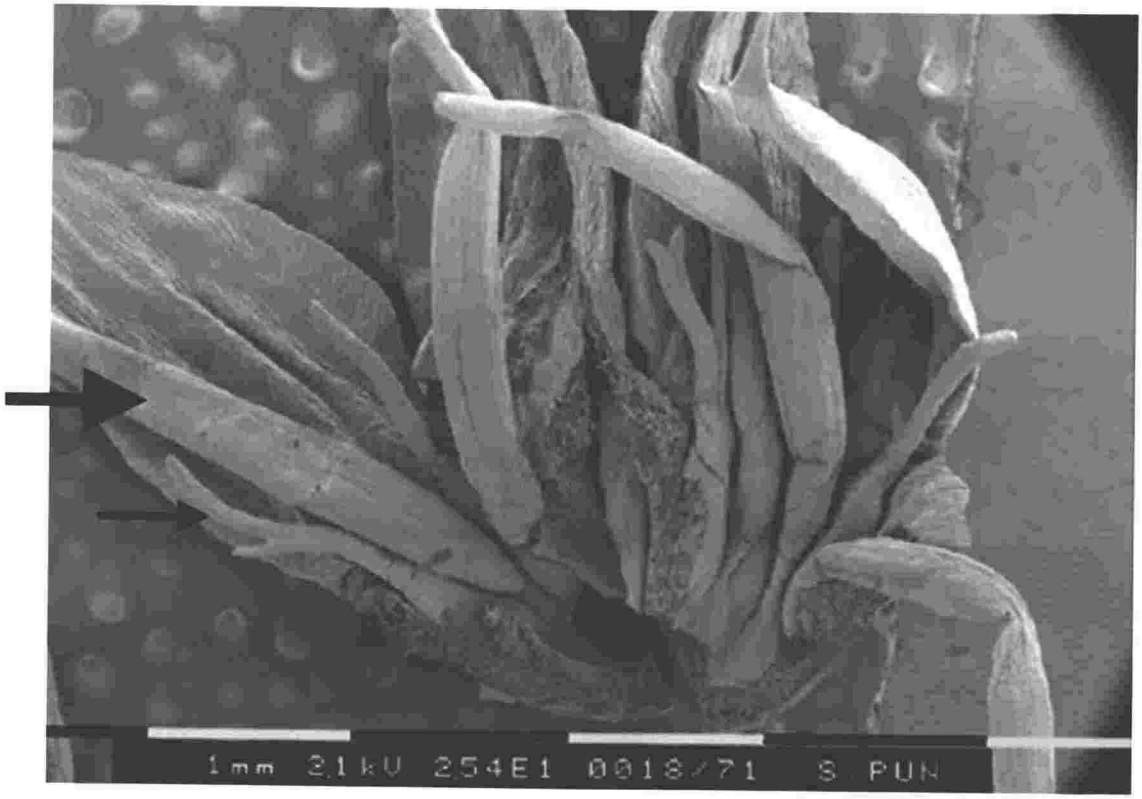

Figure 2.7

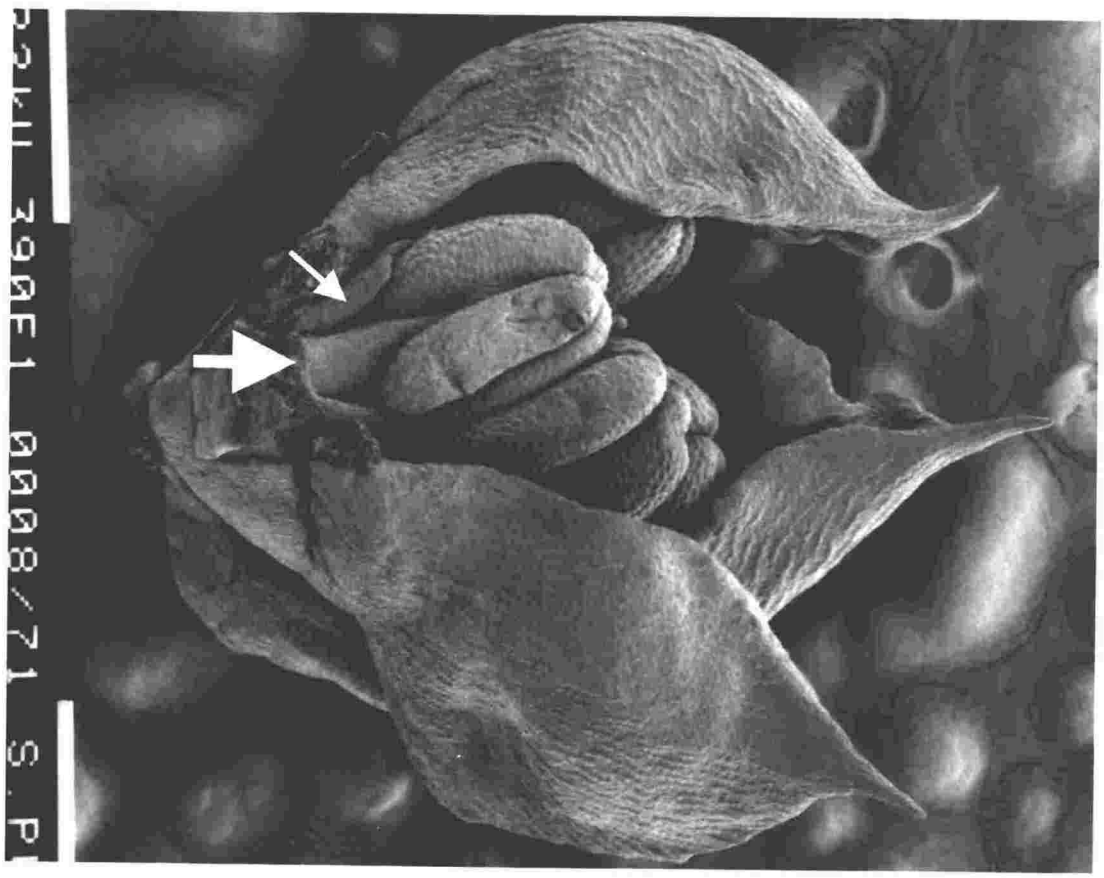

Figure 2.8

Figure 2.9. *Scleranthus pungens* transverse section of flower showing vascularized stamens (s), non-vascularized nectary filaments (n), and gynoecium (g). Scale bar 100 μm.

Figure 2.10. *Scleranthus pungens* transverse section of flower showing vascularized stamens (s), vascular bundle (v) and non-vascularized nectary filaments (n). Scale bar 100 μm.

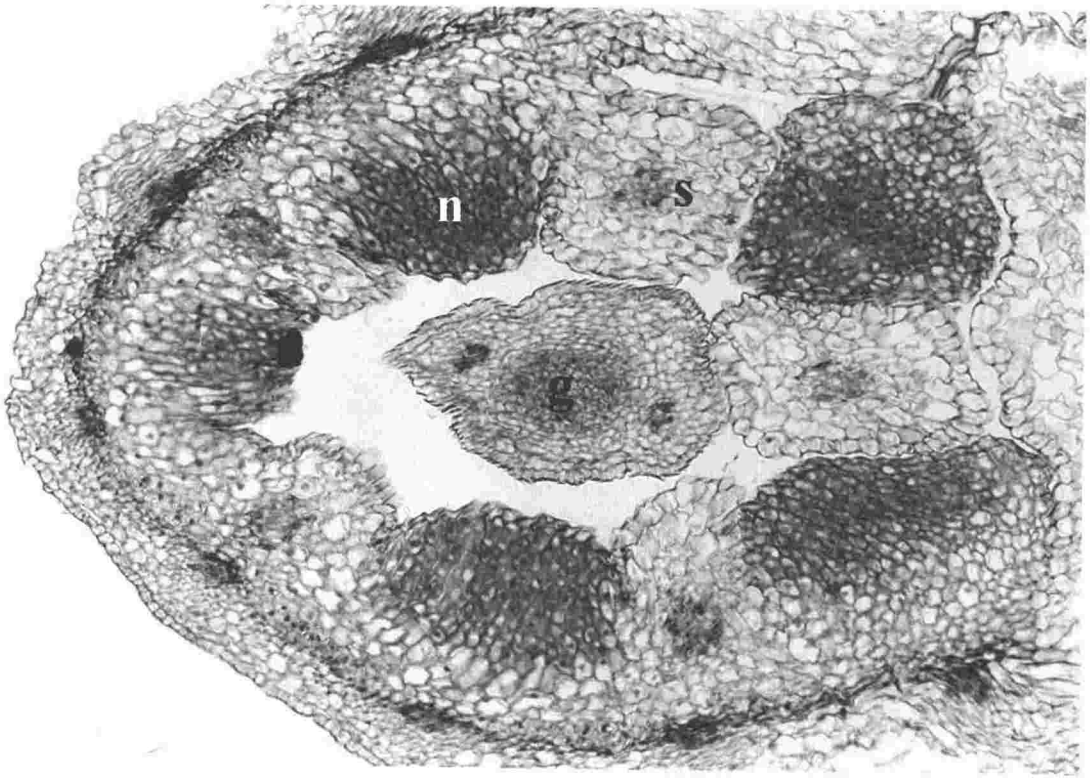

Figure 2.9 ——

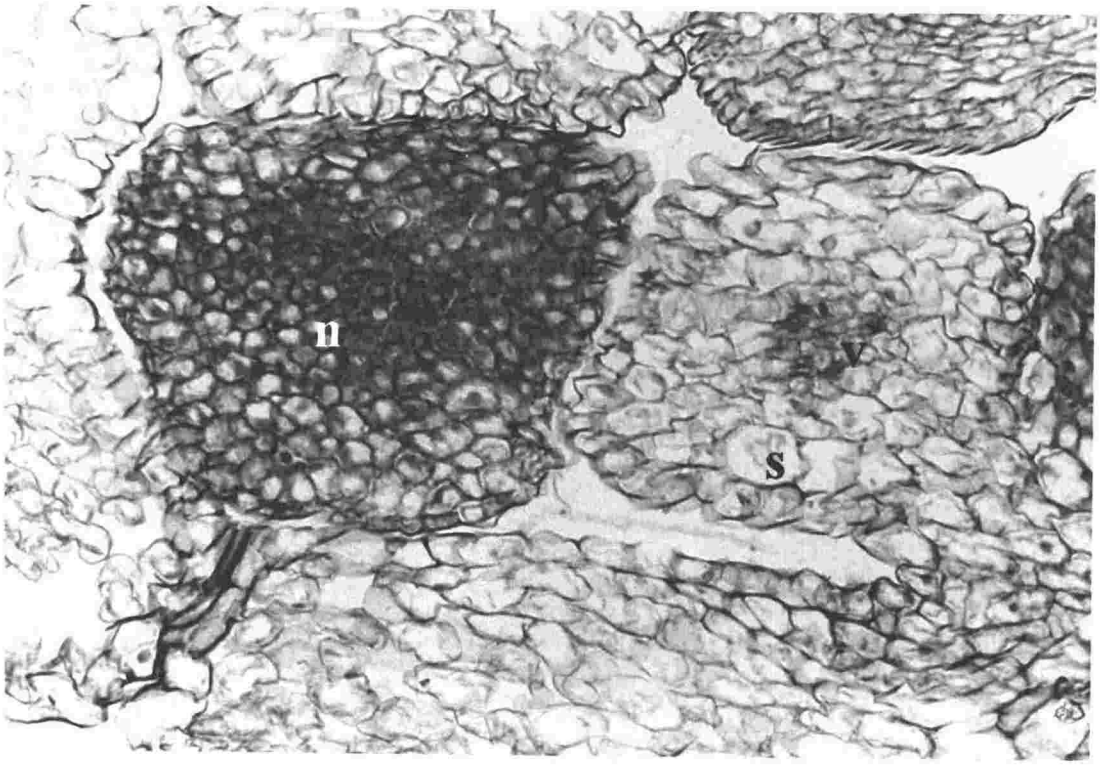

Figure 2.10 ——

Figure 2.11

Figure 2.12. *Scleranthus perennis* flower with stamens and sepals removed to show nectary tissue flanking stamen filaments. Scale bar 100 μm.

Figure 2.13. *Scleranthus perennis* close up of nectary tissue. Stomatal pore, at arrow. Scale bar 10 μm.

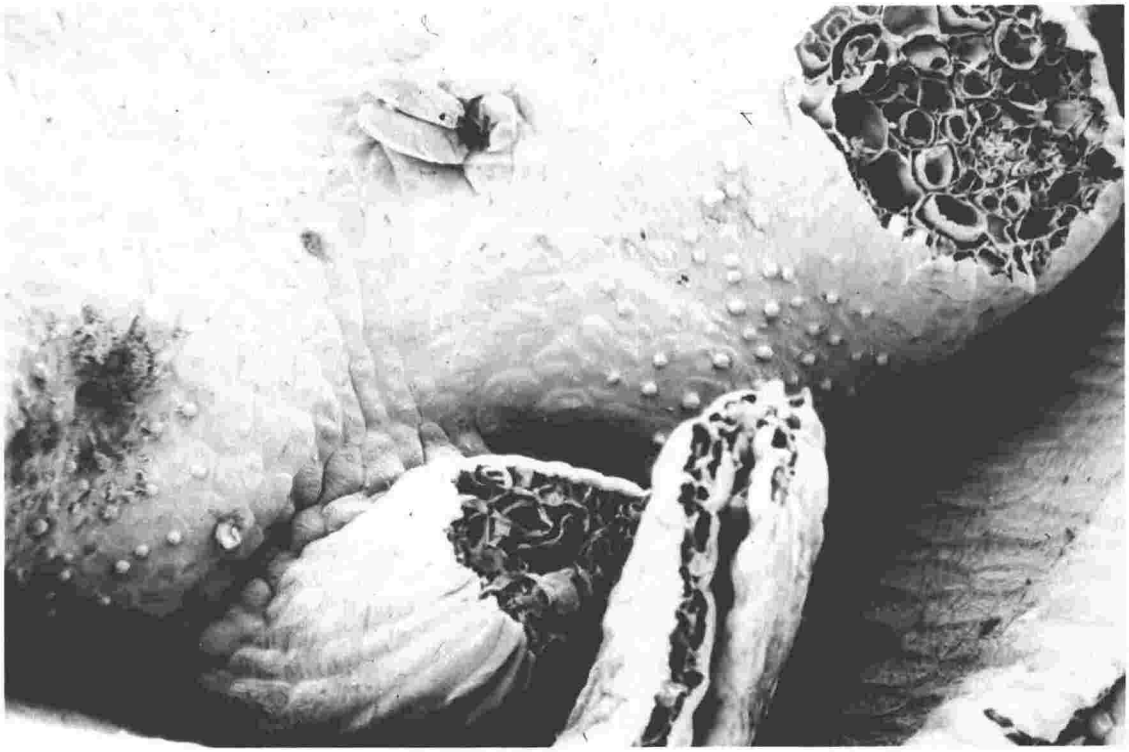

Figure 2.12

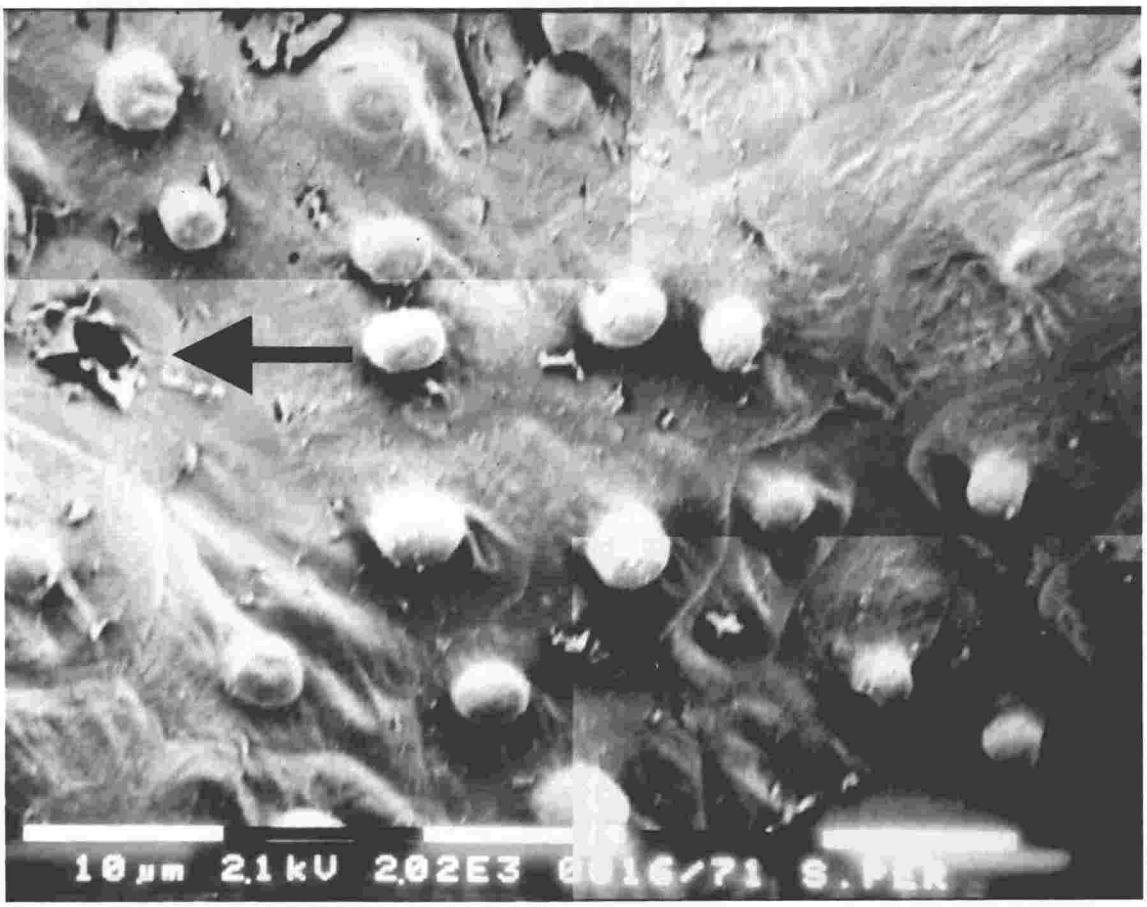

Figure 2.13

Figure 2.14. *Scleranthus perennis* section through connective of stamen showing druses. Scale bar 100 μm.

Figure 2.15. *Scleranthus annuus* section through perigynum showing rectangular prisms in soft tissue. Scale bar 100 μm.

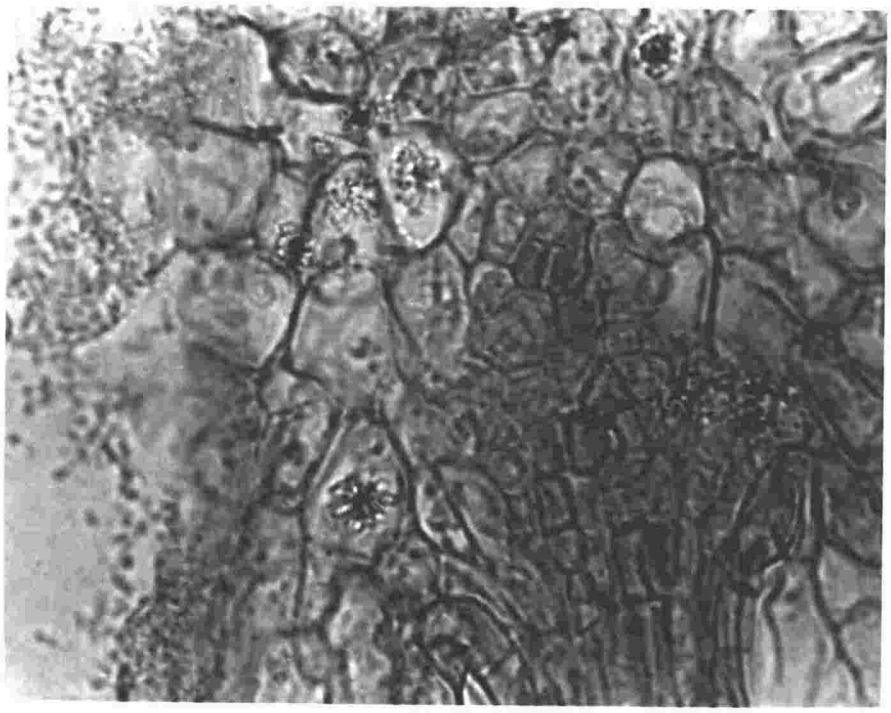

Figure 2.14

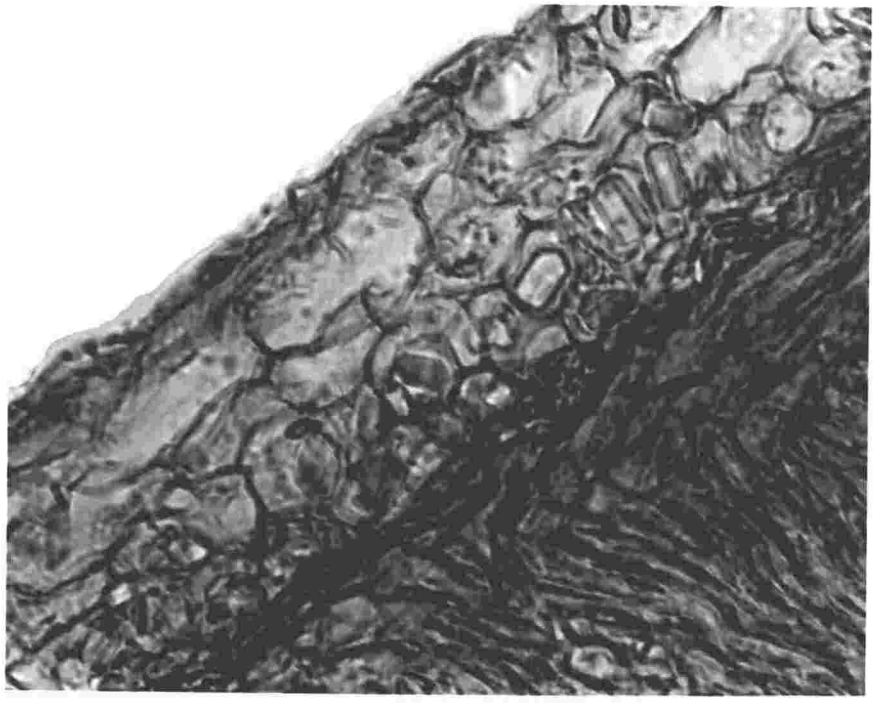

Figure 2.15

2.3.1.2 Gynoecium

The gynoecium consists of two carpels fused beneath their styles to form a single compound ovary. In *S. annuus, S. perennis*, and *S. brockiei* flowers or buds were occasionally observed with gynoecia consisting of three fused carpels. In most species, styles are sunken into the top of the ovary as a result of overgrowth by thick walled cells around the top of the ovary, as is typical in the family. In *S. pungens,* the overgrowth of styles by ovary cells is limited to the portion of the gynoecium corresponding with the lateral surface of its constituent carpels (Figure 2.16). In *S. biflorus* (Figure 2.16) and *S. uniflorus* the ovary tapers into the styles and there is no overgrowth of the styles by ovary cells.

The styles of *S. perennis* and *S. annuus* are lined along their ventral surfaces by rows of papillate stigmatic cells which extend to cover the whole of the apical portion of the style (Figure 2.16). In *S. biflorus*, *S. brockiei, S. uniflorus* (not illustrated), *S. pungens, S. minusculus* and *S. fasciculatus* stigmatic papillae are usually restricted to the apical portion of the style although they are sometimes more extensive on the ventral surface (Figure 2.16). The ovary is completely surrounded by the floral cup in *S. annuus* and *S. perennis* so that only the styles are visible in undissected flowers (Figures 2.3 and 2.4). In *S. minusculus, S. brockiei* and *S. fasciculatus* the ovary is pyriform its thickened top protrudes above the floral cup (Figures 2.1, 2.17 and 2.18). In flowers of *S. biflorus* and *S. uniflorus*, the nectary projects as a cylinder (broken where the filament of the single stamen is inserted) which surrounds the lower part of the styles

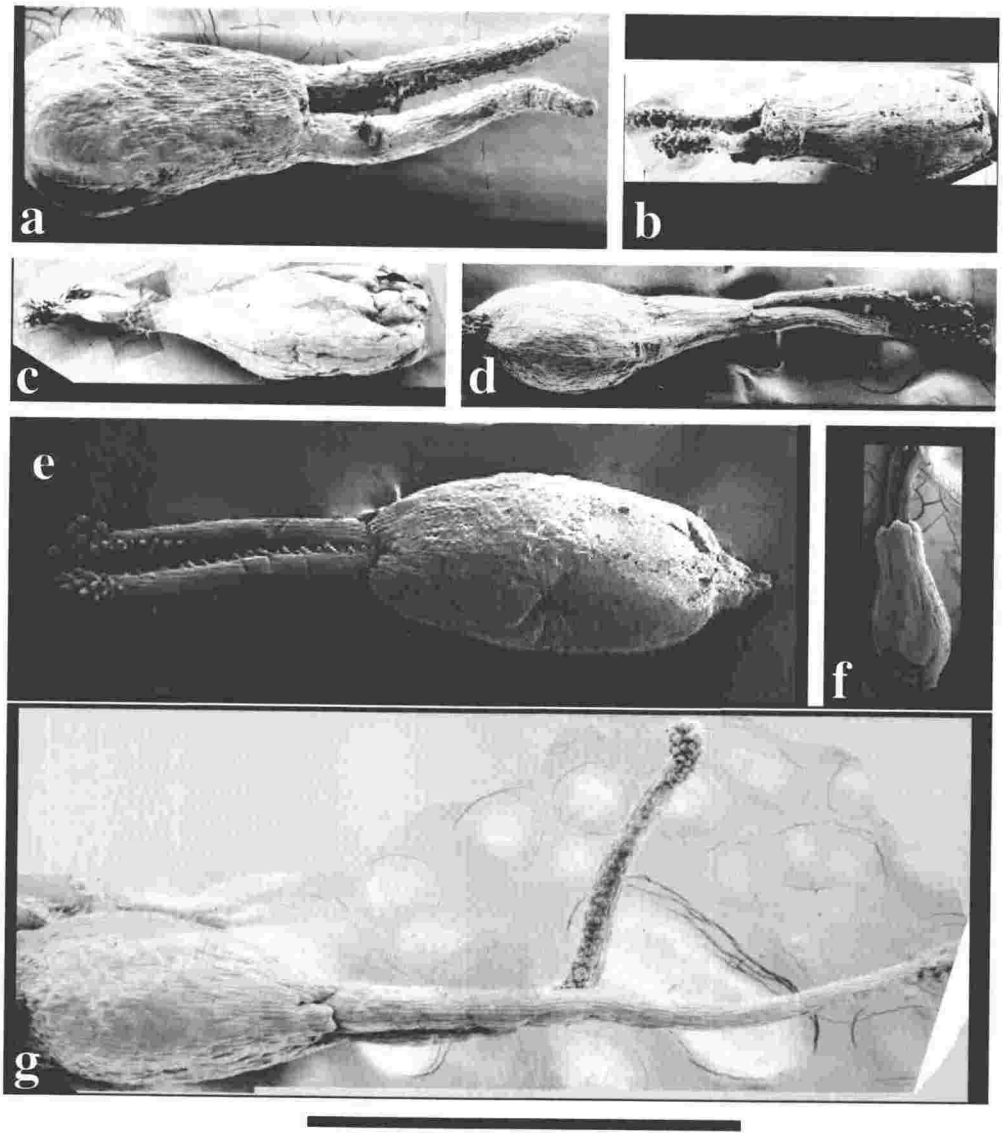

Figure 2.16. Gynoecia of *Scleranthus* species. **a,** *S. pungens*. **b,** *S. brockiei*. **c,** *S. fasciculatus*. **d,** *S. biflorus*. **e,** *S. annuus*. **f,** *S. minusculus*. **g,** *S. perennis*. Scale bar 1mm.

Figure 2.17. *Scleranthus fasciculatus* stamen and styles of flower at anthesis. Note papillate surface of ovary. Scale bar 100 μm.

Figure 2.18. *Scleranthus brockiei* flower at anthesis. Scale bar 10 mm.

Figure 2.17

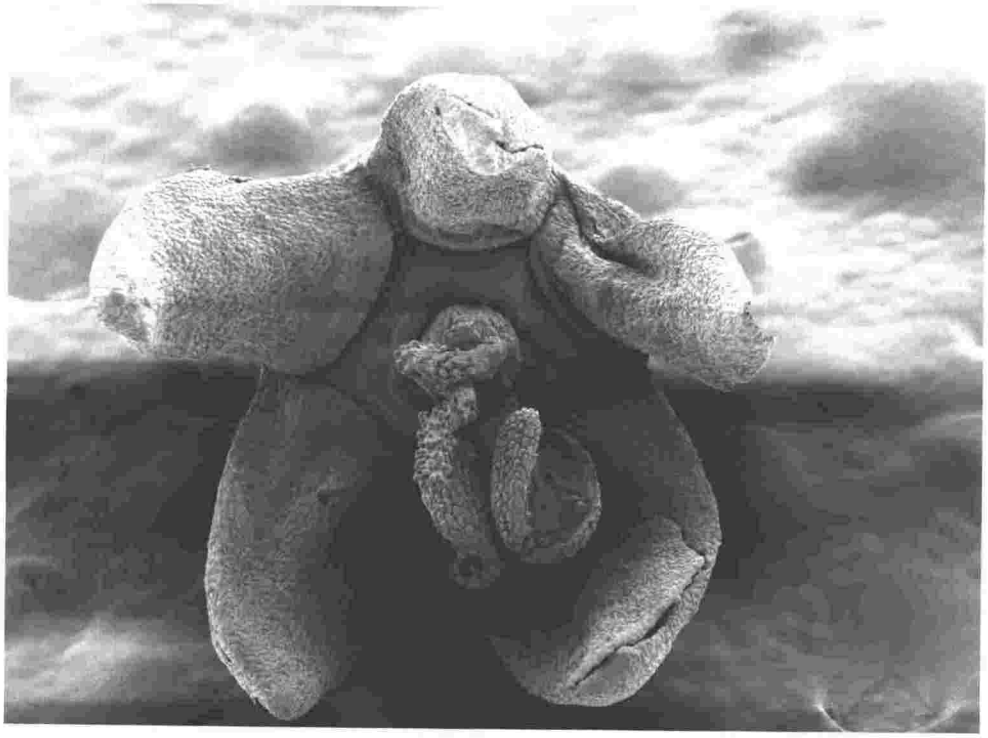

Figure 2.18

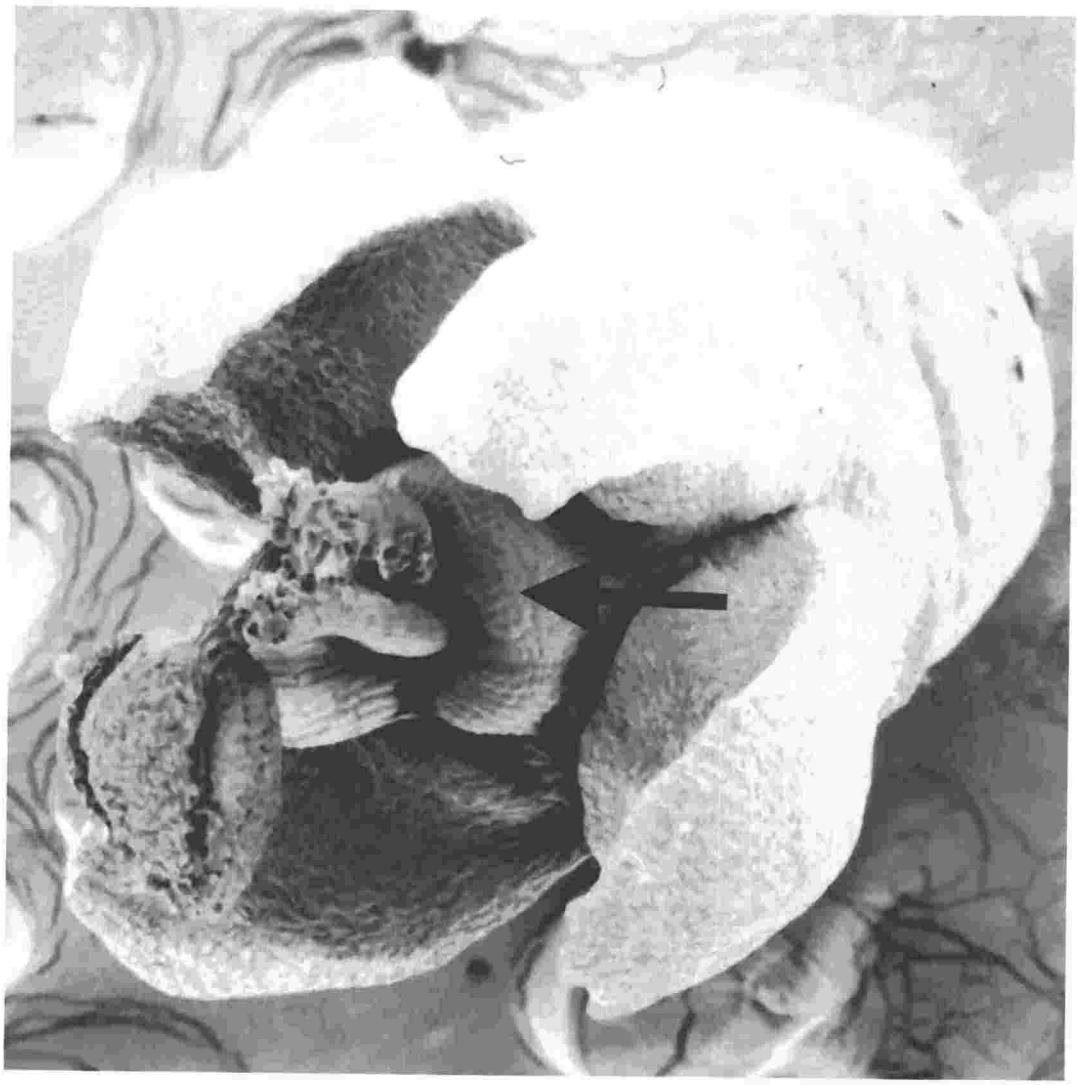

Figure 2.19

(Figure 2.19). The ovary of all species is unilocular and encloses a single basally-attached ovule (Figures 2.20 – 2.24). The ovule is more or less campylotropous in shape but the funicle is curved so that the micropyle points toward the styles and is in contact with a plug of transmission tissue forming an obdurator (Figure 2.20, 2.23, 2.24). In a single flower bud of *S. annuus* a second smaller, and probably abortive, ovule was once observed inserted alongside a normally developed ovule.

Table 2.1. Distribution of some floral characters among *Scleranthus* species as described in text [1] discussed below. [2] With cells at top of ovary papillate

Species	Ovary shape	Styles sunken into ovary	Nectary	Stigmatic surface of styles	Perigynum fibers[1]	Fibrous sepal laterals[1]
S. perennis	Ovoid	Yes	Papillate disk	Apex and ventral side	Tangential	Yes
S. annuus	Ovoid	Yes	Papillate disk	Apex and ventral side	Tangential	Yes
S. pungens	Ovoid	Partially	Filaments	Apex (mostly)	Longitudinal	No
S. minusculus	Pyriform	Yes	Disk	Apex	Longitudinal	No
S. diander	Ovoid	Yes	Disk	Apex	Longitudinal	No
S. fasciculatus	Pyriform	Yes[2]	Disk	Apex	Longitudinal	No
S. singuliflorus	Pyriform	Yes[2]	?	Apex	Longitudinal	No
S. brockiei	Pyriform	Yes[2]	Disk	Apex	Longitudinal	No
S. biflorus	Ovoid	No	Tube	Apex	Longitudinal	No
S. uniflorus	Ovoid	No	Tube	Apex	Longitudinal	No

2.3.1.3 Floral Vascularization

In *S. perennis* (and other pentamerous *Scleranthus* species), the vascular

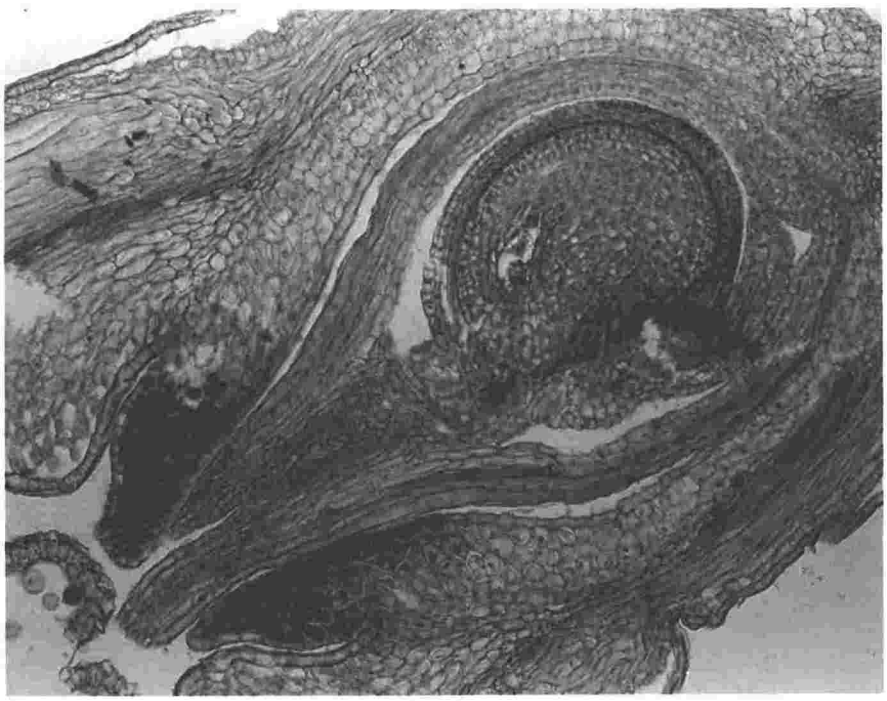

Figure 2.20

Figure 2.21. *Scleranthus perennis* longitudinal section through flower at anthesis showing ovule with line of elongated cells leading from micropyle to embryo sac. Scale 100 μm.

Figure 2.22. *Scleranthus perennis* section of ovule showing micropylar portion of nucellus with region of elongated cells. Scale bar 50 μm.

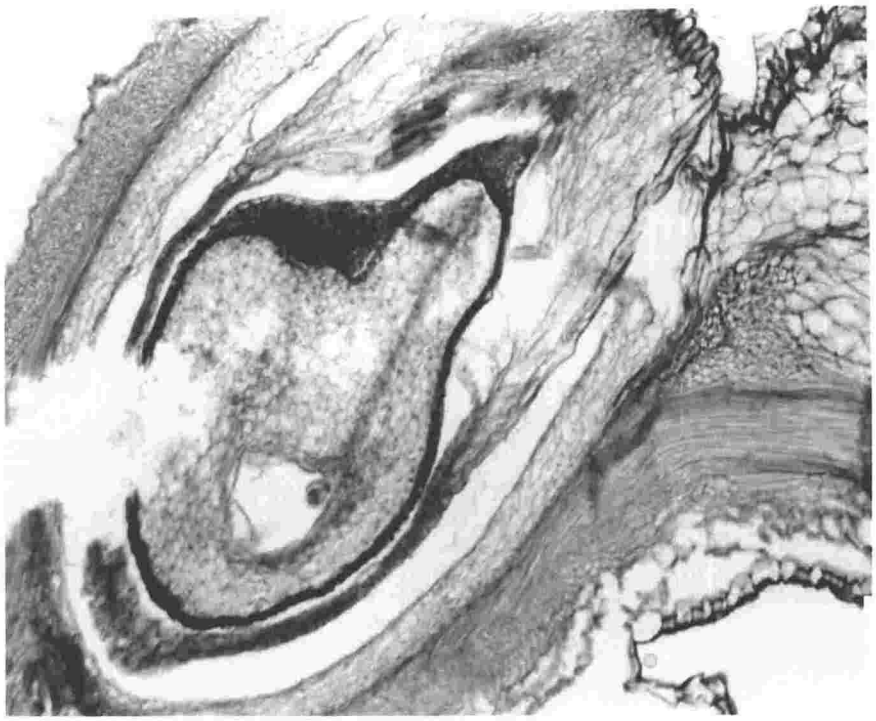

Figure 2.21

Figure 2.22

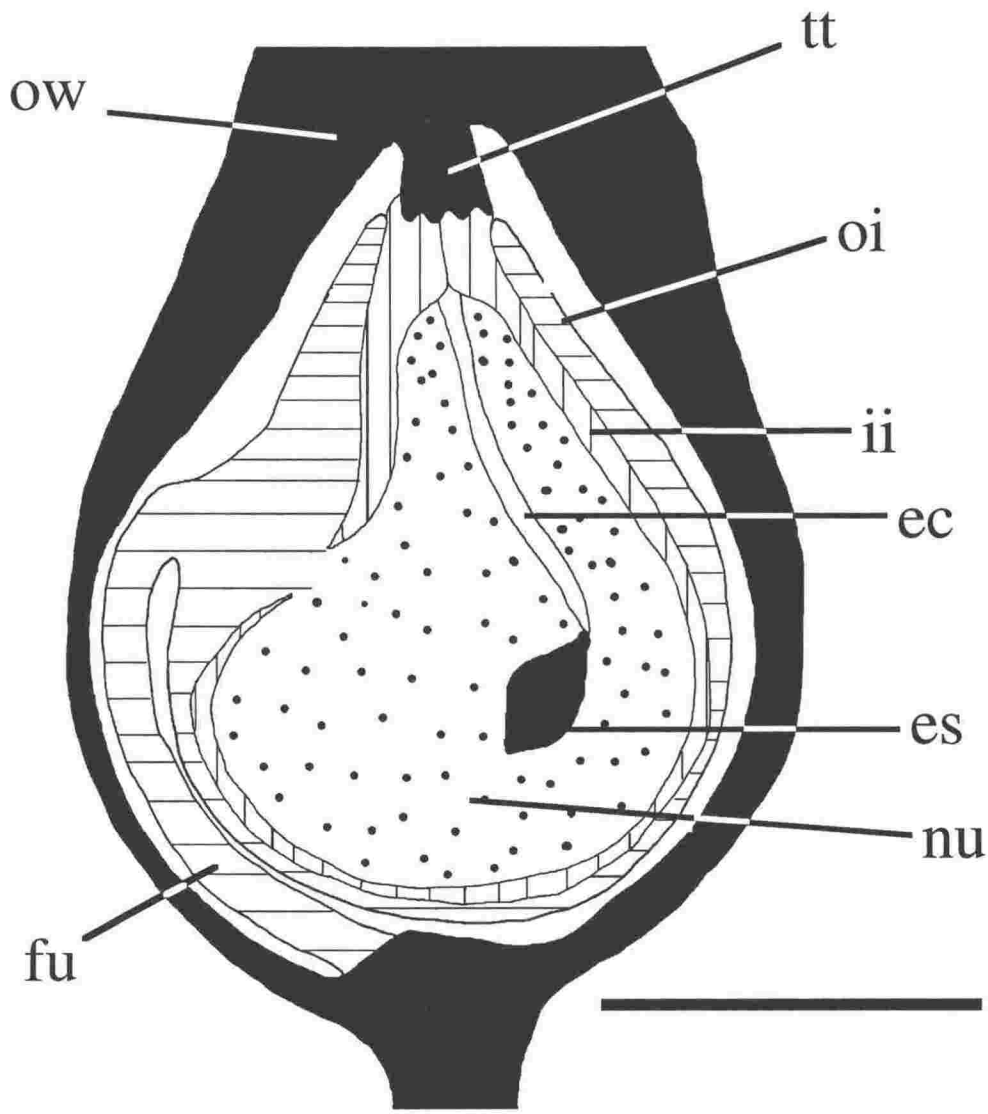

Figure 2.23. Mature ovary of *Scleranthus perennis.* tt, transmission tissue; ow, ovary wall; oi, outer integument, ii, inner integument; ec, zone of elongated cells; es, embryo sac; nu, nucellus; fu, funicle. Scale is 0.2 mm.

cylinder divides at the base of the flower to form ten equal veins (Figure 2.24, 2.29a). About two-thirds of the way up the floral cup each vein divides unevenly into an outer (larger) and inner (smaller) vein (Figures 2.27, 2.28, 2.29b). The inner veins become stamen traces. Those outer veins on sepal radii become median sepal veins, those on (inferred) petal radii become sepal lateral veins after branching dichotomously at the base of adjacent sepals (Figure 2.29c). In species with fewer than ten stamens, stamen veins only develop on radii where stamens are located (Figure 2.29d).

Two vascular traces run up the fused ovary, one on each side, and into the styles without branching (Figure 2.28 and 2.29). A well-developed trace supplies the ovule, running the length of the funicle from the base of the ovary. The connection (if any) of the carpel vasculature to the stele is obscure. In some specimens it was possible to trace vessels through the gynophore into the soft tissue at the base of the floral cup. It was not possible to determine whether these traces originated in the soft tissue or connected with adjacent sepal/stamen veins.

Sepal median and lateral veins branch at the base of the sepals but second order veins are short and restricted to the lower part of the sepals. Sepal veins are associated with extensive fibrous tissue (Figure 2.30). In all species this fibrous tissue is fused across veins in the lower part of the sepal but becomes associated with individual vascular bundles (sepal median and two sepal laterals) in the upper portion of the sepals of *S. perennis* and *S. annuus* (Figures 2.28 and 2.30). In the Australasian species, including those with larger sepals, sepal vasculature is reduced. In all Australasian material examined only the sepal median vein is associated with fibers. The two sepal lateral veins are either absent, or not associated with fibers (Figure 2.30).

Figure 2.24. *Scleranthus perennis* transverse section through perigynum showing 10 veins and tangentially aligned fibres. Scale bar 100 μm.

Figure 2.25. *Scleranthus annuus* transverse section through perigynum showing veins and tangentially aligned fibres. Scale bar 100 μm.

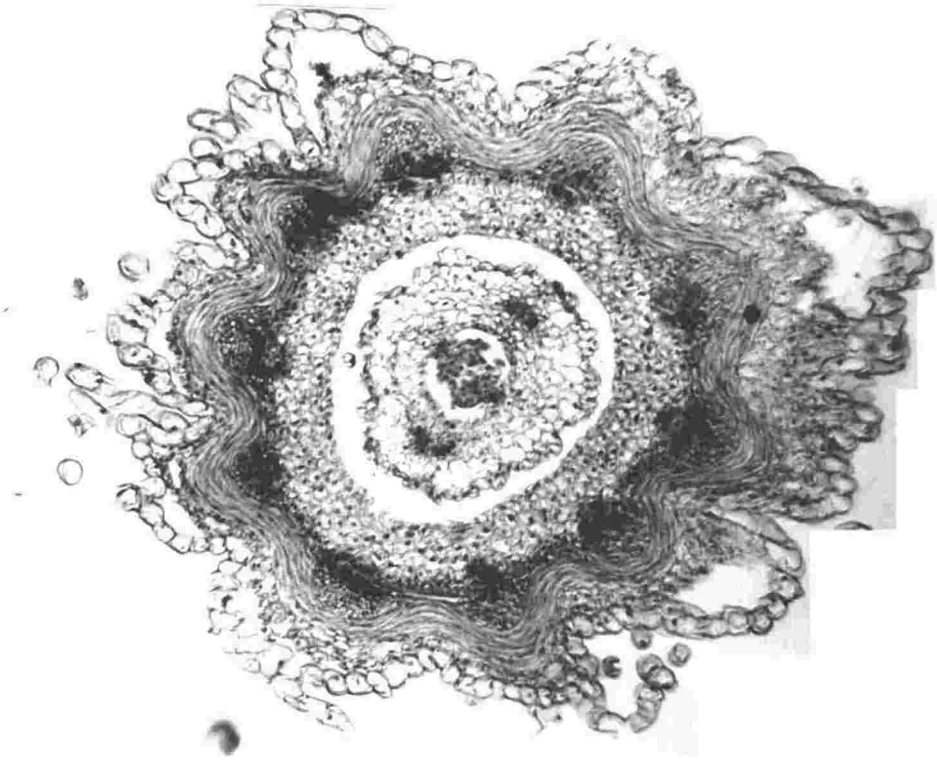

Figure 2.24

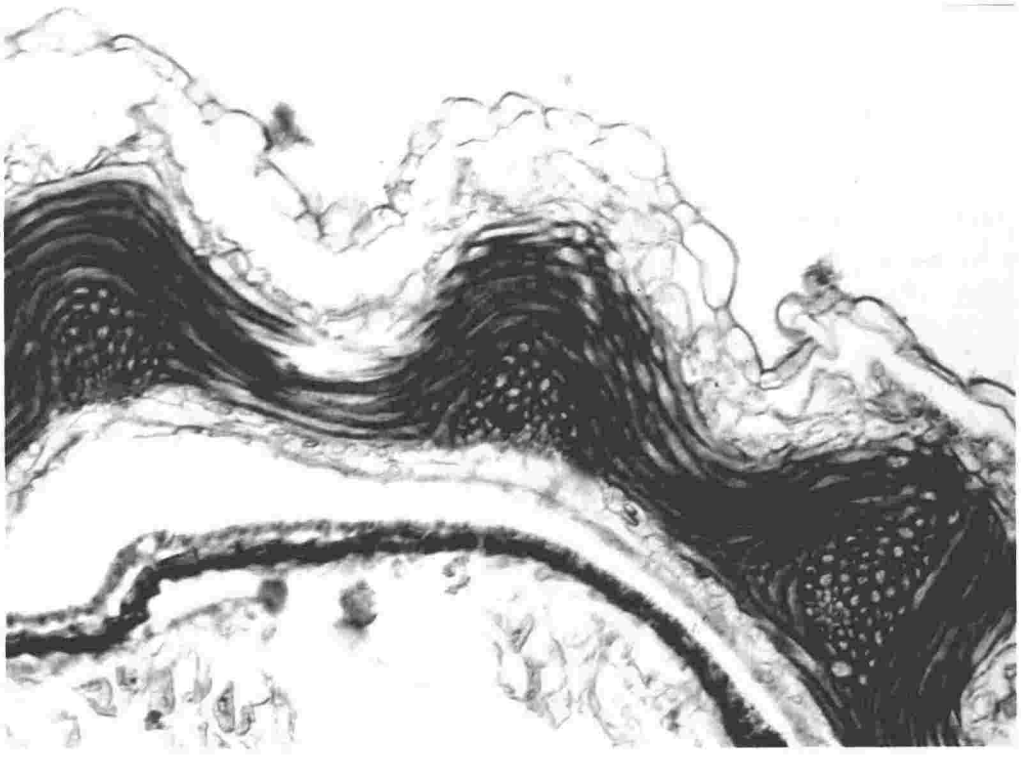

Figure 2.25

Figure 2.26. *Scleranthus perennis* transverse section through perigynum near departure of sepals showing stamen veins, dark staining nectary tissue, ovary tissue surrounding base of styles and splitting of commissural sepal marginal veins (on right). Scale bar 100 μm.

Figure 2.27. *Scleranthus perennis* transverse section through base of sepals showing separate, fibrous, sepal marginal vein at arrow. Scale bar 100 μm.

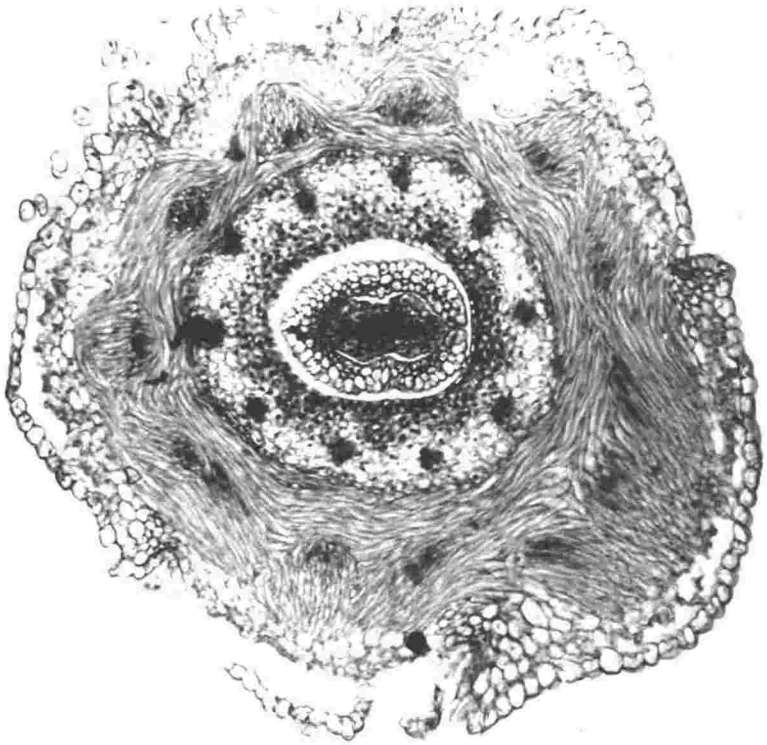

Figure 2.26

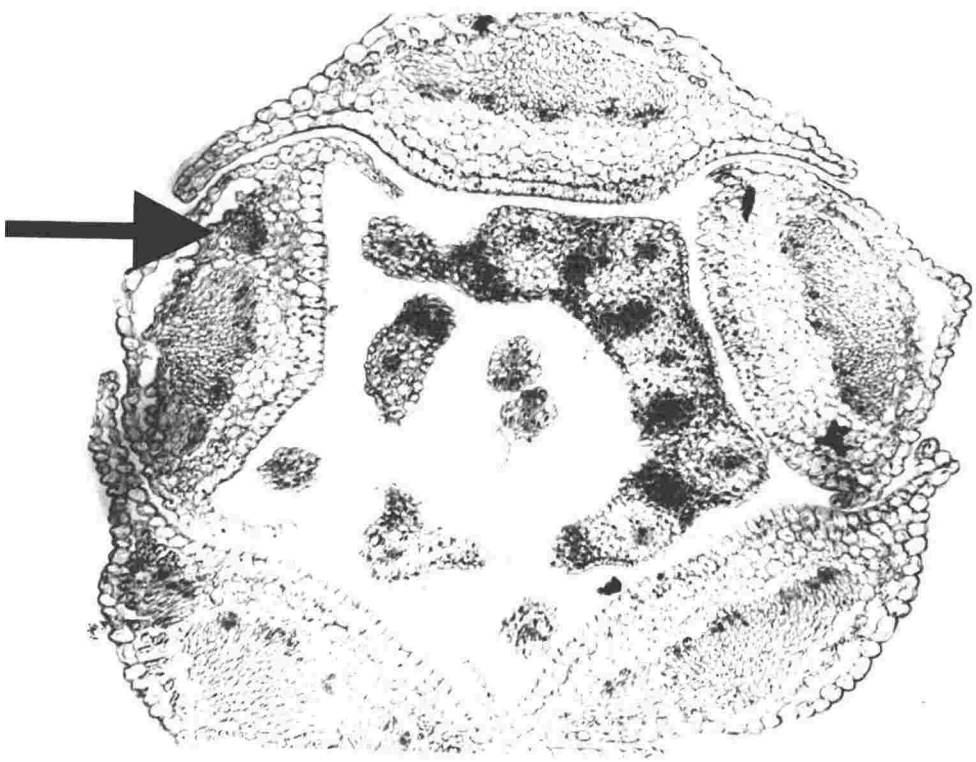

Figure 2.27

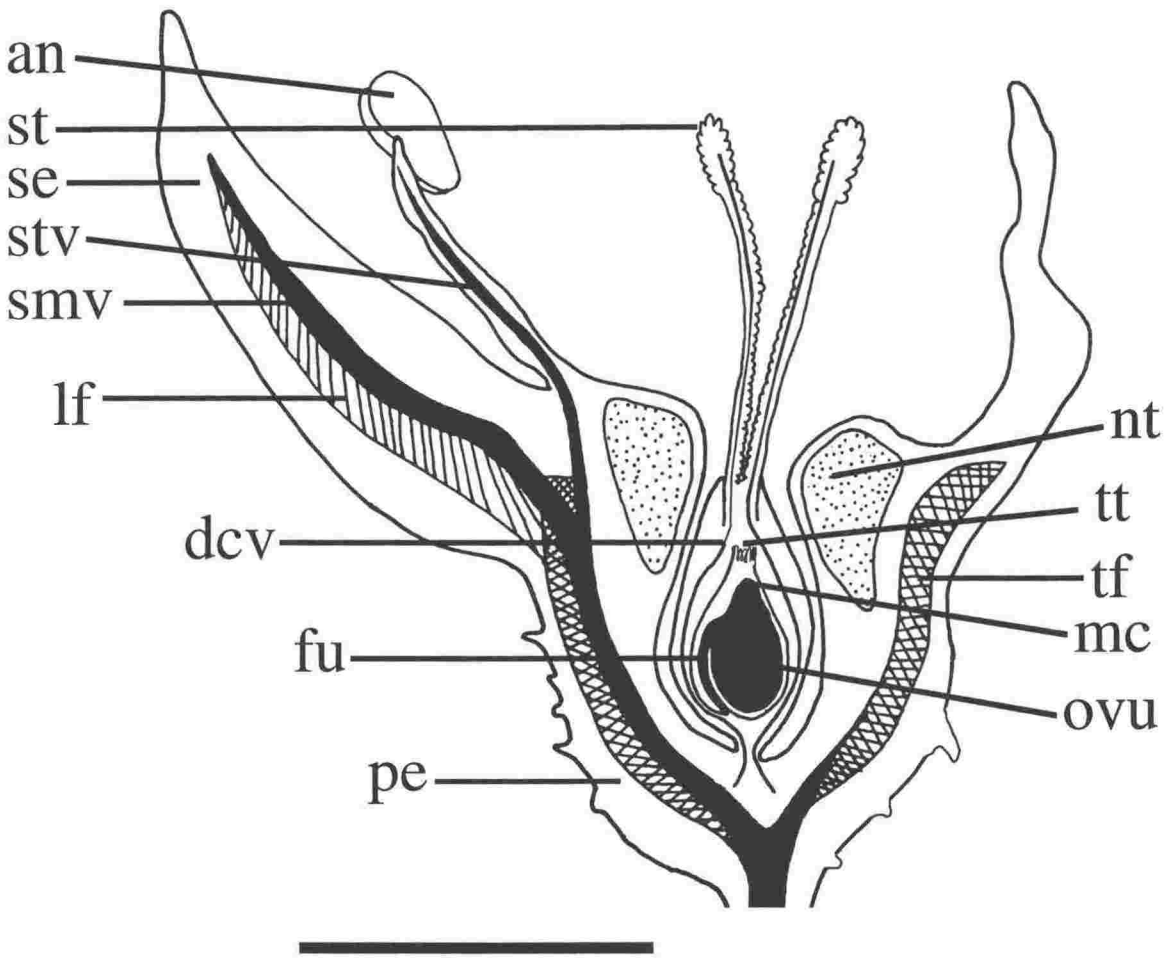

Figure 2.28 Diagramatic interpretation of longitudinal section of *Scleranthus perennis* flower showing vasculature and relation of floral organs. an, anther. st, stigma. se, sepal. stv, stamen vein. smv, sepal median vein. lf, longitudinal fibres. dcv, dorsal carpel vein. fu, funicle. pe, perigynum. ovu, ovule. mc, micropyle. tf, tangential fibres. nt, nectary tissue. tt, transmission tissue. Scale 1mm.

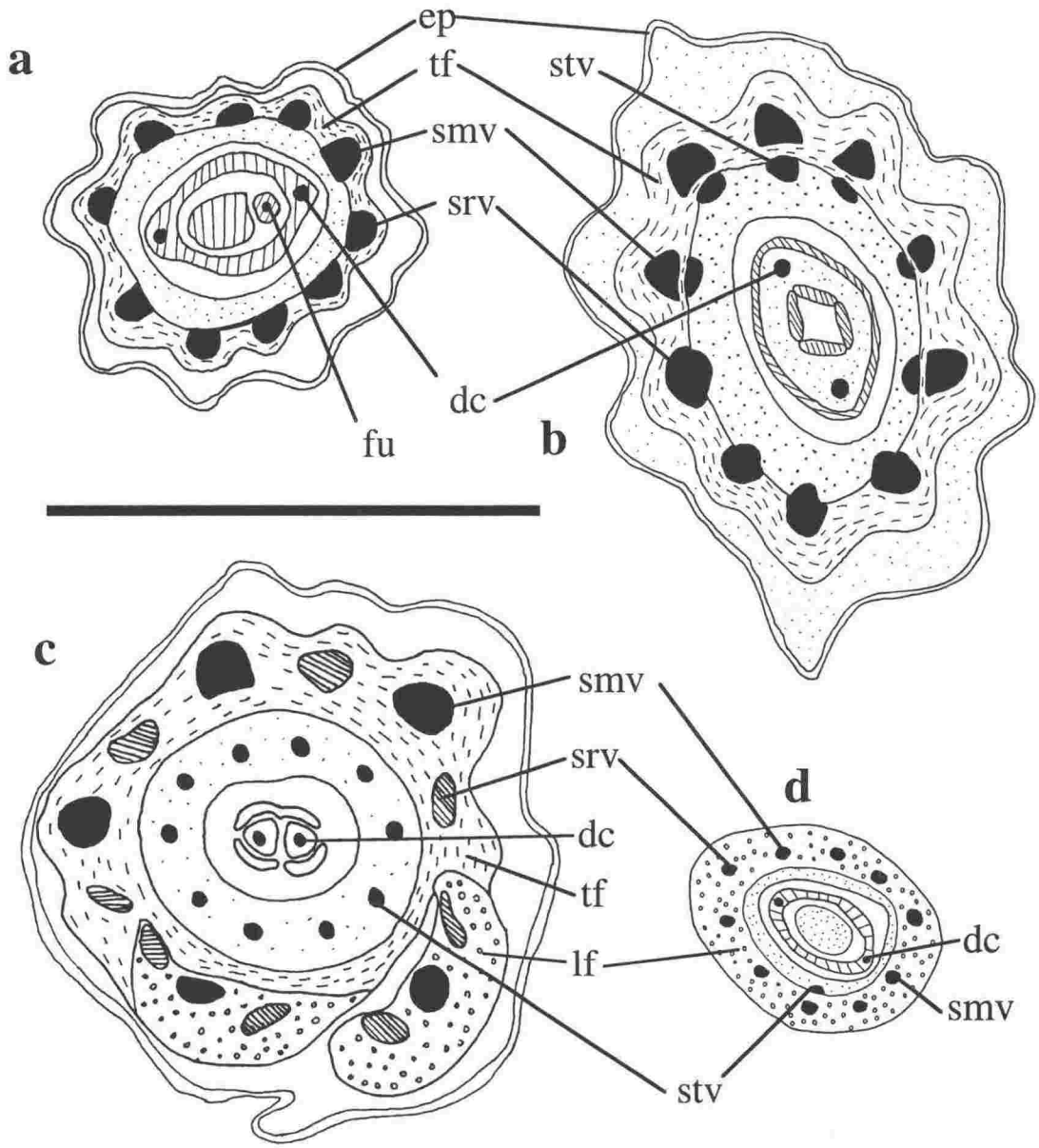

Figure 2.29 Diagrammatic representation of transverse sections through *Scleranthus* flowers showing vasculature. **a**, *S. perennis,* section through perigynum below divergence of stamen and sepal veins. **b,** *S. perennis,* section through perigynum on slight angle showing departure of stamen veins from sepal veins. **c,** *S. perennis,* section through top of perigynum and base of sepals showing departure of sepal marginal veins. Note longitudinal fibres in sepals and continuation of dorsal carpel veins into styles which are shown surrounded by ovary cells. **d,** *S. brockiei* section through perigynum showing single stamen vein and longitudinal fibres in perigynum. Scale 1mm. smv, sepal median vein. srv, sepal marginal vein (coalescent in perigynum) . stv, stamen vein. dc, dorsal carpel vein. fu, funicle. ep, epidermis. lf, longitudinal fibre. tf, tangential fibre.

S. biflorus has four sepals instead of five and in some flowers of this species the sepal lateral veins are lacking, poorly developed or consist of isolated parts (in both sepals and perigynum).

During the development of flowers of *S. perennis* and *S. annuus* a zone of tangentially aligned fibers differentiates weaving around the sepal veins

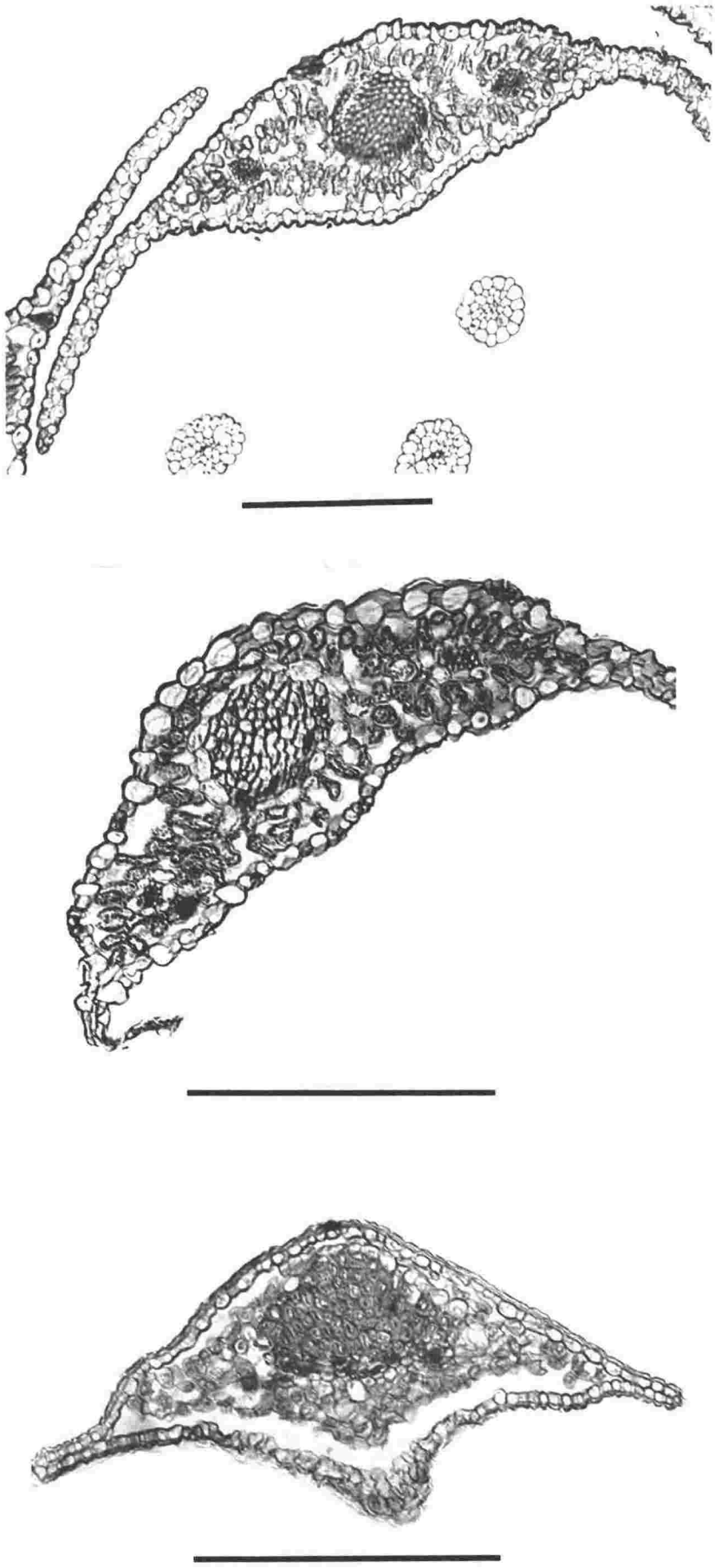

Figure 2.30

Figure 2.31. *Scleranthus perennis* development of flowers as revealed by SEM. **a,** floral meristem after initiation of two bracts (br). **b,** initiation of sepals (K) below floral apex (*). **c,** Bud with stamen primordia (A) and sepal primordia (K, numbered in order of initiation). **d,** Slightly later stage to c showing greater growth rate of stamen primordia adjacent to sepals 3, 4 and 5. Also shows asymmetric floral apex * during initiation of carpel. **e,** Initiation of two carpels on elongated floral apex. **f,** Bud undergoing initiation of gynoecium consisting of three fused carpels. Scale bars all 100 μm.

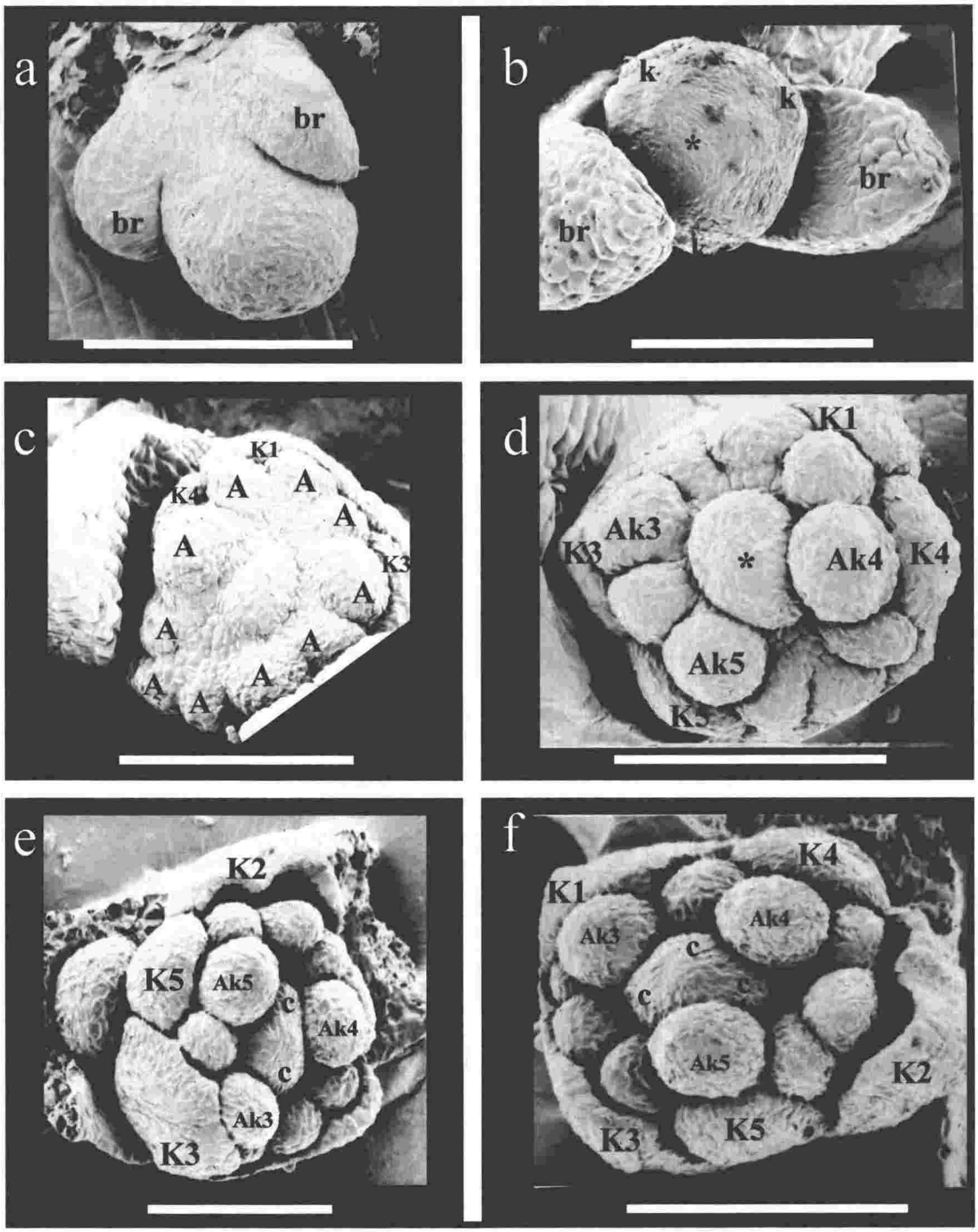
a
br
br
b
k
k
*
br
br
c
K1
K4
A
A
A
A
K3
A
A
A
A
A
A
d
K1
Ak3
K3
*
Ak4
K4
Ak5
K5
e
K2
K5
Ak5
c
Ak4
c
Ak3
K3
f
K4
K1
Ak3
Ak4
c
c
c
Ak5
K2
K3
K5

Figure 2.31.

Figure 2.32. Continuing development of *Scleranthus perennis* flowers. **a,** Dissected bud showing fusion of carpels due to zonal growth around the base of floral apex, now transformed into the ovule (o). One stamen (A) and sepal (K) also shown. **b,** Bud with sepals removed showing differential growth of antisepalous (Ak) and alternisepalous (Ac) stamens and effect of whorls. Note young bud in axil of subtending bract (removed). **c,** young gynoecium showing elongation of styles. Scale bars all 100 μm.

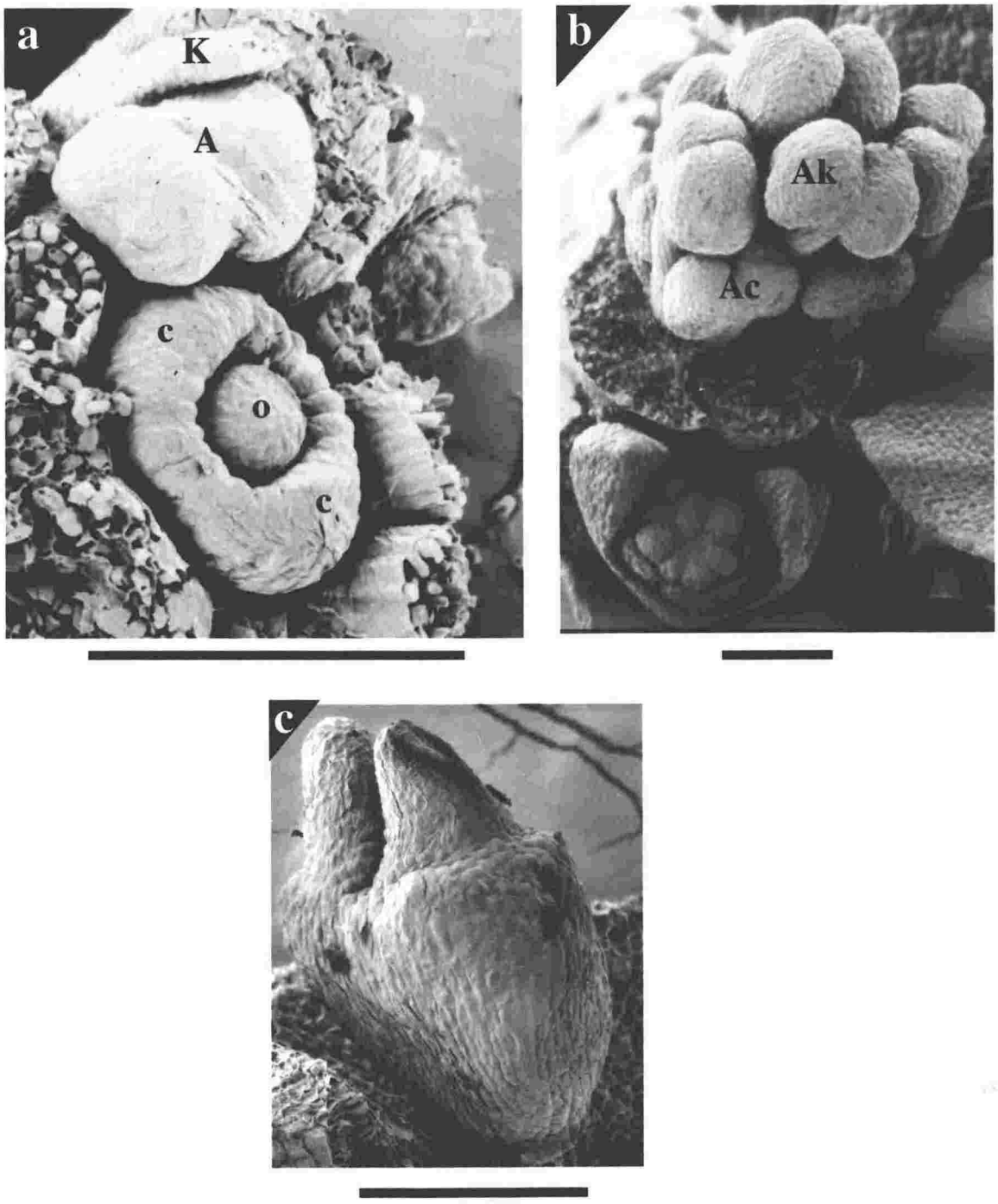

Figure 2.32.

Figure 2.33. Development of flowers of *Scleranthus annuus* as revealed by SEM. **a,** Initiation of sepals (K) and stamen (A) and staminodes (A') on floral apex. **b,** Bud showing stamen primodia adjacent to sepals 4 and 5 and smaller staminode primordia in other positions. Floral apex at (*). **c,** Bud showing initiation of carpel on one side of asymmetric floral apex (*). **d,** Bud showing sequential initiation of carpels either side of floral apex (*) and large staminode primordium adjacent to sepal 3. **e**, Gynoecium showing enclosure of ovule (not visible) by zonal growth and elongation of styles. Scale bars all 100μm.

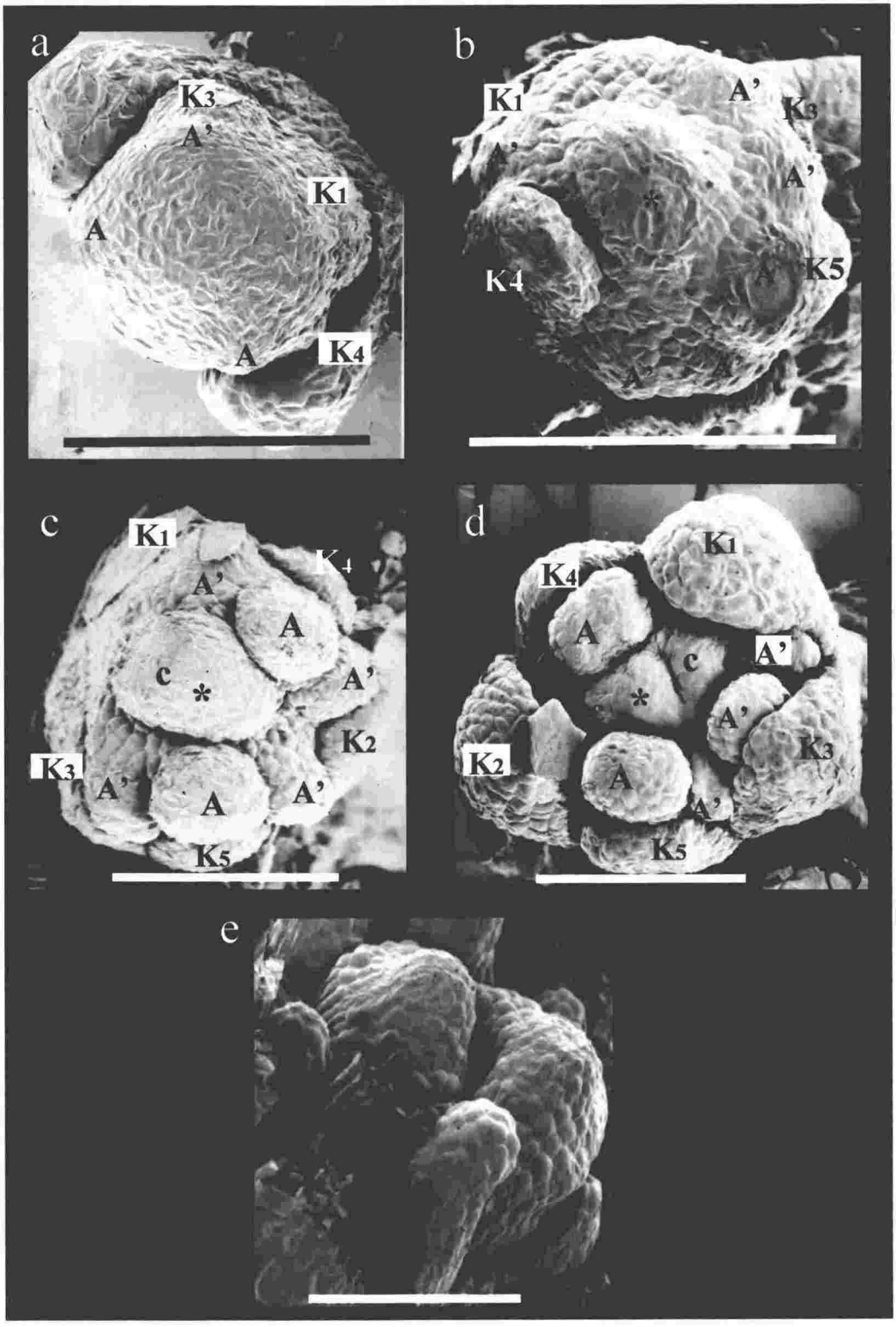
a
K3
A'
K1
A
A
K4
b
K1
A'
K3
A'
A'
*
K4
K5
A'
c
K1
K4
A'
A
c
*
A'
K2
K3
A'
A
A'
K5
d
K1
K4
A
c
A'
*
A'
K3
K2
A
A'
K5
e

Figure 2.33.

2.3.2.2 Section *Mniarum*

The floral apex of *S. brockiei* is smaller and does not extend radially to the extent that those of *S. perennis* and *S. annuus* do. The single stamen seems to be initiated almost simultaneously with sepals four and five, adjacent to sepal 4 (Figure 2.34a). Sepal and stamen primordia at this stage are much closer to the remaining floral apex than in the European species, there being no spaces or any sign of reduced primordia where stamen positions are unfilled. Young buds of *S. brockiei* and *S. biflorus* are covered by their associated bracts far more completely than those of the larger-flowered species and the bracts are strongly curved at their tips.

In both sections of the genus intercalary growth elevates the stamens and sepals above the gynoecium forming the floral cup and resulting in the apparent fusion of these parts in both sections.

2.3.3 Ontogeny of the Gynoecium

Development of the gynoecium is essentially similar in all species examined. Carpels are initiated as protrusions on the flanks of the floral apex (Figure 2.31d, e, f, 2.33c, d, 2.34b, Figure 2.49). In most buds examined two carpels are formed opposite each other but in occasional buds three carpels are initiated in a triangle (Figure 2.31f). In *S. annuus*, the carpels are clearly initiated successively (Figure 2.33d) but this was not observed in other species, although in *S. perennis* the floral apex was observed to pass through an asymmetric stage probably representing the initiation of a carpel on one side (Figure 2.31d). Although initiated separately, the two (rarely three) carpels quickly become linked by a ring of growth so that they come to sheath the

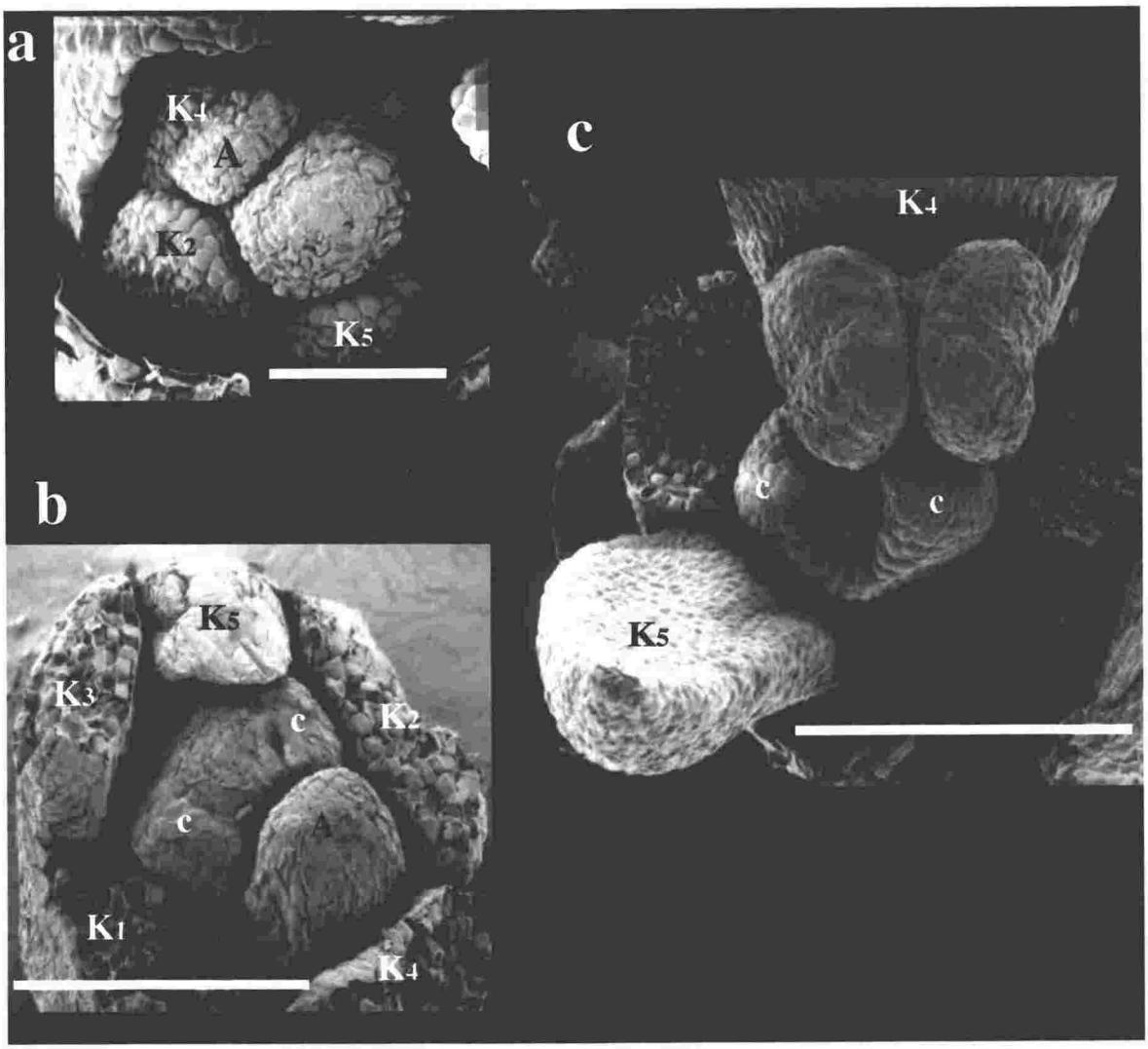

Figure 2.34. Flower development of *Scleranthus brockiei*. **a,** *S. brockiei* young bud showing sepal (K) primordia and single stamen primordium (A). **b,** *S. brockiei* bud with 4 sepals removed showing initiation of carpels (c), single stamen primordium (A) and last initiated sepal (K5). **c,** *S. brockiei* bud with 3 sepals removed showing gynoecium with two carpel primordia and girdling primordium, single stamen and last initiated sepals (K4 and K5). Scale bars 100 μm.

remainder of the floral apex, forming a cup-like structure (Figure 2.32a, 2.33e, 2.34c).

The first initiated carpel is usually alternisepalous being oriented between sepals one and three. The second initiated carpel is oriented directly opposite the first (therefore usually on the same radius as sepal 2). After the initiation of the carpels the remainder of the floral apex develops directly into the single basal ovule (Figure 2.32a, 2.34c). The ovule, therefore, is not derived from carpellary tissue. As a result of its derivation directly from the floral apex, the ovule extends past the developing carpels during the very early stages of its development.

The styles begin to protrude from the top of the gynoecium around the time the carpel walls over-reach and close around the developing ovule (Figure 2.33e). Closure of the gynoecium is primarily achieved through proliferation of tissue around the carpel margins, especially at the base of the styles (Figure 2.35). In the mature carpel the soft tissue closing the carpel will mix with the transmission tissue (see below) but in sectioned material the two can be seen to arise from different regions. Around this time, the gynoecium becomes enclosed by the floral cup.

With the exceptions of *S. biflorus* and *S. uniflorus* the styles become sunken by overgrowth of the cells at the top of the ovary, which later become thick-walled (Figure 2.16). Although the bases of the styles in *S. biflorus* and *S. uniflorus* do not become covered externally by ovary cells, cells at the top of the ovary do become thick-walled. After the styles have elongated for a period, epidermal cells at their tips become papillate, forming a stigmatic surface.

Figure 2.35. *Scleranthus perennis* transverse section through top of ovary showing closure by cell proliferation. Scale bar 100 μm.

Figure 2.36. *Scleranthus perennis* transverse section through ovary showing transmission tissue emanating from positions alternate to styles. Scale bar 100 μm.

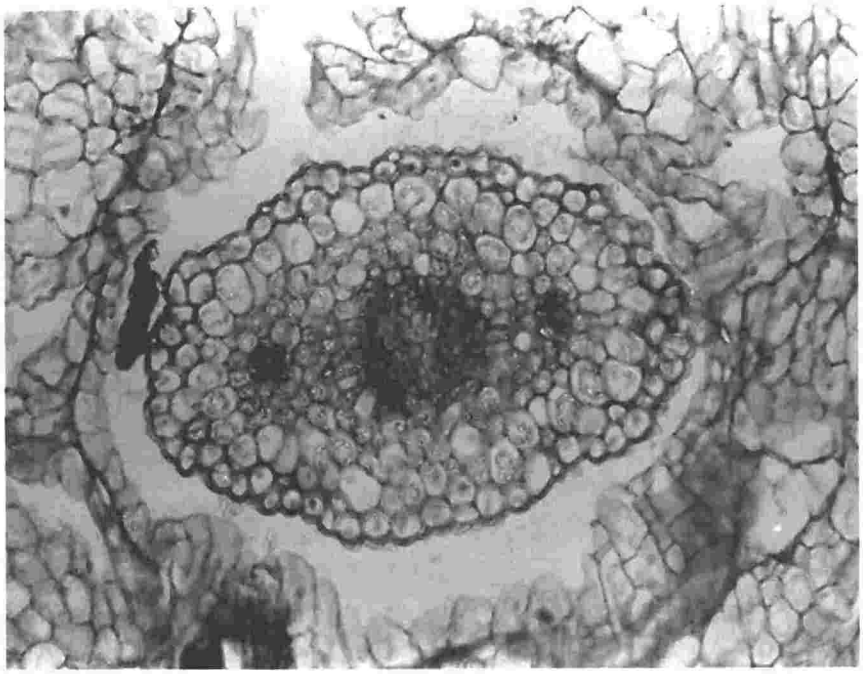

Figure 2.35

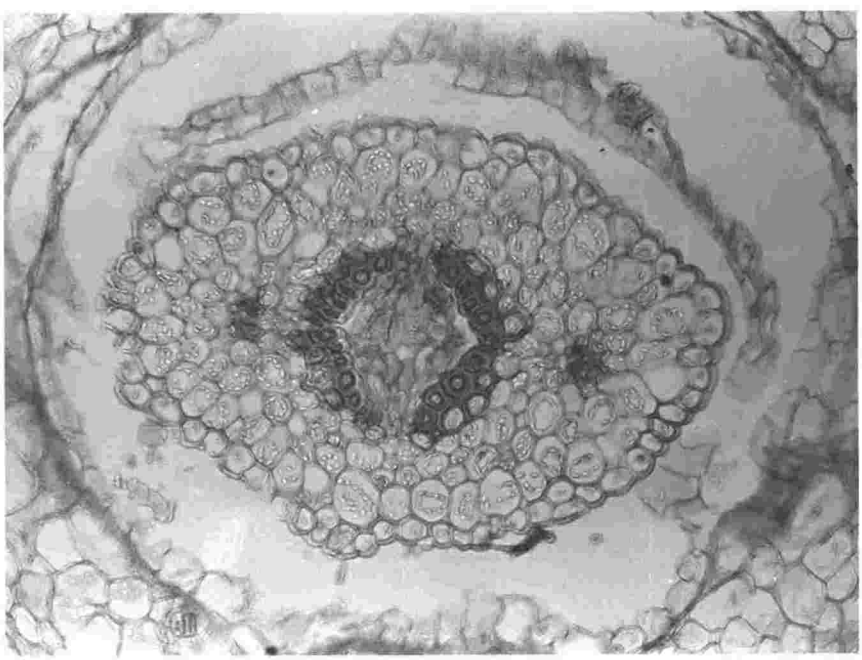

Figure 2.36

After the styles have arisen and the carpel closed, transmission tissue develops, growing from the ovary wall in the position where septa would be in Caryophyllaceae with septate ovaries (Figure 2.36). Transmission tissue is restricted to the uppermost portion of the ovary wall where it forms a plug linking the styles and the micropyle of the ovule. At the base of the gynoecium is a short stalk (gynophore) formed by intercalary growth late in its development.

2.3.4 Embryology

2.3.4.1 Anther and pollen development

Anther development is essentially similar in all *Scleranthus* species examined. The anther wall conforms to the basic type of Davis (1966) in which both secondary parietal layers contribute to the middle wall (Figure 2.37a, 2.38). Sporogenous cells do not undergo mitotic increase but develop directly into pollen mother cells (Figure 2.39). As outlined by Maheswari and Krishna (1990) for *Sagina* and *Drymaria* the pollen mother cells (PMCs) of *Scleranthus* do not separate or round off prior to meiosis and their walls do not become conspicuously thickened (Figure 2.37b and Figure 2.39). However, despite the close association of PMCs during meiosis, tetrads do not aggregate after division as reported for those Caryophyllaceae (Figure 2.41 and Figure 2.42). Meiosis of PMCs (Figure 2.37b, Figure 2.40, and Figure 2.41) results in tetrahedral tetrads of spherical microspores embedded in a common wall (Figure 2.42). Middle wall layers of the anther are completely degenerated by this time. Microspores are released from their tetrads while still very thin-walled. By the time the microspore divides the pollen wall has attained most of

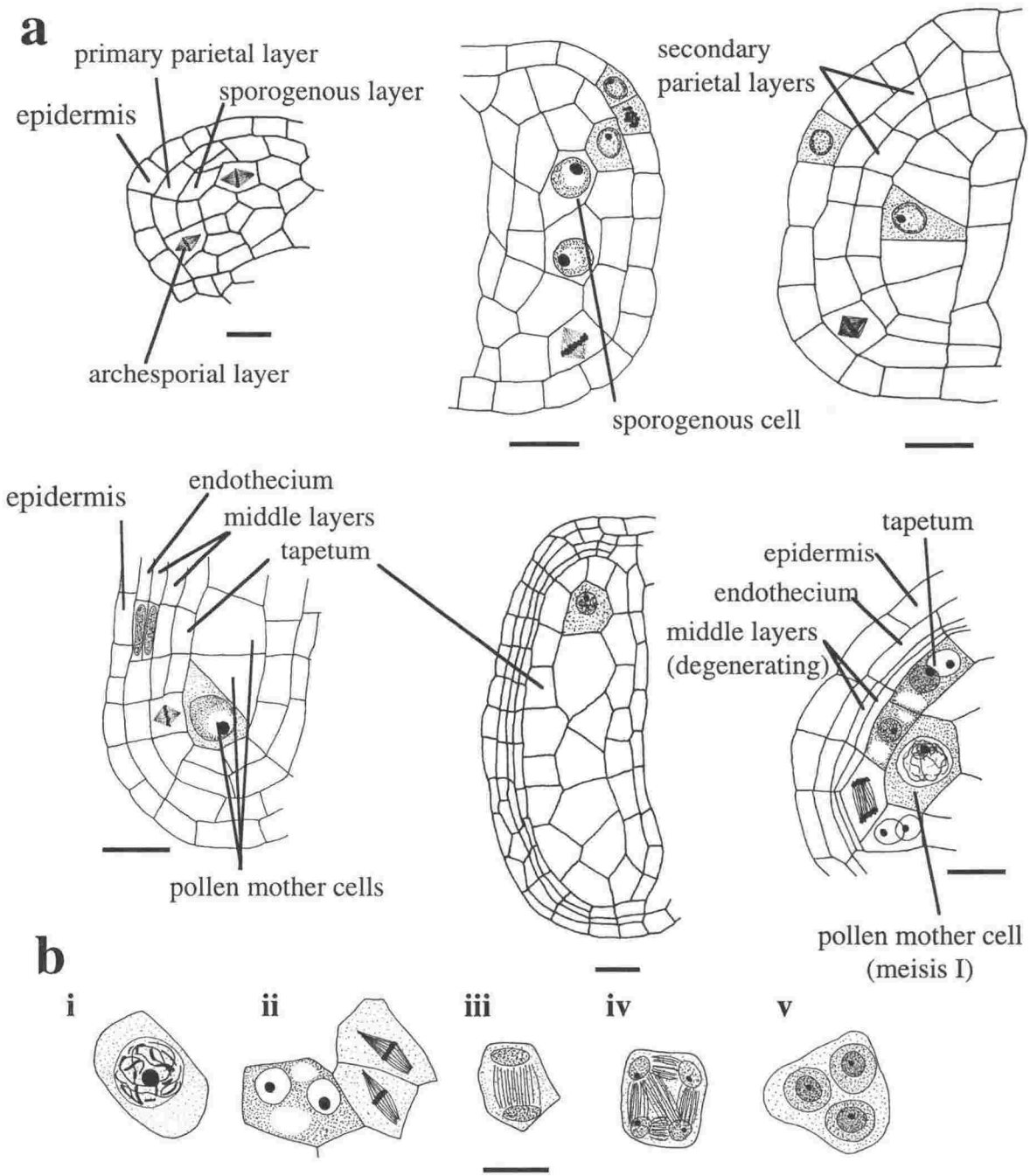

Figure 2.37. **a.** Successive stages in development of *Scleranthus perennis* anther wall and sporogenous tissue to meiosis of pollen mother cells. Other species are similar in all aspects. **b,** *Scleranthus* meiosis of PMCs. **i,** prophase I. **ii,** two PMCs at metaphase I with adjacent binulceate tapetal cell. **iii,** telophase I. **iv,** formation of spindle between nuclei prior to wall formation. **v,** tetrahedral tetrad of microspores (three shown) in callose walls. Scale bars 10 μm.

Figure 2.38. *Scleranthus perennis* section through developing anther showing division of cell in primary parietal layer (arrow). Scale bar 10 μm.

Figure 2.39. *Scleranthus perennis* section through developing anther showing anther wall with developing tapetum (*), and pollen mother cells (arrow). Scale bar 10 μm.

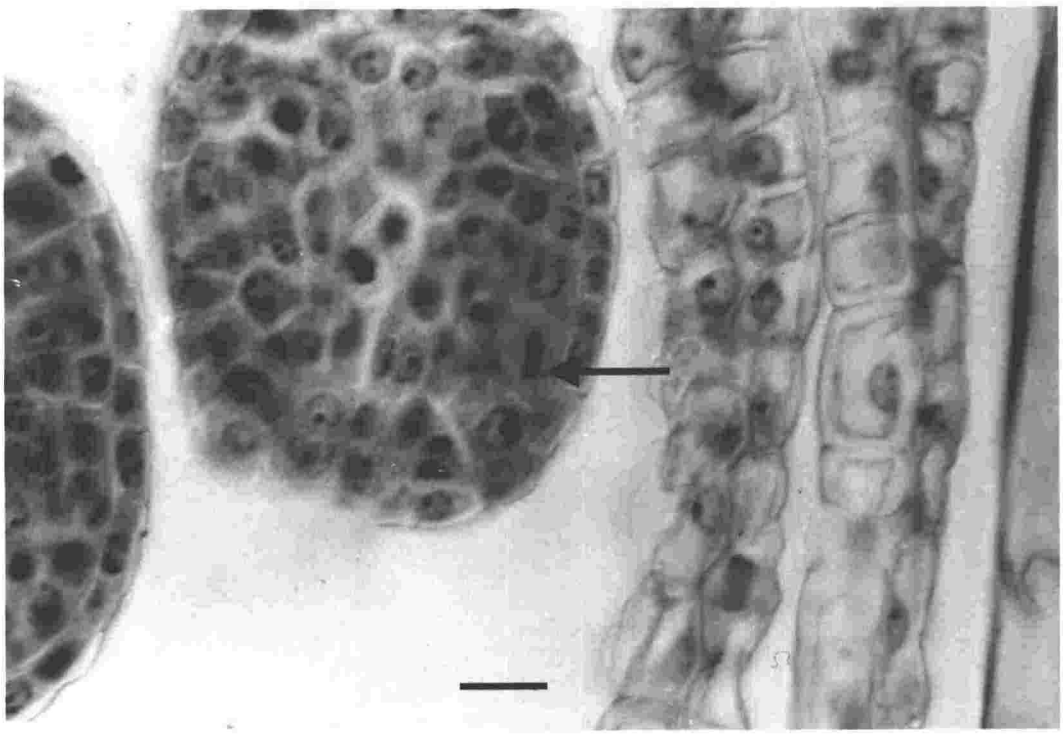

Figure 2.38

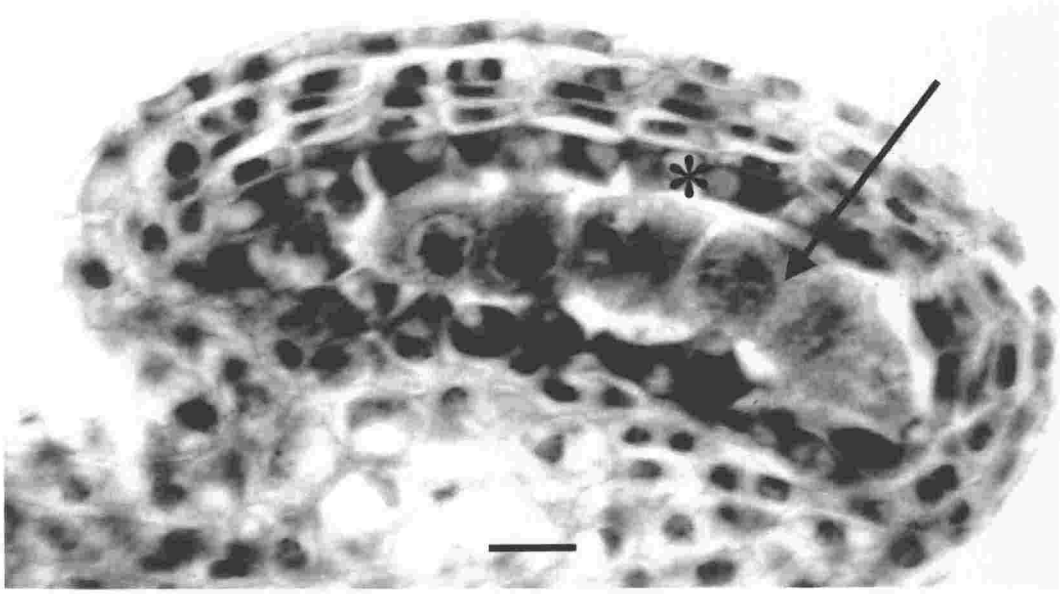

Figure 2.39

Figure 2.40. *Scleranthus annuus* section through anther showing pollen mother cells during meiosis and 2-nucleate tapetal cells. Scale bar 10 μm.

Figure 2.41. *Scleranthus perennis* section through anther showing formation of spindles between daughter nuclei at end of meiosis II (large arrow) and wall formation by furrowing (small arrow). Scale bar 10 μm.

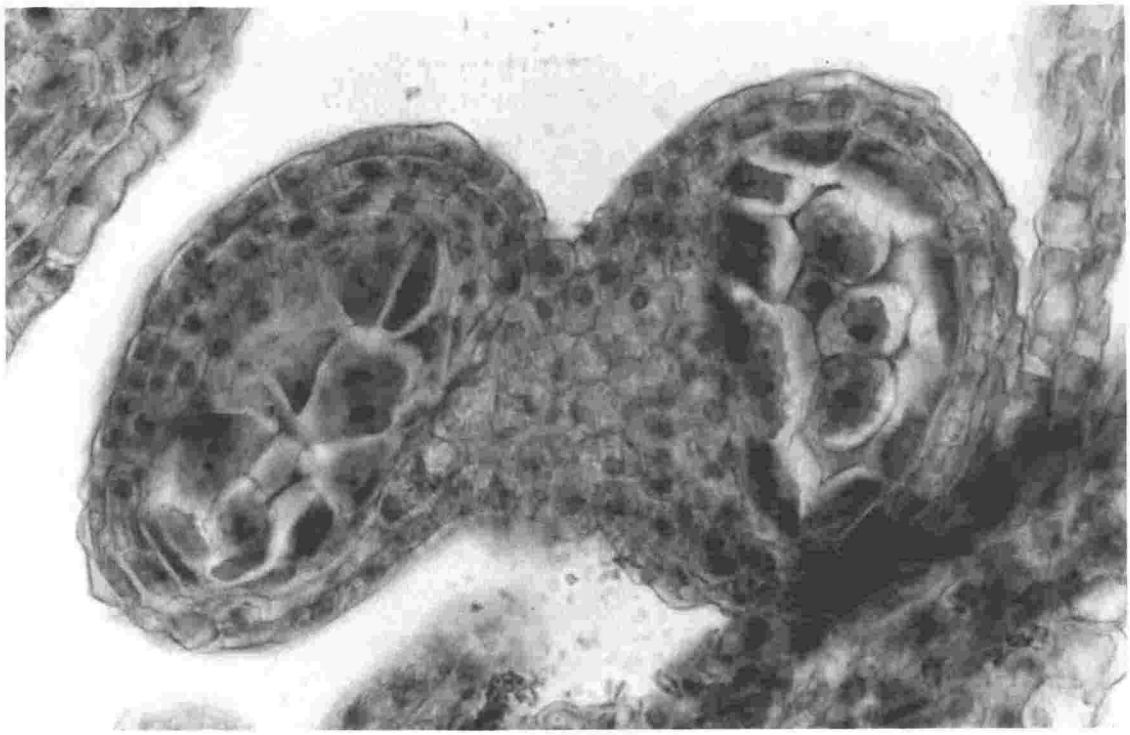

Figure 2.40

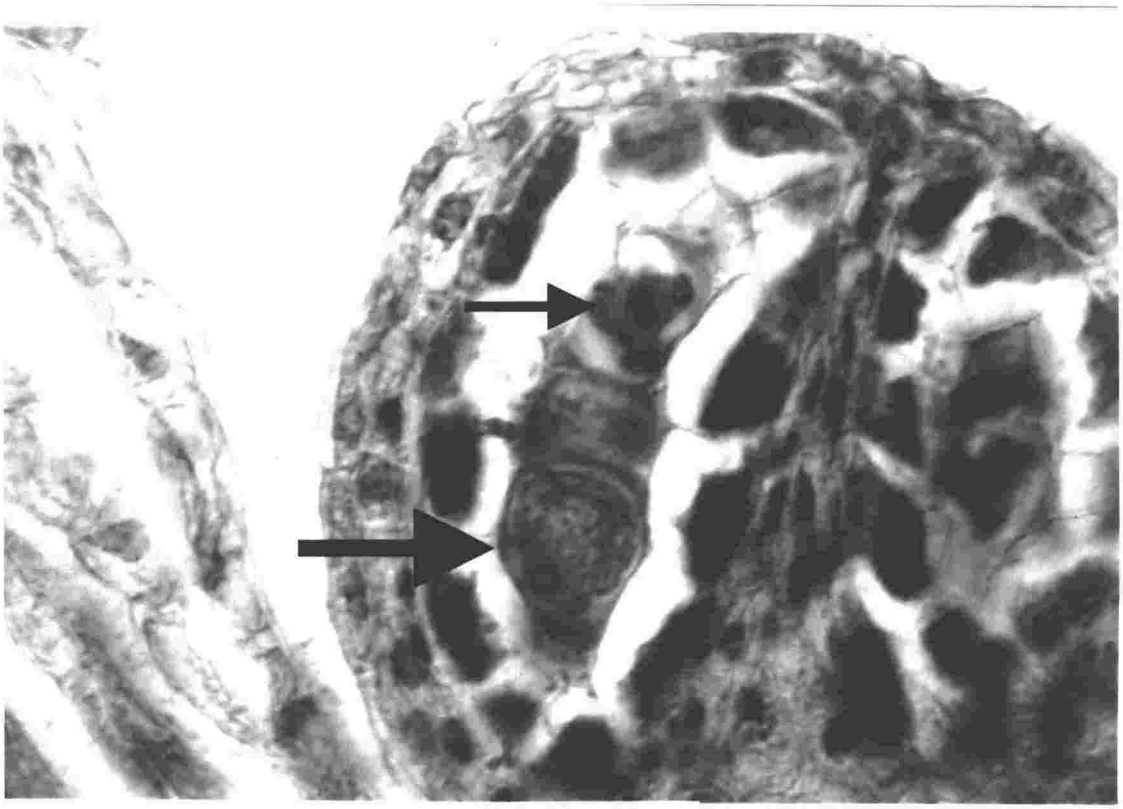

Figure 2.41

Figure 2.42. *Scleranthus perennis* tetrahedral tetrads of microspores embedded in pollen mother cell wall. Scale bar 10 μm.

Figure 2.43. *Scleranthus perennis* section through anther showing division of microspore. Scale bar 10 μm.

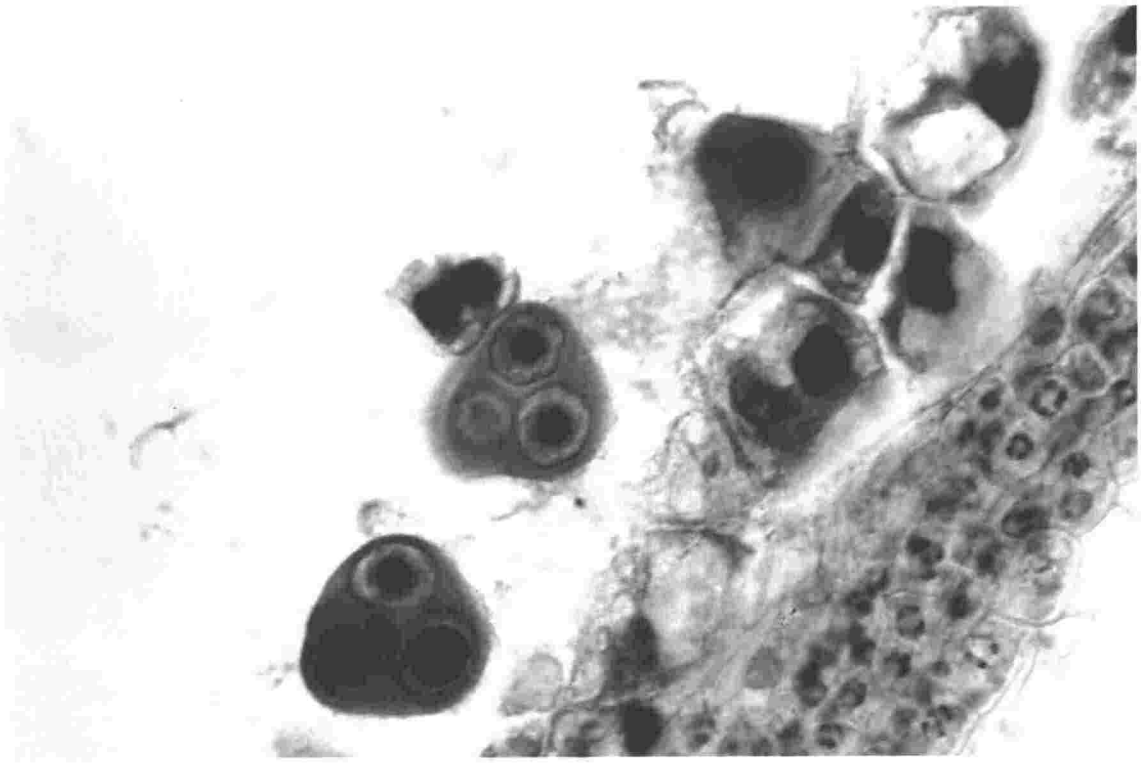

Figure 2.42

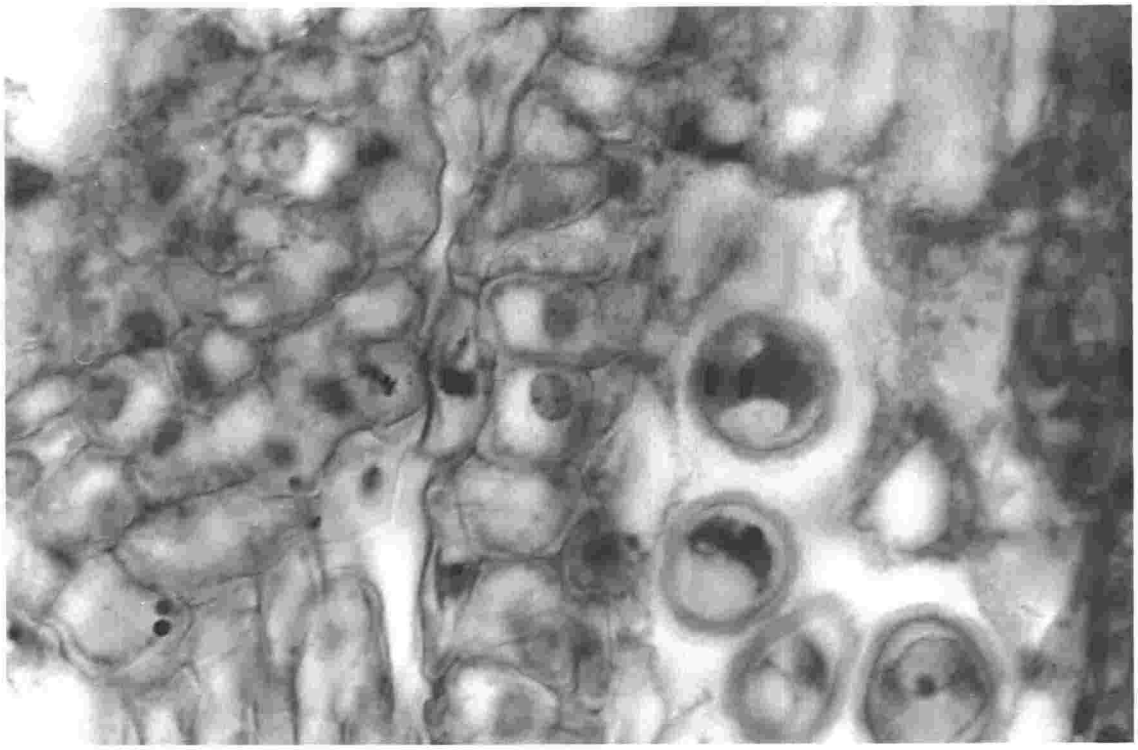

Figure 2.43

Figure 2.44. *Scleranthus pungens* section through anther showing two celled pollen grains. Dividing microspore at arrow. Scale bar 10 μm.

Figure 2.45. *Scleranthus perennis* section through anther showing three celled pollen grain and mature anther wall.

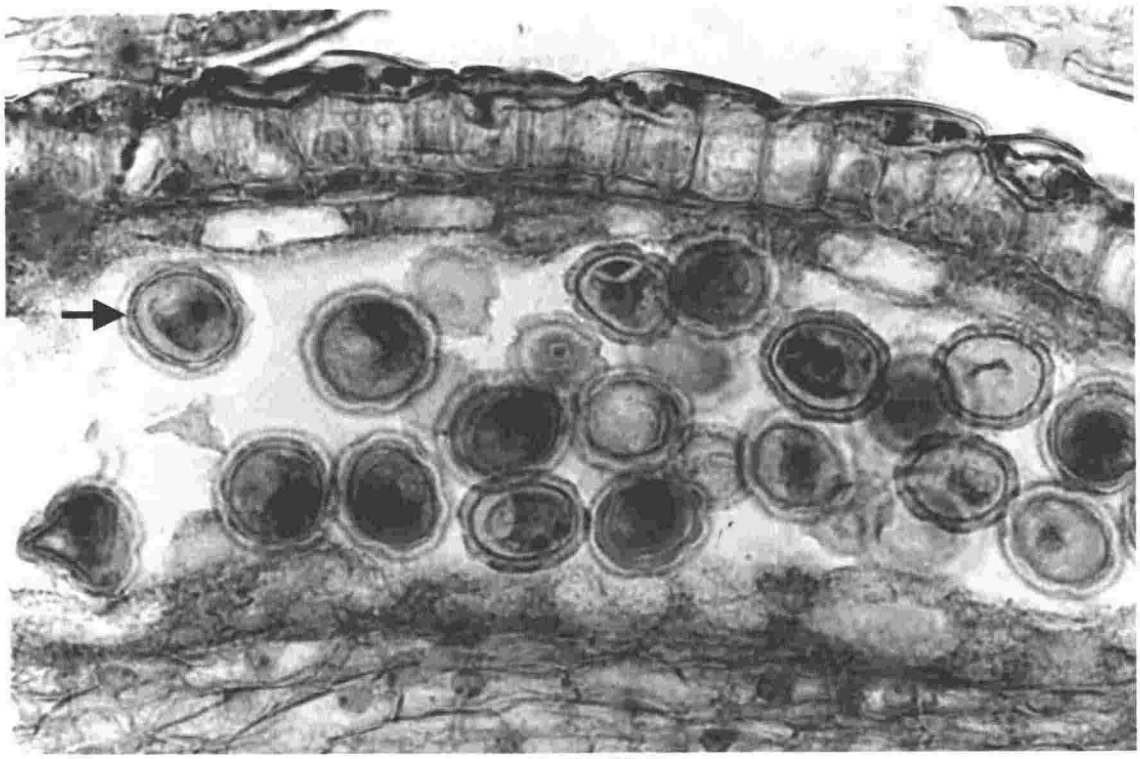

Figure 2.44

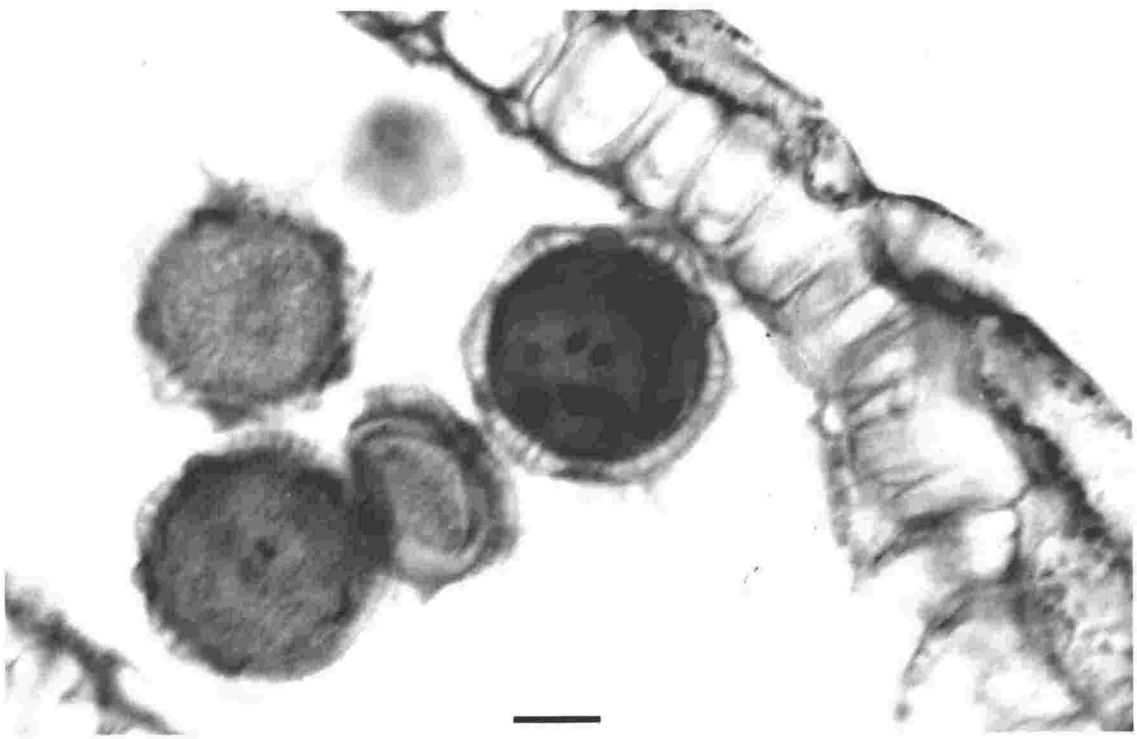

Figure 2.45

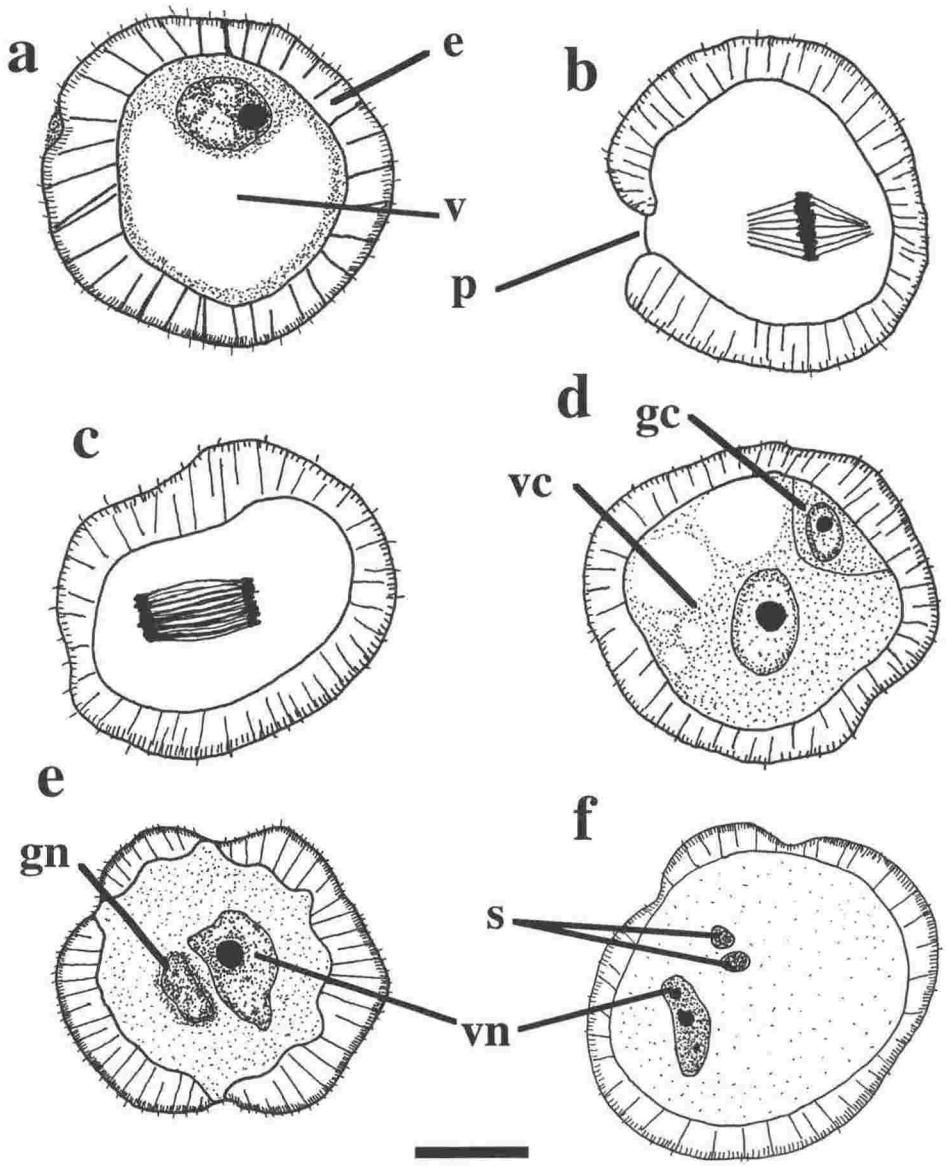

Figure 2.46 *Scleranthus perennis*. Development of pollen grains. **a,** single celled microspore with large vacuole (v) and thick exine wall (e). **b and c,** division of microspore. **d,** two celled pollen grain with generative cell (gc) and vegetative cell (vc). **e,** pollen grain staining obscures cell structure but vegetative cell nucleus (vn) and generative cell nucleus (gn) still visible. **f,** pollen grain at anthesis with vegetative cell nucleus and two dark staining sperm (s) visible. Note pores (p) visible in some sections cause uneven appearance of wall thickness. Scale 10 μm.

its mature thickness (Figure 2.43, 2.44, and Figure 2.46a). The microspore divides in the usual manner with the generative cell formed against the wall of the pollen grain (Figure 2.46 b, c, d). Division of the microspore is accompanied by conspicuous vacuolization. After division the vacuole first breaks up and then disappears (Figure 2.44, 2.46 d, e).

From the two celled stage onwards, poor fixation and dense staining of the cytoplasm hampered observations of pollen development. However, the pollen grains are three-celled prior to anthesis (Figure 2.45, 2.46f). Sperm nuclei are small and dark-staining and the vegetative nucleus is large and has a conspicuous nucleolus. The vegetative nucleus appeared degenerate in mature pollen grains, but poor fixation is the probable cause of this. Details of the morphology of mature pollen walls are described later in this chapter.

Tapetal cells undergo nuclear division becoming bi-nucleate at about the time the pollen mother cells undergo meiosis (Figure 2.37 and Figure 2.40). Depending on the anther examined, division of tapetal nuclei continues throughout meiosis, but is usually completed by the time microspore tetrads are formed. The tapetum is of the secretory type and completely degenerates before anthesis. No Ubisch bodies were observed. The endothecium develops bands of thickening on the inner tangential and radial walls of its cells. The epidermis is persistent although some individual cells degenerate.

2.3.4.2 Ovule development

The following is the case for *S. perennis* and applies to other species of *Scleranthus* except as noted. During early ovule-development three tissue zones are evident. A dermal zone and subdermal zone, each one cell thick, are

continuous over the ovule and carpels. The remainder of the ovule is occupied by the central zone. (Figure 2.47b, and Figure 2.50) A conspicuous archesporium is not formed, but one or more cells of the subdermal zone near the apex of the ovule divide periclinally (Figure 2.47b, and Figure 2.51) to form a primary parietal cell and primary sporogenous cell (Figure 2.47d, and Figure 2.52). In all observations a single megaspore mother cell (MMC) develops (Figure 2.47e, and Figure 2.53). The formation of MMC and primary parietal cell is followed or accompanied by periclinal division of other cells in the subdermal layer often some distance from the ovule's apex (Figure 2.54, and Figure 2.55). The primary parietal cell may undergo one or more divisions but most increase in the size of the nucellus is the result of periclinal division of dermal zone cells and their derivatives.

By the time the MMC has attained conspicuous size, both integuments are formed and the ovule has begun to take on its characteristic curved shape (Figure 2.47e). Note that throughout its development the ovule is epitropous (directed toward the top of the ovary). While the megaspore mother cell is enlarging, the styles have begun to protrude conspicuously from the top of the ovary. In addition, a conspicuous growth of transmission tissue extends downward from the ovary wall, bridging the gap from the styles to the micropyle (Figure 2.20 and Figure 2.47e) and forming an obdurator. In transverse sections of flowers, the hair-like projections of the transmission tissue can be seen to arise in two short rows alternating with the dorsal carpel veins (Figure 2.36).

By the time the MMC divides, the inner integument extends past the nucellus and has become more than two cells wide in the region of the micropyle (Figure 2.47f, Figure 2.54, and Figure 2.55). The MMC at this time is buried

by several layers of cells derived mostly from the dermal layer. In all the material examined, the second meiotic division was suppressed in the micropylar diad cell giving a triad of cells of which the micropylar two degenerate (Figure 2.47f, Figure 2.54, and Figure 2.55). The chalazal cell of this triad of cells forms the functional megaspore which undergoes three rounds of nuclear division resulting in an eight-nucleate embryo sac of the *Polygonum* type (Figure 2.48 a, b, Figure 2.57 and Figure 2.58). The two polar nuclei come to lie together near the ovum before fertilization but were not observed to fuse (Figure 2.57). Starch accumulates in the embryo sac in numerous small amyloplasts (Figure 2.48a, Figure 2.57, and Figure 2.58). One or both synergids degenerate prior to fertilization.

Late in the growth of the ovule the micropylar region of the nucellus extends (by uneven cell division) to form a prominent extension, or beak, oriented toward the transmission tissue (Figure 2.21). A zone of narrow, elongate cells develops in the nucellus running from the embryo sac through the nucellar beak to the micropyle (Figure 2.21 and Figure 2.22). This zone of cells is most conspicuous in larger ovuled species (section *Scleranthus*). After fertilization, the perisperm accumulates starch in large amyloplasts. The endosperm is of the nuclear type; development was not followed long enough to observe whether wall formation occurred. The embryo develops according to the Caryophyllad type of embryogeny. The elongate zygote divides unevenly to form a large basal cell and smaller apical cell. The basal cell expands and forms the vesicular basal cell of the suspensor. The apical cell divides transversely and the lower daughter cell also becomes vesicular and

Figure 2.47. Development of ovule of *Scleranthus perennis* to megaspore tetrad stage. **a,** Formation of carpels (by division of subdermal initials, ci) on flanks of floral apex (arrow) which will transform into ovule. **b,** Young ovule with dermal (d), subdermal (s) and central (c) tissue zones. A subdermal archesporial cell near the apex of the ovule is shown dividing periclinally. **c,** Ovule after division of archesporial cell to form primary parietal (p) and sporogenous (sp) cells. At this time the inner integument is initiated by periclinal division of a cell in the dermal layer of the ovule (i). **d,** Ovule after initiation of both integuments. **e,** Ovule at megaspore mother cell (mmc) stage drawn to show positioning and orientation of the ovule within the single locule of the gynoecium and developing transmission tissue (tt). Note proliferation of subdermal derivative cells (sd). **f,** Ovule after division of mmc to form a triad of cells, the functional megaspore (fm) and two degenerating cells (dc).

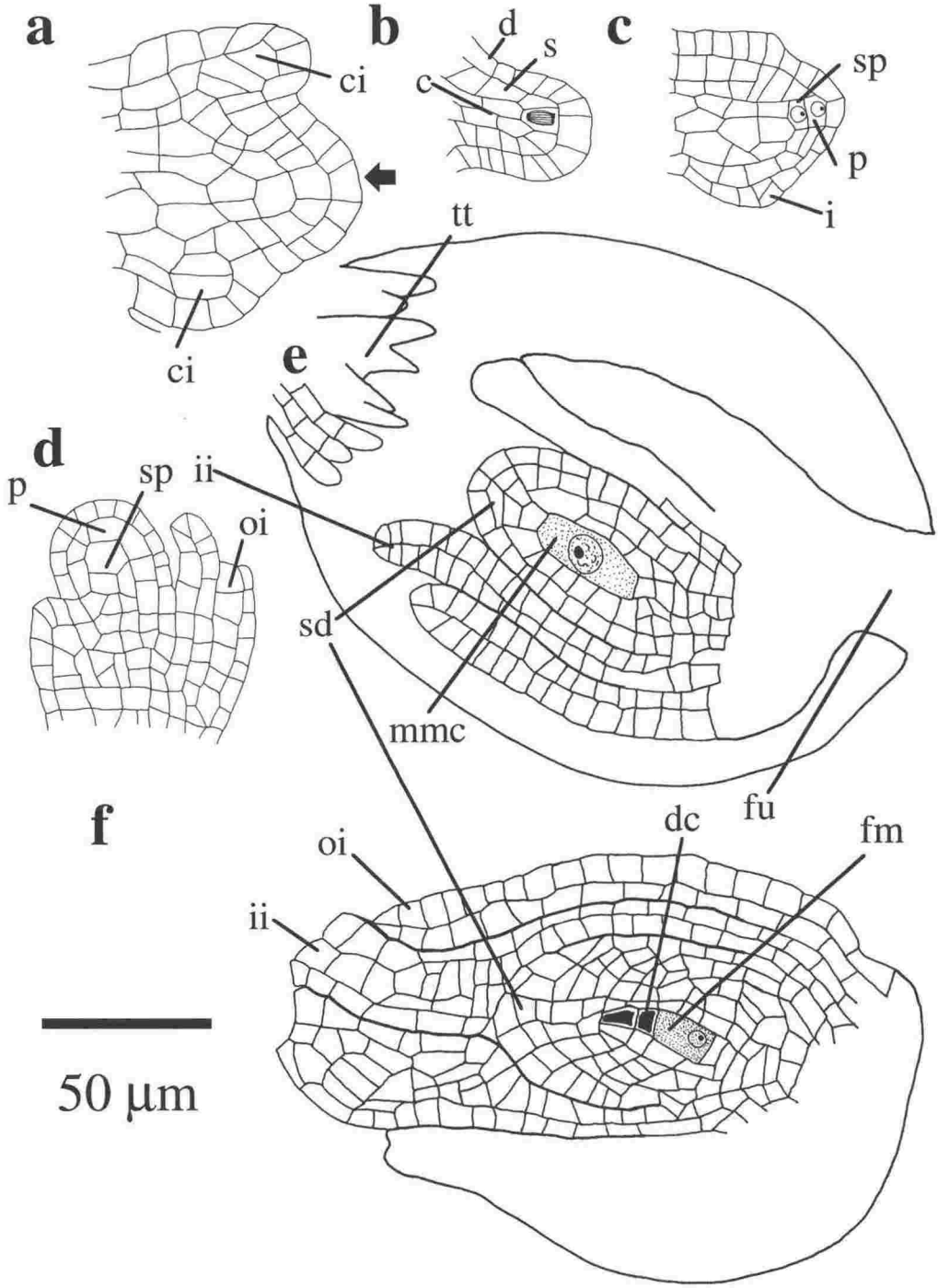

Figure 2.47

Figure 2.48. Mature embryo sac and development of embryo in *Scleranthus*. **a,** *Scleranthus minusculus* embryo sac at anthesis. an, antipodial cell; sy, synergid; pn, polar nuclei; st, starch grains. **b,** *Scleranthus minusculus* part of embryo sac showing ovum (ov) drawn from section adjacent to that depicted in **a. c,** *Scleranthus perennis* four celled embryo. ba, basal cell. **d,** *Scleranthus annuus* five celled embryo. **e,** *Scleranthus perennis* globular embryo.

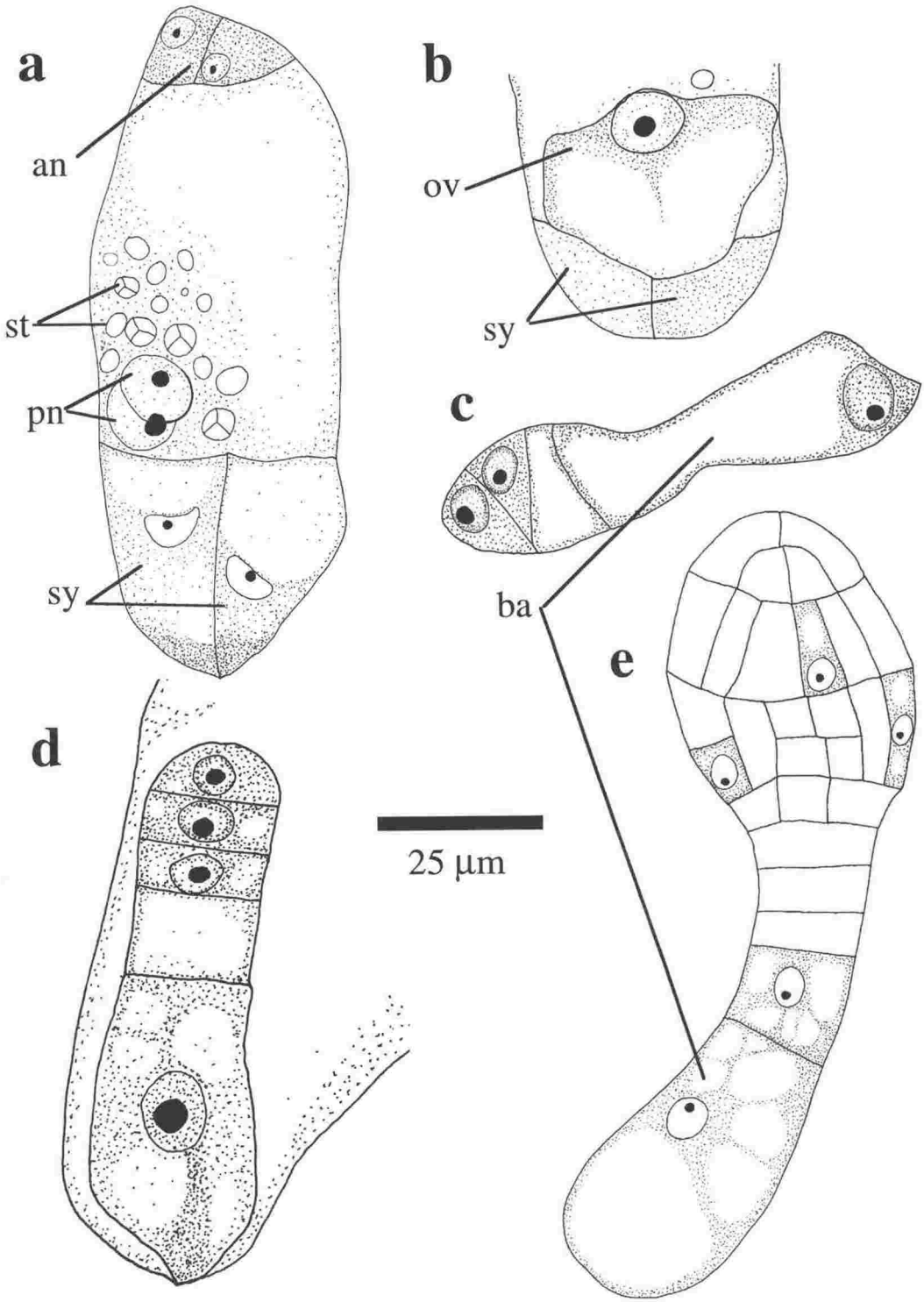

Figure 2.48.

Figure 2.49. *Scleranthus annuus* section showing formation of carpels (ca) on flanks of floral apex (*). Two stamens (st) are also shown. Scale bar 10 μm.

Figure 2.50. *Scleranthus perennis* section of flower bud showing young gynoecium encircling ovule. st, stamen; ca, carpel; ov, ovule. Scale bar 10 μm.

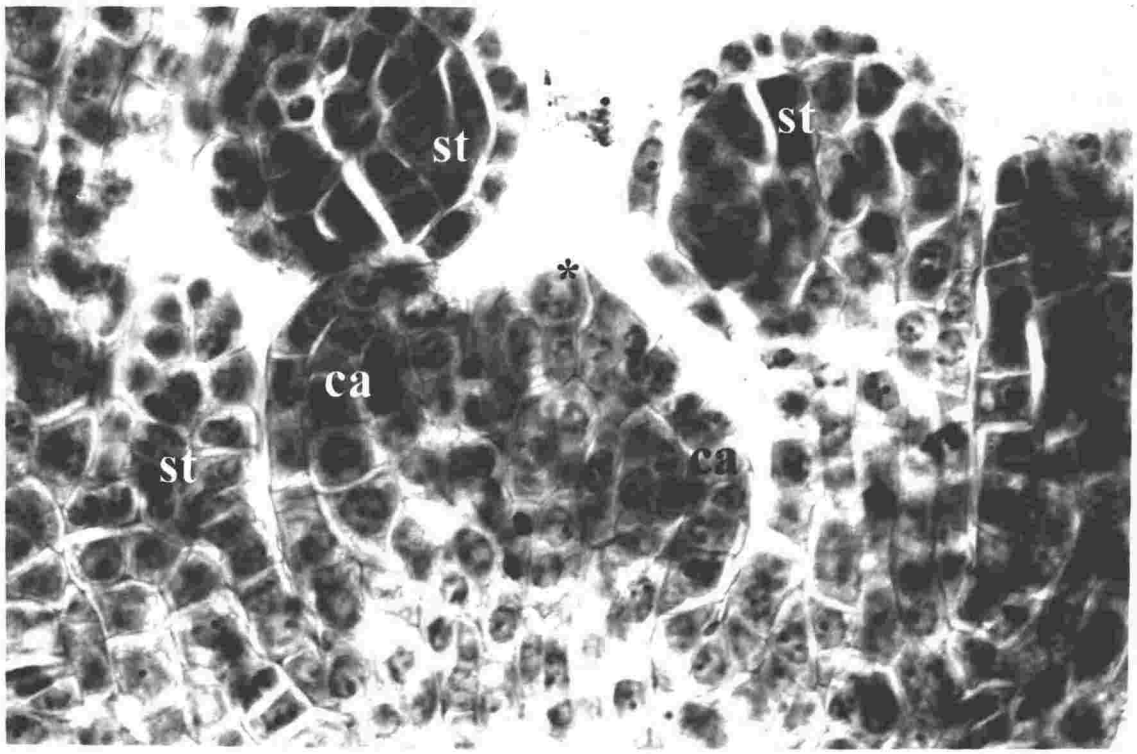

Figure 2.49

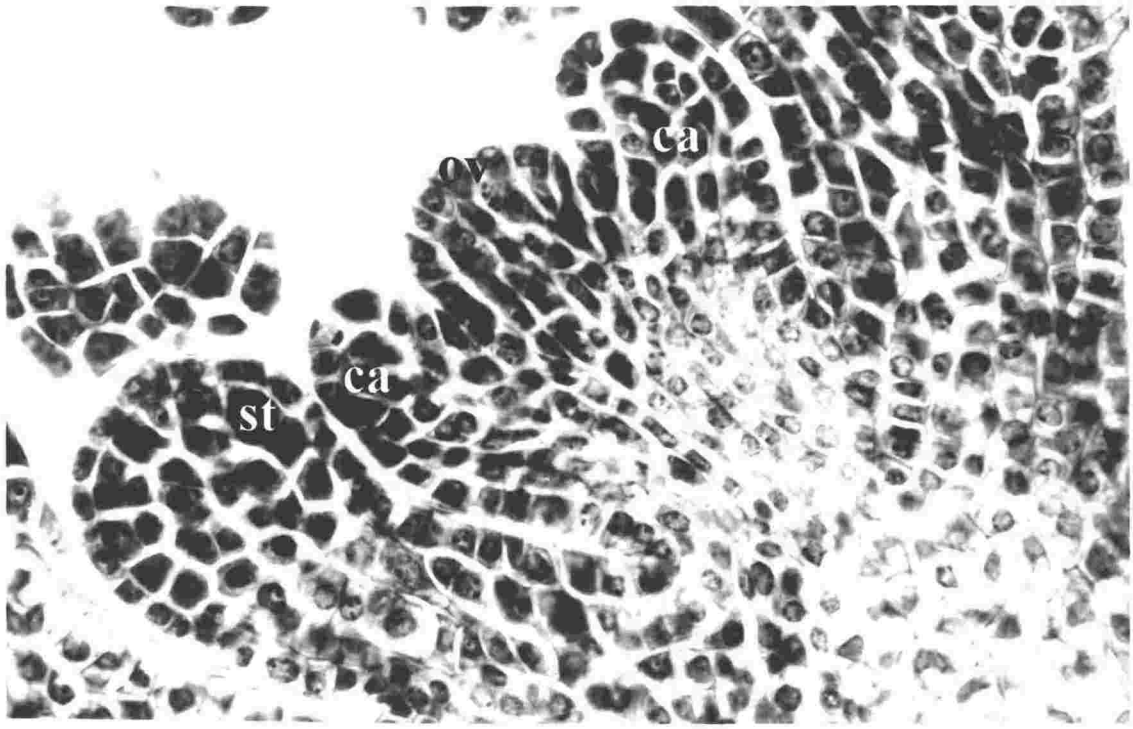

Figure 2.50

Figure 2.51. *Scleranthus perennis* section of flower bud showing ovule enclosed in gynoecium after division of subdermal archesporial cell to form primary parietal cell (dark arrow) and primary sporogenous cell (light arrow). Scale bar 10 μm.

Figure 2.52. *Scleranthus perennis* ovule and part of gynoecium after initiation of integuments. Primary sporogenous cell at arrow, transmission tissue at *. Scale bar 10 μm.

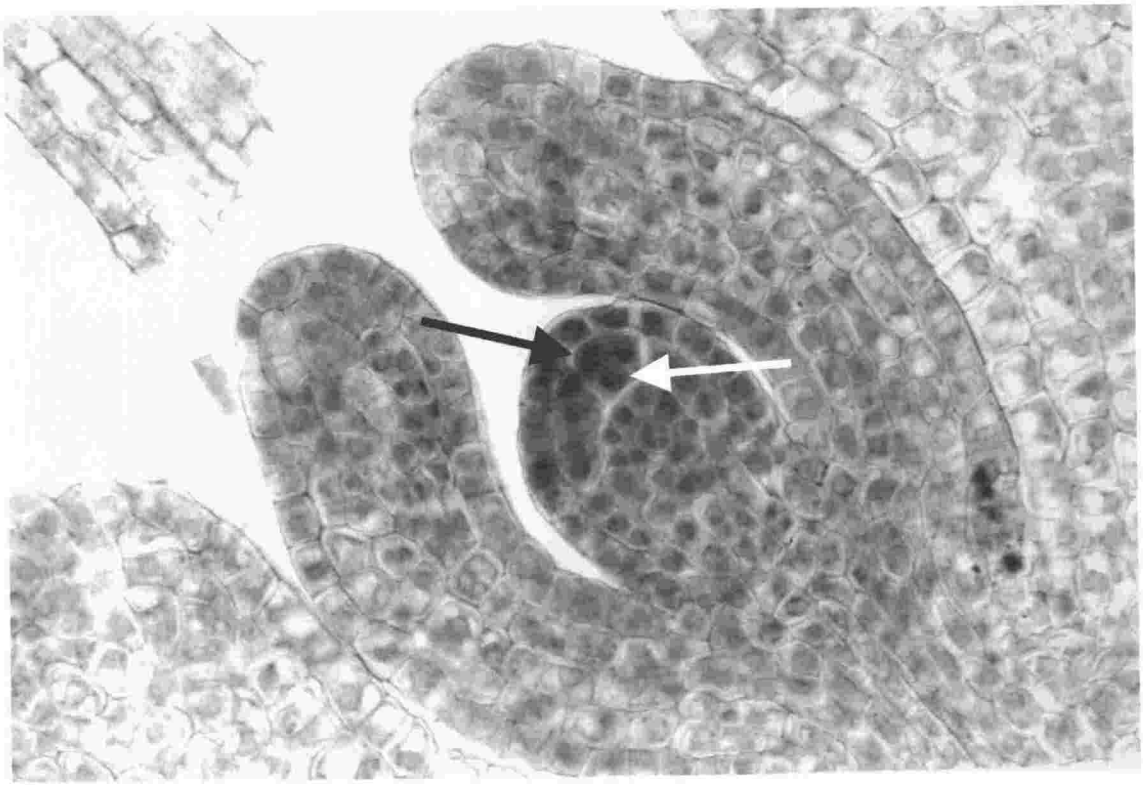

Figure 2.51

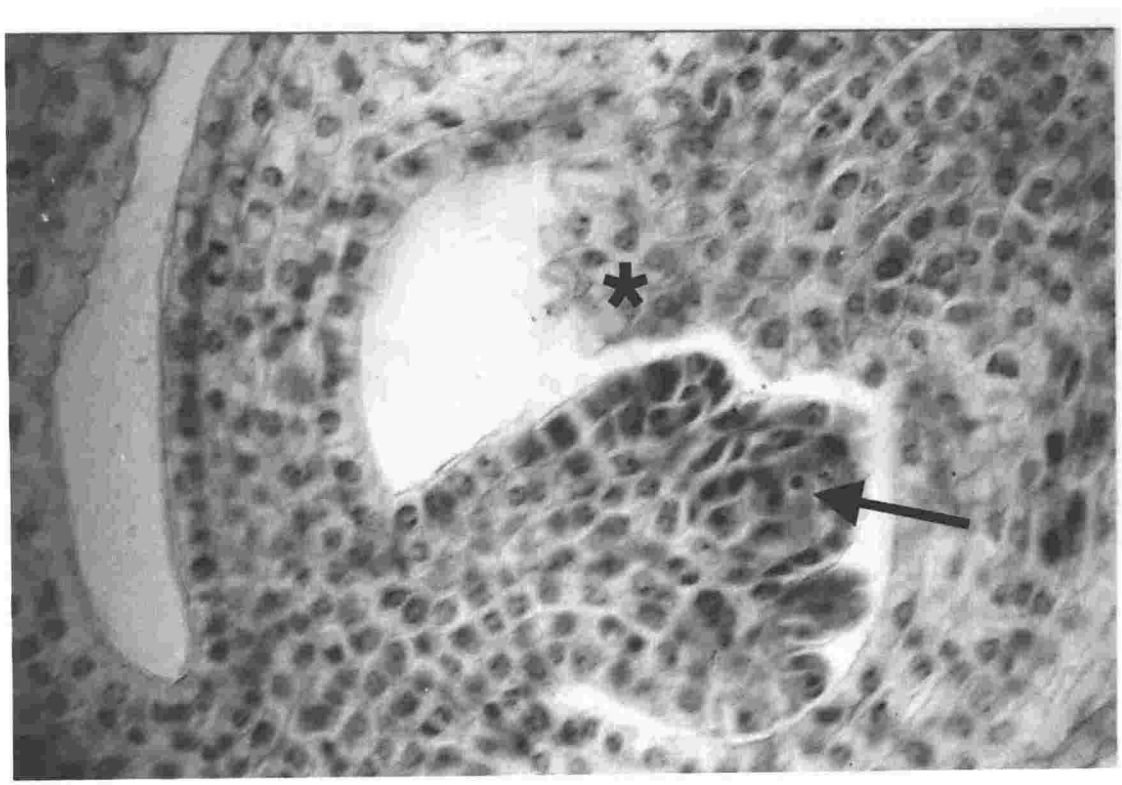

Figure 2.52

Figure 2.53. *Scleranthus perennis* ovule after differentiation of megaspore mother cell. A pair of cells recently formed by periclinal division of a dermal cell is shown at arrow. Scale bar 10 μm.

Figure 2.54. *Scleranthus brockiei* ovule after division of MMC. Functional megaspore is shown at arrow. Note prominent nucellar beak. Scale bar 10 μm

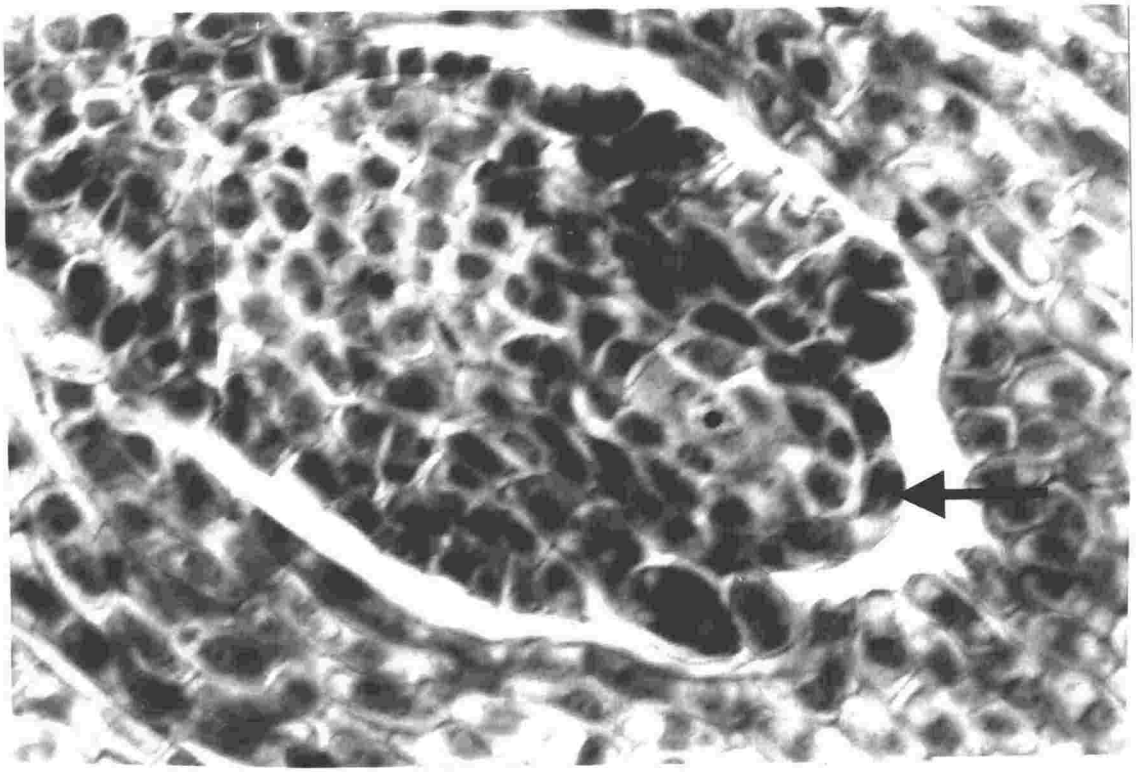

Figure 2.53 —

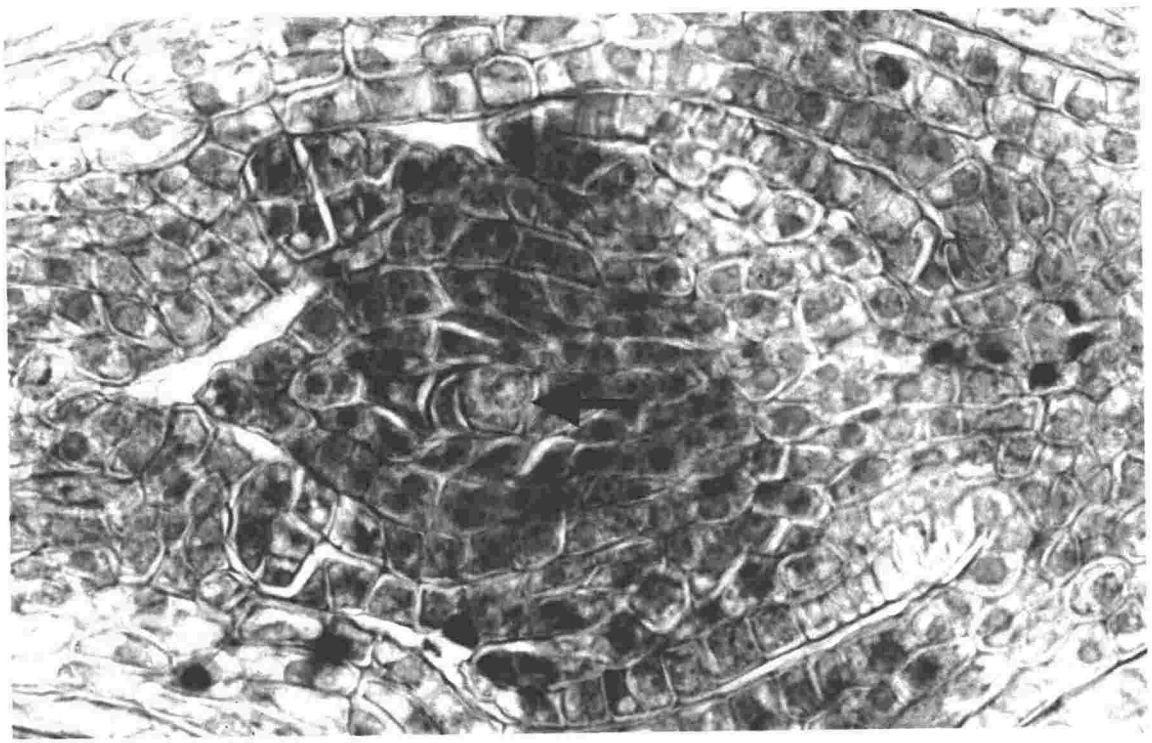

Figure 2.54 —

Figure 2.55. *Scleranthus perennis* ovule after division of mmc. Functional megaspore is shown at arrow. Note nucellar beak (n) and pseudoparietal tissue (p). Scale bar 10 μm.

Figure 2.56. *Scleranthus annuus* five celled linear embryo. Note vesicular basal cell of suspensor (v). Scale bar 10 μm.

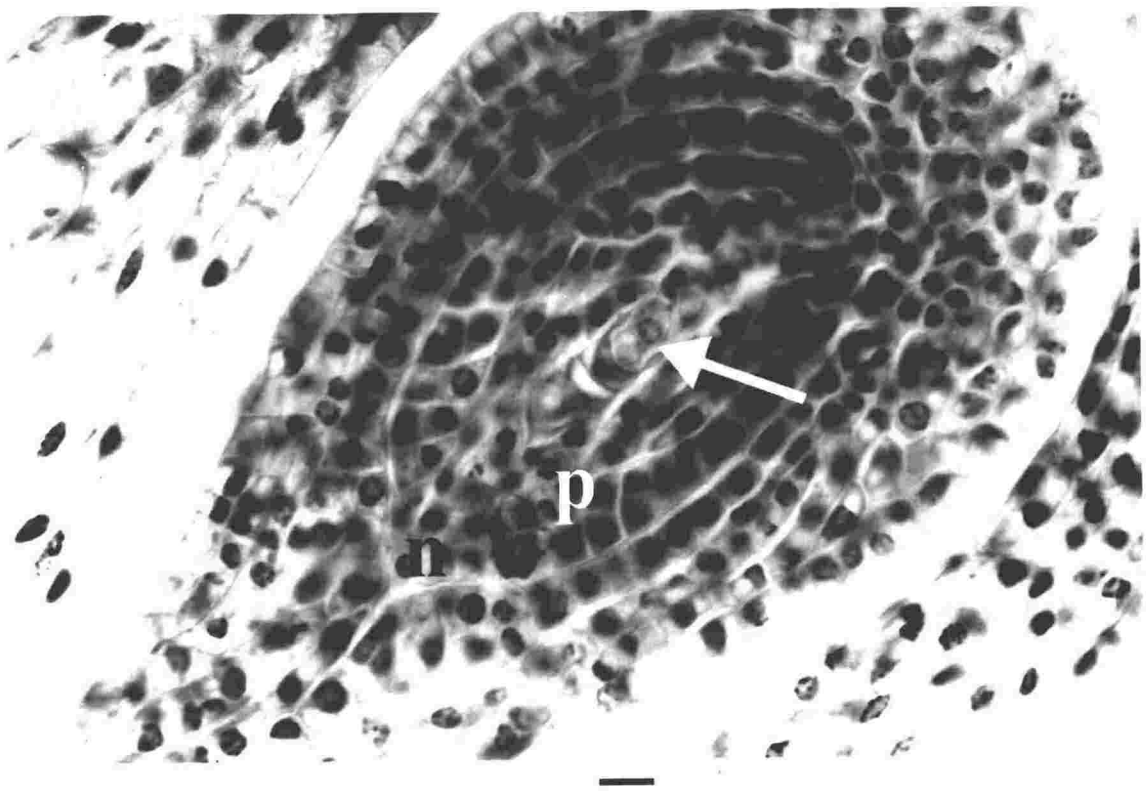

Figure 2.55

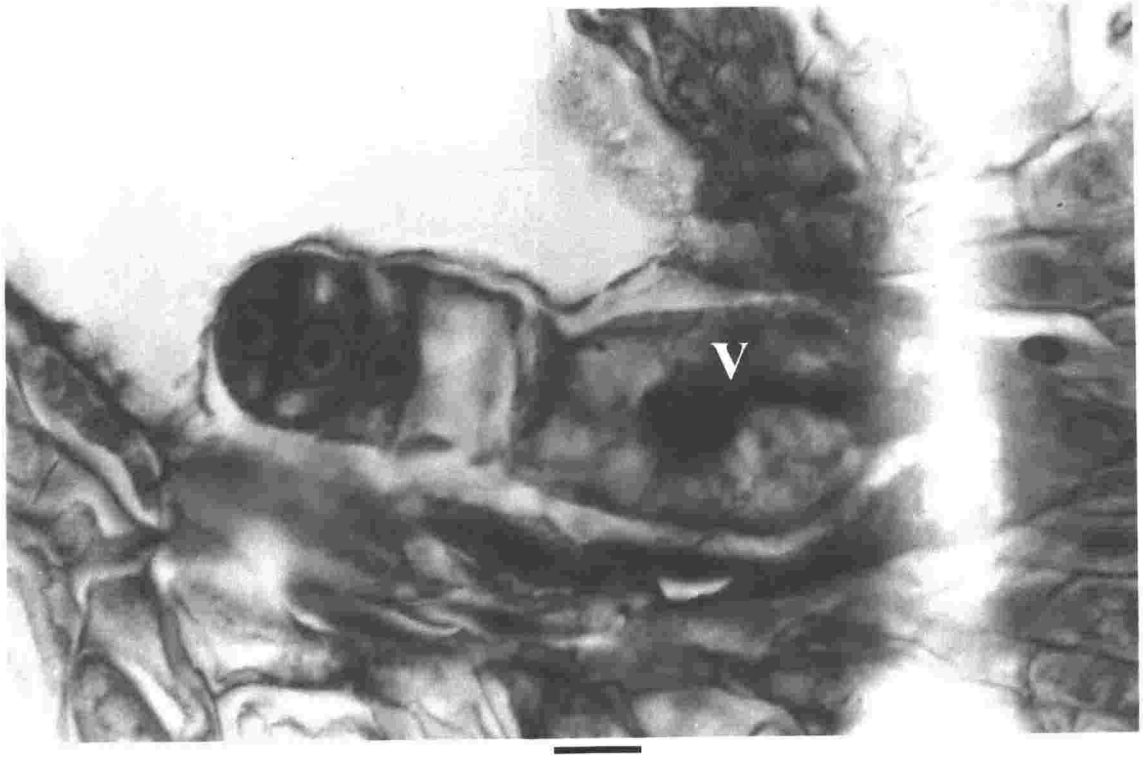

Figure 2.56

Figure 2.57. *Scleranthus perennis* embryo sac at anthesis. ov, ovum; c, central cell; pn, polar nuclei; st, starch grain; sy, synergid. Zone of elongated cells leading from micropyle is shown at arrow. Scale bar 10 μm.

Figure 2.58. *Scleranthus perennis* embryo sac at anthesis. sy, synergid with vacuole (v); c, central cell; st, starch grain; sy, synergid. Scale bar 10 μm.

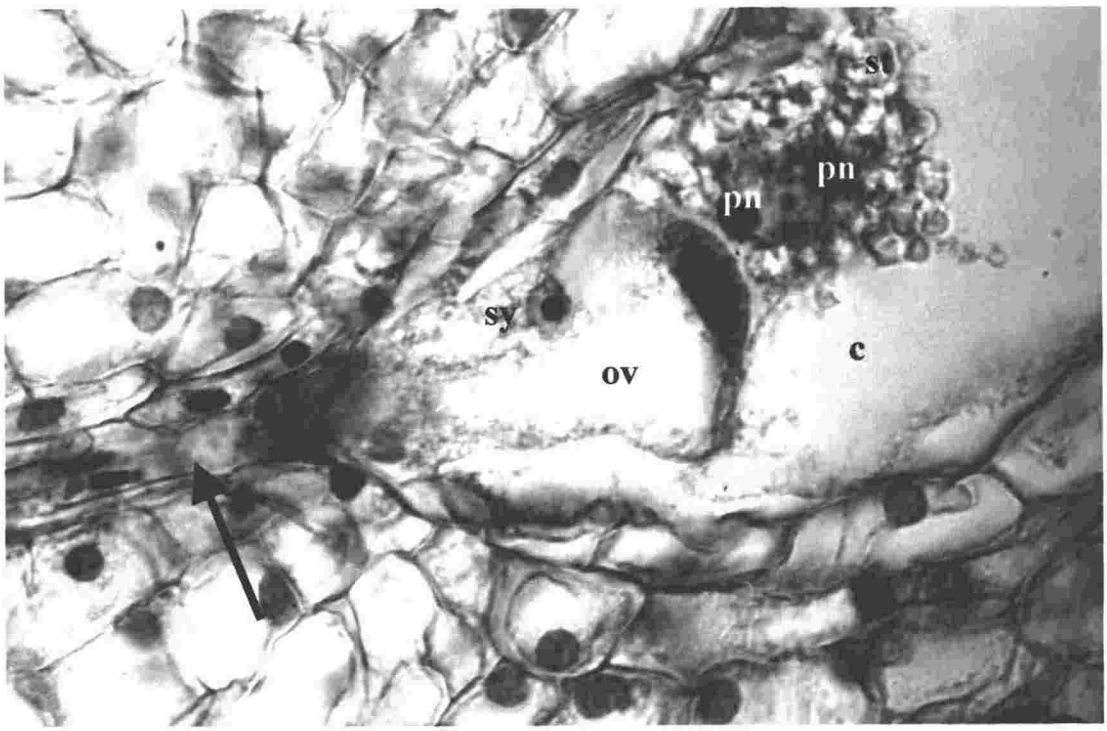

Figure 2.57

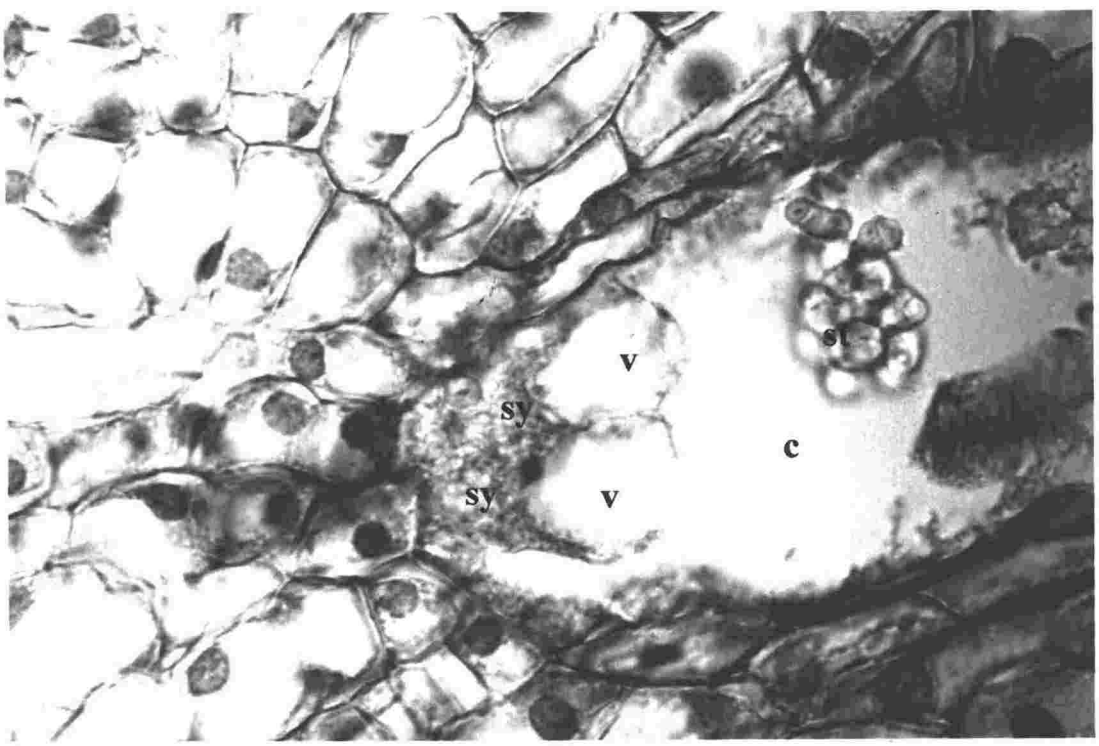

Figure 2.58

does not subsequently divide. The apical cell of this three-celled linear embryo divides and a four- (Figure 2.48c) and then five-celled linear embryo (Figure 2.48d, and Figure 2.56) are formed. The first longitudinal division in the embryo occurs at this time in the second tier. Continuing cell division results in the development of the spherical embryo illustrated (Figure 2.48e). As the embryo sac develops a differentiated group of small cells with darkly-staining walls in the area where the funicle meets the nucellus is apparent in sections(Figure 2.21: see discussion). Close examination of these cells shows them to be continuous with the inner integument. This region of cells was not observed in the ovules of section *Mniarum* plants.

As in other Caryophyllaceae (Johri *et al.*, 1992) the seed coat is formed from the outer layer of the outer integument and the inner layer of the inner integument. Cells of these layers become progressively laden with dark staining compounds (Figure 2.21). The intervening integumentary layers degenerate. In no case and at no stage was any airspace observed between the integuments. Ovule development in *S. annuus* is identical to that of *S. perennis*. *S. biflorus* and *S. brockiei* also follow essentially similar paths except that the ovule at anthesis is smaller and has a less-prominent micropylar extension (compare Figure 2.20 and 2.21) due to reduced cell division in the nucellus. In these species of section *Mniarum,* the ovule is cramped by the ovary so that the micropyle is sometimes curved slightly away from the transmission tissue (Figure 2.20).

2.3.5 Pollen morphology

Pollen grains of *S. perennis, S. annuus, S. pungens, S. fasciculatus, S. minusculus, S. uniflorus, S. biflorus,* and *S. brockiei* were examined by SEM

(Figure 2.61). All species have the spherical/polyhedral pantoporate grains with spinulose and tubuliform punctate ektexine typical of many genera of Caryophyllaceae. Spinules were observed on grains of all species varying in number and size within species and sometimes within anthers. Perforations in pollen tectum also varied from less than 100 nm to about 500 nm in diameter but were typically 100 to 200 nm. A strongly concave mesoporal exine was usually observed in *S. perennis*, but grains were often spherical in other species. Variation is apparently due to differences in storage and preparation of material, and also the exact stage of pollen development. Pore size and number probably also contribute to this variation.

2.3.5.1 Australasian species

All the Australasian species examined had very similar grains and no distinguishing characters where observed among them. Detailed observations of 24 acetolysed grains of *S. biflorus* and *S. brockiei* pollen from a number of flowers revealed pollen diameters ranging from 23 μm to 34 μm (mean of 28 μm) in *S. biflorus* and 22 μm to 32 μm (mean of 27 μm) in *S. brockiei*. Pore numbers varied from grain to grain with ranges of 15 to 22 pores in *S. biflorus* and 18 to 23 in *S. brockiei.* Pores were around 3 μm in diameter with only small variation evident. The exine of both species was more or less uniformly 2.5 μm thick. SEM examination of pollen from other Australasian species suggests that these characters are typical for all. SEM and LM observations both revealed a high proportion of collapsed, shrunken or otherwise aberrant grains in section *Mniarum* and in *S. minusculus* but not in *S. pungens*. One grain from *S. brockiei* and another in *S. minusculus* were observed to have some pores that were elongated approaching colpi (Figure 2.60). Pollen of

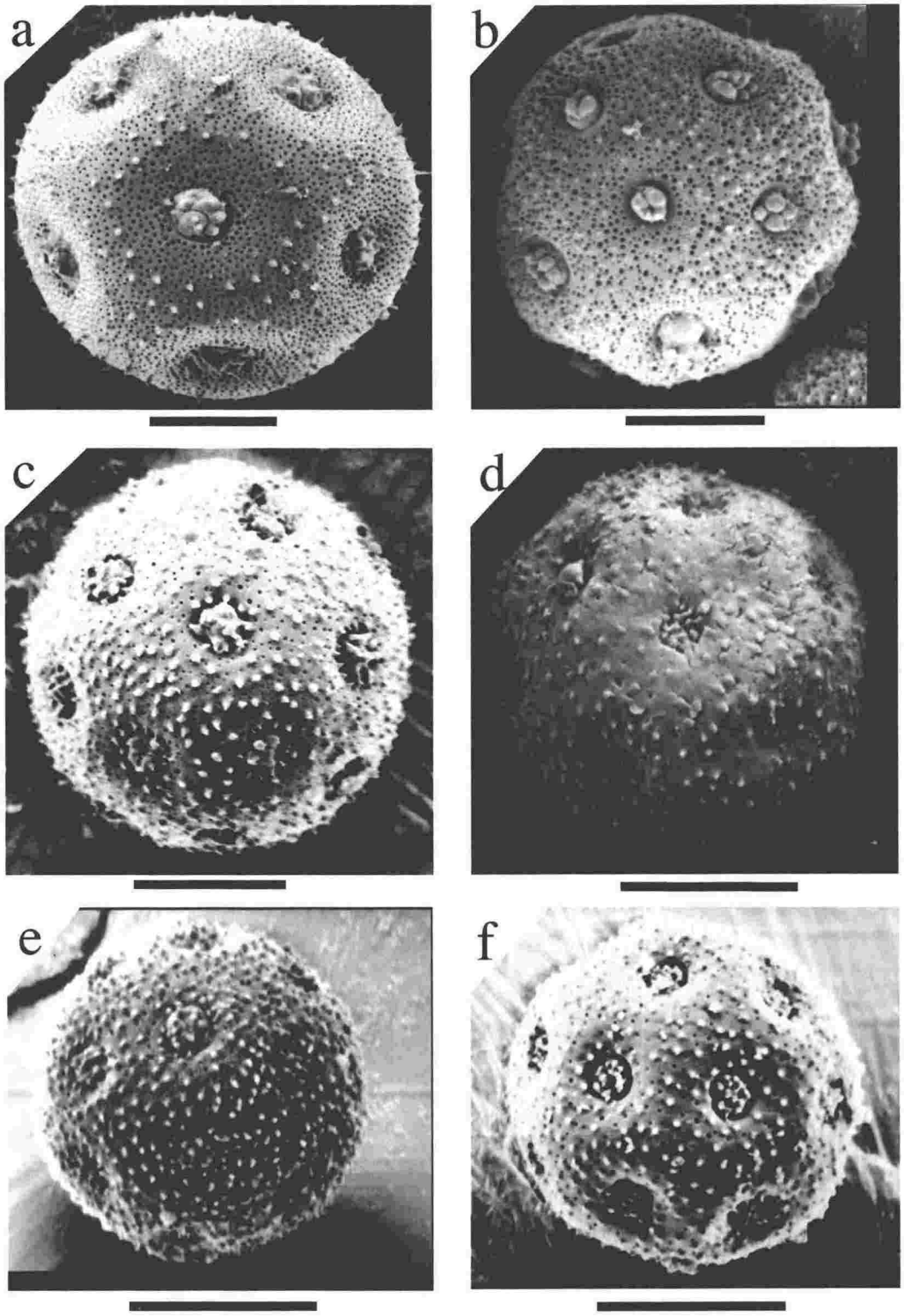

Figure 2.59 Pollen grains of **a,** *S. perennis*. **b,** *S. annuus*. **c,** *S. uniflorus*. **d,** *S. brockiei*. **e,** *S. minusculus*. **f,** *S. pungens*. Scales 10 μm

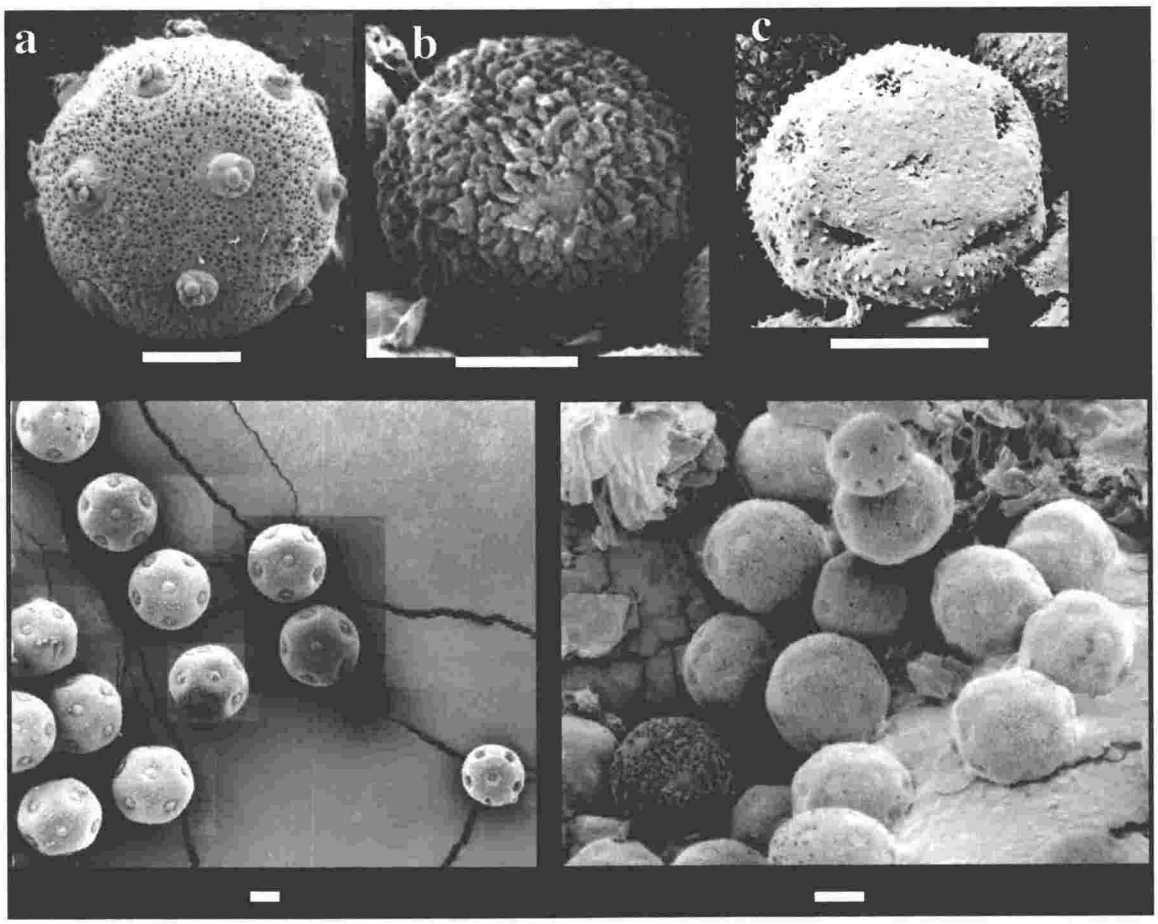

Figure. 2.60 Variation in *Scleranthus* pollen grains. **a,** *S. annuus* grain with small spinules. **b,** *S. biflorus* grain with unusual tubular exine. **c,** *S. brockiei* grain with colpate pores. **d,** group of *S. perennis* grains showing size variation. **e,** group of *S. biflorus* grains showing size variation. Scales all 10 μm

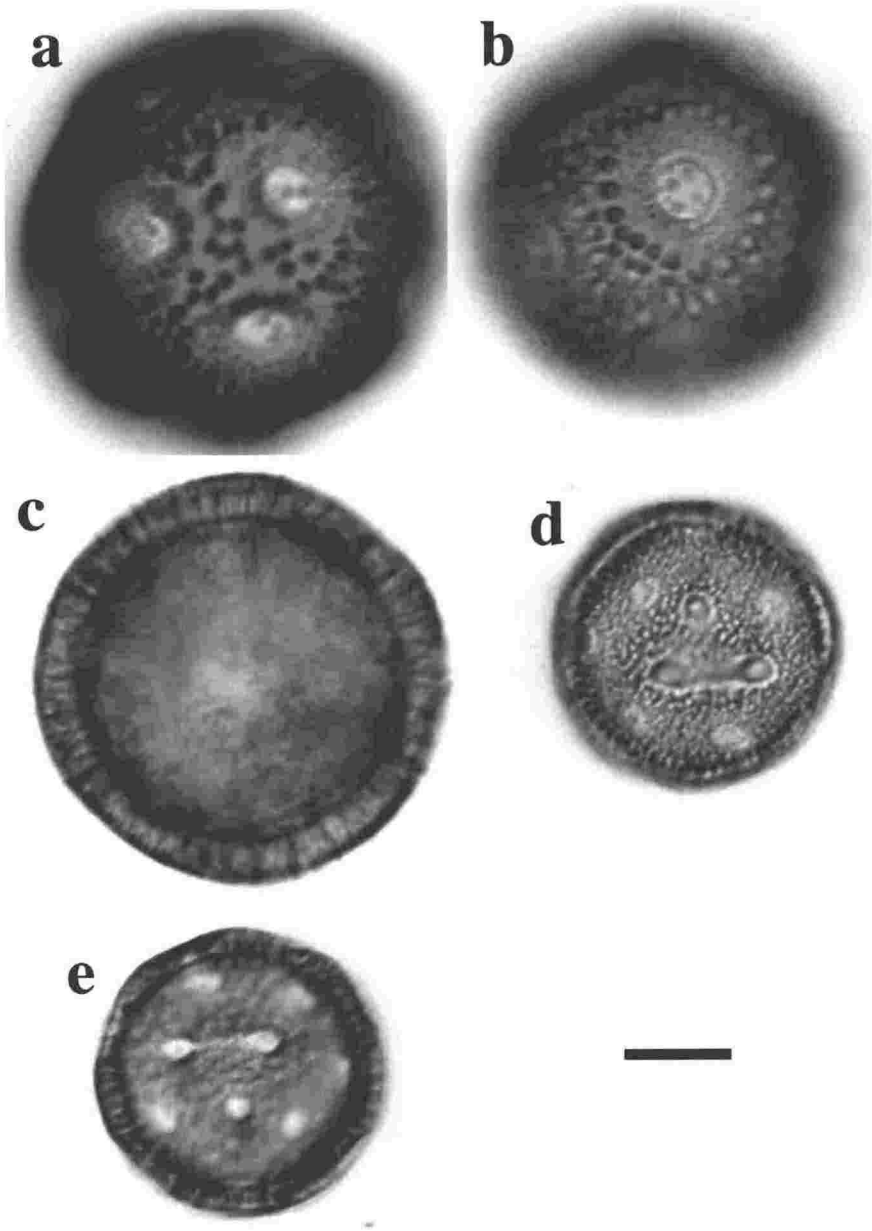

Figure 2.61. *Scleranthus* acetolysed pollen grains photographed under light microscopy. **a.** *S. annuus* surface view **b.** *S. perennis* surface view. **c.** *S. annuus* median optical section. **d.** *S. biflorus* surface view. **e.** *S. biflorus* median optical section. Scale bar 10 μm.

Australasian species examined under SEM was typically coated in acellular material from the anther, which hampered observations.

2.3.5.2 European species

Detailed observations of 10 acetolysed grains of *S. annuus* and 25 grains of *S. perennis* were made under LM. Pollen of these two European taxa examined was larger-grained than the Australasian taxa with diameter ranging from 30 to 37 μm (mean of 35 μm) for *S. perennis* and 32 to 40 μm (mean of 36 μm) for *S annuus*. Pore number is uniformly 12 in *S. perennis* but varied from 16 to 20 in the *S. annuus* grains examined. Pore diameter was also larger, averaging about 5 μm in *S. perennis* and 4.5 μm in *S. annuus*. Exine was thicker than in the Australasian species at about 3.5 μm. Apart from occasional grains of *S. perennis* which stood out as especially small, pollen of both European species was well formed and in contrast to Australasian species had little coating from the anther.

Pollen viability of single flowers of *S. perennis* and *S. brockiei* was assessed by cytoplasmic staining (Alexander, 1969). A few very small *S. perennis* grains (less than 30 μm in diameter) were observed that lacked stainable cytoplasm but otherwise all grains had stainable cytoplasm. In *S. brockiei* collapsed grains had no stained cytoplasm but spherical grains all stained.

2.3.6 Pollen ovule ratios

Species of *Scleranthus* have similar numbers of pollen grains produced in each anther. Counts for two *S. biflorus* anthers were 123 and 129 grains; two anthers from different flowers of *S. minusculus* had 156 and 183 grains. Both these sets of counts fall within the range of pollen grain number per anther in

S. perennis (33 – 203 at a mean of 92) observed by Svensson (1985). However, the much larger anthers of *S. pungens* contain considerably more pollen. Three anthers from the same flower of *S. pungens* were estimated to contain 1250, 1510 and 1430 grains each. Pollen/ ovule ratios (P/O) in *Scleranthus* are equal to the number of pollen grains produced per flower because each flower produces only one ovule. P/Os for the single-stamened species are therefore likely around 100 (at least for some specimens), approximately 200 to 300 for the two-stamened species, 1000 for ten-stamened *S. perennis* and more than 6000 for five-stamened *S. pungens.*

2.3.7 Cladistic analysis

A data matrix of 29 characters (including 5 autapomorphies) was assembled for *Scleranthus* species (see appendix 1) by combining data from this study with characters from a previous cladistic analysis of *Scleranthus* (West and Garnock-Jones, unpublished). This was analyzed without reference to outgroups using PAUP *4.0b2. Nine shortest trees were obtained through an exhaustive search. One of these trees is shown below (Figure 2.62) with character changes traced by MacClade. Shortest trees were 43 steps long and all had Consistency index 0.814, Rescaled Consistency index of 0.700 and Retention Index of 0.860.

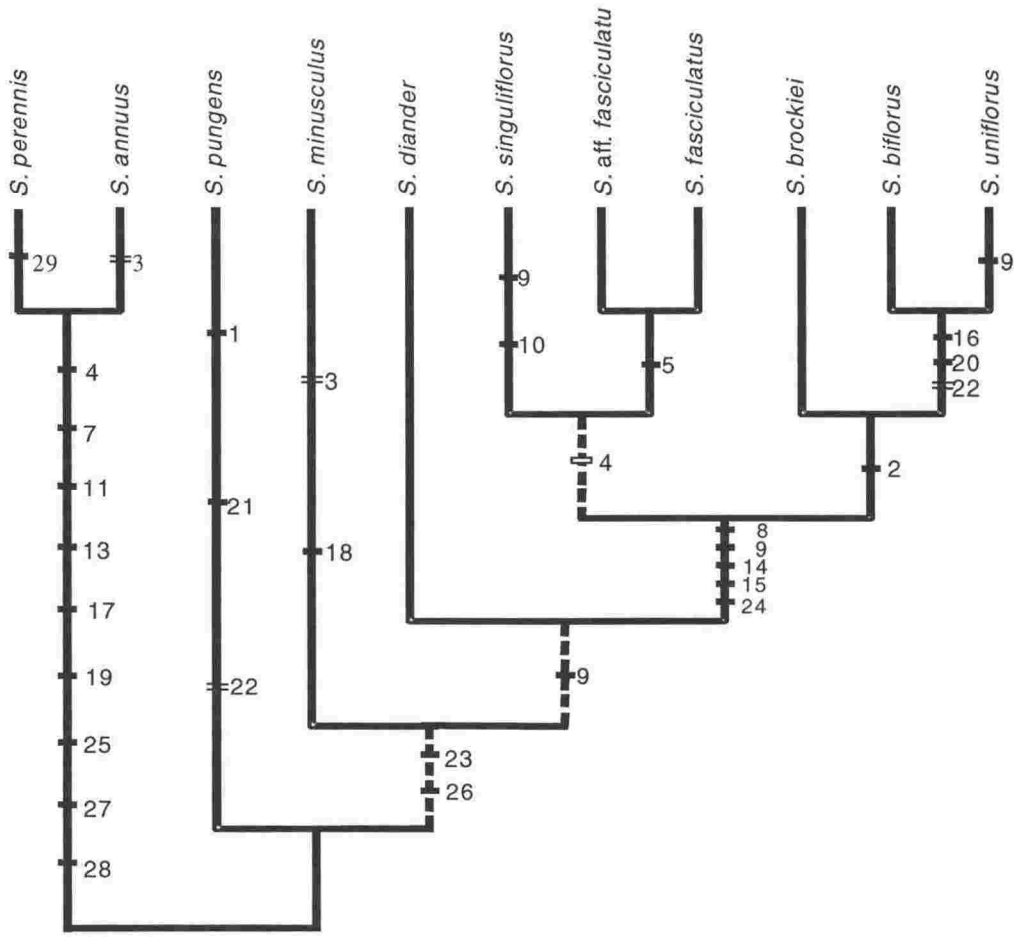

Figure 2.62. One of nine most parsimonious trees found by a PAUP* 4.0b2 exhaustive search for *Scleranthus* morphological characters with character state changes traced by MacClade. Solid bars indicate synapomorphic character state changes, open boxes indicate reversals, and parallel lines indicate homoplasious character state changes. Branches collapsing in the consensus of all shortest trees are shown with dashed lines. Note because this tree is rooted without reference to outgroup, the characters shown as synapomorphies of the European species are each equally likely to be synapomorphies of the Australasian species. If the root were attached in another position the same argument would apply.

2.4 Discussion

2.4.1 Flower development

Scleranthus conforms to the basic pattern of floral development of the Caryophyllaceae outlined by Hofmann (1994). Early development of the gynoecium is very similar in all species of *Scleranthus* examined. Two carpels are initiated separately but become fused around their bases. No traces of septa occur, except for the development of transmission tissue in the apical portion of the ovary. The transmission tissue is highly localized and not copious, only being obvious in sections, which is probably why Hofmann (1994) states that it is absent in *Scleranthus annuus*. Closure of the ovary occurs earlier than in any of the multi-ovulate Alsinoideae examined by Hofmann (1994), probably because of the reduction of the placental column.

The single ovule is not borne by either carpel, instead developing directly from the floral apex and becoming enclosed in the gynoecium after both are initiated. This is also the case in a number of other plants including *Corrigiola littoralis* (Caryophyllaceae), *Chenopodium album* (Chenopodiaceae), Primulales and Juncaceae (Sattler, 1974). The inadequacy of the concept of carpel (an appendage bearing ovules) to describe gynoecia such as these and others is discussed by Sattler (1974). The term carpel has been used here despite the fact that the organs in question are sterile because of their apparent homology with free ovulate carpels and because of their separate (sequential) initiation. However, the framework outlined by Sattler (1974) is more useful for understanding and describing the evolutionary changes leading to the morphology of the *Scleranthus* gynoecium. If the gynoecium is considered to be comprised of gynoecial appendages and placenta, then derivation of the *Scleranthus* gynoecium from multiovulate Caryophyllaceae requires

principally the reduction of placental development to the formation of a single ovule, while the gynoecial appendages have remained largely unchanged except in scale. In Sattler's (1974) terminology the ovary in *Scleranthus* is formed by zonal growth linking the gynoecial primordia, from which the styles and stigmas are derived.

Reduction of stamen number in *S. annuus* results both from failure of primordia to arise, and from failure of primordia to develop fully. Conversely in *S. brockiei, S. biflorus* and *S. fasciculatus* a single stamen primordium (rarely two) arises and develops into a fertile stamen. Stamen primordia were not observed to increase in number by subdivision of primordia nor were primordia observed to be reabsorbed. No signs of aborted stamen primordia or staminodes were observed in the two-stamened species *S. minusculus.* In *S. perennis* and *S. annuus* stamen/staminode primordia adjacent to sepals 3, 4 and 5 exceed primordia at other positions during early stages of development. These are also the most fertile stamen positions in *S. annuus* (Svensson, 1988). Also the single fertile stamen of *S. brockiei* is inserted adjacent to sepal 4.

2.4.2 Embryology

In most embryological respects *Scleranthus* is very similar to the other Caryophyllaceae that have been examined. Some variation between my observations and previous publications is likely to be the result of differences in preparation or interpretation of material. Too few studies of anther development in other Caryophyllaceae have been published for firm relationships to be drawn from the little variation that is reliably evident. The number of middle wall layers and the type of wall development

(monocotyledonous, dicotyledonous or basic type) reported vary in the family. This is the first report of the basic type of wall development in the Caryophyllaceae I am aware of. According to Jorhi *et al.* (1992) within the Caryophyllales only the Aizoaceae has the basic type of anther wall development. It seems unlikely that the basic type of anther wall development could be derived from a more reduced type given that the middle wall layers are considered to be relictual (Davis, 1966). Both the monocotyledonous and dicotyledonous types of anther development are reported for different species of *Stellaria* (Johri *et al.*, 1992) indicating that the number of divisions of parietal layers in Caryophyllaceae anthers is relatively free to vary, though why it should do so is unclear.

Although ovule and megagametophyte development is better studied in the Caryophyllaceae than is anther development, few clear variations in embryological characters have been established. *Scleranthus* displays no unusual features prior to fertilization. The development of the embryo is reported as varying in the family but in *Scleranthus* is similar, at least until the spherical embryo stage, to that in *Dianthus* as reported by Buell (1952). One apparently variable aspect is the number of cells making up the suspensor, and whether one or two develop into large vesicular cells (discussed briefly in Buell, 1952). The similarity of ovules and anthers and their development among the *Scleranthus* species examined was striking and provides considerable evidence favoring the monophyly of the genus although reducing this similarity to discrete characters for numerical analysis is problematic.

The group of thick walled cells differentiating between the funicle and nucellus in *S. perennis* is most likely part of the inner integument rather than a separate tissue. However there is some resemblence to a hypostase (*sensu*

Johri *et al.*, 1992). Hypostase tissues have been speculated to be involved in water conservation in the developing ovule and seed. However, Buell (1952) rejects such a role for a tissue in *Dianthus* which closely resembles that observed in *S. perennis*. She suggests instead that it may have a role in seed abscission. This is unlikely in *Scleranthus* because the seed does not abscise from the funicle under normal circumstances, instead germinating within the surrounding floral tissues.

2.4.3 Floral vasculature

The pattern of vascularization in *Scleranthus* is in keeping with that outlined for the Caryophyllaceae by Thomson (1942). Vascular supply to the ovary is reduced to two dorsal carpel veins. The lack of a developed placental column with vascular supply in *Scleranthus* has apparently resulted in the absence, or near absence, of a vascular stele above the departure of fused sepal/stamen traces. It was not possible to determine conclusively whether the dorsal carpel veins were linked to adjacent sepal/stamen veins or whether they are unconnected, but in some flowers at least they do continue at least into the soft tissue below the gynoecium.

The comparatively large sepals of some species are generally assumed to function in pollinator attraction. However most growth of the sepals in the two-stamened species *S. minusculus* occurs post-anthesis and they are narrow-margined throughout. This suggests that they do not act as pollinator attractants in this species and perhaps have a role in dispersal or development of seeds. Staminodes in *Scleranthus annuus* were not associated with vascular tissue and there was no evidence of vestigial traces representing absent floral parts in any of the material.

Some specimens of *S. biflorus* show further reduction in vascularization. Sepal lateral veins in this species are often poorly developed or absent. Because of the reduced size of the sepals in this species (and others of section *Mniarum*) and the lack of alternisepalous stamens, the sepal marginal veins are redundant and their loss, or partial loss, is to be expected. The lack of petals and reduction of the gynoecium preclude useful taxonomic comparison with individual species studied by Thomson (1942) as these were, on the whole, larger flowers and all had petals. However, the anatomy of *Scleranthus* flowers can be derived by a series of reductions from the examples of Alsinoideae she examined.

2.4.4 Pollen morphology

Pollen of *Scleranthus* is similar to that of many Caryophyllaceae described by other workers (e.g., Nowicke, 1975) and is of little utility in assessing relationships with other genera. Pollen of *Scleranthus* does fall into two groups, primarily defined by diameter, pore size and exine thickness. Variation within pollen from single anthers in characters such as spinule size and tectum perforation size (especially in Australasian species) makes comparison of these characters problematic. The very similar grains of the Australasian species examined support the suggestion that they are a closely related group within the genus. Pollen characters for *Scleranthus* fall within the range of observations of Argentine *Stellaria* and *Arenaria* species (Volponi, 1987). The pollen of these genera resemble closely the grains of Australasian species of *Scleranthus.* Considerable size variation was also found in the South American Caryophyllaceae pollen although most of this variation was described as pollen dimorphism with "big" and "small" grains.

Although pollen of most species of Caryophyllaceae is pantoporate, as is that of *Scleranthus,* colpate pollen is found in some members of subfamily Paronychioideae and also in some *Minuartia* species (subfamily Alsinoideae). The presence of occasional grains with colpi in *Scleranthus* suggests the transition from pantoporate to colpate grains is not a great step in the family and could have been achieved several times in independent lineages.

2.4.5 Pollen/ovule ratios

P/Os are closely associated with the degree of cross-pollination of a plant (Cruden, 1977). Cruden (1977) suggested this results from the efficiency of varying pollination mechanisms. Data from his study are summarized below in Table 2.2.

Table 2.2. Adapted from Cruden (1977).

Breeding system	Sample size	Mean P/O	Range P/O
Cleistogamy	6	4.7	2.7 – 6.7
Obligate autogamy	7	27.7	18.1 – 39.0
Facultative autogamy	20	168.5	54.1 – 396.9
Facultative xenogamy	38	796.6	245 – 2558.6
Xenogamy	25	5859.2	1062 - 19525

Because pollen/ovule ratios are influenced by a variety of factors (Preston, 1986), they cannot be used uncritically as predictors of breeding system. However, the range of P/Os observed for *Scleranthus* species is difficult to explain without invoking shifts in relative rates of outcrossing. The lowest P/Os I estimated for *Scleranthus* specimens (around 100:1 for section

Mniarum) fall within the range of P/Os of those plants described by Cruden as having facultative autogamous breeding system. This implies that they undergo at least occasional outcrossing. This may be misleading. Because the flowers are uniovulate, functional limits on the size of anthers (and therefore number of pollen grains) may mean that higher than otherwise P/O ratios are maintained in these presumably highly selfing species. This may be reflected in the number of morphologically aberrant or aborted pollen grains observed in anthers of these species (see section of pollen morphology above). However, reports of hybrids between these species (Williamson, 1956) may provide evidence that at least rare cross-pollinations do occur.

The P/O of multi-staminate species traditionally included in section *Scleranthus* range from those consistent with facultative autogamy (the two-stamened species *S. annuus, S. diander* and *S. minusculus* with P/O around 300) to facultative xenogamy (*S. perennis* with P/O around 1000) and xenogamy (*S. pungens* with P/O around 6000). Lloyd (1992) has provided a simple model of selection for selfing or outcrossing which predicts selection for either extreme rather than intermediates. Several factors, not included in the model, were advanced to explain the occurrence of many intermediate strategies adopted by plants. One of these factors is the "best of both worlds" situation provided by delayed autogamy, where self-pollination takes place after an opportunity for cross-pollination. This probably applies in the case of *S. perennis* where protandry and floral movements provide for certain autogamy after there is opportunity for cross-pollen to be delivered by pollinators (Svensson, 1985). Temporal (dichogamy) and spatial (herkogamy) separation of male and female phases in flowers may also be the result of selection against interference between pollen release and receipt (Lloyd and Webb, 1986; Webb and Lloyd, 1986). The movement of stamens away from

stigmas as the latter become receptive can also be explained as reducing interference between the functions of pollen receipt and presentation (an example of movement herkogamy: Webb and Lloyd, 1986). It is not hard to imagine that the ten stamens of *S. perennis* could interfere with the small stigmatic areas, reducing the chance of contact with pollinators bearing cross pollen, if they did not move away from the center of the small flowers. In *S. pungens,* the agent of pollination is unknown but the high P/O of this plant and its elaborated nectaries suggest it is largely outcrossing. From a phylogenetic view, the unusually large size (for the genus) of *S. pungens* anthers is seemingly incongruent with the presence of only one whorl of stamens. This information, along with the reduction of sepal vasculature of all Australasian species, suggest that *S. pungens* may have been derived from a less out-crossing ancestor with lower pollen production. If a predominantly selfing ancestor is hypothesized for all the Australasian species, then the reduction in pollen diameter (and therefore volume and reproductive cost to the parent plant) could be construed as an adaptation to increased self-fertilization. However no parallel trend is observed in *S. annuus* as compared the more out-crossing *S. perennis*. The two stamened species (*S. diander, S. minusculus* and *S. annuus*) must achieve a degree of outcrossing, at least in some situations, to allow continued selection for pollen production beyond what is required for self pollination, unless functional limitations on reduction of the androecium in these species are more rigorous than in section *Mniarum* or they are more recently derived from outcrossing ancestors. In the case of *S. annuus*, Svensson (1985) reported no sightings of pollinator activity in pure stands of *S. annuus* but did observe occasional visits (of ants) to *S. annuus* flowers growing alongside *S. perennis* plants (Svensson, 1990b). A recent origin of *S. annuus* from *S. perennis*-like ancestors has been suggested previously (Svensson, 1990a).

It is perhaps surprising that nectar should be produced by plants as apparently adapted for autogamy as those of section *Mniarum* of *Scleranthus*. The amount of nectar produced by these plants is much less than is produced by *S. perennis* and no candidates for pollinating animals have been observed associated with the plants. *S. annuus* also produces small amounts of nectar but no pollinator activity was observed during several hundred hours of observation by European investigators (Svensson, 1990b). Since loss of nectar does not facilitate autogamy its selection is likely to be governed only by metabolic economy (Garnock-Jones, 1980). The production of nectar by the *Scleranthus* plants with reduced pollen production (stamen number) is therefore likely relictual and reflects recent derivation of these forms from more outcrossing progenitors. In the absence of hard data about the rate of out-crossing in any species, the above discussion must be considered speculative and preliminary. Study of the pollination of *S. pungens* is a necessary prerequisite for understanding the evolution of Australasian *Scleranthus* as is genetic estimation of outbreeding rates in different species.

2.4.6 Relationships among species

Difficulty in selecting appropriate outgroups for character polarization/tree rooting hinders cladistic analysis of *Scleranthus*. Characters such as stamen number and arrangement of whorls, sepal size and shape, and pollen features are all variable amongst genera such as *Stellaria, Arenaria, Colobanthus* and *Sagina*, which are possible sister groups. Character polarization by reference to a hypothetical generalized Alsinoid ancestor or arbitrarily selected outgroup is unjustified because of the high degree of apparent parallelism and possible reversal of characters associated with reduced flowers in the Caryophyllaceae.

Consequently, I have presented the results of cladistic analysis in an arbitrarily rooted form. However European and Australasian species groups both possess likely apomorphies (for example, tangential perigynum fibers and loss of an alternisepalous stamen ring respectively) and can therefore be suggested as monophyletic sister taxa without resort to purely phenetic argument. The nine most parsimonious trees found by the exhaustive search differed principally over relationships between the Australian members of section *Scleranthus* and also the position of *S. singuliflorus* within section *Mniarum.* All the trees support the monophyly of section *Mniarum* (*sensu* West and Garnock-Jones, 1986) but nest it within the Australasian members of section *Scleranthus* (*sensu* West and Garnock-Jones, 1986), which are morphologically distinct from their European counterparts. Section *Scleranthus* is therefore paraphyletic as currently circumscribed and relationships would be better reflected if all the Australasian natives were included in section *Mniarum* (as in Pax and Hoffman, 1934). The two four-sepaled species in the genus, *S. biflorus* and *S. uniflorus,* also share unique carpel morphology with the styles not overgrown by cells of the ovary. Also the nectary disk of these species is tubular and envelops the base of the styles and top of the ovary. The relationship between these taxa is further discussed in chapter 3 of this thesis in terms of possible paraphyly of *S. biflorus*.

In summary this study of the floral morphology of *Scleranthus* provides no compelling information on its relations to other genera but all the aspects examined were consistent with placement in the Alsinoideae. Floral characters do support the monophyly of the genus, especially the anatomy and morphology of the gynoecium and all aspects of embryology. Pollen and some floral characters divide the genus into Australasian and European species groups, both of which possess morphological apomorphies.

Concurrently with this study, I have examined DNA sequence of the ITS1 and ITS2 regions of the 18S - 26S nuclear ribosomal DNA repeat unit from the majority of *Scleranthus* species and other Caryophyllaceae. The results of this work are reported in chapter 3 of this thesis and discussed along with the morphological characters reported here.

2.5 References

Alexander, M. P. (1969): Differential staining of aborted and non-aborted pollen. Stain Technology. 44: 117-122.

Bittrich, V. (1993): Caryophyllaceae. In K. Kubitzki, J. Rohwer, and V. Bittrich (Eds.), "The families and genera of vascular plants. Volume 2. Magnoliid, Hamamelid, and Caryophyllid families". Springer Verlag, Berlin.

Buell, K. M. (1952): Developmental morphology in *Dianthus*. I. Structure of the pistil and seed development. American Journal of Botany 39(3): 194 - 210.

Chorney, K. (1986): Caryophyllaceae. In J. P. Jessop and H. R. Toelken (Eds.) "Flora of South Australia". South Australian Government Printing Division, Adelaide.

Cruden, R. (1977): Pollen ovule ratios; a conservative indicator breeding systems in flowering plants. Evolution 31: 32 - 46.

Davis, G. L. (1966): Systematic embryology of the Angiosperms. John Wiley and Sons. New York.

Garnock-Jones, P. J. (1980): Change of adaptations from entomophily to autogamy *in Parahebe linifolia* (Scrophulariaceae). Plant Systematics and Evolution 137: 195 - 201.

Garnock-Jones, P. J. (1981): Checklist of dicotyledons naturalized in New Zealand. 8. Aizoaceae, Caryophyllaceae, and Portulacaceae. New Zealand Journal of Botany 19: 57-65.

Hoffman, U. (1994): Flower morphology. In H. -D. Behnke and T. J. Mabry (Eds.), "Caryophyllales: evolution and systematics", pp 235-246. Springer-Verlag, Berlin.

Hutchinson, J. (1974): The families of flowering plants. Oxford University Press.

Johri, B. M., Ambegokar, K. B., Srivastava, P. S. (1992): Comparative Embryology of Angiosperms. Springer-Verlag.

Lloyd, D. G. (1992): Self- and cross-fertilization in plants II. The selection of self-fertilization. International Journal of Plant Science 153(3): 370 –380.

Lloyd, D. G., and Webb, C. J. (1986): The avoidence of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. New Zealand Journal of Botany 24: 135 - 162.

Maddison, W. P, and Maddison, D. R. (1992): MacClade version 3.04. Sinauer Associates Inc. Sunderland Massachusetts.

Maheswari, D. H., and Krishna, S. V. (1990): Embryology *of Sagina procumbens* and *Drymaria cordata*. Journal of Japanese Botany 66(7): 211-219.

Moar, N. T. (1993): Pollen grains of New Zealand dicotyledonous plants. Manaaki Whenua Press. New Zealand.

Nowicke, J. W. (1975): Pollen morphology of the Centrospermae. Grana 15: 51-77.

Nowicke, J. W. (1994): Pollen morphology and exine ultrastructure. In H. -D. Behnke and T. J. Mabry (Eds.), "Caryophyllales: evolution and systematics", pp 235-246. Springer-Verlag, Berlin.

Pax, F., and Hoffman, K. (1934): Caryophyllaceae. In A. Engler and K. Prantl (Eds.), Die natürlichen pflanzenfamilien, Bd 16c. Engelmann: Leipzig.

Preston, R. E. (1986): Pollen-ovule ratios in the Cruciferae. American Journal of Botany 73(12): 1732 - 1740.

Sattler, R. (1973): Organogenesis of flowers. A photographic text-atlas. Toronto.

Sattler, R. (1974): A new approach to gynoecial morphology. Phytomorpholgy 24: 22-34.

Sell, P. D. (1964): Flora Europaea 1: 148-149. Cambridge University Press.

Svensson, L. (1985): An estimate of pollen carry over by ants in a natural population of *Scleranthus perennis* L. (Caryophyllaceae). Oecologia (Berlin) 66:273-377.

Svensson, L. (1988): Inbreeding, crossing and variation in stamen number in *Scleranthus annuus* (Caryophyllaceae), a selfing annual. Evolutionary Trends in Plants 2(1): 31-37.

Svennson, L. (1990a): Distance dependent regulation of stamen number in crosses of *Scleranthus annuus* (Caryophyllaceae) from a discontinuous population. American Journal of Botany 77(7): 889 - 896.

Svensson, L. (1990b): Floral morphology and the effects of crossing distance in *Scleranthus*. PhD dissertation. University of Lund. Sweden.

Swofford, D. L. (1999): PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Thomson, B. F. (1942): The floral morphology of the Caryophyllaceae. American Journal of Botany 29: 333-349.

Volponi, C. R. (1987): Palynological study of Argentine species of *Arenaria* L. and *Stellaria* L. (Caryophyllaceae). Candollea 42 (2): 545 - 551.

Webb, C. J., and Lloyd, D. G. (1986): The avoidence of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. New Zealand Journal of Botany 24: 163 - 178.

West, J. G. and Garnock-Jones, P. J. (1986): Evolution and Biogeography of *Scleranthus* (Caryophyllaceae) in Australasia. In Barlow, B. G. (Ed.) "Flora and Fauna of Alpine Australasia". CSIRO 1986.

Williamson, P. A. (1956): The genus *Scleranthus* in New Zealand. Rec. Domin. Mus. Wellington 3: 13-17.

Chapter 3: Phylogeny and biogeographical history of *Scleranthus* (Caryophyllaceae) inferred from ITS nucleotide sequence data

Abstract

Scleranthus is a genus of about 12 species of herbaceous plants or small shrubs native to Eurasia and Australasia. *Scleranthus* is shown here to be monophyletic and consists of European and Australasian clades which diverged within the last 10 million years. Biogeographic implications of this dating, and alternative hypotheses explaining the disjunct North-South distribution of the genus, are discussed. Within the family Caryophyllaceae, *Scleranthus* ITS2 sequences have greater similarity to sequences from representatives of the subfamilies Alsinoideae and Caryophylloideae than to sequences from representatives of the subfamily Paronychioideae. Morphological and ITS sequence data sets are significantly incongruent and trees derived from them differ over relationships among Australasian species. Hybridization and introgression are invoked to explain this discordance.

3.1 Introduction

The genus *Scleranthus* comprises 11 named species (West and Garnock-Jones, 1986) of semi-herbaceous annuals or perennials, and dwarf woody perennial shrubs. *Scleranthus* plants bear small mostly inconspicuous flowers without petals. The sepals are green, sometimes with white margins. Perigyny is well developed in all species and the sepals and floral cup are persistent, forming

part of the dispersal unit along with the fruit.

The genus is usually divided into section *Scleranthus* and section *Mniarum* (West and Garnock-Jones, 1986). Flowers of section *Scleranthus* plants are comparatively large, multi-staminate and borne in clusters. Flowers of section *Mniarum* plants are smaller, usually monandrous, and borne either singly or in pairs. The fruits of section *Mniarum* plants are presented on elongated peduncles. Ants are implicated in pollination of *S. perennis* (Svensson, 1985) which is a comparatively large-flowered species of *Scleranthus* with high pollen production. Ants or other pollinators may also be important in the breeding systems of other multi-staminate *Scleranthus* species. Species of the genus with reduced stamen number and flower size, especially members of section *Mniarum*, are generally assumed to be highly selfing.

Section *Scleranthus* contains 3 species endemic to Europe, North Africa and western Asia (*S. perennis, S. uncinatus, and S. annuus*: Sell, 1964) and 3 species endemic to Australia (*S. diander, S. pungens* and *S. minusculus*: West and Garnock-Jones, 1986). Eurasian native *S. annuus* is naturalized in many parts of the world including New Zealand (Garnock-Jones, 1981). Section *Mniarum* is restricted to Australasia and New Guinea with *S. biflorus* and *S. brockiei* occurring in both New Zealand and Australia, *S. singuliflorus* found in Australia and New Guinea, *S. fasciculatus* native in Australia (and naturalized in New Zealand), and *S. uniflorus* is endemic to New Zealand. An unnamed Australian species similar in morphology to *S. fasciculatus* (referred to as *S. 'slender'* in West and Garnock-Jones, 1986) is referred to here as *S.* aff. *fasciculatus*.

Recent consideration of morphological character distribution among *Scleranthus* species suggested that section *Mniarum* is monophyletic with a

number of synapomorphies, but suggested that section *Scleranthus* is probably paraphyletic or even polyphyletic as currently circumscribed to include both European and Australasian species (West and Garnock Jones, 1986). Pax and Hoffman (1934) had previously included all of the Australasian species of *Scleranthus* in section *Mniarum.* Evaluation of alternative phylogenetic hypotheses in the group is hampered by the small number of macromorphological characters available due to the highly reduced habit and flowers of the plants.

Chloroplast *ndh*F sequence analysis indicates that *Scleranthus* is part of a clade comprising subfamilies Alsinoideae and Caryophylloideae of the family Caryophyllaceae (see chapter 4). While *Scleranthus* is sometimes placed in subfamily Paronychioideae (=family Illecebraceae: e.g., Hutchinson 1974), most authors include the genus in the subfamily Alsinoideae, although it should be noted that this subfamily is possibly paraphyletic with respect to Caryophylloideae. Bittrich (1993) includes *Pentastemonodiscus monochlamydeus* along with *Scleranthus* in tribe Scleranthea of the subfamily Alsinoideae.

West and Garnock-Jones (West and Garnock-Jones, 1986) formulated four possible historical explanations for the Europe-Australasia disjunction in *Scleranthus* as hypotheses.

1. *Scleranthus* is monophyletic and had widespread progenitors throughout Europe-Asia-Australia which have subsequently disappeared from most of Asia resulting in the present day disjunct distribution.

2. *Scleranthus* originated from progenitors in Europe and later spread through

or across Asia into Australasia.

3. *Scleranthus* originated in Australasia and then spread through or across Asia into Europe.

4. The Northern-and Southern Hemisphere species of *Scleranthus* had different progenitors whose descendants evolved along parallel or convergent lines.

Hypotheses 1, 2 and 3 require the disappearance of progenitors from Asia or extreme long distance dispersal while hypothesis 4 implies polyphyly of the genus *Scleranthus*. Of critical importance in assessing hypotheses 1, 2 and 3 will be determining whether the Australian species of section *Scleranthus* are more closely related to the European species or to the species of section *Mniarum* (West and Garnock-Jones, 1986), in which case section *Scleranthus* as currently circumscribed would be paraphyletic. The possibility of multiple progenitors of the genus *Scleranthus* (hypothesis 4) can be assessed by investigating the relationships between the genus *Scleranthus* and possible sister taxa within the Caryophyllaceae. Specifically this research aims to test these four hypotheses and investigate the wider relationships of the genus by answering the following questions:

1. Is the genus *Scleranthus* monophyletic?
2. Are sections *Mniarum* and *Scleranthus* monophyletic as defined in West and Garnock-Jones (1986)?
3. What are the closest relatives of *Scleranthus*?

To answer these questions the Internal Transcribed Spacer regions (ITS1 and

ITS2) of the 18S - 26S nuclear ribosomal DNA (nrDNA) repeat were sequenced in a number of *Scleranthus* species and potential outgroups. Sequence from this region of DNA has been used successfully in phylogenetic studies in a number of angiosperm families including the Caryophyllaceae (Baldwin *et al.,* 1995). The nrDNA repeat unit is present in many thousand copies in nuclear genomes of plants (Hamby and Zimmer, 1992) thereby facilitating its amplification by Polymerase Chain Reaction (PCR) from preserved or ancient material. The DNA sequence uniformity of the many thousands of copies is maintained by a concerted evolutionary process in which unequal crossing over and gene conversion have been implicated (Baldwin *et. al.*, 1995). In at least some cases concerted evolution is incomplete and multiple sequence types co-exist in the genome (MacAvoy and Garnock-Jones, personal communication; Zhanga and Sang 1999)

Nuclear ITS sequences can also be used at higher taxonomic levels although alignment becomes difficult due to the accumulation of indels among sequences from divergent taxa. However, phylogenetic information can still be obtained from such highly divergent sequences because of the presence of conserved regions of sequence within ITS2 which are shared across taxonomic boundaries (Hershkovitz and Zimmer, 1996). Relationships among genera of Caryophyllaceae are poorly understood (Bittrich, 1993) with subfamilial placement of several genera disputable. I have compared ITS2 sequences from a wide sample of Caryophyllaceae in order to assess the phylogenetic relationships of *Scleranthus* and assess possible sister group relationships. Sequences from several other problematic genera (*Spergularia*, *Drymaria* and *Pycnophyllum*) were also included.

I have also been engaged in a survey of morphological characters of

Scleranthus aimed at addressing the same questions about its evolution. Cladistic analysis of these characters is reported elsewhere (Chapter 2), and they are analyzed in conjunction with ITS characters here.

3.2 Materials and methods

Species from which sequence was gathered in this study are shown below in table 1.

Table 1. Samples from which sequence was gathered in this study.

Species	Origin	Voucher #
Scleranthus annuus	Sweden	WELTU, 19658
Scleranthus perennis	Sweden	WELTU, 19659
Scleranthus biflorus (NZ)	New Zealand	WELTU, 19660
Scleranthus biflorus (AUST)	Australia	*AML, 2033*, CANB
Scleranthus brockiei (NZ)	New Zealand	WELTU, 19661
Scleranthus brockiei (AUST)	Australia	*AML, 1974*, CANB
Scleranthus uniflorus	New Zealand	WELTU 19662
Scleranthus fasciculatus	Australia	*AML, 2032*, CANB
S. aff. *fasciculatus*	Australia	WELTU 19663
Scleranthus singuliflorus (PNG)	Papua New Guinea	CHR 341457
Scleranthus singuliflorus (AUST)	Australia	CHR 344858
Scleranthus diander	Australia	CHR 344856
Scleranthus pungens	Australia	CHR 302074,
Scleranthus minusculus	Australia	*West, 5009*, CANB
Arenaria benthamii	Texas	# pending
Habrosia spinuliflora	Kurdistan	*Bornmuller*, 11506, B
Pycnophyllum bryoides	Chile	*Werdmann*, 1026, B
Pentastemonodiscus monochlamydeus	Afghanistan	*Rechinger 117834*, B
Cerastium glomeratum	New Zealand	WELTU, 19664
Sagina procumbens	New Zealand	WELTU, 19665
Drymaria laxiflora	Texas	*Clement*, 0223, TEX
Polycarpon tetraphyllum	New Zealand	WELTU, 19666
Colobanthus brevisepalus.	New Zealand	WELTU, 19547

DNA was extracted from up to 200 µg fresh or 20 µg dried plant tissue using the CTAB method (Doyle and Doyle, 1987) and purified by phenol/chloroform extraction according to Sambrook *et al.* (1989). Samples were ground in preheated mortar and pestles with 1 mL extraction buffer at 60

°C. Mortar and pestles were previously washed with detergent, hypochlorate bleach and then distilled water. After grinding, samples in buffer were transferred to 1.5 µL plastic micro-centrifuge tubes and incubated in a water bath at 60° C for 30 minutes. Plastic micro-centrifuge tubes were then centrifuged briefly to settle particulate matter and supernatants transferred to fresh tubes. Next, 375 µL buffered phenol and 375 µL of 24:1 chloroform/isoamyl alcohol were added to sample tubes and the contents mixed by inversion. Tubes were centrifuged for 15 s at high speed to separate phases and the aqueous phases removed by pipetting to new plastic micro-centrifuge tubes. The aqueous phases were then re-extracted with an approximately equal volume of chloroform/isoamyl alcohol and removed to new plastic micro-centrifuge tubes. DNA was precipitated by adding approximately 1mL of cold isopropanol to samples and storing at -20 °C for at least 1 hour and up to overnight.

After precipitation, DNA was pelleted by centrifugation for 4 minutes at 10 000 *g* in a Sorval MC12V centrifuge. Supernatant was removed and the pellets washed in 70% alcohol and air-dried. The DNA was then resuspended in 200 µL of sterile TE or distilled water and 100 µL of phenol and 100 µl chloroform isoamyl alcohol added and mixed by inversion. Phases were separated and the aqueous phases recovered to fresh plastic micro-centrifuge tubes. This step was then repeated up to 3 times. Samples were then extracted with 200 µL of 24:1 chloroform isoamyl alcohol mixture and precipitated with 400 µL isopropanol for 1 hour to overnight at -20 °C. After precipitation, DNA samples were pelleted as above and washed twice with 70% ethanol. DNA was air-dried and resuspended in 50 µL sterile TE or distilled water.

Part of the nrDNA repeat unit containing ITS1, ITS2 and the intervening 5.8s RNA gene was amplified by PCR using the primers detailed in table 2 and Figure 3.1. For DNA extracted from fresh material the primers ITS4 or ITS28cc and ITS5 were used. ITS4 and ITS5 produced DNA that gave unreadable sequence for some specimens but use of ITS28cc and ITS5 rectified this problem. Degraded DNA from herbarium specimens was amplified using primers ITS3 and ITS28cc or ITS2 and ITS5.

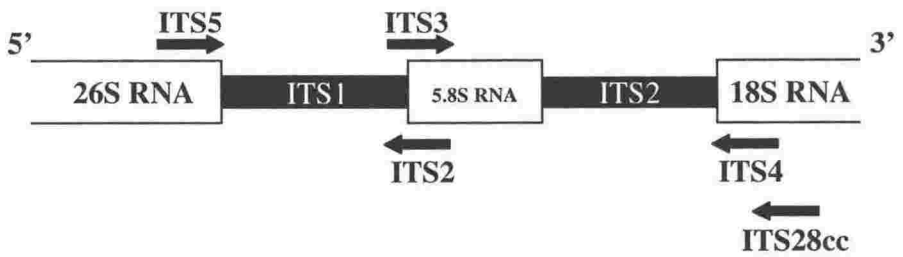

Figure 3.1. Position of primers used for PCR and sequencing

Table 2. Sequences of primers used in this study. [1]Primers based on White *et al.* (1990). [2]Primer from S. Wagstaff (personal communication)

Primer	Sequence (5' to 3')
ITS2[1]	gCTgCgTTCTTCATgCATgC
ITS3[1]	gCATCgATgAAgAACgCAgC
ITS4[1]	TCCTCCgCTTATTgATATgC
ITS5[1]	ggAAgTAAAAgTCgTAACAAgg
ITS28cc[2]	CgCCgTTACTAggggAATCCTTgTAAg

Single stranded DNA for dideoxy sequencing was obtained by one of two methods.

1. Asymmetric PCR reactions were carried out directly on genomic DNA samples using pairs of primers (each supporting amplification in different directions as in Figure 3.1) in 1:10 molar ratio. PCR reactions contained 1 unit of Taq DNA Polymerase (Pharmacia Biotech), 2.5 μL 10 × reaction buffer (500mM KCl, 15 mM $MgCl_2$, 100 μM Tris-HCl pH 9.0), 10nM each primer, 42.5 μM $MgCl_2$, 25ng BSA and H_2O to make up a total volume of 25 μL. Cycling conditions were 30 seconds at 97 °C for denaturation, 45 seconds at 48 °C primer annealing, 45 seconds plus 4 seconds each subsequent cycle at 72 °C for extension. The mixed double and single stranded PCR products products were electrophoresed on 1% agarose using TBE buffer (Sambrook *et al.* 1989) to check size, quality, and quantity of amplification products.

2. Alternatively PCR reactions were conducted with one 5' biotinylated primer and one non-biotinylated version of the other primer (Hultman *et al.* 1989). Cycling conditions for PCR were as detailed above. Double stranded PCR products were electrophoresed on 1% agarose using TBE buffer (Sambrook *et al.* 1989) to check size, quality, and quantity of amplification products. Single stranded DNA was then recovered by the use of Dynal m-280 Streptavidin Dynabeads™ according to the manufacturer's instructions.

Sequencing of amplified single stranded DNA was conducted using ^{35}S labeled dATP in conjunction with US biochemicals Sequenase™ version 2.0 DNA sequencing kit (P/N 70770) according to the manufacturers instructions. Reaction products were separated by electrophoresis on denaturing polyacrylamide gels (Sambrook *et al.* 1989). Following this, gels were fixed in a mixture of 15% ethanol and 15% acetic acid in distilled water, transferred to Whatman 3MM chromatography paper, dried in a BioRad™ model 583 gel

dryer, and exposed to Kodak Biomax film for up to 3 days. Auto-radiograms were developed in Kodak Xray Developer Number 2.

Sequence was read with a sonic digitizer and checked manually using DNAStar Lazergene computer package (DNAStar Inc.). Alignment of sequences was accomplished manually in the case of *Scleranthus* sequences using the program XESEE (Cabot, 1997). ITS2 sequences of Caryophyllaceae were compared using the guide tree function of ClustalW (Thomson, 1994; Hershkovitz and Zimmer, 1996) running on an IBM-PC-compatible computer. This program constructs a dendrogram based on pairwise sequence comparisons thus circumventing the need to construct a single alignment. ClustalW parameters for the guide-tree shown were, gap opening penalty 15.00, gap extension penalty 6.66.

Phylogenetic trees were generated from *Scleranthus* and *Sagina* (as outgroup) ITS sequences (ITS1 + ITS2) alone and from *Scleranthus* ITS sequences combined with morphological characters without outgroup using PAUP* 4.0b2 (Swofford, 1999) on an Aegis Umax computer. Maximum parsimony trees were found through exhaustive searches without character weighting. Bootstrap values were determined from 1000 replicate heuristic searches. Next, Neighbor-Joining trees were constructed from *Scleranthus* and *Sagina* ITS data using a variety of distance correction methods (uncorrected distances, JC69, or K-2-p). Character evolution was traced using the MacClade (Maddison and Maddison, 1992) software package on an Aegis Umax computer. Morphological characters used are from chapter 2 of this thesis (Chapter 2, appendix 1). Uncorrected and JC69 corrected distances used to estimate divergence times for taxa were generated from a ClustalW alignment of ITS2 sequences of *Scleranthus*, *Drymaria* and *Polycarpon*

(parameters as above) using PAUP* 4.0b2 excluding sites with gaps or missing data in pairwise calculations.

3.3 Results

High quality sequences were readily obtained from most samples using both DNA strands. Rare ambiguous sites were coded as unknown. Only a small amount of readable sequence could be generated from the limited herbarium specimen of *Pentastemonodiscus monochlamydeus* that was available. A contiguous ITS2 region of 82 nucleotides long which included 8 ambiguous sites was obtained from *P. monochlamydeus.* This short segment aligned with up to 75% nucleotide similarity with other Caryophyllaceae sequences. The presence of multiple sequence signals prevented reading any more of the sequence from auto-radiograms obtained and insufficient sample was available to attempt further sequencing.

Complete or near complete ITS 2 sequences were obtained for 7 genera of Alsinoideae and 3 genera of Paronychioideae and these were analyzed along with published sequence data from other Caryophyllaceae as shown in Table 3.2.

Table 3.2. Sequences included in Guide tree analysis. [a] sequences generated in this study. [b] sequences from Oxelman and Liden (1995). A – indicates sequences not yet submitted to genbank.

Taxon	Genbank accession #
Arenaria benthamii [a]	-
Cerastium glomeratum [a]	-
Colobanthus brevisepalus [a]	-
Habrosia spinulifolia [a]	-
Pycnophyllum bryoides [a]	-
Sagina procumbens [a]	-
Scleranthus pungens [a]	-
Scleranthus perennis [a]	-
Stellaria media [b]	X86899
Drypis spinosa	X86900
Agrostemma githago [b]	X86895
Dianthus seguieri	U30973
Psammosilene tunicoides [b]	X86897
Silene rotundifolia [b]	X86887
Saponaria calibrica [b]	X86898
Vaccaria hispanica [b]	X86896
Drymaria laxiflora [a]	-
Polycarpon tetraphyllum [a]	-
Spergularia marina [a]	-

3.3.1 ITS Evidence of Relationships within Caryophyllaceae

It was not possible to confidently align sequences for ITS 2 across most generic boundaries, even with the aid of ClustalW. Variation in length of ITS2

is the result of insertion/deletion events in which short strings of nucleotides are lost or gained in a lineage (Baldwin *et al.* 1995). Other types of structural rearrangement within the nrDNA repeat, and the high proportion of substituted nucleotide sites in variable regions, may have contributed to difficulty in aligning highly divergent sequences. Where alignment of sequences cannot be achieved with confidence trees derived may be incorrect because most tree building methods assume homology of aligned sites. Therefore, instead of reconstructing phylogeny from a single optimized alignment of multiple sequences, where homology of sites is assumed at each position, I have employed the approach developed by Hershkovitz and Zimmer (1996) of producing a dendrogram based on pairwise comparisons of sequences using the guide-tree function of ClustalW. This method groups sequences on the basis of shared strings of nucleotides. Pairs of sequences are assessed for phenetic similarity only. Thus similarities revealed are not assessed for homology, and are not necessarily synapomorphic. Varying either the values of alignment parameters, or the taxa included in this analysis affects the tree produced, especially ordering of the short internal branches. However, the process does provide an objective method for comparing and communicating sequence similarities. The dendrogram produced with the alignment parameters described in the methods section is shown in Figure 3.2.

The star-like pattern of Figure 3.2 shows that little hierarchy exists in ITS2 sequence variation among genera belonging to the subfamilies Alsinoideae and Caryophylloideae. Most groupings were unstable if alignment parameters were altered. However, several close similarities are evident in the dendrogram and these were resistant to changes in alignment parameters. The two *Scleranthus*

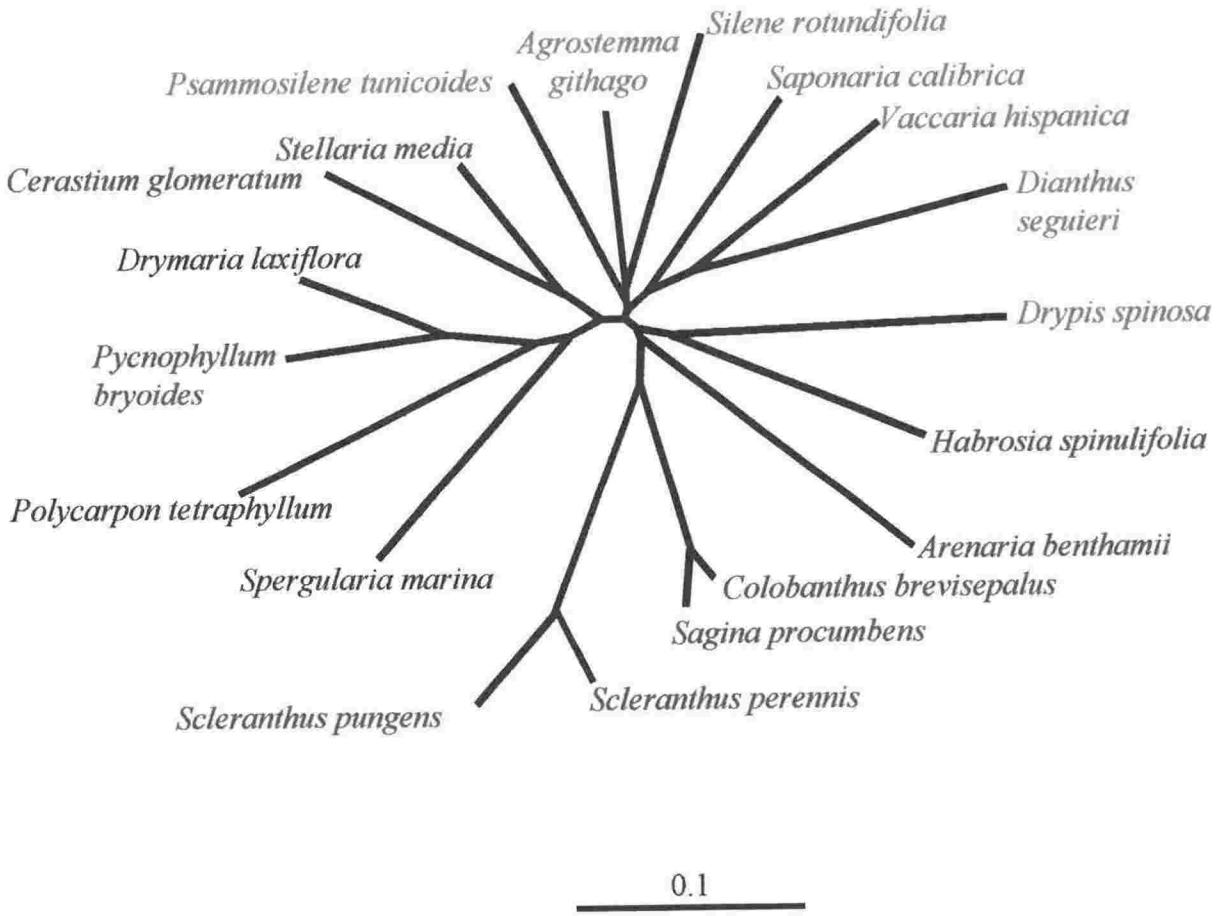

Figure 3.2. ClustalW guidetree for Caryophyllaceae ITS2 sequences. Alsinoideae shown in green, Caryophylloideae in red and Paronychioideae in black. Scale is proportion of nucleotides different in pairwise alignment.

sequences included are very similar to each other but dissimilar to all the other sequences. The *Colobanthus* and *Sagina* sequences were also very similar to each other. The ITS2 sequence of *Pycnophyllum bryoides* has higher sequence identity in pairwise alignments with Polycarpeae sequences, especially *Drymaria* (with which it groups in the dendrogram), than with Alsinoideae sequences. The *Spergularia marina* sequence has similarities with both the sequences of Alsinoideae and Caryophylloideae on one hand and with the sequences of the Polycarpeae and *Pycnophyllum* on the other, showing no strong affinity for either group in the guide tree analysis.

3.3.2 Phylogenetic relationships of species within the genus *Scleranthus* based on ITS sequences

3.3.2.1 Analysis of *Scleranthus* ITS sequences

Complete ITS1 and ITS2 sequences were obtained for 10 of the 11 named species recognized by West and Garnock-Jones (1986), the unnamed Australian *Scleranthus* species referred to as *S.* aff. *fasciculatus* and the outgroup *Sagina procumbens.* This latter species belongs to the group which had ITS2 sequences most similar to *Scleranthus* sequences in the guide-tree analysis reported above. The species *S. singuliflorus* is variable with morphologically distinguishable Papua New Guinean and Australian races (P. J. Garnock-Jones, personal communication) and both of these were included in this study. Two *Scleranthus* species, *S. brockiei* and *S. biflorus,* occur in New Zealand and Australia. Sequence gathered from New Zealand and Australian plants of both *S. brockiei* and *S. biflorus* was included in analysis.

The ITS1 and ITS2 sequences gathered from *Scleranthus* species were both readily aligned requiring no gaps. Alignment of *Scleranthus* sequences with

the outgroup *Sagina* sequence required the introduction of five gaps and in places alternative alignments were possible. The Neighbor-Joining and maximum parsimony trees derived from this alignment had identical topology and small changes to the alignment did not change the topology of either tree. Sequence from *S. diander* had five sites at which double signal was present indicating that at least two different ssDNA products were amplified from the sample. All these sites correspond to sites varying between *S. minusculus* and *S. biflorus/uniflorus.* At three of these five sites where double signal occurred, the signal was notably stronger for one base than for the other. Cross contamination is an unlikely explanation for the double signal because no samples of *S. minusculus* were in our possession at the time the *S. diander* sequence was gathered. Because of this double sequence, *S. diander* sequence has been excluded from the tree building analyses outlined below.

The single shortest tree for *Scleranthus* and *Sagina procumbens* sequences found by PAUP* 4.0b2 in an exhaustive search is shown in Figure 3.3. Note the large number of substitutions inferred along the branch separating the outgroup, *Sagina* from the *Scleranthus* samples. Bootstrap values for this tree may be low because of the extreme distance to the outgroup as is evidenced by the much higher bootstrap values achieved when the *Scleranthus* sequences are analyzed alone (below). This tree has a Consistency Index (CI) of 0.784, Retention Index (RI) of 0.486 and Rescaled Consistency Index (RC) of 0.665 ignoring variable but uninformative sites. The data were also analyzed without inclusion of the *Sagina* (outgroup) sequence and the resulting unrooted tree is shown in Figure 3.4. Removing *Sagina* sequence reduces the level of homoplasy considerably as is reflected in the higher CI of

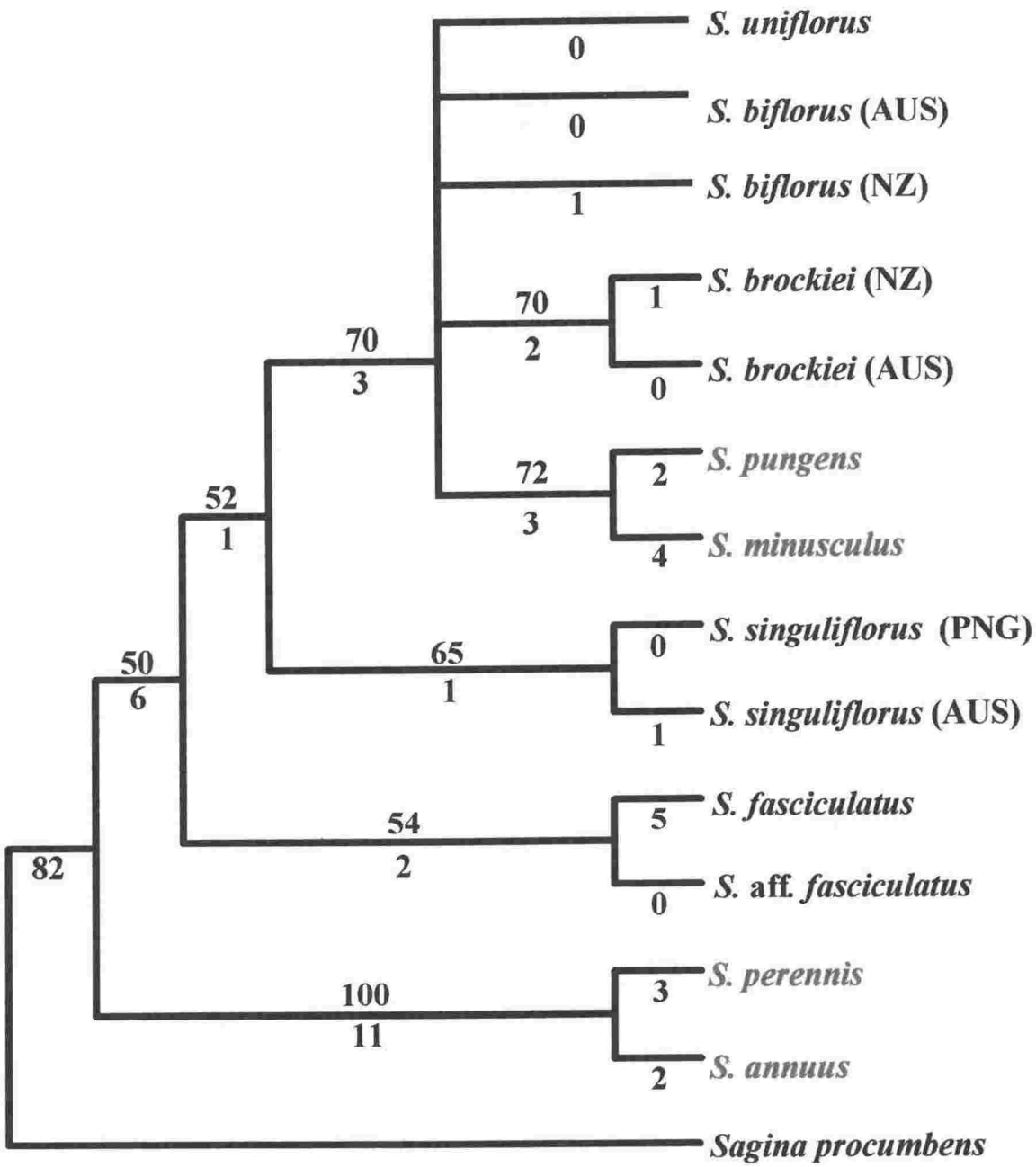

Figure 3.3. Shortest tree for *Scleranthus* sequences from PAUP exhaustive search. Numbers above branches are bootstrap percentages, numbers below branches are minimum branch lengths. Note long branch length to outgroup (*Sagina*). Taxa in red are section *Scleranthus*.

0.875, RI of 0.94, and RC of 0.797 ignoring variable but uninformative sites. This tree is also shown rooted along the long-branch separating the European and Australasian species as Figure 3.5. Note the higher bootstrap values in Figure 3.5 over Figure 3.3, presumably due to removal of *Sagina* sequence from the analysis. Shortest trees returned by PAUP* 4.0b2 for either data set (including or excluding *Sagina*) nest the Australian species which have been previously included in section *Scleranthus* within the species of section *Mniarum*. Note that there is no possible rooting of the tree shown in Figure 3.4 which would make section *Scleranthus* (*sensu* West and Garnock-Jones, 1986) strictly monophyletic.Three additional steps are required to force monphyly of section *Scleranthus* (*sensu* West and Garnock-Jones, 1986).

3.3.2.2 Combined Analysis of nrDNA and Morphological characters

The degree of congruence between morphological and ITS character sets was assessed by Partition Homogeneity Test as implemented in PAUP* 4.0b2. The two data sets (partitions) were found to be significantly incongruent (P=0.0095 from 10 000 bootstrap replicates). If ITS1 and ITS2 are treated as separate partitions then ITS1 is not significantly incongruent with either of the other data sets (P=0.2000 when it is compared with ITS2, P=0.2300 when it is compared with the morphological data). In contrast, the ITS2 and morphological data are significantly incongruent (P=0.0100). This apparent discrepancy (ITS1 not significantly incongruent with either data set, ITS2 significantly incongruent with morphology) arises because ITS1 does not vary sufficiently among Australian species of *Scleranthus* to provide a statistically significant phylogenetic signal when treated alone. Consequently I have not treated the two ITS regions separately any further.

Their significant incongruence implies that it is likely that the morphological

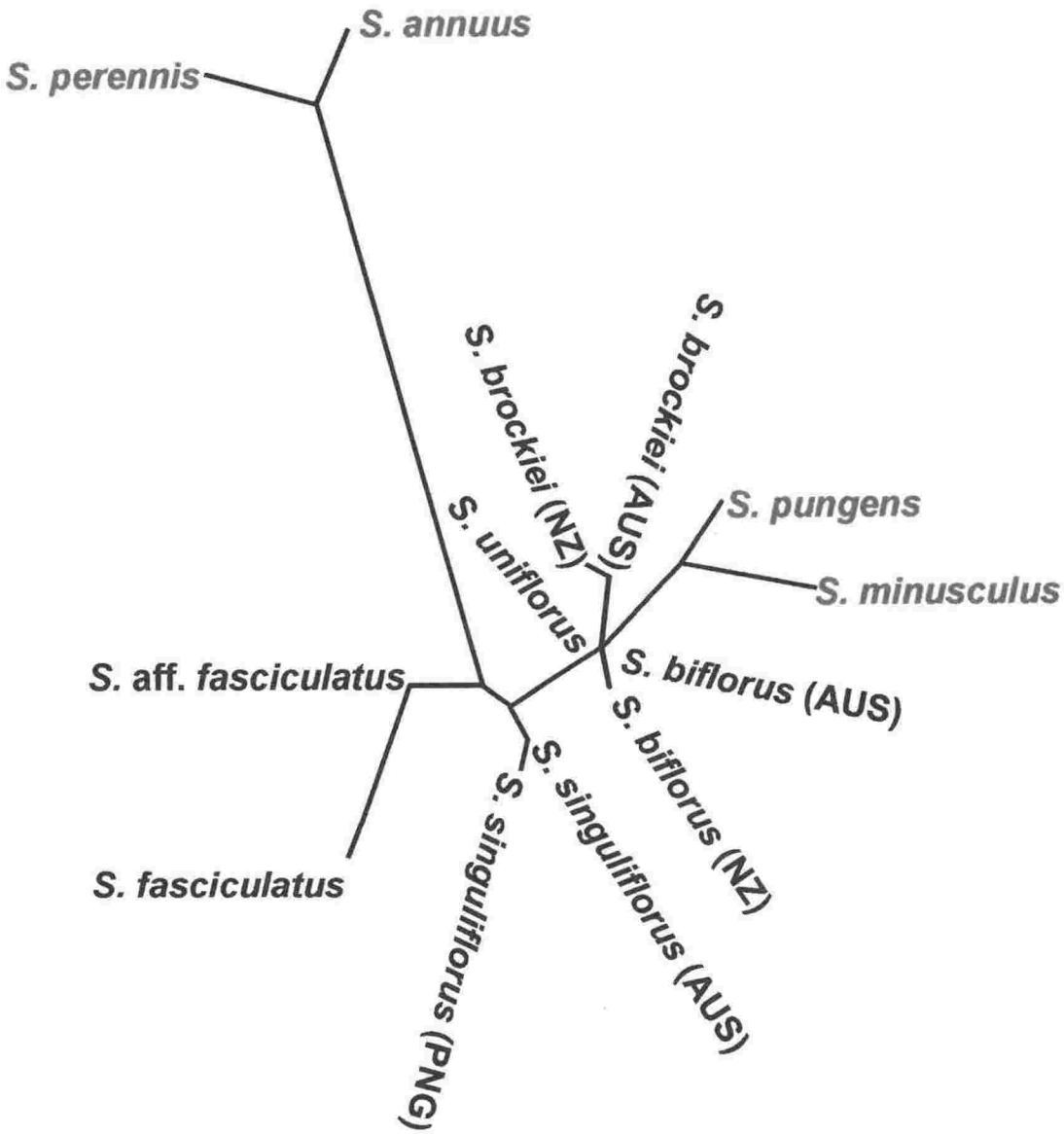

Figure 3.4. Unrooted phylogram for *Scleranthus* ITS sequences. Species in red are section *Scleranthus*, those in black are section *Mniarum*.

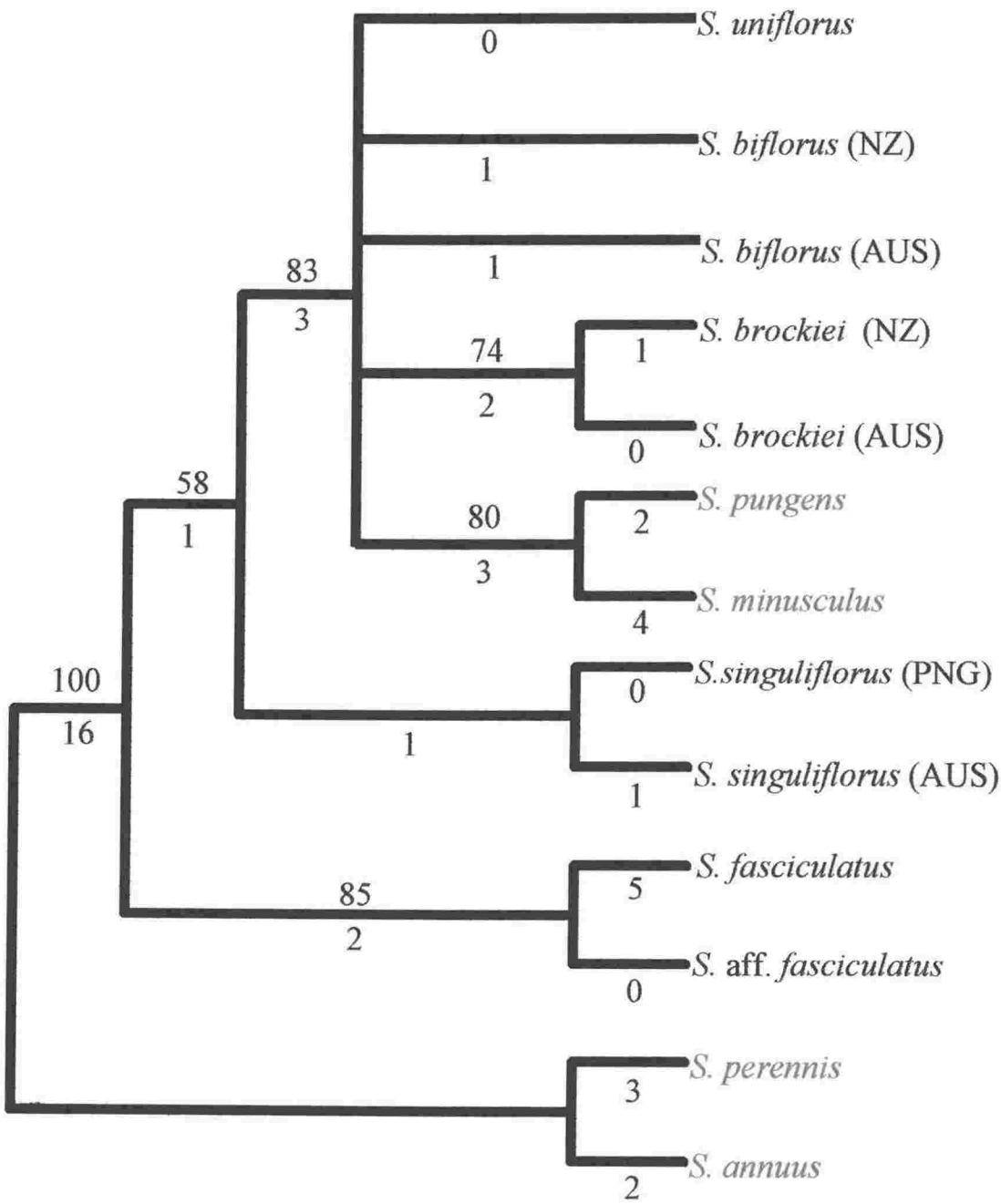

Figure 3.5. Tree in Figure 3.4 rooted to a make European and Australasian species groups monophyletic. Numbers above branches are bootstrap percentages, numbers below branches are minimum branch lengths. Section *Scleranthus* shown in red, Section *Mniarum* in black.

and molecular data sets do not share a common evolutionary history and that one or both are positively misleading. As shown by Bull *et al.* (1993) combining data from incongruent partitions can reduce the probability of returning the correct tree. However, a combined analysis has been conducted despite incongruence for several reasons: first, neither data set alone fully resolves relationships within Australasian species of *Scleranthus*; second, incongruence in the partitions reflects their support of alternative positions for the Australasian species traditionally included in section *Scleranthus* (as sister group to section *Mniarum* or nested within section *Mniarum*), the data are otherwise congruent (Partition Homogeneity test score of P=1.0000 from 10 000 bootstrap replicates when *S. pungens* and *S. minusculus* are excluded); third, the data sets (ITS1+ITS2 and morphology) are both small and include little homoplasy, so their relative support for different clades in the combined tree can readily be assessed through character mapping; fourth, the combined tree includes elements of both individual analyses and thus provides a third explicit phylogenetic hypothesis that can be evaluated against the known biology of the plants.

Combined morphological and ITS sequence data for *Scleranthus* were analyzed using PAUP* 4.0b2 exhaustive parsimony search without character weighting. A single shortest tree (shown as Figure 3.6) was found which combined elements of trees generated from morphological characters only (see Chapter 2, Appendix 1 and Appendix 2) and the ITS only tree reported above. The combined tree groups those species which were included in section *Mniarum* by West and Garnock-Jones (1986) together as sister clade to *S. minusculus* and *S. pungens* whereas ITS-only trees placed *S. pungens* and *S. minusculus* within the species of section *Mniarum.* Also the combined

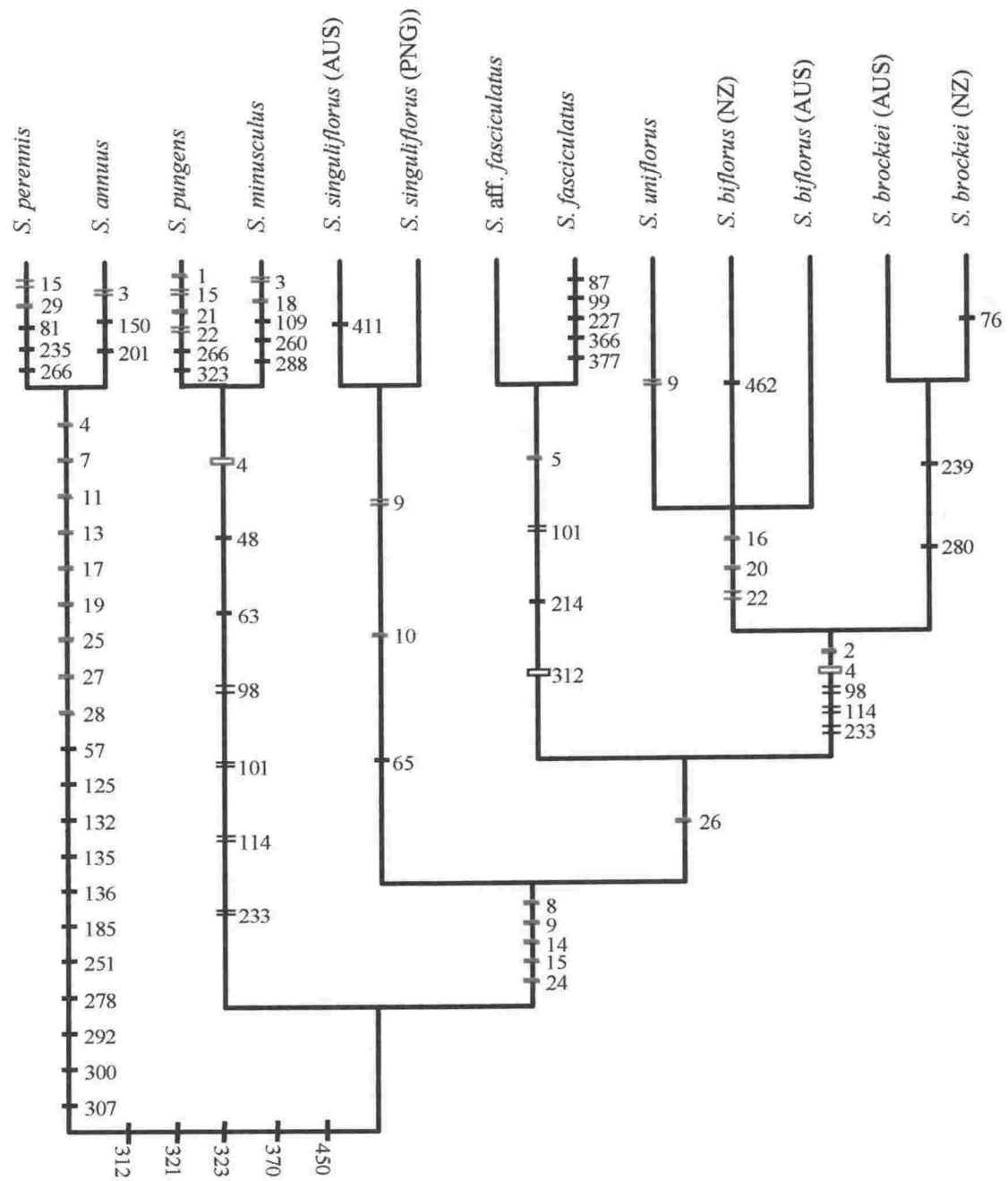

Figure 3.6. Combined ITS and morphology tree for *Scleranthus* with unambiguous character state changes traced by MacClade. Morphological characters are shown in red, ITS characters in black. Solid bars indicate apomorphic character state changes, parallel lines indicate homoplasious changes and open open boxes indicate reversals

ITS and morphology data tree has greater resolution among the species of section *Mniarum*, grouping *S. biflorus* and *S. uniflorus* together as sister clade to *S. brockiei* whereas these relationships are unresolved by ITS alone. The combined data tree has CI of 0.769, RI of 0.846, and RC of 0.688

3.3.3 Date Estimates

Estimates for the divergence date of European and Australasian *Scleranthus* clades and for the earliest divergence among extant Australasian species were made by comparing ITS sequence divergence within *Scleranthus* with the divergences between *Scleranthus* sequences and those of *Polycarpon tetraphyllum* and *Drymaria laxiflora* (Polycarpeae). According to *ndh*F sequence analysis, The Polycarpeae (including *Drymaria* and *Polycarpon*) is part of the sister group to subfamilies Alsinoideae and Caryophylloideae, (including *Scleranthus,* see Chapter 4). Although a number of lineages belonging to subfamilies Alsinoideae and Caryophylloideae are closer relatives of *Scleranthus,* they are not useful for dating purposes because no independent information is available as to when these lineages diverged from the lineage leading to *Scleranthus*. Figure 3.7 shows phylogenetic relationships between the taxa discussed above and used to estimate dates for divergence events between *Scleranthus* lineages.

The ITS2 sequences of *Drymaria laxiflora*, *Polycarpon tetraphyllum* and 10 *Scleranthus* sequences were aligned using ClustalW and distances corrected according to the JC-69 model by PAUP* 4.0b2. The JC-69 model was used because it makes few explicit assumptions about the pattern of nucleotide substitution.

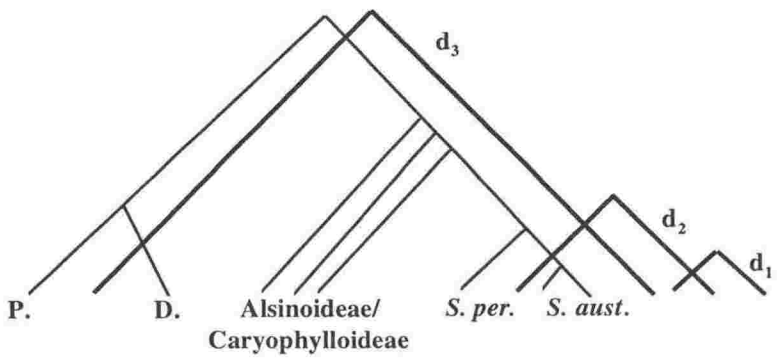

Figure 3.7. Relationship of taxa used to estimate times since divergence of *Scleranthus* lineages P. = *Polycarpon*, D. = *Drymaria*, *S. per.* = *Scleranthus perennis*, S. *aust.* = Australasian *Scleranthus* species (8 sequences included). Distances used in calculations to estimate for divergence events dates, as discussed in text, are shown as d_1, d_2, and d_3.

In the shortest (i.e., maximum parsimony) tree for *Scleranthus* ITS data (Figure 3.5) the earliest dichotomy within the Australasian group is between the *S. fasciculatus*-*S.* aff. *fasciculatus* clade and the remaining Australasian species. In Figure 3.7 (above), distances between ITS2 sequences of plants belonging to these Australasian clades are defined as d_1, the distances between sequences of European and Australasian species are d_2, and the distances between sequences of *Scleranthus* species and the two Polycarpeae species are d_3. Because of the stochastic nature of molecular evolution, and possibly because of rate variation between lineages, nucleotide substitution rates for ITS2 differ between different pairs of species across each node. For example, d_1 calculated as the distance between *S. singuliflorus* (PNG) and *S. fasciculatus* is different from d_1 calculated as the distance between *S.* aff.

fasciculatus and *S. biflorus,* despite these lineages diverging at the same node. For this reason, a range of figures is given here for each set of comparisons. Averaging these figures is inappropriate because some terminal taxa will have shared histories for some of the time since their divergence from the node in question. Consequently, different calculations of d_1, d_2, and d_3 can not provide independent estimates of the rate of nucleotide substitution or the time elapsed since a divergence event. As a result, mean values of d_1, d_2, or d_3 would differ widely depending on which terminal taxa were included in calculations.

It is known that ITS2 is subject to considerable among-sites rate variation (Hershkovitz and Zimmer, 1996). Distance estimates of highly divergent ITS2 sequences calculated as number of sites varying (n) divided by the total number of sites in the alignment (n_t) may be underestimates because of the presence of several short regions of relatively conserved sequence. The number of sites free to vary in a sequence (n_v) can be estimated by simply excluding all sites invariant over all taxa in the alignment. Distances can then be calculated as the number sites varying, divided by the number of sites free to vary (n/n_v). However, where uncorrected distances are greater than 0.75 Jukes-Cantor corrected distances cannot be calculated from them because the calculation then requires the logarithm of a negative number. For this reason PAUP* 4.0b2 returned "undefined" distance estimates for two of the pair-wise comparisons between Polycarpeae and *Scleranthus* species. The range of distance estimates of d_3 shown for "variable sites only" in table 3 includes only those for which PAUP calculated a result.

Table 3. The range of distances between pairs of sequences for taxa as shown in Figure 3.7. Figures in brackets for "variable sites only" are uncorrected distances.

	All sites in alignment (n/n_t)	Variable sites only (n/n_v)
d_1	0.028 - 0.052	0.065 – 0.125 (0.031 - 0.115)
d_2	0.052 - 0.082	0.124 - 0.205 (0.104 - 0.178)
d_3	0.375 - 0.451	1.890 - 3.64 (0.688 - 0.788)

The rate at which substitutions accumulate in a DNA molecule may be estimated as half of the distance between sequences from two lineages divided by the time elapsed since the lineages diverged (where this time is known and where rates of substitution are assumed to be the same in each lineage). Fossil pollen attributable to the Caryophyllaceae appears in the middle Oligocene (Muller, 1981). Pollen similar to modern Alsinoideae/Caryophylloideae and pollen similar to modern Paronychioideae are also found in deposits of similar age. I have used this evidence as the basis for assuming that Paronychioid and Alsinoid/Caryophylloid lineages diverged about 35 million years ago, just prior to the appearance of these pollen types in the fossil record. If this is taken as the true date for divergence of Alsinoideae and Paronychioideae, then nucleotide substitution rates derived from corrected pair-wise distances between *Scleranthus* and the Polycarpeae species vary from 5.34 to 6.44 $\times 10^{-9}$ substitutions per site per year using all sites (n/n_t), and from 27.0 to 52.0 $\times 10^{-9}$ substitutions per site per year for variable sites only (n/n_v: 9.83 to 11.3 $\times 10^{-9}$ for uncorrected distances).

If substitutions have accumulated at a more or less constant rate in all

Caryophyllaceae lineages, then the length of time elapsed since any two lineages within *Scleranthus* diverged can be estimated by dividing half the distance between the sequences of pairs of species representing each lineage by the rate of substitution. Table 4 summarizes the range of estimated dates derived from the substitution rates above, with all possible pairs of species separated by the divergence events considered (with the exclusions mentioned above).

Table 4. Estimates of length of time since events in *Scleranthus* evolution in millions of years. Figures in brackets are times calculated from uncorrected distances.

Divergence event	Estimate from all sites (n/n_t)	Estimate from variable sites only (n/n_v)
Divergence of European and Australasian *Scleranthus* lineages	4.0 – 7.7	1.2 -3.8 (4.6 - 9.1)
First divergence within extant Australasian *Scleranthus* species	2.2 – 4.9	0.6 - 2.3 (1.4 - 5.9)

3.4 Discussion

3.4.1 Relationships among Caryophyllaceae

3.4.1.1 *Pentastemonodiscus*

The short length of ITS2 sequence that was obtained for *Pentastemonodiscus* was not enough to place this genus firmly in any subfamily. I suggest that *Pentastemonodiscus* is probably not closely related to *Scleranthus* as its ITS2

sequence was not found to be recognizably more similar to *Scleranthus* ITS2 sequence than to those of other Caryophyllaceae by inspection or pairwise comparison. However, the sequence is broadly similar to the other Caryophyllaceae sequences suggesting it does belong in the family. Plants of *Pentastemonodiscus* and *Scleranthus* do not share any notable morphological apomorphies and their suggested grouping (Bittrich, 1993) appears to be based on their sharing a reduced vegetative habit and small, apetelous, uniovulate flowers with two parted gynoecia. Unlike *Scleranthus*, *Pentastemonodiscus* is described as having anatropous ovules and is not perigynous. Further, *Pentastemonodiscus* has connate styles which are otherwise found only in *Pycnophyllum* amongst Alsinoideae (Bittrich, 1993 but see below regarding placement of *Pycnophyllum*). Therefore I suggest that placement of *Pentastemonodiscus* in Paronychioideae should be considered.

3.4.1.2 Relationships between genera within the family Caryophyllaceae

ITS sequences could not be unambiguously aligned along their entire length across most genera of Caryophyllaceae because of a combination of the effects of the accumulation of indels and the high divergence of variable regions. However, similarity information can be recovered through pair-wise comparisons. This allows sequences to be grouped according to possession of shared strings of nucleotides. Within the Caryophyllaceae this approach has allowed us to identify closely similar sequences and to attribute taxa of uncertain position to major groupings previously identified. Thus Figure 3.2 should not be interpreted as a phylogenetic tree, but as a network of similarities where relationships can reasonably be inferred for taxa whose sequences are closely similar. The ITS2 sequences of Alsinoideae (excluding *Pycnophyllum*) and Caryophylloideae species sampled are more similar to each other than to *Polycarpon* or *Drymaria* of the Paronychioideae. Within the

Alsinoideae-Caryophylloideae group little hierarchical structure in ITS2 similarity is evident. This may be an artifact which is in part due to "saturation" of variable nucleotide sites, but may also reflect a rapid radiation of these lineages from a common ancestor some time in the relatively distant past. This latter suggestion is in keeping with the results of an analysis of Caryophyllaceae *ndh*F sequence variation (Chapter 4). The sequences obtained for this gene from Caryophyllaceae are more conserved than Caryophyllaceae ITS2 sequences, but were nevertheless unable to resolve relationships reliably between most genera of Caryophylloideae and Alsinoideae.

3.4.1.3 *Pycnophyllum*

In this analysis, the ITS2 sequence of *Pycnophyllum* is most similar to that of *Drymaria* of the Polycarpeae. The genus *Pycnophyllum* contains about 17 species from the Andes (Bittrich, 1993). Mattfeld (Mattfeld, 1934, cited in Bittrich, 1993 and in Skipworth, 1961) included *Pycnophyllum* along with *Lyallia* and *Hectorella* in tribe Pycnophylleae of subfamily Alsinoideae. Skipworth (1961) supported a close relationship between *Lyallia* and *Hectorella,* but rejected a relationship between either of them and *Pycnophyllum* or placement of them in Caryophyllaceae. He also rejected placement of *Lyallia* and *Hectorella* in Portulacaceae, and instead erected a new family, Hectorellaceae (Phillipson and Skipworth, 1961). While placement of *Hectorella* and *Lyallia* remains disputed (for discussion see Garnock-Jones, 1988), their relationship with *Pycnophyllum* is apparently no longer argued. This leaves *Pycnophyllum* as the sole genus of the tribe Pycnophylleae. The plants of *Pycnophyllum* differ from the remainder of the Alsinoideae in having connate styles, a condition common in Paronychioideae. The plants of *Pycnophyllum* also differ from most of the

Alsinoideae in that they lack nectary glands at the base of their stamens and their leaves are free at their bases (Bittrich, 1993). Despite the limitations of the guide tree analysis, the ITS2 sequence data provide strong evidence suggesting *Pycnophyllum* should be transferred to Polycarpeae.

Conversely, the *Spergularia* sequence obtained shares similarity with members of Alsinoideae and Paronychioideae although its exact position is not determined here. Inclusion of *Spergularia* in the Alsinoideae/Caryophylloideae group is strongly supported by chloroplast *ndh*F sequence data (chapter 4, this thesis).

3.4.1.4 *Colobanthus* and *Sagina*

Colobanthus and *Sagina* have very similar ITS2 sequences suggesting a very recent common ancestor for these plants. The close relationship between these genera has been previously noted (Crow, 1978), but they can be distinguished by aspects of floral morphology including positioning of styles (on the same radii as sepals in *Sagina,* on alternate radii with sepals in *Colobanthus*), and differing nectaries (B. V. Sneddon personal communication). The more widespread and variable *Sagina* is possibly paraphyletic with respect to *Colobanthus* (P. J. Garnock-Jones, personal communication). Relationships between the two genera need to be resolved and the appropriate taxonomic rank to assign each group assessed in this light.

3.4.1.5 *Scleranthus*

ITS sequences are consistent with monophyly of *Scleranthus*. In addition to their much greater similarity in pair-wise comparisons, reflected in the guide-tree network (Figure 3.2), and the very large number of character state changes inferred along the branch separating *Sagina* from the *Scleranthus* species in Figure 3.3, a close relationship of all the *Scleranthus* species is supported by the fact that no gaps or other rearrangements were required to align their sequences. Aspects of floral anatomy, especially gynoecial morphology and ontogeny, also support the monophyly of *Scleranthus* (Chapter 2).

3.4.2 Relationships within *Scleranthus* based on ITS sequences

3.4.2.1 European and Australasian clades

Within *Scleranthus* both ITS sequence and morphology datasets suggest that there is a basal dichotomy between European and Australasian clades (Figure 3.5). Section *Scleranthus* is shown by these results to be at least paraphyletic (as in the morphology-only and combined morphology and ITS analyses) or polyphyletic (ITS alone). The Australasian members of section *Scleranthus* should therefore be transferred to section *Mniarum*. Morphological support for the grouping of *Scleranthus* into European and Australasian clades is based on characters of floral anatomy, pollen and inflorescence (see Chapter 2).

3.4.2.2 Origin of *S. diander*

The *S. diander* ITS sequence obtained in this study included 5 sites (four in ITS2 and one in ITS1) where double banding indicated the presence of multiple sequence types. The pattern can be interpreted as an additive combination between those sequences found in *S. minusculus* and *S.*

biflorus/uniflorus, perhaps suggesting a hybrid origin for *S. diander* with members of these species or progenitors as parents. As *S. uniflorus* is endemic to New Zealand it can probably be eliminated as a likely parent of the Australian endemic *S. diander.* West and Garnock-Jones (1986) described *S. diander* as providing "*morphological ... and ecological links*" between Australasian species presently included in section *Scleranthus* and section *Mniarum.* Such links might be explained by hybridization. Evidence relating to hybridization, if not hybrid speciation, in the group should be considered when evaluating both morphological and sequence character-based phylogenetic reconstructions. Alternatively, as *S. diander* is sometimes sympatric with members of section *Mniarum,* I may, by chance, have sampled a hybrid or back-crossed individual, although nothing in the morphology of the specimen suggests it is atypical of *S. diander.* A further possibility is that the observed double sequence is indicative of the presence of multiple ITS sequence types in *S. diander.* Further sampling of both *S. diander* and other Australasian species and cloning of individual ITS sequence types from *S. diander* will be critical in evaluating these hypotheses.

3.4.3 Data combining and Incongruence

The combined morphology/ITS data analysis conducted in this study must be interpreted with caution, as the two data partitions were significantly incongruent. Although I am conscious of the strong arguments against combining incongruent data, like Messenger and McGuire (1998) I feel that full exploration of the data is facilitated by a combined analysis even in the presence of partition incongruence. Further, I note that the otherwise compelling argument of Bull *et al.* (1993) against combining incongruent data sets begins with the remark:

"*For any modest set of well-known taxa, the systematist may have access to nucleic acid sequence from many different genes plus a variety of morphological, biochemical, and physiological characters.*"

The present study is of a poorly known group where only a limited set of morphological characters and a single set of nucleotide sequence characters are available. The choice here is not which data to combine in order to give the best chance of obtaining a totally correct species tree, but rather how best to combine two partially resolved and partly complementary phylogenetic hypotheses. The data partitions (ITS and morphological data sets) are only incongruent in support for alternative relationships among Australian species

greater resolution than either morphological or molecular subsets of the data. Australian species *S. pungens* and *S. minusculus* do not share any unique apomorphic morphological character states. However, the monophyly of this pairing is well supported by three ITS nucleotide substitution characters and is preserved in the shortest tree derived from the combined data. This pairing is also consistent with the consensus of shortest parsimony trees derived from the morphological characters alone (Chapter 2). The three *Scleranthus* species native to New Zealand (*S. brockiei, S. biflorus*, and *S uniflorus*) appear as a monophyletic group in the shortest tree derived from the morphology-only and the combined ITS/morphology data set, although their relationships are unresolved in the ITS-only trees. The ITS sequences of the New Zealand species are very similar (see especially the unrooted phylogram, Figure 3.4). Although these species lack ITS sequence synapomorphies, their monophyly.is still consistent with the data Within this group, *S. biflorus* and *S. uniflorus* especially share several morphological synapomorphies implying the derivation of *S. uniflorus* from the more widespread *S. biflorus* (Chapter 2, this thesis; West and Garnock-Jones, 1986).

However, in contrast to morphological characters, the ITS data provide evidence that *S. pungens* and *S. minusculus* are nested among the monandrous members of section *Mniarum.* Paraphyly of section *Mniarum* (*sensu* West and Garnock-Jones, 1986) is unlikely in the light of the considerable morphological similarity of these species, which share a number of derived characters of flowers and fruiting structures not shared by *S. pungens* and *S. minusculus*. Paraphyly of section *Mniarum* with respect to *S. minusculus* and *S. pungens,* if accepted, would imply either the convergent evolution of several single-stamened lineages or, more likely, a reversion to larger infloresences, larger flower size and increased stamen number in the *S.*

minusculus - *S. pungens* clade. Such a series of reversals is difficult to exclude because many of the characters involved (stamen number, calyx size, sepal margins, and inflorescence flower number) are probably functionally related to pollination and are unlikely to be independent. However, not all the characters linking the single stamened species have obvious functional connection (tops of ovaries papillate, peduncles elongating in fruit) and no synapomorphies were found linking *S. pungens* or *S. minusculus* with any subset of the section *Mniarum* species in a morphological study (Chapter 2). Likewise, convergence of the monandrous species as a result of adaptation to autogamy would explain some, but not all, of the characters linking this group.

3.4.3.2 Hybridization and Introgression

Although previously unconsidered as significant evolutionary agents in this group, hybridization and introgression may have played a major role in the evolution of *Scleranthus* in Australasia. They provide an explanation of the observed discordance between morphological and ITS-based phylogenies (see earlier). The ITS sequence reported in this study suggest that *S. diander* is possibly of hybrid origin or perhaps hybridizes with members of section *Mniarum.* Hybrids between New Zealand native species (Williamson, 1956) and between European species (Sell, 1964) are believed to occur naturally. Plant species of recent hybrid origin are known to display additive patterns of parental ITS characters (Reiseberg, 1991). However, it is probable that over time processes of concerted evolution result in homogenization of ITS sequence types in stabilized hybrids (Woolfe and Elisens, 1994). The variation in signal strength of the additive pattern of ITS sequence in *S. diander* observed in this study suggests that this process may have already begun acting on independent nucleotide sites. The final outcome would be a single sequence displaying a non-additive combination of parental characters. This

same process could account for the distribution of ITS characters in Australasian *Scleranthus,* particularly the 3 nucleotide apomorphies shared between the *S. minusculus-S. pungens* clade and the *S. biflorus/brockiei/uniflorus* group. If this is the case then a chloroplast DNA phylogeny should resemble more closely the morphology derived trees, especially as the single-stamened species are much more likely to have provided the female parent than the male parent of any hybrids (see Chapter 2, this thesis, for a discussion of pollen ovule ratios and sex allocation). If ITS nucleotide sequence data reflect the true pattern of relationships among *Scleranthus* species, and reticulate evolution has not been a major factor, then a chloroplast DNA nucleotide sequence phylogeny should resemble the ITS phylogeny. Full elucidation of the evolutionary history of the Australasian species of *Scleranthus* will likely require population level sampling of highly variable nuclear and chloroplast molecular markers.

3.4.3.3 Lineage sorting

An alternative explanation of the ITS nucleotide sequence tree topology, that also rejects paraphyly of the group of monandrous species (section *Mniarum sensu* West and Garnock-Jones, 1986), is that different members of a suite of ancestral ITS polymorphisms have become variously fixed in different Australasian *Scleranthus* lineages, with the result that the gene tree and species tree are discordant (lineage sorting). Because the ITS regions for relatively few individuals of each species (in most cases only one) have been sequenced, the level of variation within each species is still unknown. However, the ITS sequences determined for two *S. biflorus* specimens, one from New Zealand the other from Australia, differed by only one substitution. Likewise *S. brockiei* specimens from Australia and New Zealand differed by only one substitution. Further, ITS trees are in full agreement with

morphology in grouping several *Scleranthus* species or sub-specific entities: the two geographical races of *S. singuliflorus* (whose ITS sequences differ by only one nucleotide substitution); *S. fasciculatus* and *S.* aff. *fasciculatus; S. minusculus* and *S. pungens,* indicating that ITS sequences do have resolving power at this taxonomic level.

3.4.4 Chromosome counts

Chromosome counts for the Australian endemic species remain an important gap in knowledge of *Scleranthus.* European species include diploid and tetraploid examples on a base of x=11 while the three New Zealand species of section *Mniarum* and *S. fasciculatus* all have 2n=48. However, it seems likely that all the Australasian species might share 2n=48, in which case the additional counts would be of limited phylogenetic value (other than to provide additional support for the monophyly of the Australasian species group). It would at least be worth examining *S. diander* in this regard in order to test the possibility that it is an allopolyploid.

3.4.5 Divergence times

I have estimated the time of the divergence of European and Australasian *Scleranthus* species and of the radiation of Australasian *Scleranthus* species based on ITS nucleotide substitution rates. Use of ITS2 nucleotide substitution rates calculated from other Angiosperm groups was rejected because evolutionary rates vary by an order of magnitude or more among groups of plants with differing biology (Suh *et al.*, 1993). The estimates given here are entirely reliant on assumptions about the timing of the divergence of the lineage ancestral to *Drymaria* and *Polycarpon* from the lineage ancestral to

subfamilies Alsinoideae and Caryophylloideae. The figure of 35 million years ago for this latter event is subject to considerable error, including ambiguity of the relationships of fossil taxa to extant lineages. However, the fossil record of the Caryophyllaceae does not allow more reliable dating.

Further, the alignment of ITS sequences used to estimate distances is arbitrary in places. Thus, some nucleotide sites compared in distance calculation may not have been homologous. Substitution rate variation between lineages can also introduce inaccuracy to estimates of divergence times, as a strict molecular clock is not evident for the Caryophyllaceae ITS2 data. For this reason, ranges of values based on the minimum and maximum observed distances for different pairs of species separated at a node have been given.

Simple calculation of distances as the number of sites varying between two sequences divided by the total number of sites is unrealistic because the substitution rate is known to vary markedly among different nucleotide positions in ITS2. Therefore distances based on all ITS2 sites rather than just variable sites alone will certainly underestimate distances between the Polycarpeae species and *Scleranthus*, causing estimates of the time since divergence events within *Scleranthus* to be too great. For this reason, distances calculated from variable sites only probably give more realistic estimates of the time elapsed since divergence events. However, an additional problem arises because correction of distances for multiple substitutions (By JC69 or similar method) may be unreliable when uncorrected distances are large (see Kumar *et al.*, 1993). This is evident in part from the greater range of date estimates derived from corrected distances over uncorrected distances, when invariant sites are excluded. However, in the absence of more realistic models of ITS sequence evolution more accurate distance corrections are not

possible. In any case, the date estimates presented here do allow sufficient confidence to exclude hypotheses invoking relatively ancient events in the explanation of *Scleranthus* distribution.

3.4.6 Biogeography

The extant distribution of the genus must be explained by either an extreme case of long distance dispersal, or through retraction of progenitor species, with a once greater old-world range, away from Asia. Extinction of *Scleranthus* from Asia seems unlikely because through this region exist habitats similar to those inhabited by the genus today and it is likely that this has been the case for millions of years. Clearly, *Scleranthus* is capable of long distance dispersal despite lacking any obvious adaptations to facilitate it. Two well-distinguished species, *S. biflorus* and *S. brockiei,* occur in both Australia and New Zealand indicating that *Scleranthus* propagules have crossed the Tasman sea at least twice. Vicariance explanations for the shared species would be inconsistent with the lack of divergence between Ithe TS sequences of *S. biflorus* specimens from Australia and New Zealand (the Tasman sea having opened some 80 million years ago). Sequences gathered for all three species native to New Zealand differed by no more than five sites for ITS1 and ITS2 combined. However, it is much harder to imagine direct dispersal occurring between Europe and Australasia. Further, even if such cross-global dispersal were possible, it remains hard to explain the absence of *Scleranthus* from so much of the world.

The species *S. uniflorus* and *S. biflorus* share several morphological apomorphies (nectary shape, gynoecium without overgrowth of styles by ovary cells and four rather than five parted calyx). The more restricted *S.*

uniflorus is endemic to New Zealand whereas *S. biflorus* is found in New Zealand and Australia. It is parsimonious to conclude that *S. uniflorus* has evolved from *S. biflorus* ancestors since the dispersal of the latter to New Zealand from Australia. This leaves *S. biflorus* as a paraphyletic species. Several justifications can be advanced for recognizing paraphyletic groups at species level within a cladistic framework (Donoghue and Cantino, 1988). Perhaps the most powerful of these is if the organisms concerned form an interbreeding population. Although gene flow through continual seed dispersal of *S. biflorus* from Australia to New Zealand cannot be excluded, there is no current evidence to support it either. However, *S. biflorus* is morphologically indistinguishable in Australia and New Zealand and probably constitutes a biological species (*sensu* Mayr, 1963) in that the plants are *potentially* interbreeding. I do not, therefore, propose to attempt to recognize strictly monophyletic species groups in place of a wider definition of *S. biflorus* including allopatric populations.

West and Garnock-Jones (1986) suggested a Miocene radiation of Australasian section *Scleranthus* and a Pleistocene origin of section *Mniarum.* While a Quaternary origin of section *Mniarum* is supported by the ITS data a significantly older radiation of section *Scleranthus* (*sensu* West and Garnock-Jones, 1986) in Australasia is not. The small divergence between ITS sequences for Australasian *Scleranthus* species in general indicate that all the extant species probably share a common ancestor more recent than the Miocene, probably within the last 5 million years.

In summary, the divergence of extant European and Australasian clades within the genus probably occurred no more than 10 million years ago and may have been much more recent. In the time between the divergence of the progenitors

of *Scleranthus* from other Alsinoideae and the divergence of the extant European and Australasian clades *Scleranthus* may either have had a much greater range than at present, or may have been restricted to part of its present range, or may even have occurred only in an area where it is no longer found. This period in *Scleranthus* history is not reflected in the phylogeny of the extant species because they are apparently the result of more recent radiations in Europe and Australasia. Fossil evidence is unlikely to be forthcoming because the output of pollen from *Scleranthus* is low. However, even if *Scleranthus* had contributed significantly to the pollen record, then its similar appearance to pollen of many other species of Caryophyllaceae would preclude its recognition. The extant Australasian species of *Scleranthus* probably all evolved in response to climate change in the Quaternary and may not have a long history in the area as was suggested by West and Garnock-Jones (1986). Hybridization and introgression seem to have played a role in the distribution of characters among extant Australasian species and have been invoked here to explain the discordance of molecular and morphological phylogenies.

3.5 References

Baldwin, B. G., Sanderson, M. J., Porter, M. J., Wojciechowski, M. F., Cambell, C. S., Donoghue, M. J. (1995): The ITS region of nuclear ribosomal DNA: a valuable source of evidence on Angiosperm phylogeny. Annals of the Missouri Botanical Garden. 82: 247-277.

Bittrich, V. (1993): Caryophyllaceae. In K. Kubitzki, J. Rohwer, and V. Bittrich (Eds.) "The families and genera of vascular plants". Volume 2. Magnoliid, Hamamelid, and Caryophyllid families. Springer Verlag, Berlin.

Bull, J.J., Huelsenbeck, J. P., Cunningham, C. W., Swofford, D. L., Waddell, P. J. (1993): Partitioning and combining data in phylogenetic analysis. Systematic Biology 42(3): 384-397.

Cabot, E. (1998): The Eyeball Sequence Editor. Version 3.2. Computer program.

Crow, G. E. (1978): A taxonomic revision of *Sagina* (Caryophyllaceae) in North America. Rhodora 80: 1-91.

Donoghue, M. J., and Cantino, P. D. (1988): Paraphyly, ancestors, and the goals of taxonomy. A botanical defense of Cladism. Botanical Review 54(2).

Doyle, J. J., and Doyle, J. L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11-15.

Garnock-Jones, P. J. (1981): Checklist of dicotyledons naturalized in New

Zealand. 8. Aizoaceae, Caryophyllaceae, and Portulacaceae. New Zealand Journal of Botany 19: 57-65.

Garnock-Jones, P. J. (1988): Caryophyllaceae. In Webb, Sykes, and Garnock-Jones (Eds.) Flora of New Zealand volume 4, p. 474.

Hamby, R. K., and Zimmer, E. A. (1992): Ribosomal RNA as a phylogenetic tool in plant systematics. In P. S. Soltis, D.E. Soltis, and J. J. Doyle (Eds.) "Molecular systematics of plants" pp. 50-91. Chapman & Hall, New York.

Hershkovitz, M. A., and Zimmer, E. A. (1996): Conservation patterns in Angiosperm rDNA ITS2 sequences. Nucleic Acids Research 24(15): 2857-2867.

Hultman, T., Stahl, S., Hornes, E., Uhlen, M. (1989): Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. Nucleic Acids Research 17 (13): 4937 - 4946.

Hutchinson, J. (1974): The families of flowering plants. Oxford University Press.

Kumar, S., Tamura, K., Nei, M. (1993): MEGA: Molecular Evolutionary Genetics Analysis, version 1.01. The Pensylvania State University, University Park, PA 16802.

Maddison, W. P, and Maddison, D. R. (1992): MacClade version 3.04. Sinauer Associates Inc. Sunderland Massachusetts.

Mattfeld, J. (1934): Nachtrag zu den Caryophyllaceae. In A. Engler, and K Prantl (Eds.) "Die natürlichen pflanzenfamilien. 2nd edn.. Leipzig: Englemann 16c: 365-367.

Mayr, E. (1963): Animal species and evolution. The Belknap Press of Harvard University Press. Cambridge, Massachusetts.

Messenger, S. L., and McGuire, J. A. (1998): Morphology, molecules and the phylogenetics of Cetaceans. Systematic Biology 47(1): 90-124.

Muller, J. (1981): Fossil pollen records of extant angiosperms. Botanical Review 47: 1-142.

Oxelman, B., and Liden, M. (1995): Generic boundaries in the tribe Sileneae (Caryophyllaceae) as inferred from nuclear ribosomal DNA sequences. Taxon 44: 525-542.

Philipson and Skipworth, J. P. (1961): Hectorellaceae: a new family in Dicotyledons. Transactions of the Royal Society of New Zealand (Botany) 1: 31.

Reiseberg, L. H. (1991): Homoploid reticulate evolution in *Helianthus*: evidence from ribosomal genes. American Journal of Botany 78: 1218-1237.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989): Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.

Sell, P. D. (1964): *Scleranthus* (Caryophyllaceae). Flora Europaea 1: 148-149. Cambrdige University Press.

Skipworth, J. P. (1961): The taxonomic position of *Hectorella caespitosa* Hook. f. Trans. Roy. Soc. N. Z. (Bot.) 1(3): 17-30.

Suh, Y., Thien, L. B., Reeve, H. E., and Zimmer, E. A. (1993): Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. American Journal of Botany 80: 1042-1055.

Swofford, D. L. (1999): PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Svensson, L. (1985): An estimate of pollen carry over by ants in a natural population of *Scleranthus perennis* L. (Caryophyllaceae). Oecologia (Berlin) 66:273-377.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673 - 4680.

West, J. G. and Garnock-Jones, P. J. (1986): Evolution and Biogeography of *Scleranthus* (Caryophyllaceae) in Australasia. In Barlow, B. G. (Ed.) "Flora and Fauna of Alpine Australasia". CSIRO 1986.

White, T. J., Bruns, T., Lee, S., Taylor, J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. Innis, D. Gelfand, J. Sninsky, and T. White (Eds.) "PCR Protocls: A Guide to methods and Applications".

Williamson, P. A. (1956): The genus *Scleranthus* in New Zealand. Rec. Domin. Mus. Wellington 3: 13-17.

Woolfe, A. D., and Elisens, W. J. (1994): Nuclear ribosomal DNA restriction-site variation in *Penstemon* section *Peltanthera* (Scrophulariaceae): an evaluation of diploid hybrid speciation and evidence for introgression. American Journal o f Botany 81(12): 1627-1635.

Zhanga, D., Sang, T. (1999): Physical mapping of ribosomal RNA genes in peonies (Paeonia, Paeoniaceae) by fluorescent in situ hybridization: implications for phylogeny and concerted evolution. American Journal of Botany 86: 735-

Chapter 4: Subfamilial relationships within Caryophyllaceae as inferred from 5' *ndh*F sequences

Abstract

Sequences of the 5' end of the chloroplast *ndh*F gene for 15 species of Caryophyllaceae are analyzed by parsimony and Neighbor-Joining analyses. Three major clades within Caryophyllaceae are identified, with little or no support for monophyly of traditionally recognized subfamilies. The first of the three major clades identified (Clade I) comprises part of subfamily Paronychioideae tribe Paronychieae. The second (Clade II) contains the remainder of the Paronychioideae species sampled and includes members of tribe Paronychieae together with members of tribe Polycarpeae. Paronychieae is thus apparently polyphyletic and Polycarpeae at least paraphyletic. The third clade (Clade III) comprises members of subfamilies Alsinoideae and Caryophylloideae. The genera *Scleranthus* and *Spergularia* are confirmed as part of the Alsinoideae/Caryophylloideae clade (Clade III), while *Drymaria* is part of Paronychioideae Clade II. Morphological characters previously used to delimit subfamilial groupings in the Caryophyllaceae are apparently unreliable estimators of phylogeny.

4.1 Introduction

For classification to serve as a useful tool in the exploration as well as description of living systems it should reflect the phylogenetic relationships between the organisms. At this time sub-familial taxonomy in the Caryophyllaceae is only loosely based on phylogenetic relationships

(Bittrich, 1993). Three subfamilies (Paronychioideae, Alsinoideae and Caryophylloideae) have been widely accepted within the Caryophyllaceae, although delimitation of each has varied between authors. Subfamily Paronychioideae, or a part of it, has sometimes also been recognized as a distinct family, the Illecebraceae (e.g., Hutchinson, 1973). Of the three subfamilies of Caryophyllaceae, only Caryophylloideae is well supported by morphological apomorphies (possession of a calyx tube, corolla scales, closed petal venation and seed morphology: Bittrich, 1993) and the relationships between the three subfamilies are unclear. The subfamilies Alsinoideae and Caryophylloideae, taken together, have been suggested to form a monophyletic group. Bittrich (1993) suggests that chromosome numbers and embryogeny type (Solanad in at least some Paronychioideae, generally Caryophyllad in Alsinoideae and Caryophylloideae) are important characters supporting the monophyly of Caryophylloideae and Alsinoideae together. However, embryogeny type has not been widely surveyed in Paronychioideae and the Caryophyllad type is possibly ancestral in the family as whole.

It has also been suggested that Caryophylloideae themselves are derived from "advanced" Alsinoideae (on the basis of chromosome numbers: Bittrich, 1993), thus making Alsinoideae paraphyletic. Bittrich (1993) argues that most Alsinoideae possess nectary glands on the base of their stamens and that this is an apomorphy for the subfamily. The presence or absence of stipules is also an important character in the family. Bittrich (1993) circumscribes Paronychioideae to include all the stipulate species in the family. Some weight has also been attached to fruit characters, especially the indehiscent fruits of many Paronychioideae are contrasted with the capsules of most Alsinoideae and Caryophylloideae. However, several genera usually included in the Alsinoideae have indehiscent fruits (e.g., *Scleranthus,*

Habrosia, and *Plettkea*: Bittrich, 1993) and some capsulate genera are often included in Paronychioideae (e.g. *Polycarpon, Spergularia,* and *Loeflingia*).

Within the three Caryophyllaceae subfamilies, genera have been assigned to various tribes, although the bases for these associations are often weak. The most recent classification scheme of Bittrich (1993) is presented here for discussion.

Table 4.1. Classification of the Caryophyllaceae (from Bittrich 1993).

subfamily	tribe	total genera	number of genera sampled in this study
Caryophylloideae	Caryophylleae	**17**	**1**
	Sileneae	**6**	**1**
Alsinoideae	Alsineae	**28**	**4**
	Scleranthеae	**2**	**1**
	Geocarpeae	**1**	**0**
	Pycnophylleae	**1**	**0**
	Habrosieae	**1**	**0**
Paronychioideae	Paronychieae	**15**	**4**
	Polycarpeae	**16**	**4**
	Corrigioleae	**2**	**0**

As part of a program of molecular systematics directed at *Scleranthus* using nrDNA sequences (reported elsewhere) I have become interested in intergeneric relationships between Caryophyllaceae. Specifically I needed to explore the relationships of *Scleranthus* in order to use appropriate outgroups in phylogenetic analysis.

One of the problems confounding systematic study of the Caryophyllaceae using floral characters is the apparent convergent evolution of important characters such as indehiscent fruits, perigynous flowers and loss of petals. It is therefore valuable to apply DNA-based systematics in the Caryophyllaceae. The chloroplast genome has provided data for phylogenetic reconstruction principally through restriction site mapping and sequencing of the ribulose biphosphate carboxylase large subunit gene (*rbc*L). Restriction site mapping requires relatively large quantities of fresh (or at least, well-preserved) plant tissue. Moreover, restriction site data sets cannot accommodate the addition of new taxa without comparing raw data (Olmstead and Palmer, 1994). Due to the relatively slow rate of evolution of the molecule, *rbc*L sequence analysis is limited in its ability to resolve intra-familial phylogenetic relationships. Gene sequences from taxa that diverged relatively recently may not have accumulated enough substitutions to allow reliable phylogenies to be reconstructed from *rbc*L data. Within the family Caryophyllaceae, *rbc*L sequences are known to be too conserved to allow reconstruction of robust phylogenies (Manhart and Rettig, 1994).

Recently a number of other chloroplast and nuclear encoded genes have received attention as sources of phylogenetic information (reviewed by Baldwin, 1995). Of these; *ndh*F, *mat*K, and nuclear ITS1 and ITS2 regions all have significantly faster rates of substitution than *rbc*L. Nuclear ITS sequences are readily amplified and sequenced in Caryophyllaceae, but evolve too rapidly, accumulating numerous insertions/deletions in addition to single nucleotide substitutions, to allow alignment of sequences except from closely related genera or species (Oxelman and Liden, 1991; Chapter 2, this thesis). The substitution rate of chloroplast gene *mat*K is appropriate to address systematic questions below the family level, but I am aware of no studies where it has been applied to taxa closely related to the

Caryophyllaceae. However, the 5' region of *ndh*F has been previously sequenced for a number of species in the order Caryophyllales including some representatives of the Caryophyllaceae (J. C. Clement, personal communication). These sequences indicate a higher rate of substitution for 5' *ndh*F than *rbc*L. The phylogenetic utility of *ndh*F has been previously examined for the Asteraceae (Kim and Jansen, 1995). These authors found that substitution rates within *ndh*F varied dramatically with the 3' region of 855 nucleotides evolving approximately twice as fast as the 5' region of approximately 1200 nucleotides. Kim and Jansen (1995) and other studies (Bohs and Olmstead, 1997; Neyland and Urbatsch, 1996; Terry *et al.*, 1997) have demonstrated phylogenetic utility of *ndh*F for studies below the family level.

The aim of the present study is to identify monophyletic groups within the Caryophyllaceae by using DNA sequences, to test existing hypotheses about the phylogeny of the family and to provide a framework for future more complete study. A total of 1064 nucleotide sites from the 5' region of the chloroplast *ndh*F gene from species representing 15 genera of Caryophyllaceae were sequenced. Included were representatives of all 3 subfamilies and 6 of the 11 tribes listed in Table 4.1. These sequences were subjected to phylogenetic analysis using *Mollugo verticillata* (Molluginaceae) and *Atriplex canescens* (Chenopodiaceae) as outgroup taxa. The Molluginaceae have been considered a likely sister group to the Caryophyllaceae (Bittrich, 1993), and the two families appear as sister taxa in the cladistic analysis of the order by Rodman (1994). However, analyses of *rbc*L (Manhart and Rettig, 1994) and chloroplast ORF2280 (Downie *et al.*, 1997) DNA sequences did not support this relationship, although only a single representative of Molluginaceae was included in each study. Instead, these DNA sequence studies suggest that the sister group of the

Caryophyllaceae is a clade including the families Amaranthaceae and Chenopodiaceae.

Several genera of Caryophyllaceae with uncertain taxonomic status have been included in the present study in order to resolve their true affinities and test the value of key morphological characters in estimating phylogeny in this family. The genera *Scleranthus* and *Spergularia* have previously been included in both the Paronychioideae and Alsinoideae and *Drymaria* has some characteristics of the Alsinoideae and its position in Paronychioideae has been questioned (Bittrich, 1993). In the case of *Scleranthus,* its very small, apetalous flowers and especially one-seeded indehiscent fruits are the main characters linking it to Paronychioideae, while its connate leaves and lack of stipules link it to Alsinoideae. Stipulate leaves link *Spergularia* to the Paronychioideae (Bittrich, 1993), but its capsules link it to Alsinoideae (where it is placed by Pax and Hoffman, 1934). Development of the stipules differs from other Paronychioideae in *Dryamria*, another capsulate genus included in Paronychioideae, with reservations, by Bittrich (1993). Other taxa sampled in the present study include representatives of all the tribes containing more than one genus, except that Corrigioleae (two genera) was not included.

4.2 Materials and methods

Plant species sampled in this study and their classification are shown in Table 4.2.

Table 4.2. Plants sampled in this study

Plant species	Subfamily	Tribe	Voucher
Scleranthus biflorus	Alsinoideae	Scleranthea	*WELTU 19660*
Colobanthus brevisepalus.	Alsinoideae	Alsineae	*WELTU 19547*
Cerastium glomeratum	Alsinoideae	Alsineae	*WELTU 19664*
Stellaria media	Alsinoideae	Alsineae	*WELTU 19667*
Arenaria benthamii	Alsinoideae	Alsineae	
Dianthus caryophyllus	Caryophylloideae	Caryophylleae	No voucher
Silene antirrhina	Caryophylloideae	Sileneae	*Clement 0022*
Spergularia marina	Paronychioideae	Polycarpeae	*Clement 0202*
Polycarpon tetraphyllum	Paronychioideae	Polycarpeae	*WELTU 19666*
Drymaria laxiflora	Paronychioideae	Polycarpeae	*Clement 0223*
Loeflingia squarrosa	Paronychioideae	Polycarpeae	*Clement 0123*
Dicheranthus plocamoides	Paronychioideae	Paronychioideae	*Clement 0208*
Scopulophila rixtordii	Paronychioideae	Paronychieae	*Clement 0211*
Herniaria glabra	Paronychioideae	Paronychieae	
Paronychia drummondii	Paronychioideae	Paronychieae	*Clement 0123*
Mollugo verticillata	family Molluginaceae	-	*Nesom 7480*
Atriplex canescens	family Chenopodiaceae	-	*Clement 0002*

DNA was extracted from fresh leaves by the CTAB method (Doyle and Doyle, 1987) and purified by phenol chloroform extraction (*Sambrook et al.*, 1989). Then 1μL aliquots of DNA extracts or a 1/10 dilution thereof were used in subsequent PCR reactions. To amplify the 5' region of *ndh*F versions of primers *ndh*F15 and *ndh*F8 with a number of degenerate sites were used. In addition to DNA template, PCR reactions contained 1 unit of Taq DNA Polymerase (Pharmacia Biotech), 2.5 μL 10 × reaction buffer (500mM Kcl, 15 mM $MgCl_2$, 100 μM Tris-HCl pH 9.0), 10nM each primer, 42.5 μM $MgCl_2$, 25ng BSA and H_2O to make up a total volume of 25 μL. Cycling conditions were; 96°C for 30 seconds, 50°C for 45 seconds, 70°C for 2

minutes, for a total of 30 cycles. PCR reactions were electrophoresed on 1% agarose using TBE buffer (Sambrook *et al.*, 1989) to check size, quality, and quantity of amplification products.

Three sequencing methods were used to generate the data used in this study as alternative technologies became available in our Laboratory.

1. Double stranded DNA (dsDNA) PCR products were purified by electrophoresis on 1% LMP agarose using Tris-EDTA buffer (Sambrook *et al.*, 1989) and DNA visualized by staining with ethidium bromide. Pieces of agarose containing stained DNA bands were excised from gels (gel-cuts) and melted in approximately equal volumes of sterile distilled water by heating at 70°C for 1 minute. A second round of PCR using either *ndh*F15 or *ndh*F8 and one of the internal primers *ndh*F536 or *ndh*F972R at stringent annealing temperatures (65°C) was used to produce dsDNA template for cycle sequencing. Products were purified by electrophoresis as above and DNA recovered from the agarose gel using Biorad Prep-a-gene kits (Biorad catalogue number 732-6010) according to the manufacturers guide. Between 3 and 6 µL of DNA template was used in cycle sequencing reactions with Applied Biosystems ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (P/N 402078), 3.2 nM sequencing primer, and H_2O to make up a final volume of 20 uL. Products were analyzed by electrophoresis on an ABI Prism™ 377 DNA sequencer.

2. Alternatively, PCR products were purified by electrophoresis as above and a second round of PCR carried out using modified *ndh*F8 or *ndh*F15 primers tailed with the M13 forward universal sequencing primer sequence and one of the internal primers *ndh*F536 or *ndh*F972R annealing at 65°C. Products were again purified by electrophoresis and Biorad Prep-a-gene

system (as above) and recovered DNA used as template in cycle sequencing reactions with Applied Biosystems ABI Prism™ Big Dye™ Dye Terminator Cycle Sequencing Ready Reaction Kit (P/N 4303149) and 3.2 μM M13 -21 primer or Applied Biosystems ABI Prism™ M13 -21 Big Dye™ Dye Primer Cycle Sequencing Ready Reaction Kit (P/N402136). Again extension products were separated and detected with an ABI Prism™ 377 DNA sequencer.

3. Finally, for some sequence, *ndh*F DNA was PCR amplified and gel purified as above and dsDNA product used as template for PCR with 5' biotinylated versions of either the *ndh*F8 or *ndh*F15 primer and one of the internal primers *ndh*F536 or *ndh*F972R. Single stranded DNA was recovered with Dynal m-280 Streptavidin Dynabeads™ as described in the manufacturer's instructions. Single stranded DNA from both biotinylated and unbiotinylated strands were used in sequencing reactions using ^{35}S labeled dATP in conjunction with US biochemicals Sequenase™ version 2.0 DNA sequencing kit (P/N 70770) according to the manufacturers instructions. Primers *ndh*F15, *ndh*F536, *ndh*F536R, *ndh*F972, *ndh*F972R and *ndh*F8 were used as sequencing primers. Labeled products were separated by electrophoresis. Following this, gels were exposed to Kodak Biomax™ film for up to 3 days and then autoradiograms developed with Kodak Xray Developer Number 2.

Sequences obtained from these three methods were combined. Uncertain or ambiguous sites were coded as unknown.

Position of sequencing primers and regions of sequence combined for analysis in this study are shown in Figure 4.1.

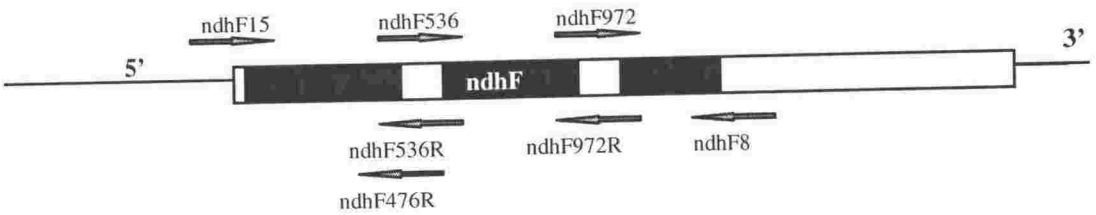

Figure 4.1. Position of primers and sequence used in this study. Shaded regions are those included in phylogenetic analysis. Primers are shown as arrows pointing in the direction of polymerization they support. For primer sequences and references see text.

In this study I used modified versions of the previously published primers, *ndh*F8 and *ndh*F15 (Olmstead and Sweere, 1994), to amplify the 5' region of *ndh*F by PCR as well as novel primers, *ndh*F476R, *ndh*F 536, *ndh*F536R, *ndh*F972, *ndh*F972R, which were used in sequencing protocols. Primer sequences as used in this study are as follows;

*ndh*F8	ATA GAT CCG ACA CAT ATA AAA TSC RGT T,
*ndh*F15	ATG GAA CAG ACA TAT CAA TAY GSR TG,
*ndh*F476R	TTG TTG ACA AGC ACT CGC AGC A,
*ndh*F536	CTC TCA ATT CGG YTA TAT KAT G,
*ndh*F536R	TCC CCT ACA CGA TTS GTY ACA A,
*ndh*F972	CTC TCA ATT GGG YTA TAT KAT G,
*ndh*F972R	CAT CAT ATA ACC CAA TTG AGA C.

All *ndh*F sequences obtained were readily alignable manually using XESEE editing program (Cabot, 1998). Phylogenetic analysis was conducted using PAUP*4.0b2 (Swofford, 1999). All shortest parsimony trees were found using Branch and Bound searches with MULPARS and steepest descent options in effect. Amino acid sequences for the *ndh*F gene product were

inferred by the program MEGA (Kumar *et al.*, 1993) using "universal" genetic code. Neighbor-Joining trees were constructed from Jukes-Cantor distances with missing data excluded only in those pairwise comparisons affected. Nucleotide frequencies and transition/transversion ratios were estimated by PAUP* 4.0b2 from a selected shortest parsimony tree by maximum-likelihood method using Hasegawa-Kishano-Yano (Hasegawa *et al.*, 1985) model of nucleotide substitution with all sites assumed to evolve at equal rate. Pairwise divergences and nucleotide frequencies are those determined by PAUP* 4.0b2. Numbers of substitutions by codon position were determined by MacClade from a selected shortest parsimony tree.

4.3 Results

Nucleotide sequence was obtained from all of the protocols described in methods, with Cycle sequencing using M13 tailed primers the most reliable and efficient (in terms of length of readable sequence returned). A total of 1064 aligned nucleotide positions from 3 non-contiguous segments of the 5' region of *ndh*F were used in phylogenetic analyses. In total 363 positions were variable in the complete data matrix (330 excluding outgroups); of these 169 (150 excluding outgroups) were parsimony informative. Including outgroups, 239 of the variable and 111 of the parsimony informative sites involved synonymous nucleotide substitutions. Pair-wise uncorrected distances calculated from the total number of aligned sites for Caryophyllaceae sampled ranged from 0.028 between *Stellaria media* and *Cerastium glomeratum* and 0.109 between *Stellaria media* and *Paronychia fastigiata.* Distances between Caryophyllaceae and outgroup taxa ranged from 0.088 between *Mollugo* and *Polycarpon,* and 0.129 between *Atriplex* and *Scleranthus.* Uncorrected pair-wise distance between outgroups *Atriplex* and *Mollugo* was 0.91. A comparison of uncorrected distances for

*rbc*L and 5'*ndh*F between Caryophyllaceae species for which both sequences are available is provided in Table 4.3. The *rbc*L sequences used are from Genbank or J. C. Clement (personal communication).

Table 4.3. Comparison of *rbc*L (bottom) and 5'*ndh*F (top) uncorrected distances.

	Polycarpon	*Herniaria*	*Cerastium*	*Stellaria*	*Silene*	*Dianthus*
Polycarpon		**0.074** **0.042**	**0.085** **0.047**	**0.089** **0.050**	**0.082** **0.042**	**0.083** **0.035**
Herniaria		-	**0.094** **0.058**	**0.099** **0.058**	**0.087** **0.051**	**0.088** **0.041**
Cerastium		-	-	**0.028** **0.017**	**0.072** **0.038**	**0.070** **0.040**
Stellaria		-	-	-	**0.076** **0.040**	**0.082** **0.036**
Silene		-	-	-	-	**0.062** **0.028**

A Branch and Bound search by PAUP *4.0 found 12 shortest trees 733 steps long for the aligned 5'*ndh*F data set. One of these is shown as Figure 4.2. A decay analysis was conducted by searching for successively longer trees up to 6 steps longer than the shortest trees. The decay value indicates the number of additional steps required to collapse a branch. Some clades were found in all trees 6 steps or less longer than the shortest trees. These are shown in Figure 4.2 as having a decay value of >6. A decay index of 0 indicates that the branch collapses in the strict consensus of shortest trees. The 733 changes in this tree are distributed among codon positions in the ratio 1.6:1:5.1 with transitions outnumbering transversions by 1.17:1. The

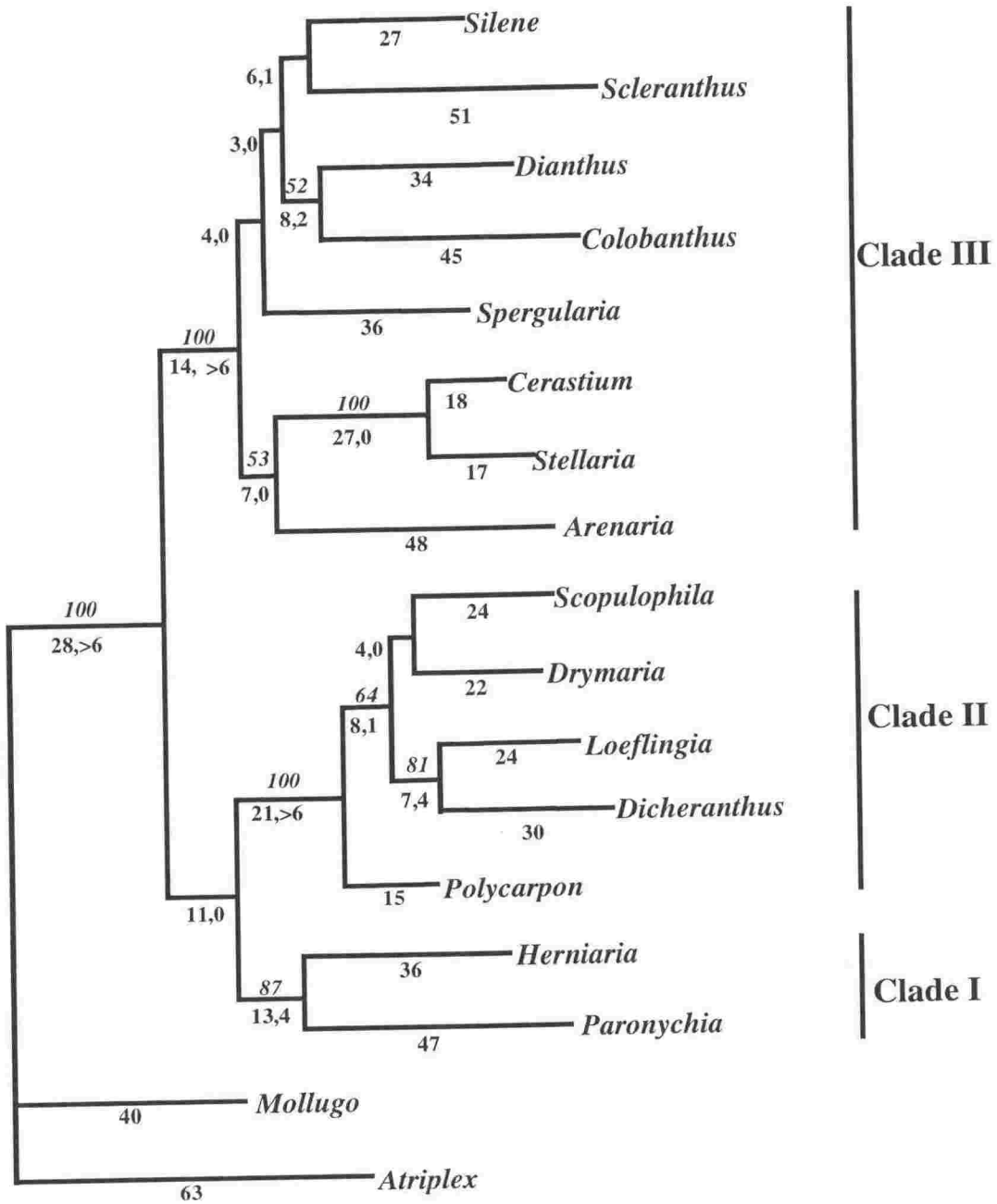

Figure 4.2. One of 12 shortest trees found for the nucleotide substitution data by a PAUP heuristic search. Numbers below branches before commas are inferred numbers of character state changes; numbers after commas are decay values. Italicized numbers above branches are bootstrap percentages where these are 50% or more. Decay values of 0 indicate branches which collapse in the strict consensus of shortest trees. Clades I, II, and III are well supported clades discussed in the text.

tree shown has a Consistency Index of 0.496, Retention Index 0.543, and Rescaled Consistency Index of 0.298.

A notable feature of this tree is the lack of support for monophyly of any of the tribes of Bittrich (1993) for which more than one species has been sampled. Species of Polycarpeae are distributed over Clade II (most species) and Clade III (*Spergularia*). Moreover, two species of Paronychieae (*Dicheranthus* and *Scopulophila*) group amongst the Polycarpeae included in Clade II. No support for the monophyly of subfamilies Caryophylloideae or Alsinoideae, or the tribe Alsineae, is provided, however no contradictory groupings are well supported either. In this analysis, *Spergularia* and *Scleranthus* group strongly with the Alsinoideae/Caryophylloideae and not with the Paronychioideae clades. However, *Drymaria* is well supported within Clade II along with the other Polycarpeae sampled

Amino acid sequences for *ndh*F gene products inferred from the nucleotide data for all taxa sampled were then subjected to parsimony analysis. A Branch and Bound search found a single shortest tree for these data. Figure 4.3 is derived from this tree by collapsing all clades not supported at the 50% level in a bootstrap analysis. As might be expected the amino acid tree is less well resolved than the nucleotide tree (with only 58 parsimony informative characters compared with 169). The Alsinoideae and Caryophylloideae still group together, although with less bootstrap support. The positions of *Herniaria* and *Paronychia* are not resolved, but the rest of the Paronychioid species still form a well supported clade, and

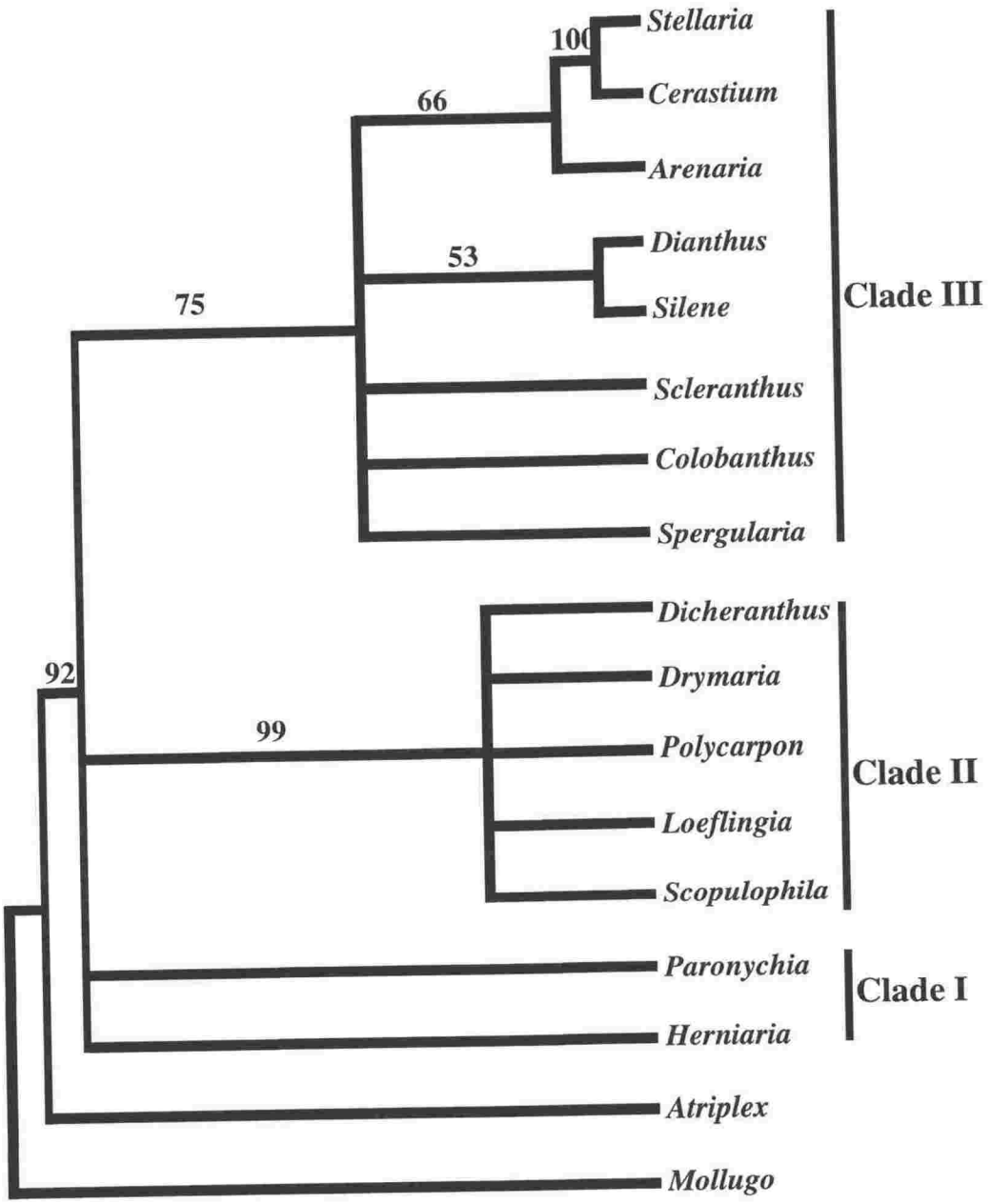

Figure 4.3. 50% parsimony bootstrap consensus for inferred amino acid substitution data. Numbers above branches are bootstrap percentages.

the grouping of *Arenaria*, *Stellaria* and *Cerastium* is more strongly supported than it is by the nucleotide substitution data. In contrast to the nucleotide tree the two Caryophylloideae taxa, *Silene* and *Dianthus* are sister taxa in this tree with 53% bootstrap support.

A Neighbor-Joining tree (Figure 4.4) was also constructed using the program MEGA (Kumar *et al.*, 1993) for the nucleotide substitution data set using Jukes-Cantor distances. This tree is identical to the parsimony tree except that it groups *Spergularia* with *Scleranthus* and *Silene*. However, branch lengths within the Alsinoideae/Caryophylloideae clade are very short and probably not significant except for the branch leading to *Cerastium* and *Stellaria*.

4.4 Discussion

4.4.1 Sequencing methods

Several DNA sequencing methods were used in this study to recover data from PCR products, in part because of problems obtaining sequence from several of the primers used in a number of taxa. The *ndh*F gene lacks internal regions of conserved sequence, which makes designing sequencing primers to work over a range of species difficult. Cycle sequencing appeared to be much more vulnerable to mismatches between primer and template sequences. However, despite this, consistent results have been obtained by using M13 sequence tailed primers in the amplification of target DNA and M13 sequencing primers during dye terminator cycle sequencing chemistry.

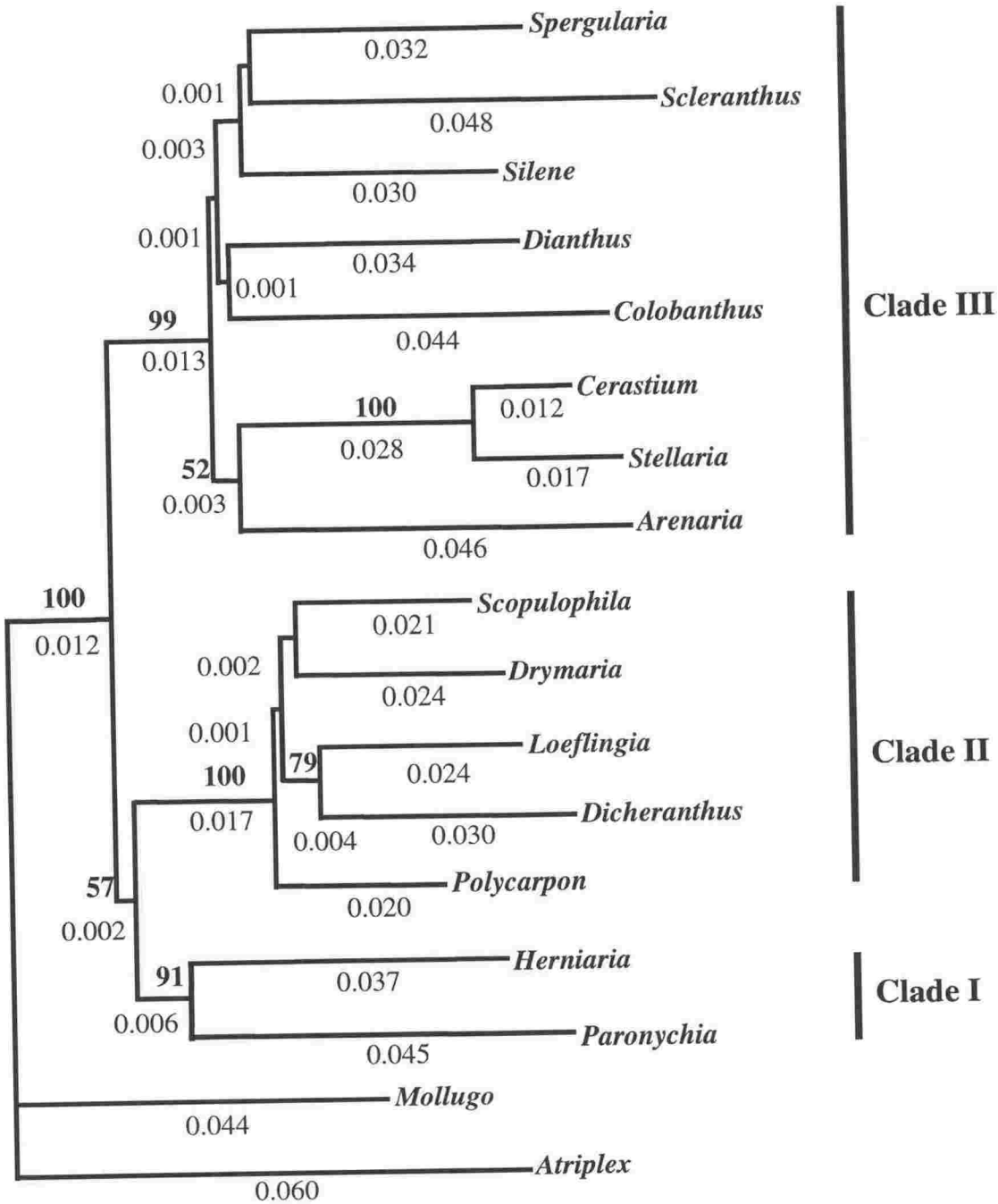

Figure 4.4. Neighbor-Joining tree for nucleotide substitution data. Branch lengths are shown below branches and numbers above branches are bootstrap percentages.

4.4.2 Comparison with *rbc*L

The rate of nucleotide substitution in 5' *ndh*F sequences in the Caryophyllaceae is approximately twice as fast as that for *rbc*L (see table 4.3). This is in contrast to the Asteraceae where 5' *ndh*F evolved at a similar rate to *rbc*L (Kim and Jansen, 1994).

4.4.3 Paronychioideae (Clades I and II)

Trees derived from nucleotide substitution data using Neighbor-Joining and parsimony analyses showed broad agreement while parsimony analysis of inferred amino-acid sequences provides generally more conservative hypotheses of phylogenetic relationships. Three major clades (Clade I, II, and III) are evident in the consensus of the shortest trees from the parsimony analyses (Figure 4.2) and in the Neighbor-Joining tree (Figure 4.4) derived from all the nucleotide substitution data. Clade II comprises part of subfamily Paronychioideae. It includes genera usually ascribed to either tribe Polycarpeae (*Polycarpon, Loeflingia, Drymaria*) or tribe Paronychieae (*Dicheranthus* and *Scopulophila*). This grouping is very well supported by both bootstrap and decay analyses. The tribe Paronychieae is apparently a polyphyletic grouping of taxa which have evolved indehiscent fruits with reduced ovule number in parallel. The position of *Drymaria* as a member of Paronychioideae and not Alsinoideae is also supported by its inclusion in Clade II (Figures 4.2, 4.3, 4.4). Clade I contains *Herniaria* and *Paronychia,* the other representatives of subfamily Paronychioideae included in this study. Both of these genera have indehiscent fruits with low numbers of ovules and are traditionally part of the tribe Paronychieae. This clade is well supported by the bootstrap and decay analyses (Figure 4.2, 4.4) suggesting that this part of the Paronychieae does constitute a monophyletic group.

Additional sampling from subfamily Paronychioideae is needed to establish tribal affinity of individual genera.

Monophyly of the Paronychioideae as a whole (excluding *Spergularia* and in all likelihood its close relative *Spergula*) is not established in this study. Paronychioideae appear as a monophyletic group in the Neighbor-Joining tree but not in the consensus of shortest parsimony trees or the amino acid substitution tree. This sub-family is not well defined by morphological characters likely to be apomorphic, (based on comparison with potential outgroups) and may be a paraphyletic assemblage of old lineages within the family.

4.4.4 Alsinoideae and Caryophylloideae (Clade III)

Clade III comprises representatives of subfamilies Caryophylloideae and Alsinoideae along with *Spergularia*. Monophyly of the two subfamilies together and inclusion of the genera *Spergularia* and *Scleranthus* are all well supported by bootstrap and decay analysis. However, none of the analyses was able to resolve relationships between genera within this clade reliably, with the exception of the close sister group relationship of *Stellaria* and *Cerastium*. The data suggest a 'star phylogeny' for this group where rapid radiation of extant lineages occurred without being reflected in DNA sequence because of the stochastic nature of nucleotide substitution fixation. This is well illustrated by the Neighbor-Joining tree (Figure 4.4) where internal branch lengths are very short relative to those leading to terminal taxa.

The two genera of Caryophylloideae sampled here, *Dianthus* and *Silene*, do appear as a monophyletic group in the consensus of shortest trees found by

PAUP for the amino acid data (Figure 4.3), but not in any of the shortest trees found by PAUP, or in the Neighbor-Joining tree, for the full nucleotide substitution data. Subfamily Caryophylloideae is usually considered to be well defined by floral characters (including possession of a calyx tube, corolla scales, petal venation and seed morphology: Bittrich, 1993). The full substitution data do not support monophyly of the Caryophylloideae, instead weakly grouping *Dianthus* with *Colobanthus* and grouping *Silene* with *Scleranthus* and *Spergularia.* This topology implies multiple origin of the Caryphylloideae from Alsinoideae and is thus hard to reconcile with the morphology of the plants and is unlikely to be a true reflection of their relationships.

It has previously been suggested that the subfamily Alsinoideae may be paraphyletic because there is a lack of apomorphic characters uniting its members (Bittrich, 1993). Perhaps it is significant that the shortest parsimony tree in which the Alsinoideae appear as a monophyletic group is 6 steps (five steps if *Spergularia* is allowed to be part of this clade) longer than the shortest parsimony tree found for all nucleotide substitution data (using the constraints function of PAUP*4.0). All shorter trees have *Dianthus* and *Silene* nested somewhere within the Alsinoideae although not necessarily together.

4.4.5 Evaluation of 5' *ndh*F and possible further study

Very large DNA sequence data sets are likely to be required to fully resolve this part of the Caryophyllaceae phylogeny. A data set produced by combining *rbc*L and 5' *ndh*F sequences was unable to resolve relationships between the four taxa *Polycarpon, Stellaria, Dianthus* and *Silene* despite including 255 variable characters among the genera (Smissen, unpublished).

It is possible that additional data from the more rapidly evolving 3' end of the *ndh*F gene or from *mat*K might provide better resolution and make greater taxon sampling in this group useful. However, careful examination of morphological characters and a cladistic treatment of these might provide more insight than DNA sequencing within current practical limitations (especially the small number of well studied fast evolving chloroplast genes).

Although 5' *ndh*F does not fully resolve relationships within subfamilies it has self-evident utility in assessing the affinities of problematic genera. This study has clearly indicated the subfamilial positioning of *Drymaria*, *Scleranthus* and *Spergularia*. It has also provided compelling data supporting a clade composed of subfamilies Alsinoideae and Caryophylloideae, and rejecting the monophyly of the Paronychioideae tribes Polycarpeae and Paronychieae accepted in Bittrich (1993). A number of other genera could be usefully sampled in a larger data set. Obtaining 5' *ndh*F sequences for the two genera of the third tribe of Paronychioideae, the Corrigioleae, which contains the two genera *Corrigiola* and *Telephium* would clearly be desirable, especially as these have also been placed in Molluginaceae (Gilbert, 1987; but see Downie *et al.*, 1997). Inclusion of sequences from these two genera might also improve resolution of relationships between the other tribes of Paronychioideae. Nuclear ITS sequences indicate that the Andean genus *Pycnophyllum* may be sister group to *Drymaria* (Paronychioideae) and not a member of the Alsinoideae where it is usually included (Chapter 2, this thesis). Corroboration of this relationship using nucleotide sequences from the independently inherited chloroplast genome would be worthwhile.

In conclusion, it is clear that chloroplast gene sequences, including *ndh*F, have much to offer for phylogenetic study of the Caryophyllaceae. A larger

study, with longer or more rapidly-evolving sequences of DNA and wider taxon sampling, could lay the framework for a stable subfamilial classification. This would facilitate more finely focused evolutionary studies of individual genera or groups of closely related genera such as study of *Scleranthus* (this thesis) and work on Hawaiian Alsinoideae (W. Wagner pers. comm.).

4.5 References

Baldwin, B. G., Sanderson, M. J., Porter, M. J., Wojciechowski, M. F., Cambell, C. S., Donoghue, M. J. (1995): The ITS region of nuclear ribosomal DNA: a valuable source of evidence on Angiosperm phylogeny. Annals of the Missouri Botanical Garden. 82: 247-277.

Bittrich, V. (1993): Caryophyllaceae. In K. Kubitzki, J. Rohwer, and V. Bittrich (Eds.), The families and genera of vascular plants. Volume 2. Magnoliid, Hamamelid, and Caryophyllid families. Springer Verlag, Berlin.

Bohs, L., and Olmstead, R. G. (1997): Phylogenetic relationships in Solanum (Solanaceae) based on *ndh*F sequences. Systematic Botany 22(1): 5-17.

Cabot, E. (1998): The Eyeball Sequence Editor. Version 3.2. Computer program.

Downie, S. R., Katz-Downie, D. S., and Cho, K.-J. (1997): Relationships in the Caryophyllales as suggested by phylogenetic analyses of partial

chloroplast DNA ORF2280 homolog sequences. American Journal of Botany 84(2): 253-273.

Gilbert, M. G. (1987): The taxonomic position of the genera Telephium and Corrigiola. Taxon 36: 47-49.

Hasesgawa, M., Kishino, H., and Yano, T. (1985): Dating the human-ape split by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22: 160-174.

Hutchinson, J. (1974): The families of flowering plants. Oxford University Press.

Kim, K. –J., and Jansen, R. K. (1995): *ndh*F sequence evolution and the major clades in the sunflower family. Proceedings of the National Academy of Sciences U.S.A. 92: 10379-10383.

Kumar, S., Tamura, K., Nei, M. (1993): MEGA: Molecular Evolutionary Genetics Analysis, version 1.01. The Pensylvania State University, University Park, PA 16802.

Manhart, J. R., and Rettig, J. H. (1994): Gene sequence data. In H. -D. Behnke and T. J. Mabry (Eds.), "Caryophyllales: evolution and systematics, pp 235-246. Springer-Verlag, Berlin.

Neyland, R., and Urbatsch, L. E. (1996): Phylogeny of subfamily Epidendroideae (Orchidaceae) inferred from *ndh*F chloroplast gene sequences. American Journal of Botany 83(9): 1195-1206.

Olmstead, R. G., and Palmer, J. D. (1994): Choroplast DNA systematics: a review of methods and data analysis. American Journal of botany 81: 1204-1224.

Olmstead, R. G., and Sweere, J. A. (1994): Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. Systematic Biology 43: 467-481.

Oxelman, B., and Liden, M. (1995): Generic boundaries in the tribe Sileneae (Caryophyllaceae) as inferred from nuclear ribosomal DNA sequences. Taxon 44: 525-542.

Pax, F., and Hoffman, K. (1934): Caryophyllaceae. In A. Engler and K. Prantl (Eds.), Die natürlichen pflanzenfamilien, Bd 16c. Engelmann: Leipzig.

Rodman, J. E. (1994): Cladistic and Phenetic studies. In H. -D. Behnke and T. J. Mabry, Eds., Caryophyllales: evolution and systematics, pp. 279-301. Springer-Verlag, Berlin.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989): Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.

Swofford, D. L. (1999): PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sinauer Associates, Suderland, Massachusetts.

Terry, R. G., Brown, G. R., Olmstead, R. G. (1997): Examination of subfamililal phylogeny in Bromeliaceae using comparative sequencing of the plastid locus *ndh*F. American Journal of Botany 54(5): 664-670.

Chapter 5: Synthesis

5.1 Taxonomy

5.1.1 *Scleranthus* phylogeny

The data gathered and analysed in this thesis are consistent with the hypothesis that *Scleranthus* is monophyletic (section 2.4.6, 3.3.1, and 3.4.1.5). Characters of seed and ovary anatomy provide morphological evidence of common descent (section 2.3.6), and ITS sequences suggest recent derivation of extant *Scleranthus* species from a common ancestor (section 3.3.3 and 3.4.3). A phylogeny of *Scleranthus* based on morphological characters suggests that the basal dichotomy in the genus is between European and Australasian species (section 2.3.7 and 2.4.6). In morphology-based cladograms section *Scleranthus* (*sensu* West and Garnock-Jones, 1986) is paraphyletic (Figure 2.62). Its Australian members are part of a clade including the exclusively Australasian section *Mniarum*, either as sister group or as a paraphyletic assemblage within which section *Mniarum* is nested. Section *Mniarum* (*sensu* West and Garnock-Jones, 1986) is supported as monophyletic in these cladograms. Trees generated from ITS sequence data concur with morphology-derived trees over the basal dichotomy into European and Australasian *Scleranthus* clades (section 3.3.2.1 and 3.4.2.1). However, the two data sets disagree over relationships among the Australasian species (section 3.4.2). In contrast to the morphology-derived trees the ITS data do not support the monophyly of section *Mniarum* (section 3.3.2.1 and 3.4.3.1). Comparison of ITS sequences also raises the possibility of a hybrid origin of *S. diander* (section 3.3.2.1 and 3.4.2.2). I suggest that incongruence between morphology and ITS sequence characters is the result of hybridization and introgression or of ancient hybrid speciation events (section 3.4.3.2). Regardless of relationships within Australasian species of *Scleranthus*, the

Australian members of section *Scleranthus* (*sensu* West and Garnock-Jones, 1986) should be included in section *Mniarum* as in the treatment of the genus by Pax and Hoffman (1934).

5.1.2 Subfamilial groups in Caryophyllaceae

While neither ITS nor *ndh*F sequence analyses were able to identify a sister group for *Scleranthus,* both (and especially the latter) do confirm that *Scleranthus* is correctly placed in subfamily Alsinoideae, not Paronychioideae (section 3.4.1.2 and 4.4.4). Further, analysis of *ndh*F sequences provides strong support for the hypothesis that the subfamilies Alsinoideae and Caryophylloideae together form a monophyletic group and that the genus *Spergularia* (included in Paronychioideae by Bittrich, 1993) is also part of this clade (section 4.4.4). Molecular characters may not be as useful in phylogenetic study of this Alsinoideae-Caryophyloideae clade as cladistic analysis of morphological characters because it seems to approach a "star phylogeny" where extant lineages are the result of a rapid ancient radiation (section 4.4.4). Within the subfamily Paronychioideae (whose monophyly excluding *Scleranthus* and *Spergularia* is not established) the tribe Paronychieae is shown to be polyphyletic and part of its membership should be transferred to Polycarpeae (section 4.4.3). Further study (including better sampling) of Paronychioideae should be a high priority for the systematics of the family and may result in changes to subfamily level taxonomy and nomenclature (section 4.4.5).

5. 2 Biogeography

The phylogenetic conclusions outlined in section 5.1.2 above enable one to reject those historical explanations of *Scleranthus'* extant native distribution that require independent origins of European and Australasian groups of species. The comparatively recent radiation of extant *Scleranthus* species, which is suggested by their closely similar ITS sequences (section 3.4.1.5), also argues against the long Australasian history of the group as postulated in West and Garnock-Jones (1986). From data presented here it is not possible to conclude with any certainty where the genus originated or the subsequent direction of dispersal. However, there is no evidence against the hypothesis that *Scleranthus* originated in the Northern Hemisphere and spread into Australia via Asia in the late Pliocene or early Pleistocene (section 3.4.6). This is at least consistent with the date estimates derived from ITS sequences section 3.4.5. Under this latter hypothesis it still remains to be explained why no trace of *Scleranthus* is presently found in East or South East Asia.

Other Caryophyllaceae native to Southern floras may also have Northern Hemisphere origins. ITS sequences indicate the Southern genus *Colobanthus* is very recently diverged from (or derived from within) the Northern Hemisphere genus *Sagina.* After establishing in the Southern Hemisphere, especially in New Zealand, *Colobanthus* has undergone rapid radiation in the newly created alpine and sub-alpine habitats. Raven (1973) invokes adherence of minute *Colobanthus* seeds to mud on bird's feet to account for its dispersal. Although *Scleranthus* propagules are larger than those of *Colobanthus*, a similar explanation might be proposed for the apparent dispersal of *Scleranthus*, especially dispersal across the Tasman Sea. Some bird species are known to make this journey (Dorst, 1956). *Scleranthus* is certainly a recent arrival in New Zealand, having dispersed

from Australia at least twice (i.e., independently for the two shared species *S. brockiei* and *S. biflorus*: section 3.4.6). New Zealand endemic *S. uniflorus* was probably derived from *S. biflorus* subsequent to the latter's arrival in New Zealand (section 3.4.6). A less parsimonious alternative is that *S. uniflorus* arose in Australia, dispersed across the Tasman Sea independently of *S. biflorus*, and subsequently became extinct in Australia. The Caryophyllaceae does have a long history in the Southern Hemisphere, as evidenced by the presence of pollen attributed to the family in Oligocene deposits in New Zealand (Muller, 1981). However, as Caryophyllaceae pollen of different lineages is difficult to distinguish, this does not necessarily indicate that any extant lineages have a long history in the area.

5.3 Molecules, morphology and data combining

The widespread application of DNA sequence data to phylogeny reconstruction has led not only to an increase in the number of characters and sets of characters available to systematists, but also to new theoretical challenges inherent in the handling of data from different sources. Controversy exists over whether data from different sources should be analyzed separately (e.g., Miyamoto and Fitch, 1995), combined (e.g., Nixon and Carpenter, 1996), or combined only if not significantly incongruent (e.g., Bull *et al.*, 1993). It is not my intention to review the various arguments that have been put forward by different authors around this issue. However, one aspect of the debate revolves around the recognition that different molecules evolve under different rules and may have different histories (e.g., fast versus slow nucleotide substitution rates, organellar vs. nuclear genomes). This has led some authors to the position that incongruence between data partitions should be assessed before they are combined (Bull *et al.*, 1993). This strategy provides the opportunity to detect cases where different data sets reflect different evolutionary histories

(i.e., through introgression, horizontal transfer paralogy, or lineage sorting,) as well as maximizing the chance of returning a correct estimate of phylogeny. Although by no means universally accepted (see de Queiroz *et al.*, 1995 for review and attempt at a compromise position), such arguments are powerful when applied to combining multiple DNA sequence data sets. Combining sequence data from genes that have evolved at different rates can reduce the chance of returning the correct estimate of phylogeny in simulation studies under certain conditions (Bull *et al.*, 1993).

One of the advantages of DNA sequences as a source of characters for phylogenetic studies is the potential for modeling the processes by which nucleotide substitutions occur. The genetic basis of morphological character change can seldom be addressed in practice. This makes the search for "process partitions", or sets of sequence data whose evolution can be more precisely modeled separately from other sequences a worthwhile pursuit. Several authors have questioned the reality of process partitions (for example, Kluge and Wolf, 1993) and consequently the validity of dividing data into groups for analysis on the basis of congruence. However, as de Queiroz *et al.* (1995) point out, molecular studies do seem to provide "identifiable classes of evidence". I agree that this is true of gene sequences, at least to the extent that an elaborated evolutionary model might apply to some sets of data better than to others. When this is the case, separate analysis of data partitions provides the best opportunity to model the evolution of each, although not necessarily that of the organisms from which the sequences have been obtained.

However, it is doubtful whether any set of morphological characters could be said to have a single history in this sense, or whether any of them have evolved consistently with respect to any single set of assumptions about evolutionary process. Barret *et al.* (1991) point out that the problem of

combining heterogeneous data precedes the application of molecular biology to systematic problems. Clearly mitochondrial, chloroplast and nuclear genes can all contribute to phenotype. While most heritable morphological variation in plants is probably due to variation in nuclear genes, little is likely to be the result of simple mutations in single genes. Some phenotypic characters are probably the result of mutation in several genes, while in other cases a suite of phenotypic characters can be altered by mutation of a single gene. In any case, different nuclear genes are subject to different constraints and selection and are inherited independently in most cases, allowing for different histories and certainly for different evolutionary processes to act on them. Phenotypic characters are subject to influences other than genetics because interactions between organisms and their environment and between co-existing organisms (e.g. symbiotic and host-parasite relationships) have important roles in development. These issues do not necessarily reduce the value of phenotypic characters in tracing phylogenetic relationships between species or higher level taxa, but they do prevent the practical modeling of their evolution along probabilistic lines.

The resistance of some morphologists to molecular systematics, and the early enthusiasm of some molecular systematists for their tools, seems to have created a partly artificially founded debate over how much weight should be applied to each type of data. One of the legacies of this debate, and of the now widespread acceptance of molecular data among systematists, seems to be the treatment of complex morphological data sets as analogous to single gene sequences. While the "more is better" approach to data combining may be inappropriately crude when applied to multiple gene sequences, it may be a necessary prerequisite to assembling data sets based on morphology. Indeed "traditional" plant systematics has tended to regard floral morphology, palynology, embryology, leaf anatomy, and so

on as independent sources of data. Adoption of numerical taxonomic methods has enabled the possibility of treating morphological data of different types together in a single objective analysis. In fact, such combining is usually necessary to accumulate sufficient characters for numerical analysis to be meaningful. Excluding molecular data from such analysis simply because they are not congruent may not be any different from *a-posteriori* exclusion of characters from any other source. Certainly, a difficult group might provide a morphological data set that could easily be split into incongruent data partitions by separating various subsets of characters on an otherwise arbitrary basis. Such an exercise might well be a useful way of exploring data by allowing examination of the effect of removing certain characters or sets of characters on the tree returned. This might in turn provide support for hypotheses of non-independence of some group of characters and indicate a reevaluation of character coding or weight. However suggested morphological data partitions such as hard and soft body parts, larval and adult morphology, head and body are unlikely to fulfill the assumptions of data partitions, being themselves composed of heterogeneous characters.

Once morphological and DNA sequence data sets are combined the opportunity for greater rigor in analysis of the sequence data (through the use of explicit models of evolution) is lost, or at least reduced (with current tools for analysis), regardless of congruence. As Siddall (1997) points out:

"it is not clear to me what form of reasoning could justify the combined analysis of morphological and molecular data together under an HKY85 [nucleotide substitution] *model".*

However, a cladistic "total evidence" approach does allow the integration of data from different sources to trace organismal phylogeny, rather than

the potentially unconnected evolution of process partitions (particularly the creation of a myriad of "gene trees"). Separate analysis of DNA sequences allows better understanding of the evolution of these characters, just as investigation of the genetic underpinnings of morphological characters can improve their interpretation and treatment (although it is seldom practical to do so). However, unless these characters can be integrated into a combined analysis they cannot contribute to reconstructing organismal phylogeny. It remains unclear what criteria the proponents of conditional data combining believe should be used to pick between alternative trees supported by incongruent data sets. Presumably, incongruent partitions are simply ignored when a majority of partitions that are not significantly incongruent have been gathered. If the approach of taxonomic congruence (Miyamoto and Fitch, 1995) is used, then strict consensus trees will be largely unresolved whenever one data set or more supports trees which are widely different from others. If resolved phylogenies are ever to be produced in such circumstances, then incongruent data will have to be excluded (arbitrarily) from analysis, or a tree chosen on the basis of ad-hoc argument.

Specifically where reticulate evolution is suspected (or known) molecular data can result in "gene trees" which differ from "species trees". Morphological data can also yield incorrect trees under such circumstances, as can any data type where phylogeny reconstruction assumes a strict divergent branching pattern. However, provided that the molecular evidence does not swamp morphological evidence through greater numbers of characters, which can be addressed through weighting (Chippindale and Wiens, 1994), combined analysis can still provide increased resolution when DNA sequence and morphological data are available. Clearly the independence of DNA sequence characters from a single gene is suspect because their inheritance is not independent even if the accumulation of

substitutions within the molecule is independent. This alone could provide justification for down-weighting DNA sequence characters in a combined analysis. Molecules which have passed between lineages through hybridization and introgression can still accumulate apomorphies after (or before) reticulation has occurred and provide phylogenetic signal at different levels of a real data analysis.

In a morphological data set for a group of organisms in which reticulation has occurred, characters may have different histories. Unlike gene sequences from a single locus, morphological data sets cannot be assumed to constitute a data partition evolving under a single set of conditions or reflecting a single evolutionary history. Combining morphological data with a small set of sequence characters, such as the nrDNA characters used in this study can, at worst, increase the number and proportion of characters evolving under one set of conditions or with one particular history. Thus, provided that the incongruence between data partitions is acknowledged, there seems to be no compelling reason not to combine data. What must be emphasized is the importance of character mapping and assessment of support for groupings in cladograms, not only on the basis of numbers of characters, but on the reliability of the characters and the feasibility of biological implications. This subjective element of all morphological character systematics does not disappear through the addition of DNA sequence characters.

McDade (1995), in discussing cladistic analysis of groups where hybridization may have occurred, has suggested that morphological cladograms be used as a "reality check" on those derived from other sources. Placing greater reliance on morphology than on single gene sequence data sets is in part a recognition that morphological data sets are heterogeneous assemblages of "process partitions" (independent

characters) and the extent to which they provide clear phylogenetic signal is dependent on the congruence of multiple partitions. This approach seems sensible in the case of this study of *Scleranthus*, although reticulation was not anticipated in advance, and is consistent with unweighted combined analysis in this study because morphology dominates over sequence data where the two are significantly incongruent (section 3.4.3). A higher number of DNA sequence characters might force down-weighting of these to prevent swamping.

I believe that if morphological character data are accepted as appropriate for phylogeny reconstruction, then data combining (regardless of congruence) is, at worst, a necessary evil. Incongruence between DNA sequence data and morphology, if statistically significant, raises questions about the reliability of one or other or both data sets and indicates a need for caution. Nixon and Carpenter (1996) note:

"...homogeneity among characters or sets of characters (partitions) is neither the null hypothesis nor an assumption of parsimony analysis of any data set. If that were the case, then we would only be able to use parsimony to analyze data sets that lacked homoplasy, since homoplasy is indicative of 'heterogeneity' among characters!"

In some circumstances data combining may require down-weighting (of which exclusion is an extreme case) of some subset of data on the grounds that they are unreliable, especially where their independence is suspect. When no objective basis can be found to weight (favor) some characters over others, it may be necessary to advance multiple phylogenetic estimates through separate analyses pending acquisition of additional data. Whether multiple trees are actually more useful than an unresolved or inaccurate combined tree is another matter.

In this study of *Scleranthus*, examination of micromorphology proved useful in providing a number of phylogenetically informative characters from the highly reduced flowers characteristic of the genus. However, these additional characters largely served to increase the confidence of existing hypotheses based on smaller numbers of characters (section 2.4.6). In contrast, nrDNA characters gave further support to some relationships evidenced by morphology (e.g. paraphyly of section *Scleranthus, sensu* West and Garnock-Jones, 1986: section 3.4.2.1) while challenging others (notably the monophyly of section *Mniarum, sensu* West and Garnock-Jones, 1986: section 3.4.3). Seeking biological explanations for the disagreement between data partitions is perhaps the greatest challenge to be faced in combined data studies, but is one that will sometimes yield serendipitous results. The position of Brower, DeSalle and Vogler (1996), that:

"explaining incongruence is not the task of systematics"

reduces cladistics to dogma and begs the question of who should address the real historical processes that have shaped biological evolution, and what use is systematics if it cannot account for them. Whether combined or separate analyses best reveal disagreement in data sets will depend on the data examined and on how results are displayed. Perhaps ironically, the massive expansion of molecular systematics has provided the impetus for renewed study of morphology as their complementary use is shown to provide far more insight into biology than the production of trees from either one alone. As is often stressed, the best solution to data incongruence is the collection of more data. As is discussed in section 3.4.3.2 of this thesis, additional DNA sequence or other macro-molecular information is

most likely to establish the true pattern of evolution in Australasian *Scleranthus*.

5.4 References

Barret, M., Donoghue, M. J., Sober, E. (1991): Against Consensus. Systematic Zoology 40(4): 486-493.

Bittrich, V. (1993): Caryophyllaceae. In K. Kubitzki, J. Rohwer, and V. Bittrich (Eds.) "The families and genera of vascular plants". Volume 2. Magnoliid, Hamamelid, and Caryophyllid families. Springer Verlag, Berlin.

Brower, A. V. Z., DeSalle, R., and Vogler, A. (1996): Gene trees, species trees and systematics: A cladistic perspective. Annual Review of Ecology and Systematics. 27: 423-50.

Bull, J.J., Huelsenbeck, J. P., Cunningham, C. W., Swofford, D. L., Waddell, P. J. (1993): Partitioning and combining data in phylogenetic analysis. Systematic Biology 42(3): 384-397.

Chippindale, P. T. and Wiens, J. J. (1994): Weighting, partitioning , and combining characters in phylogenetic analysis. Systematic Biology 43: 278-287.

de Queiroz, A., Donoghue, M. J., Kim, J. (1995): Separate versus combined analysis of phylogenetic evidence. Annual Review of Ecology and Systematics 26: 657-681.

Dorst, J. (1956): Les Migrations des Oiseaux. Payot, Paris.

Kluge, A. G., and Wolf, A. J. (1993): Cladistics, what's in a word? Cladistics 9: 183-199.

McDade, L., A. (1995): Hybridisation and phylogenetics. In P. C. Hoch and A. G. Stephenson (Eds.) "Experimental and molecular approaches to plant biosystematics". Missouri Botanical Garden.

Miyamoto, M. M., and Fitch, W. M. (1995): Testing species phylogenies and phylogenetic methods with congruence. Systematic Biology 44(1): 64-76.

Muller, J. (1981): Fossil pollen records of extant angiosperms. Botanical Review 47: 1-142.

Nixon, K. C., and Carpenter, J. M. (1996): On similtaneous analysis. Cladistics 12: 221-241.

Pax, F., and Hoffman, K. (1934): Caryophyllaceae. In A. Engler and K. Prantl (Eds.), Die natürlichen pflanzenfamilien, Bd 16c. Engelmann: Leipzig.

Raven, P. H. (1973): Evolution of subalpine and alpine plant groups in New Zealand. New Zealand Journal of Botany 11: 177-200.

Siddal, M. E. (1997): Prior agreement: Arbitration or arbitrary? Systematic Biology 46(4): 765-769.

West, J. G. and Garnock-Jones, P. J. (1986): Evolution and Biogeography

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Appendix 1: *Scleranthus* Morphological character set and coding

1. Leaf form: 0, linear to lanceolate with obtuse to acute tip; 1, spinous[1]
2. Habit: 0, diffusely branched or caesiptose herb; 1, cushion forming[1]
3. Duration: 0, perennial; 1, annual[1]
4. Stem pubescence: 0, pubescent; 1, puberulent; 2, glabrous[1]
5. Vegetative growth form: 0, lateral branches elongating; 1, lateral branches telescoped (fascicled)[1]
6. 2n chromosome number: 0, 22 or 44; 1, 48[1]
7. inflorescence internodes: 0, elongated; 1, reduced[1]
8. peduncle growth: 0, early; 1, delayed[12]
9. Inflorescence size: 0, dichasium; 1, reduced dichasium; 2, diad; 3, monad[1]
10. Bract shape: 0, lanceolate to cordate; 1, deltoid[1]
11. Perigynum: 0, with curled hairs; 1, glabrous[1]
12. Pedicels: 0, ± elongated; 1, reduced[1]
13. Perigynum fibre alignment: 0, longitudinal; 1, tangential
14. Calyx length: 0, ≥ perigynum length; 1, < perigynum length[1]
15. Sepal margins: 0, present; 1, reduced; 2, absent[1]
16. Calyx: 0, five parted; 1, four parted[1]
17. Sepal lateral veins: 0, with separate fibre bundles; 1, fibres fused with sepal median vein or absent
18. Sepals: 0, ± full size at anthesis; 1, grow conspicuously in fruit
19. Nectary: 0, with papillate cells; without papillate cells
20. Nectary shape: 0, disk; 1, tube
21. Nectary: 0, simple; 1, with dichotomising filaments
22. Styles: 0, sunken into ovary; 1, sunken only on sides alternate with style positions; 2 not sunken[3]
23. Stigmatic surface: 0, apex and ventral surface of styles; 1, ± apex of styles only
24. Cells at top of ovary: 0, smooth; 1, papillate[4]
25. Alternisepalous stamen whorl: 0, present at least as staminodes; 1, absent[5]
26. Stamen + staminode number: 0, more than 2; 1, 2; 2, 1[6]
27. Mean pollen diameter: 0, >30 µm; 1, < 30 µm[7]
28. Exine thickness: 0, < 3 µm; 1, >3µm
29. Pollen aperture number: 0, 12; 1, > 12[8]

[1]Denotes characters from West and Garnock-Jones (unpublished).

[2]Those species with monad or diad inflorescences have these born on long peduncles in fruiting stages but are nearly sessile at anthesis.

[3]This character is complicated because of the apparently intermediate form observed in *S. pungens* (character state 2).

[4]This character is absent in *S. biflorus* and *S. uniflorus* because the styles are not sunken.

[5]This character is problematic in *S. pungens* where coding depends on the interpretation of the alternisepalous organs; either as nectary filaments (that is novel structures), or staminodes.

[6]The key issue here is that in *S. annuus, S. pungens* and *S. perennis* more than two primordia form in the androecium (regardless of the number that develop into fertile stamens) while in the other species only one or two primordia form. Rare two-stamened flowers occur in those species coded as single-stamened (state 2).

[7]Pollen diameter varies extensively within anthers such that the range of diameters overlaps between species but the grains of *S. perennis* and *S. annuus* are, on average, considerably larger than those of the other species examined. Ranges and means of pollen grain diameters are discussed in Chapter 2.

[8]Pore number is fixed in *S. perennis* (at twelve) but variable within anthers in other species (although always more than twelve)

Appendix 2: Morphological character matrix for *Scleranthus* species

S. perennis	00000 00000 01100 00010 00000 0011
S. annuus	00100 00000 01101 00010 00000 0010
S. fasciculatus	00011 11120 10012 01000 00111 2100
S. **aff.** *fasciculatus*	00011 ?1120 10012 01000 00??1 2???
S. pungens	10020 ?1000 10000 01000 11001 0100
S. minusculus	00120 ?1000 11001 01100 00101 1100
S. diander	00020 ?1010 10000 01000 00?01 1100
S. uniflorus	01020 11130 10012 11001 021-1 2100
S. biflorus	01020 11120 10012 11001 021-1 2100
S. brockiei	01020 11120 10012 01000 00111 2100
S. singuliflorus'	00010 ?1131 10012 01000 00111 1???

Appendix 3: ITS2 sequences of Caryophyllaceae (alignment arbitrary)

```
                                  1 1111111112 2222222223 3333333334 4444444445
                         1234567890 1234567890 1234567890 1234567890 1234567890
Silene rotundifolia      GTCGCAACAG TTAG------ -GT--TGC-- --CGTTAAGC AAC-TT--AA
Drypis spinosa           ........GA ..TT...... ...AT...AC ..GT.GTGCA ...A.C....
Habrosia spinulifolia    ......G.GT ..TT...... .??.T..... ..GA?A.... ...A......
Arenaria benthammii      .........A ..T....... .......... ..-CGAGG.. ...TCA..GT
Cerastium glomeratum     ........G- ..T....... .TG.AC..A. ..-ACA.G.. G..AAC....
Stellaria media          ........G- ..T....... .TG.TC..A. ..-ACA.G.. ...A.C....
Colobanthus brevisepalus ......G.-- G.TC...... .AA.TC..A. ..-ACG.G.. G..A.C...C
Sagina procumbens        ......G.-- G.TC...... .AA.TC..A. ..-ACG.G.. G..A.C...T
Scleranthus perennis     .......A-- .AG....... .AG.T...A. ..-AGATGA. ...T.C...T
Scleranthus pungens      .......A-- .AG....... .AG.TC..A. ..-AGATG.. ...TCC...T
Vaccaria hispanica       ......TAG. ..T....... ..G.TC.... ..--CG.C.- -..A.C....
Dianthus seguieri        ----...AG. ..T....... .AA.T...A. ..--CA.T.. -.AAAC...T
Saponaria calibrica      .......AG. ..T....... .CC.G...T. ..--AG.... ...G.C....
Psammosilene tunicoides  ........G. ..T....... .AG.T...A. ..-..G.T.- ...T.C....
Pycnophyllum bryoides    ........G. ...-...... ..GGTC..GA ..--CA.G.. ...AC...CG
Drymaria laxiflora       ........T. ..T-...... ..??TC..AA ..--CA.G.. ...AC....-
Spergularia marina       ........G. ..TTGAGCAA C.GCCC..GC TAG.CG.GC. G..GCGTC.G
Polycarpon tetraphyllum  ........G. ..T-..G... .-GGC...AA ..--CG.G.. .G.GCA.C.G
Agrostemma githago       ........G. ..T-...... ..CGT...AC ..--CG.G.. ...T.C...T
```

```
                                                                          1
                         5555555556 6666666667 7777777778 8888888889 9999999990
                         1234567890 1234567890 1234567890 1234567890 1234567890
Silene rotundifolia      AGGGTCCTTC GAGCTCACGT TGG-CATCGT CACGCGCGGC TGGTTG-CTT
Drypis spinosa           T....T.... ....?ACA.C AA...G.GT. GC..T.T.A. A.........
Habrosia spinulifolia    .........A. ......GAAA .C.A..AA.. G.T..AT.A. G.........
Arenaria benthammii      .......... ......TAAA .-.A..AA.. GCT.....A. G........A
Cerastium glomeratum     T......... .....C-TAC ATT...AA.G GTT.....A. C......A..
Stellaria media          . ........A. ....C.GTAG ..T...AG.A GCT.....-. C......T..
Colobanthus brevisepalus .......... .......A.. .T....AA.. GCTA..T.A. G.........
Sagina procumbens        .......... .......A.. .T....AA.. GCTA..T.A. G.........
Scleranthus perennis     ........A. A...A.CTT. ......AA.. .CTA.A..A. G.........
Scleranthus pungens      ........A. A.....CTT. ......AA.. .C.A....A. G........C
Vaccaria hispanica       .......AA. ....G.-ACA A......AAA G.TT..A... A......T.A
Dianthus seguieri        T......AA. ....C.-ACA C--.--.AAC ACAC...AA. G......T..
Saponaria calibrica      ........G. ...TC.-AA. GTA.GCAA.G GTTC...A.. G......T..
Psammosilene tunicoides  ........A. A.....-ACA .TC.GGCA.G .GT.....A. G......T.C
Pycnophyllum bryoides    ........G. ......G.AA G....GG..C GCT..-.... -..A..ATA.
Drymaria laxiflora       .......... ......T.TA G....GAGTC GCT..A..A. -..A..AT..
Spergularia marina       .......... ....G.-..G ..A...AG.G GCT.....A. ......A...
Polycarpon tetraphyllum  .......... .......... .....GGT.A GG...A..A. --A...AGA.
Agrostemma githago       ........C. ....A..... ..A...AA.C G.T....... G.........
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```
                          1111111111 1111111111 1111111111 1111111111 1111111111
                          0000000001 1111111112 2222222223 3333333334 4444444445
                          1234567890 1234567890 1234567890 1234567890 1234567890
Silene rotundifolia       GCTTGGCCGA G---GCCTT- -GTTCACCAC CTATCGCCAC A?CATCCCGA
Drypis spinosa            A.GC....T. .......... ....T..... ...A...... TG.GG.TGC.
Habrosia spinulifolia     ..AC....A. .......... .......... .A.A.....T .G..G.T.A.
Arenaria benthammii       ..AC...A.. C...?..... .......... ....-????? ?.??G.T.A.
Cerastium glomeratum      C.AC...... ....A..... ...A.T.... ........G. GG..A.....
Stellaria media           ...C....A. .......... .......... ........G. GG..G.T...
Colobanthus brevisepalus  ..AC....T. A......... .......... .......... .AA.G.T.AG
Sagina procumbens         ..AC....T. A......... .......... .......... TAA.G.T.AG
Scleranthus perennis      ..AC...AA. A......... ..C..CT... ........G. GAA.GAA...
Scleranthus pungens       ..AC...AA. .......... .......... ........G. GAA.GAA...
Vaccaria hispanica        ..AC...... A......... .......... ........G. GAG.A.TTAG
Dianthus seguieri         A.AC....T. A........A A......... ........G. GG..AATATT
Saponaria calibrica       ...C...... .......... .......... .A.A....G. .G..A.T.CG
Psammosilene tunicoides   .......... .AAG...A.. .......... ...A....G. GG..G.T.AG
Pycnophyllum bryoides     TGAG.A.... .......G.. .......... .A......G. GG..G.T...
Drymaria laxiflora        TGAG...... A......G.. ...A...... .A......G. GG..G.T...
Spergularia marina        AG----..TG .......G.. ...A.T.... .ATA....G. GA..G.T...
Polycarpon tetraphyllum   A--------- -.......-. ....A-.... ...AT...GA G---G.T.C.
Agrostemma githago        ...CA...A. .......... .......... ........G. GA....A.A.

                          1111111111 1111111111 1111111111 1111111111 1111111112
                          5555555556 6666666667 7777777778 8888888889 9999999990
                          1234567890 1234567890 1234567890 1234567890 1234567890
Silene rotundifolia       TGCTGA--GG GCTCCAATTT TAGGCCAGCC GCGACCGGTG AGTCACGGGA
Drypis spinosa            C..CCT..T. .......... .......A.. ..CC...... ..GG......
Habrosia spinulifolia     ...CAT..T. .......... .......A.. ..CC...... ..G-......
Arenaria benthammii       ...CT...A. .......... .......A.. .T.C...... ..G.....-.
Cerastium glomeratum      ...CTT..A. .....TTA.. .......A.. ...C...... ..G.......
Stellaria media           ...CTT..A. .......A.. .......A.. ..AC...... ..G.......
Colobanthus brevisepalus  ...CTC..T. .......A.. .......A.. ..CC...A.. ..GG......
Sagina procumbens         ...CTC..T. .......A.. .......A.. .TCC...... ..AG......
Scleranthus perennis      ...CTT..T. .......A.. .......... ..CC.G.... ..G.G.....
Scleranthus pungens       ...CTT..T. .......A.. .......A.. .TTTTG.... ..GTG...-.
Vaccaria hispanica        .A.CAC.... ......T... .......A.. ..AC.G.... ..G.......
Dianthus seguieri         .A.C.T.... .......G.. ..A....A.. ..AC...... .T.......
Saponaria calibrica       ...C.T..T. ......TA.. .......... ...C...... ..G.......
Psammosilene tunicoides   ...C.T.G-. .....G.A.. ..........- ...C...... ..G.......
Pycnophyllum bryoides     C..C..G.-. -....CGA.. .......... .T.C...... ..G.T.....
Drymaria laxiflora        C..C.TG.-. .....CTA.. .......A.. .T.C.T.... ..G.T.....
Spergularia marina        ...C.TG.-. T.....G... .......... ...C...... ..G.......
Polycarpon tetraphyllum   C..C.TTA-. .....GTA.. .......A.. ...C...... ..G.T.....
Agrostemma githago        C..C.TG.-. .....CT... .......... ...C...... ..G.......
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                          2222222222 2222222222 2222222222 2222222222 2222222222
                          0000000001 1111111112 2222222223 3333333334 4444444445
                          1234567890 1234567890 1234567890 1234567890 1234567890
Silene rotundifolia       GACCA-TCCT CCTT-CCCCT -CCA--CTCA GAGTGG-ATT T-GGGGGAGA
Drypis spinosa            AG......A. ......G.-. AA.....G.T AG...-.-.G ..A...A...
Habrosia spinulifolia     AG......A. ...A...... T......G.. AGA....-.G A.........
Arenaria benthammii       AG........ .......... C..C..A... AG.....GAG A.........
Cerastium glomeratum      A......... ...CC..T.A A.T....CG. AGTG.-.T.G ..-....G.T
Stellaria media           AG......T. ....C...A. C......G.. AG...-.G.G ..-....G-T
Colobanthus brevisepalus  A.......T. ...C...... T......A.T AG...-.G.. ..-.......
Sagina procumbens         A......... ...C....T. T......A.T AG...-.G.. ..-...-..-
Scleranthus perennis      AG........ ..CCT...T. T.A....C.G TG.CT-.G.G .A.........
Scleranthus pungens       AG........ ..CCT...T. T.A....A.. TG.CT-.G.G .A.....G..
Vaccaria hispanica        AG......A. .......... C.A-....G. TT.G.-.T.G ..........
Dianthus seguieri         AT......A. .........A C.A-..A.GT ..T..-.G.G ..........
Saponaria calibrica       AG........ .......... CAAT..A... AG...ATT.G ..A.......
Psammosilene tunicoides   AG........ .......... C.A-.....G AG...-.GGG .........?
Pycnophyllum bryoides     .G......T. ....C...A. C.A-.....G AG.....G.G ..........
Drymaria laxiflora        .G........ ....C...A. C..-..TC.G TG.....GAA ..........
Spergularia marina        AG......-- .G.CCT..TC C..-.A.G.G .G.....G.G ..........
Polycarpon tetraphyllum   ........T. ...CC...GC C..GCA.G.. .G.....TGG ..........
Agrostemma githago        AG........ ....C...AC C..-.A.A.. AG...A.GCG ......A...
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                          2222222222 22
                          5555555556 66
                          1234567890 12
Silene rotundifolia       CGCGATGCGT GA
Drypis spinosa            .......... ..
Habrosia spinulifolia     ....------ --
Arenaria benthammii       TCGA...... ..
Cerastium glomeratum      T......... ..
Stellaria media           T......... ..
Colobanthus brevisepalus  .......... ..
Sagina procumbens         .......... ..
Scleranthus perennis      T..A....A. ..
Scleranthus pungens       T..A....A. ..
Vaccaria hispanica        ...A...... ..
Dianthus seguieri         .......... ..
Saponaria calibrica       .......... ..
Psammosilene tunicoides   .......... ..
Pycnophyllum bryoides     .......... ..
Drymaria laxiflora        .......... ..
Spergularia marina        .......... ..
Polycarpon tetraphyllum   .......... ..
Agrostemma githago        .......... ..
```

Appendix 4: ITS2 sequence alignment used for divergence date estimation

```
                                  1 1111111112 2222222223 3333333334 4444444445
                         1234567890 1234567890 1234567890 1234567890 1234567890
                         GGGGTCGCAA A--TAGGAGT TGCAAGATGA CAACT-TCAT AGGGTCCTAC
S. perennis              .......... .......... .........G .......... ..........
S. fasciculatus          .......... .......... .........G .......... ..........
S. aff. fasciculatus     .......... .......... .........G ........G. ..........
S. singuliflorus (AUS)   .......... .......... .........G .......... ..........
S. uniflorus             .......... .......... .........G .......... ..........
S. biflorus (NZ).......  .......... .......... .........G .......... .........G
S. brockiei (NZ)         .......... .......... C........G ......C... ..........
S. pungens               .........G .......... C........G ......C... ..........
S. minusculus            .......... CTG.TT.??. C....C.A.G ....-.A.TA .........T.
Drymaria laxiflora       .......... CGG.TT.G.C .....CGA.G ..G.GCA..G .........T.
Polycarpon tetraphyllum

                                                                              1
                         5555555556 6666666667 7777777778 8888888889 9999999990
                         1234567890 1234567890 1234567890 1234567890 1234567890
                         AAGCACCTTT TGGCAAAGTC CTACACGACG GGTTGCTTGC ACGGCAAAAG
S. perennis              ....T..... C......... ..G.G..... .......... .........G.
S. fasciculatus          ....T..... .......... ....G..... .......... .........G.
S. aff. fasciculatus     ....T..... .......... .......... .......... .........G.
S. singuliflorus (AUS)   ....T..... .......... .C........ .......C.. .........G.
S. uniflorus             ....T..... .......... .C........ .......C.. .........G.
S. biflorus (NZ)         ....T..... .......... .C........ .......C.. .........G.
S. brockiei (NZ)         ....T..... .......... .C..G..... .......C.. .........G.
S. pungens               ....T..... .......... .C..G..... ..C....C.. .........G.
S. minusculus            G...T.TC.A G...G.GTCG ..G....... .A.GAT..TG .G...CG...
Drymaria laxiflora       G...T.ACG. ....GGT.AG GCG......- ----AT.GAG .T------.--
Polycarpon tetraphyllum

                         1111111111 1111111111 1111111111 1111111111 1111111111
                         0000000001 1111111112 2222222223 3333333334 4444444445
                         1234567890 1234567890 1234567890 1234567890 1234567890
                         CCTTGCTCCT CACCTATCGC CGCGAAAGAA CGATGCCTTT -GGCTCCAAA
S. perennis              .....T..AC .......... .......... .......... ..........
S. fasciculatus          .....T..AC .......... .......... .......... ..........
S. aff. fasciculatus     .....T..AC .......... .......... .......... ..........
S. singuliflorus (AUS)   .....T..AC .......... .......... .......... ..........
S. uniflorus             .....T..AC .......... .......... .......... ..........
S. biflorus (NZ)         .....T..AC .......... .......... .......... ..........
S. brockiei (NZ)         .....T..AC .......... .......... .......... ..........
S. pungens               .....T..AC .......... .......... .......... ..........
S. minusculus            .....T..AC .......... .......... .......... ..........
Drymaria laxiflora       ..G..TA.AC ....A..... ....GC..CT ...C...G.G .......CT.
Polycarpon tetraphyllum  ---..T.-AC ......AT.. ..A.G---CT .C.C...G.. A......GT.
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```
                                1111111111 1111111111 1111111111 1111111111 1111111112
                                5555555556 6666666667 7777777778 8888888889 9999999990
                                1234567890 1234567890 1234567890 1234567890 1234567890
                                TTTAGGCCAG CCG-CCCCGG GTGAGGCGCG GGAAGCCATC CTCCCCTCCC
S. perennis                     .........A .......... .......... ........A. ..........
S. fasciculatus                 .........A .......... .......... ........A. ..........
S. aff. fasciculatus            .........A .......... .......... .......... ..........
S. singuliflorus (AUS)          .........A .......... .......... .......... ..........
S. uniflorus                    .........A .......... .......... .......... ..........
S. biflorus (NZ)                .........A .......... .......... .......... ..........
S. brockiei (NZ)                .........A .......... .......... .......... ..........
S. pungens                      .........A .......... .......... .......... ..........
S. minusculus                   .........A .......... .......... .......... ..........
Drymaria laxiflora              .........A ....TG..T. .......T.. ...G...... ....TTC...
Polycarpon tetraphyllum         .........A .....G..C. .......T.. ...GA..... T...T.C...

                                2222222222 2222222222 2222222222 2222222222 22
                                0000000001 1111111112 2222222223 3333333334 44
                                1234567890 1234567890 1234567890 1234567890 12
                                TTTCAACC-- CGTGGCTGTG TAGGGGGAGA TGCAATGCAT GA
S. perennis                     .C........ .A........ .......G.. .---------- --
S. fasciculatus                 .......... .A........ .......G.. .---------- --
S. aff. fasciculatus            .......... .A........ .......G.. .---------- --
S. singuliflorus (AUS)          .......A.. .A........ .......G.. .---------- --
S. uniflorus                    .......A.. .A........ .......G.. .---------- --
S. biflorus (NZ)                .......A.. .A...A.... .......G.. .---------- --
S. brockiei (NZ)                .......A.. .A........ .......G.. .---------- --
S. pungens                      .......A.. .A........ .-......G.. .---------- --
S. minusculus                   A.C.CT.-.. .....TG.GA AT........ C..G....G. ..
Drymaria laxiflora              GCC.CG.ACG .AG..TG... GT........ C..G....G. ..
Polycarpon tetraphyllum
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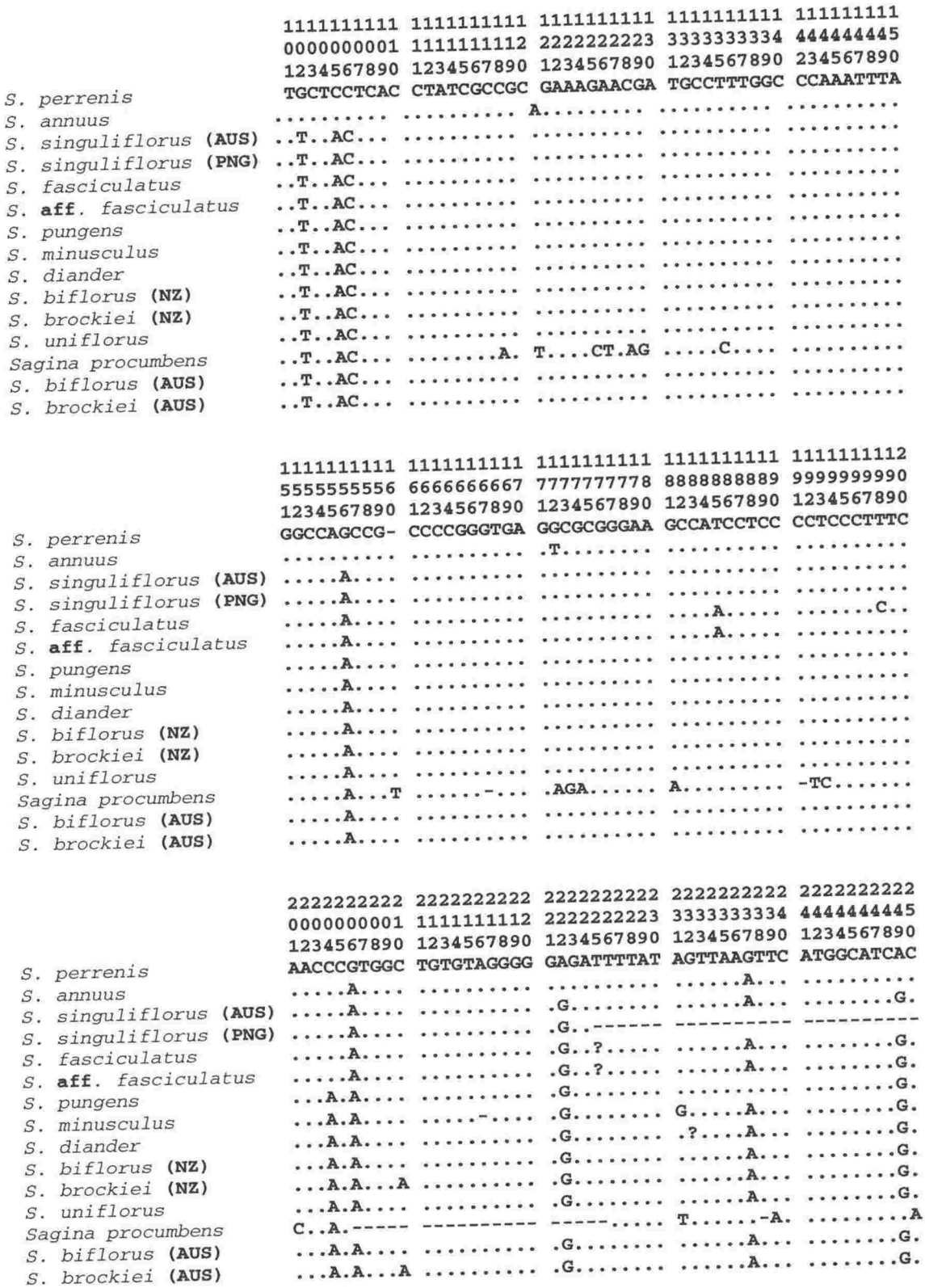
Appendix 5: Aligned *Scleranthus* ITS1 and ITS2 sequences

```
                          1 1111111112 2222222223 3333333334 4444444445
                 1234567890 1234567890 1234567890 1234567890 1234567890
                 GGGGTCGCAA ATAGGAGTTG CAAGATGACA ACTTCATAGG GTCCTACAAG
S. perrenis      .......... .......... .......... .......... ..........
S. annuus        .......... .......... .......G.. .....G.... ..........
S. singuliflorus (AUS) .......... .......... .......G.. .....G.... ..........
S. singuliflorus (PNG) ???????.. .......... .......G.. .....G.... ..........
S. fasciculatus  ???????.. .......... .......G.. .......... ..........
S. aff. fasciculatus .......... .........C. .......G.. ...C...... ..........
S. pungens       .......... .........C. .......G.. ...C...... ..........
S. minusculus    .......... .........?. .......G.. ...?...... ..........
S. diander       .......... .......... .......G.. .......... ..........
S. biflorus (NZ) .......... .......... .......G.. .......... ......G...
S. brockiei (NZ) .......... .......... .......G.. .......... ..........
S. uniflorus     .....?...G CGTTC.A.C. ...CGA.G.G ..A....... .....T.G..
Sagina procumbens .......... .......... .......G.. .......... ..........
S. biflorus (AUS) .......... .......... .......G.. .......... ..........
S. brockiei (AUS)
                                                                        1
                 5555555556 6666666667 7777777778 8888888889 9999999990
                 1234567890 1234567890 1234567890 1234567890 1234567890
                 CACCTTTTGG CAAAGTCCTA CACGACGGGT TGCTTGCACG GCAAAAGCCT
S. perrenis      .T........ .......... .......... .......... .....G....
S. annuus        .T........ .......... .......... .......... .....G....
S. singuliflorus (AUS) .T........ .......... .......... .......... .....G....
S. singuliflorus (PNG) .T.....C.. .........G .G........ .......... .....G....
S. fasciculatus  .T........ .......... .G........ .......... .....G....
S. aff. fasciculatus .T........ .........C. .G........ ....C..... .....G....
S. pungens       .T........ .........C. .G.......C ....C..... .....G....
S. minusculus    .T........ .........C. .?.......? ....C..... .....G....
S. diander       .T........ .........C. .......... ....C..... .....G....
S. biflorus (NZ) .T........ .........C. .......... ....C..... .....G....
S. brockiei (NZ) .T........ .........C. .......... ....C..... .....G....
S. uniflorus     .T.AAG..T. ......G... .GT....... .......... ..CT......
Sagina procumbens .T........ .........C. .......... ....C..... .....G....
S. biflorus (AUS) .T........ .........C. .......... ....C..... .....G....
S. brockiei (AUS)
```


1111111111 1111111111 1111111111 1111111111 111111111
0000000001 1111111112 2222222223 3333333334 444444445
1234567890 1234567890 1234567890 1234567890 234567890
TGCTCCTCAC CTATCGCCGC GAAAGAACGA TGCCTTTGGC CCAAATTTA
S. perrenis .... A..... ....
S. annuus ..T..AC... ....
S. singuliflorus (AUS) ..T..AC... ....
S. singuliflorus (PNG) ..T..AC... ....
S. fasciculatus ..T..AC... ....
S. aff. fasciculatus ..T..AC... ....
S. pungens ..T..AC... ....
S. minusculus ..T..AC... ....
S. diander ..T..AC... ....
S. biflorus (NZ) ..T..AC... ....
S. brockiei (NZ) ..T..AC... ....
S. uniflorus ..T..AC... .....A. T....CT.AG .....C.... .....
Sagina procumbens ..T..AC... ....
S. biflorus (AUS) ..T..AC... ....
S. brockiei (AUS)
1111111111 1111111111 1111111111 1111111111 1111111112
5555555556 6666666667 7777777778 8888888889 9999999990
1234567890 1234567890 1234567890 1234567890 1234567890
GGCCAGCCG- CCCCGGGTGA GGCGCGGGAA GCCATCCTCC CCTCCCTTTC
S. perrenis .... .T..... ....
S. annuus .....A.... ....
S. singuliflorus (AUS) .....A.... ....A..... .....C..
S. singuliflorus (PNG) .....A.... ....A..... .....
S. fasciculatus .....A.... ....
S. aff. fasciculatus .....A.... ....
S. pungens .....A.... ....
S. minusculus .....A.... ....
S. diander .....A.... ....
S. biflorus (NZ) .....A.... ....
S. brockiei (NZ) .....A.... ....
S. uniflorus .....A...T .....-... .AGA..... A..... -TC.....
Sagina procumbens .....A.... ....
S. biflorus (AUS) .....A.... ....
S. brockiei (AUS)
2222222222 2222222222 2222222222 2222222222 2222222222
0000000001 1111111112 2222222223 3333333334 4444444445
1234567890 1234567890 1234567890 1234567890 1234567890
AACCCGTGGC TGTGTAGGGG GAGATTTTAT AGTTAAGTTC ATGGCATCAC
S. perrenis .....A.... ....A... .....
S. annuus .....A.... ..... .G..... .....A... .....G.
S. singuliflorus (AUS) .....A.... ..... .G..----- ---------- ----------
S. singuliflorus (PNG) .....A.... ..... .G..?..... .....A... .....G.
S. fasciculatus .....A.... ..... .G..?..... .....A... .....G.
S. aff. fasciculatus ...A.A.... ..... .G..... ....G.
S. pungens ...A.A.... .....-.... .G..... G.....A... .....G.
S. minusculus ...A.A.... ..... .G..... .?....A... .....G.
S. diander ...A.A.... ..... .G..... .....A... .....G.
S. biflorus (NZ) ...A.A...A ..... .G..... .....A... .....G.
S. brockiei (NZ) ...A.A.... ..... .G..... .....A... .....G.
S. uniflorus C..A.----- ---------- -----..... T.....-A. .....A
Sagina procumbens ...A.A.... ..... .G..... .....A... .....G.
S. biflorus (AUS) ...A.A...A ..... .G..... .....A... .....G.
S. brockiei (AUS)

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                          2222222222 2222222222 2222222222 2222222222 2222222223
                          5555555556 6666666667 7777777778 8888888889 9999999990
                          1234567890 1234567890 1234567890 1234567890 1234567890
                          GCCCAT-CCG CCAGACAACC TAGCGCACGG ACACAACACA CTATGAGTTC
S. perrenis               .......... .......... .......... .......... ..........
S. annuus                 A......... ..G....... G......T.. ..G....... .A.C......
S. singuliflorus (AUS)    ?????????? ??G....... G......T.. ..G....... .A.C......
S. singuliflorus (PNG)    A......... ..G....... G......T.. .......... .A.C......
S. fasciculatus           A......... ..G....... G......T.. .......... .A.C......
S. aff. fasciculatus      A......... ..G....... G......T.. ..G....... .A.A......
S. pungens                ........T. ..G....... G......T.. ..G....... .A.C......
S. minusculus             ?......... ..G....... G......T.. ..G....... .A.C......
S. diander                A......... ..G....... G......T.. ..G....??? ???C......
S. biflorus (NZ)          .......... ..G....... G......T.. ..G....... .A.C......
S. brockiei (NZ)          A......... ..G....... G......T.. ..G....... .A.C......
S. uniflorus              A...T.CAAA ..G.CT.... G-..CA.A.. .T.G.G.... .-..T....T
Sagina procumbens         A......... ..G....... G......T.. ..G....... .A.C......
S. biflorus (AUS)         .......... ..G....... G......T.. ..G....... .A.C......
S. brockiei (AUS)

                          3333333333 3333333333 3333333333 3333333333 3333333333
                          0000000001 1111111112 2222222223 3333333334 4444444445
                          1234567890 1234567890 1234567890 1234567890 1234567890
                          ATGTTCCTTG ACGCTTTTAG CGCCGGGGTT CGTTGAATTG TCAAGCTAAC
S. perrenis               .......... .......... .......... .......... ..........
S. annuus                 .......... .......... .......... .......... C.........
S. singuliflorus (AUS)    .......... .......... .......... .......... C.........
S. singuliflorus (PNG)    .......... .......... .......... .......T... C.......G..
S. fasciculatus           .......... .......... .......... .......... C.........
S. aff. fasciculatus      .......... .......... .......... .......... C.........
S. pungens                .......... .......... .......... .......... C.........
S. minusculus             .......... .......... .......... .......... C.........
S. diander                .......... .......... .......... .......... C.........
S. biflorus (NZ)          .......... .......... .......... .......... C.........
S. brockiei (NZ)          .......... .......... .......... .......... C.........
S. uniflorus              .......... .......... .......... ....TTT... C...A.-G..
Sagina procumbens         .........A .........C. .......... .......... C.........
S. biflorus (AUS)         .......... .......... .......... .......... C.........
S. brockiei (AUS)

                          3333333333 3333333333 3333333333 3333333333 3333333334
                          5555555556 6666666667 7777777778 8888888889 9999999990
                          1234567890 1234567890 1234567890 1234567890 1234567890
                          ATCCCACTGG TGGGAGGCTG CCCGACTCAG GCAAGAGGCA CGCGAGGGCA
S. perrenis               .......... .......... .......... .......... ..........
S. annuus                 .......... .......... .......... .A........ ..........
S. singuliflorus (AUS)    .......... .......... .......... .......... ..........
S. singuliflorus (PNG)    .......... .......... .......... .......... ..........
S. fasciculatus           .......... .......... .......... .......... ..........
S. aff. fasciculatus      .......... .......... .......... .......... ..........
S. pungens                .......... .......... .......... .......... ..........
S. minusculus             .......... .......... .......... .......... ..........
S. diander                .......... .......... .......... .......... ..........
S. biflorus (NZ)          .......... .......... .......... .......... ..........
S. brockiei (NZ)          .......... .......... .......... .......... ..........
S. uniflorus              .......... .......... .......... .......... ..........
Sagina procumbens         G.T.TG---- ---------- ---------- ---------- ..A.CAA...
S. biflorus (AUS)         .......... .......... .......... .......... ..........
S. brockiei (AUS)         .......... .......... .......... .......... ..........
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```
                          4444444444 4444444444 4444444444 4444444444 4444444444
                          0000000001 1111111112 2222222223 3333333334 4444444445
                          1234567890 1234567890 1234567890 1234567890 1234567890
                          CAAGGGCGCC AAGCGAACCC ACTGCACCTG TGAGGGATGA ACATGTTCGC
S. perrenis               .......... .......... .......... .......... ..........
S. annuus                 .......... .......... G......... .......... ..........
S. singuliflorus (AUS)    .......... .......... G.......?? ?????????? ??????????
S. singuliflorus (PNG)    .......... .......... G......... .......... ..........
S. fasciculatus           .......... .......... G......... .......... ..........
S. aff. fasciculatus      .......... .......... G......... .......... ..........
S. pungens                .......... .......... G......... .......... ..........
S. minusculus             .......... .......... G......... .......... .......???
S. diander                .......... .......... G......... ..G....... ..........
S. biflorus (NZ)          .......... .......... G......... .......... ..........
S. brockiei (NZ)          .......... .......... G......... .......... ..........
S. uniflorus              .......... .......... G......... .......... ..........
Sagina procumbens         ..CAA.---- ---------- ---------. ..GATTG.T. ..........
S. biflorus (AUS)         .......... .......... G......... .......... ..........
S. brockiei (AUS)         .......... .......... G......... .......... ..........
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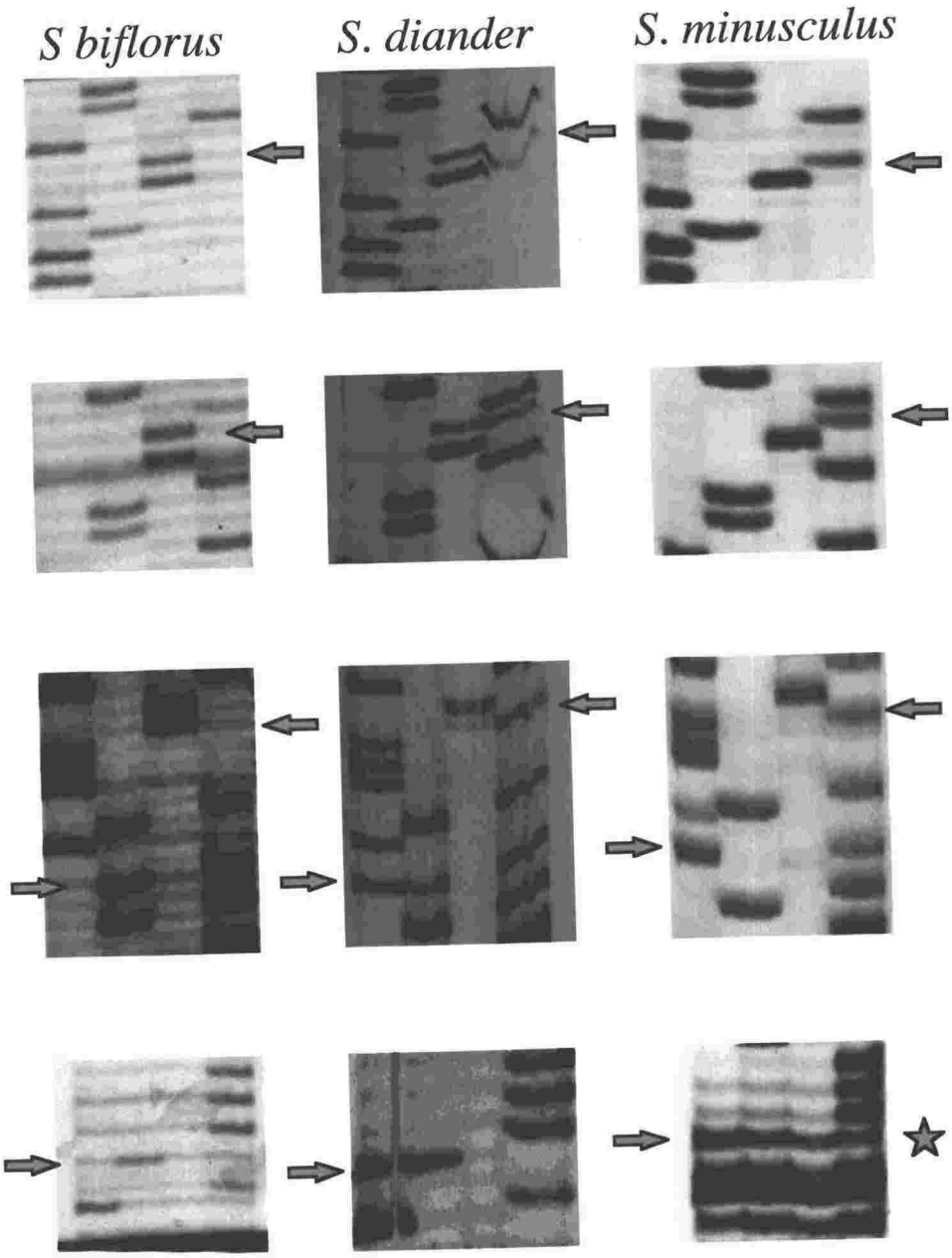
                          4444444444 4444444444 4444
                          5555555556 6666666667 7777
                          1234567890 1234567890 1234
                          CGGTCGTTCT GCTGAGCAGG TTTC
S. perrenis               .......... .......... ....
S. annuus                 .......... ........?? ????
S. singuliflorus (AUS)    ?????????? ?????????? ????
S. singuliflorus (PNG)    .......... .......... ....
S. fasciculatus           .......... .......... ....
S aff. fasciculatus.......... .......... ....
S. pungens                .......... .......... ....
S. minusculus             .......... .......... ....
S. diander                ?????????? ?????????? ????
S. biflorus (NZ)          .......... .......... ....
S. brockiei (NZ)          .......... .......... ....
S. uniflorus              .......... .......... ....
Sagina procumbens         T......... ...A...... ....
S. biflorus (AUS)         .......... .......... ....
S. brockiei (AUS)         .......... .......... ....
```

Appendix 6: Comparison of double sequence in *S. diander* with *S. biflorus* and *S. minusculus* sequences



* This site is determined as a'G' by cycle sequencing (G-C intrastrand pairing causes stop artifacts on gel)

Appendix 7: Aligned Caryophyllaceae *ndh*F sequences

```
                                  111 111 111 122 222 222 223 333 333 333
                          123 456 789 012 345 678 901 234 567 890 123 456 789
Mollugo verticillata      --A ATA GGA ATC GGA CTT CTA CTT TTT CCT ACG GCA ACA
Scopulophila rixtordii    ... ... ..G G.G ... ... ..C ... ... ..G ..A ... ...
Loeflingia squarrosa      ... ... ..G G.G ... ... ..C ... ... ..G ..A ... ...
Spergularia marina        ... ... ... G.G ... ... ..C T.. ... ..G ..A ... ...
Polycarpon tetraphyllum   ... ... ..G G.G ... ... ..C ... ... ..G ... ... ...
Hernaria glabra           ... ... ... G.A ... ... ..C ... ... ..G ... ... ...
Drymaria laxiflora        ... ..C ..G G.G ... ... ..C ..C ... ..G ... ... ...
Silene antirrhina         ... ... ... G.G ... ... ..C ... ... ..A ..A ... ...
Dianthus caryophyllus     ... ... ... G.G ..C ... ..C ... ... ..G G.A .TG ...
Dicleranthus plocamoides  ... ... ..G G.G ... ... ..C ... .GG ..G ... A.. ...
Paronychia drummondii     ... ... ... G.G ... ... ..C T.. ..C ..G ... T.. ...
Arenaria benthamii        ... ... ... G.G ... ... ..C ... ... ..G ..A ... .T.
Cerastium glomeratum      ... ... ... G.G ..G ... ... ... ... ..G .GA ... ...
Stellaria media           ... ... ... G.A ..G ... ... ... ... ..G ..A A.. ...
Atriplex canescens        ... ... ... TCT ... ... T.. T.. ... ..G .TA ... ...
Colobanthus brevisepalus  ... ... ... G.G ... ... ..C ... ... ..G ..A ... ...
Scleranthus biflorus      ... ... ... ..G ... ... ..C ... ... ..A ..C ... ...

                          444 444 444 455 555 555 556 666 666 666 777 777 777
                          012 345 678 901 234 567 890 123 456 789 012 345 678
Mollugo verticillata      AAA AAT CTT CGG CGT ATA TGG GCT TTT TCA AGT ATT TCG
Scopulophila rixtordii    ... ... ... ... ... ... ... ..G ... .GT ... ... ..T
Loeflingia squarrosa      ..G ... ... ... ... ... ... T.. ... .GT ... ... ..T
Spergularia marina        ... ... ... ... ... ... ... ... ... ..G ... ... .TT
Polycarpon tetraphyllum   ... ... ... ... ... ... ... T.. ... ..T ... ... ..T
Hernaria glabra           ... ... ... ... ... ..G ... ... ... ..T ... ... ..C
Drymaria laxiflora        ... G.. ... ... ... ... ... T.. ... .GT ... ... ..T
Silene antirrhina         ... .C. ... ... ..G ..G ... ... ... ..T ... ... ..T
Dianthus caryophyllus     ... ... ... ... ... ..G ... ... ... ..T ... ... ..C
Dicleranthus plocamoides  ... ... ... ..T ... ... ... TT. ... .GT ... ... ..T
Paronychia drummondii     ... ... ... ... ... ... ... ... ... ..T ... ... ..C
Arenaria benthamii        G.. ... .G. A.. ... ... ... ... ... ..T ... ... G.T
Cerastium glomeratum      ... ... ... ..T ... ... ... ..G ... ..T ... ... .TT
Stellaria media           ... ... T.. ..A ... ... ... ..C ... ..T ... ... .TT
Atriplex canescens        ... ..A ... ... ... ... ... ... ... ..C ... ... .T.
Colobanthus brevisepalus  ... ... ... ..A ... ... ... ... ... ..T ... ... ..T
Scleranthus biflorus      ... ... ... ... ... ... ... ..G ... ..T ... ... ..T

                                                      111 111 111 111 111 111
                          788 888 888 889 999 999 999 000 000 000 011 111 111
                          901 234 567 890 123 456 789 012 345 678 901 234 567
Mollugo verticillata      TTG TTA AGT ATA GTT ATG ATT TTT TCG ATG AAG CTG TCT
Scopulophila rixtordii    ... ... ... ... .C. ... C.. ... ..A C.. .T. T.. ..G
Loeflingia squarrosa      ... ... ... C.. .C. ... C.. ... ... ... .T. ... ..G
Spergularia marina        ..A ... .C. ... .C. ... ... ... ... ... .C. ... ..G
Polycarpon tetraphyllum   C.. ... ... ... .C. ... C.. ... ... C.. .T. T.. G.G
Hernaria glabra           ... ... ... ... .CA ... ... ... ... C.. .C. ... ..G
Drymaria laxiflora        ... ... ... ... .C. ... C.. ... ... C.. .T. ... ..G
Silene antirrhina         ... ... .C. ... .C. ... ... ... ... ... .C. ... G.G
Dianthus caryophyllus     ... ... .C. ... .C. ... ... ... ... ... .CA ..C ..G
Dicleranthus plocamoides  ... ... ... ... .C. ... C.. ... ... C.. CT. ... ..G
Paronychia drummondii     ... ... ..C ... .C. ... T.. ... ... G.. .T. ... ..C
Arenaria benthamii        ... ... .C. ... .C. ... ... ... ..T ..T .TA ... ..G
Cerastium glomeratum      ... ... .C. ... .C. ... C.. ... .G. ..C .C. T.. ..G
Stellaria media           ... ... .C. ... .C. ... C.. ... .G. ..C .C. T.. ..G
Atriplex canescens        ..A ... ... ... ... ... G.. ... ..C ... ..A ... G..
Colobanthus brevisepalus  ... ... .C. ... .C. ... T.. ... ... ... .TC G.. ..G
Scleranthus biflorus      ... ... .C. T.T .C. ... C.. ... ... ... .C. ... ..G
```

	111	111	111	111	111	111	111	111	111	111	111	111	111
	112	222	222	222	333	333	333	344	444	444	445	555	555
	890	123	456	789	012	345	678	901	234	567	890	123	456
Mollugo verticillata	ATT	CAG	CAA	ATA	AAT	GGT	AAT	TCT	ATT	TAT	CAA	TAT	GTA
Scopulophila rixtordii	...	..A	...	..?	..A	A..	...	...	...	...	...	...	...
Loeflingia squarrosa	...	..A	...	...	...	A.C	...	..G	...	...	...	...	...
Spergularia marina	.G.	..A	...	...	...	A..	...	..A	...	...	...	...	...
Polycarpon tetraphyllum	...	..A	...	...	...	A.C	...	.T.	...	...	...	...	...
Hernaria glabra	...	..A	...	...	...	AAC	...	..G	...	...	...	...	..C
Drymaria laxiflora	...	..A	...	...	...	A.C	...	..C	.G.	...	...	...	...
Silene antirrhina	...	..A	...	...	...	A..	...	..C	.C.	...	...	...	...
Dianthus caryophyllus	...	..A	...	...	...	A..	...	...	...	...	...	...	..C
Dicleranthus plocamoides	...	..A	...	...	...	A.C	...	..G	...	...	...	...	...
Paronychia drummondii	...	..A	...	...	...	AAC	...	...	...	...	...	...	...
Arenaria benthamii	...	..A	...	...	...	A..	...	..A	...	...	...	...	...
Cerastium glomeratum	...	..C	...	...	...	A..	...	..C	...	...	...	...	...
Stellaria media	...	..C	...	...	...	A..	...	..C	...	.G.	...	...	...
Atriplex canescens	...	..A	...	...	...	AA.	...	..C	.C.	...	...	...	...
Colobanthus brevisepalus	...	..A	...	...	...	AA.	...	..C	...	...	...	...	...
Scleranthus biflorus	...	..A	...	..C	..C	A..	C..	.T.	...	.T.	...	...	...

	111	111	111	111	111	111	111	111	111	111	111	111	111
	555	666	666	666	677	777	777	778	888	888	888	999	999
	789	012	345	678	901	234	567	890	123	456	789	012	345
Mollugo verticillata	TGG	TCT	TGG	ACT	ATT	AAT	AAT	GAT	TTT	TCT	TTA	GAA	TTT
Scopulophila rixtordii	...	...	...	..C	...	...	.C.	A..	...	...	...	...	...
Loeflingia squarrosa	...	...	...	.GC	...	...	.C.	...	...	..C	...	...	...
Spergularia marina	...	...	...	...	...	...	...	...	...	...	...	...	...
Polycarpon tetraphyllum	...	...	...	...	...	...	.C.	...	...	...	...	...	...
Hernaria glabra	...	...	...	..C	...	...	...	...	...	...	...	...	...
Drymaria laxiflora	...	...	...	..G	...	...	.C.	...	...	...	...	...	...
Silene antirrhina	...	...	...	...	...	...	...	...	...	...	..T	...	...
Dianthus caryophyllus	...	...	...	...	...	...	...	...	...	...	..T	...	..C
Dicleranthus plocamoides	...	...	...	..C	...	...	.C.	...	...	.G.	...	...	...
Paronychia drummondii	..C	..C	...	..G	...	...	...	...	...	...	...	..G	...
Arenaria benthamii	...	..A	...	...	...	...	...	...	...	..G	..T	...	...
Cerastium glomeratum	...	...	...	..C	...	...	...	...	...	..G	..C	...	...
Stellaria media	...	...	...	..C	...	...	...	...	..A	..G	..C	...	...
Atriplex canescens	...	...	...	...	...	...	...	...	...	...	..T	...	...
Colobanthus brevisepalus	...	...	...	...	...	...	...	...	...	...	...	...	...
Scleranthus biflorus	...	...	...	...	...	...	...	...	...	A..	...	...	...

	111	122	222	222	222	222	222	222	222	222	222	222	222
	999	900	000	000	001	111	111	111	222	222	222	233	333
	678	901	234	567	890	123	456	789	012	345	678	901	234
Mollugo verticillata	GGT	TAC	TTG	ATC	GAT	CCA	CTT	ACC	TCT	ATT	ATG	TCA	ATG
Scopulophila rixtordii	..C	...	...	C..	...	..G	...	..T	...	...	...	..G	...
Loeflingia squarrosa	..C	...	...	C..	...	..G	...	..A	...	...	...	..G	...
Spergularia marina	..C	...	...	T..	...	...	...	..T	...	...	...	...	G..
Polycarpon tetraphyllum	..C	..T	..A	...	...	..G	...	..T	...	...	...	..G	...
Hernaria glabra	..C	..T	...	T.T	...	...	...	..T	...	...	...	G..	G..
Drymaria laxiflora	..C	...	...	C..	...	..G	...	..T	...	...	...	..G	...
Silene antirrhina	..C	..T	...	C..	...	...	...	..T	...	...	...	.T.	G.C
Dianthus caryophyllus	..C	...	...	...	...	...	...	..T	...	...	...	...	G.T
Dicleranthus plocamoides	..C	...	...	C.A	...	..G	...	..T	...	...	...	..G	..A
Paronychia drummondii	..C	...	...	C.T	...	...	...	..T	...	...	...	...	G.A
Arenaria benthamii	...	...	...	T..	...	...	...	.GT	...	...	...	.T.	G.C
Cerastium glomeratum	..C	...	...	C..	...	...	...	..T	...	...	...	...	G.C
Stellaria media	..C	...	...	T..	...	...	...	..T	...	...	...	...	G.C
Atriplex canescens	..C	..T	...	C..	...	...	...	..T	...	...	...	.T.	G.C
Colobanthus brevisepalus	..C	..T	...	T..	...	...	...	..T	...	...	...	...	G.C
Scleranthus biflorus	...	...	...	T.T	...	...	...	..T	...	...	...	.T.	G..

	222	222	222	222	222	222	222	222	222	222	222	222	222
	333	334	444	444	444	555	555	555	566	666	666	667	777
	567	890	123	456	789	012	345	678	901	234	567	890	123
Mollugo verticillata	TTA	ATC	ACT	ACT	GTT	GCA	ATT	TTG	GTT	CTT	ATT	TAT	AGT
Scopulophila rixtordii	...	..T	...	...	...	...	...	...	...	...	...	...	...
Loeflingia squarrosa	...	..A	...	...	...	...	...	...	...	...	...	...	...
Spergularia marina	...	..T	...	...	...	...	...	...	...	...	...	...	...
Polycarpon tetraphyllum	...	..A	...	...	...	...	...	...	...	...	...	...	...
Hernaria glabra	...	..T	...	...	...	...	...	...	...	...	...	...	...
Drymaria laxiflora	...	..A	...	...	...	...	...	...	...	...	...	...	...
Silene antirrhina	...	..A	...	...	...	...	...	...	...	...	...	...	...
Dianthus caryophyllus	C..	..A	...	...	...	...	...	...	...	...	...	...	..C
Dicleranthus plocamoides	...	..A	...	...	...	...	...	...	...	...	...	...	...
Paronychia drummondii	...	...	...	...	...	...	...	...	...	...	...	...	...
Arenaria benthamii	...	..A	...	...	..A	...	...	...	...	...	...	...	..C
Cerastium glomeratum	...	...	..C	...	...	...	...	..A	...	...	...	...	..C
Stellaria media	...	..A	...	...	...	...	...	...	...	...	...	...	...
Atriplex canescens	...	..A	...	...	...	...	...	C..	...	...	...	...	...
Colobanthus brevisepalus	...	..A	...	...	...	...	...	...	...	...	...	...	..C
Scleranthus biflorus	...	..A	...	...	...	...	...	...	...	...	...	...	..C

	222	222	222	222	222	222	222	222	223	333	333	333	333
	777	777	888	888	888	899	999	999	990	000	000	000	111
	456	789	012	345	678	901	234	567	890	123	456	789	012
Mollugo verticillata	GAT	AAT	TAT	ATG	TCT	CAT	GAT	CAG	GGA	TAT	TTG	AGA	TTT
Scopulophila rixtordii	...	...	...	...	..C	...	...	..A	...	...	...	...	...
Loeflingia squarrosa	...	...	...	...	...	...	...	G..	...	...	...	...	...
Spergularia marina	...	...	...	...	.G.	...	...	..A	...	...	...	..A	...
Polycarpon tetraphyllum	...	...	...	...	...	...	...	...	...	...	...	...	...
Hernaria glabra	...	...	...	...	...	...	...	G..	...	...	...	C..	...
Drymaria laxiflora	...	..A	...	...	...	...	...	...	...	...	...	...	...
Silene antirrhina	...	...	...	...	.G.	..C	...	...	...	...	...	..A	...
Dianthus caryophyllus	...	...	...	...	.A.	...	...	GG.	...	...	...	..A	...
Dicleranthus plocamoides	...	...	...	...	...	...	...	G.A	...	...	...	...	...
Paronychia drummondii	...	...	...	...	..A	...	...	...	...	...	...	...	...
Arenaria benthamii	...	...	...	...	.G.	...	..C	...	...	...	...	..A	...
Cerastium glomeratum	...	...	...	...	.G.	...	..C	..A	...	...	...	..A	...
Stellaria media	...	...	...	...	.G.	...	..C	..A	...	...	...	..A	...
Atriplex canescens	...	...	...	...	...	...	...	..A	...	...	...	...	...
Colobanthus brevisepalus	...	...	...	...	.G.	...	...	.GC	...	...	...	..A	...
Scleranthus biflorus	...	...	...	...	.G.	...	...	...	...	...	...	..A	...

	333	333	333	333	333	333	333	333	333	333	333	333	333
	111	111	122	222	222	223	333	333	333	444	444	444	455
	345	678	901	234	567	890	123	456	789	012	345	678	901
Mollugo verticillata	TTT	GCT	TAT	ATG	AGT	TTT	TTC	AAT	ACT	TCC	ATG	TTG	GGA
Scopulophila rixtordii	...	...	...	...	...	...	..T	.C.	...	..T	...	..A	...
Loeflingia squarrosa	...	...	...	...	...	...	..T	...	...	..T	...	..A	...
Spergularia marina	...	...	...	T.?	...	...	...	...	...	...	...	..A	...
Polycarpon tetraphyllum	...	...	...	...	...	...	..T	...	...	..G	...	..A	...
Hernaria glabra	...	...	...	...	...	...	...	...	...	...	...	..A	...
Drymaria laxiflora	...	...	...	...	...	...	..T	.C.	...	..T	...	..A	..G
Silene antirrhina	...	...	...	...	...	...	...	...	...	...	...	..A	...
Dianthus caryophyllus	...	...	...	...	...	...	..T	...	...	..T	...	..A	...
Dicleranthus plocamoides	...	...	...	...	..?	...	..T	.C.	...	..T	...	..A	...
Paronychia drummondii	...	...	...	...	...	...	...	.C.	...	...	...	..A	...
Arenaria benthamii	...	...	...	...	...	...	...	...	...	...	...	..A	..G
Cerastium glomeratum	...	...	...	...	...	...	...	T..	...	...	...	..A	...
Stellaria media	...	...	...	...	...	...	...	...	...	...	...	..A	...
Atriplex canescens	...	..G	...	T..	...	...	...	..C	...	..T	...	C..	...
Colobanthus brevisepalus	...	...	...	...	...	...	...	...	...	...	...	C.A	..C
Scleranthus biflorus	...	...	...	C..	...	...	...	...	...	...	...	..A	...

	333	333	333	333	333	333	333	333	333	333	333	333	333
	555	555	556	666	666	666	777	777	777	788	888	888	889
	234	567	890	123	456	789	012	345	678	901	234	567	890
Mollugo verticillata	TTA	GTT	ACT	AGT	TCG	AAT	TTG	ATA	CAA	ATT	TAT	ATT	TTT
Scopulophila rixtordii	...	...	...	...	...	...	...	...	...	...	...	...	...
Loeflingia squarrosa	...	...	...	...	..C	...	...	...	...	...	...	...	...
Spergularia marina	...	...	...	...	..A	...	...	..T	...	...	...	...	...
Polycarpon tetraphyllum	...	...	...	...	..A	...	...	...	...	...	...	...	...
Hernaria glabra	...	..C	...	...	..T	...	...	...	...	...	...	...	...
Drymaria laxiflora	...	...	...	...	...	...	..A	...	...	...	...	...	...
Silene antirrhina	...	...	...	...	.TA	...	..A	...	...	...	...	...	.G.
Dianthus caryophyllus	...	...	...	...	..A	...	...	...	...	...	...	...	...
Dicleranthus plocamoides	...	...	...	...	...	...	...	...	...	...	...	...	...
Paronychia drummondii	...	...	..G	...	..A	...	...	G..	...	...	...	T..	...
Arenaria benthamii	...	...	...	...	..A	...	...	...	...	...	...	...	...
Cerastium glomeratum	...	...	...	...	..A	...	...	...	...	...	...	C..	...
Stellaria media	...	A..	...	.?.	..A	...	...	...	...	...	...	C..	...
Atriplex canescens	...	...	...	...	...	...	..A	...	...	...	...	...	...
Colobanthus brevisepalus	...	...	..C	...	..C	...	...	...	...	...	...	...	...
Scleranthus biflorus	...	...	...	...	..C	...	...	...	...	...	...	...	...

	333	333	333	444	444	444	444	444	444	444	444	444	444
	999	999	999	000	000	000	011	111	111	112	222	222	222
	123	456	789	012	345	678	901	234	567	890	123	456	789
Mollugo verticillata	TGG	GAA	TTA	GTT	GGA	ATG	TGT	TCA	TAT	CTA	TTA	ATA	GGC
Scopulophila rixtordii	...	...	...	...	...	...	...	..G	...	...	...	...	..G
Loeflingia squarrosa	...	...	...	...	...	...	...	..G	...	..C	...	...	..T
Spergularia marina	...	...	...	...	...	...	...	..G	...	...	...	...	..T
Polycarpon tetraphyllum	...	...	...	..C	...	...	...	..G	...	...	...	...	..T
Hernaria glabra	...	...	...	...	...	...	...	..G	...	...	...	...	..T
Drymaria laxiflora	...	...	...	...	...	...	...	..G	...	...	...	...	..T
Silene antirrhina	...	...	...	...	...	...	...	..G	...	...	...	...	..T
Dianthus caryophyllus	...	...	...	...	...	...	...	..G	...	...	...	...	..T
Dicleranthus plocamoides	...	...	...	...	...	...	...	..C	...	...	...	...	..T
Paronychia drummondii	...	...	...	...	...	...	...	..G	...	...	...	...	..T
Arenaria benthamii	...	...	...	A..	...	...	.C.	..G	...	...	...	...	..T
Cerastium glomeratum	...	...	...	...	...	...	.C.	..G	...	...	...	...	..T
Stellaria media	...	...	A..	?..	...	...	.C.	..G	...	...	...	...	..T
Atriplex canescens	...	...	...	...	...	...	..C	..G	...	...	...	..C	..T
Colobanthus brevisepalus	...	...	...	...	...	...	...	..G	...	...	...	...	..T
Scleranthus biflorus	...	...	...	...	..G	...	.C.	...	...	...	...	...	..T

	444	444	444	444	444	444	444	444	444	444	444	444	444
	333	333	333	344	444	444	445	555	555	555	666	666	666
	012	345	678	901	234	567	890	123	456	789	012	345	678
Mollugo verticillata	TTT	TGG	-TT	TAT	TGG	ATA	ACA	GGC	AGT	TTC	GAG	TTT	CGA
Scopulophila rixtordii	...	...	...	...	...	G..	...	...	...	..T	..T	...	...
Loeflingia squarrosa	...	...	...	...	...	G..	...	...	...	...	..T	...	???
Spergularia marina	...	...	...	...	...	G.G	...	..T	...	...	..T	...	..T
Polycarpon tetraphyllum	...	...	...	...	...	G..	...	...	...	...	..T	...	...
Hernaria glabra	...	...	...	...	...	...	...	..A	...	...	..T	...	A..
Drymaria laxiflora	...	...	...	...	...	C..	...	...	...	...	..T	...	..G
Silene antirrhina	...	...	...	...	...	G..	..G	...	...	..A	..T	...	..T
Dianthus caryophyllus	...	...	...	...	...	G..	..C	...	...	...	..T	...	..T
Dicleranthus plocamoides	...	...	...	...	...	G..	...	...	...	...	..T	...	..G
Paronychia drummondii	...	...	...	...	...	...	...	...	...	...	..T	...	?AG
Arenaria benthamii	...	...	...	...	...	G..	..G	...	...	...	..T	...	..C
Cerastium glomeratum	...	...	...	...	...	G..	..C	...	...	..T	..T	...	..T
Stellaria media	...	...	...	...	...	G..	...	...	...	..T	..T	...	..T
Atriplex canescens	...	...	...	...	...	...	...	..T	...	...	...	...	..G
Colobanthus brevisepalus	...	...	...	...	...	T..	...	...	...	..T	..T	...	..T
Scleranthus biflorus	...	...	...	...	...	...	..T	..A	...	...	..T	...	..T

	444	444	444	444	444	444	444	444	444	444	455	555	555
	677	777	777	778	888	888	888	999	999	999	900	000	000
	901	234	567	890	123	456	789	012	345	678	901	234	567
Mollugo verticillata	GAT	TTG	TTT	GAA	ATA	TTC	AAT	AAC	TTA	ATT	ACT	AAT	AAT
Scopulophila rixtordii	...	...	...	...	...	..T	..A	..T	...	...	.A.	...	...
Loeflingia squarrosa	...	..?	...	...	...	..T	C.A	..T	...	...	.AA	...	...
Spergularia marina	...	...	...	...	...	...	...	...	...	...	.AA	...	...
Polycarpon tetraphyllum	...	..A	...	...	...	C.T	C.A	..T	...	...	.A.	...	...
Hernaria glabra	...	...	...	...	...	...	...	..T	...	...	.A.	...	...
Drymaria laxiflora	...	...	...	...	...	G.T	..A	..T	...	...	.A.	...	...
Silene antirrhina	...	...	...	...	...	..T	.C.	...	...	...	CAA	...	C..
Dianthus caryophyllus	...	...	...	C..	...	..T	.C.	...	...	...	.AA	...	...
Dicleranthus plocamoides	...	...	...	...	G..	..T	C.A	..T	...	...	.A.	...	...
Paronychia drummondii	...	..A	...	...	...	...	.C.	...	...	...	.A.	...	...
Arenaria benthamii	...	...	...	...	.C.	...	...	...	...	...	.AA	...	...
Cerastium glomeratum	...	...	...	...	...	..T	...	.G.	...	...	.AA	...	...
Stellaria media	...	...	...	..G	...	..T	...	...	...	...	.AA	...	...
Atriplex canescens	...	...	...	...	...	...	..A	.CT	...	...	.A.	...	..C
Colobanthus brevisepalus	...	...	...	...	..T	..T	...	...	...	..A	.AA	...	...
Scleranthus biflorus	...	...	...	.C.	...	..T	...	G..	...	...	CAA	...	...

	555	555	555	555	555	555	555	555	555	555	555	555	555
	001	111	111	111	222	222	222	233	333	333	334	444	444
	890	123	456	789	012	345	678	901	234	567	890	123	456
Mollugo verticillata	GAG	ATC	AAT	TAT	TTA	TTT	TGT	ATT	TTA	TGT	GCT	TTC	TTA
Scopulophila rixtordii	...	G..	...	CT.	...	...	...	C..	...	...	A..	...	..T
Loeflingia squarrosa	...	G..	...	CT.	...	...	...	C..	..C	...	A..	...	..T
Spergularia marina	...	G..	...	CG.	...	...	...	...	...	...	A..	...	..C
Polycarpon tetraphyllum	...	G..	...	CT.	...	...	...	C..	..T	...	A..	...	..T
Hernaria glabra	.GA	G..	...	CT.	.?.	...	...	...	...	..G	...	...	..T
Drymaria laxiflora	...	G..	...	CT.	...	...	...	C..	...	...	A..	...	..T
Silene antirrhina	..A	G.T	...	CT.	...	...	...	...	...	...	A..	...	..C
Dianthus caryophyllus	...	G..	...	CT.	...	...	...	...	...	...	A..	..A	..C
Dicleranthus plocamoides	...	G..	...	CT.	...	...	...	T..	...	...	A..	..A	..T
Paronychia drummondii	...	G..	...	CT.	...	...	...	...	...	...	A..	...	.GT
Arenaria benthamii	...	...	...	CT.	..G	...	...	...	...	...	A..	...	..C
Cerastium glomeratum	.G.	G..	...	CT.	...	...	.C.	...	..T	..C	A..	C..	..C
Stellaria media	.C.	G..	...	CT.	...	...	.C.	T..	..T	..C	A..	...	..C
Atriplex canescens	...	G..	...	.CC	...	...	...	...	...	...	...	...	..G
Colobanthus brevisepalus	...	G..	...	CT.	..C	...	...	...	...	...	A..	..G	..T
Scleranthus biflorus	...	G..	...	CT.	...	...	...	..A	...	...	A..	...	.GC

	555	555	555	555	555	555	555	555	555	555	555	555	555
	444	555	555	555	566	666	666	667	777	777	777	888	888
	789	012	345	678	901	234	567	890	123	456	789	012	345
Mollugo verticillata	TTA	TTT	GTG	GGT	GCA	GTT	GCT	AAA	TCT	GCT	CAA	TTC	CCC
Scopulophila rixtordii	...	...	.CT	...	...	...	...	...	..C	..C	...	..T	...
Loeflingia squarrosa	...	...	.CT	...	...	...	...	...	..C	?.C	...	..?	?..
Spergularia marina	...	...	.CT	...	...	...	..A	...	..C	..C	...	..T	...
Polycarpon tetraphyllum	...	...	.CT	...	...	...	...	...	..C	..G	...	..T	...
Hernaria glabra	...	...	.CT	...	...	...	...	...	..C	..C	...	..T	...
Drymaria laxiflora	...	...	..T	...	...	...	...	...	..A	..C	...	..T	...
Silene antirrhina	...	...	TCT	...	...	..A	..A	...	...	..C	...	..T	...
Dianthus caryophyllus	...	...	.CT	...	...	...	..A	...	..C	..C	...	..T	..T
Dicleranthus plocamoides	...	...	.CT	...	...	A..	...	...	..C	..C	...	..T	...
Paronychia drummondii	...	...	.CT	...	...	...	...	...	..G	..C	...	..T	..A
Arenaria benthamii	...	...	.CT	...	...	...	..A	...	...	T.C	...	...	...
Cerastium glomeratum	...	...	T.T	..C	...	...	..A	...	..G	T.C	...	..T	..T
Stellaria media	...	...	T.T	...	...	...	..A	...	..G	T.C	...	..T	...
Atriplex canescens	...	...	.CC	...	...	...	...	...	..G	..C	...	..T	..T
Colobanthus brevisepalus	...	...	.CT	...	...	...	..A	...	...	..C	...	..T	...
Scleranthus biflorus	...	...	.CT	...	...	...	..A	...	..G	..C	...	..T	..G

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                          555 555 555 555 556 666 666 666 666 666 666 666 666
                          888 899 999 999 990 000 000 000 111 111 111 122 222
                          678 901 234 567 890 123 456 789 012 345 678 901 234
Mollugo verticillata      CTT CAT GTA TGG TTA CCT GAT GCT ATG GAG GGG CCT ACC
Scopulophila rixtordii    ... ... ... ... ... ..C ... ... ... ... ... ... ..T
Loeflingia squarrosa      ... ... ... ... ... ... ... ... ... ..A ... ... ..T
Spergularia marina        ... ... A.. ... ... ..C ... ... ... ... ... ..G ...
Polycarpon tetraphyllum   ... ... ... ... ... ... ... ... ... ... ... ... ..T
Hernaria glabra           ... ... ... ... ... ... ... ... ... ... ..T ... ..T
Drymaria laxiflora        ... ... ... ... ... ..C ... ... ... ... ... ... ..T
Silene antirrhina         ... ... ... ... ... ..C ... ... ... ... ... ... ..T
Dianthus caryophyllus     ... ... ... ... ... ..C ... ... ... ... ... ... ..T
Dicleranthus plocamoides  ... ... ... ... ... ... ... ... ... ..A ... ... ..T
Paronychia drummondii     ... ... ... ... ... ... ... ... ... ... ..C ... ..G
Arenaria benthamii        ... ..C ... ... ... ... ... ... ... ..A ... ... ...
Cerastium glomeratum      ... ... ... ... ... ..C ... ... ... ... ..T ... ..T
Stellaria media           ... ... ... ... ... ..C ... ... ... ... ... ... ..T
Atriplex canescens        ... ... ... ... ... ... ... ... ... ... ..A ... ..T
Colobanthus brevisepalus.           ... ... ... ... ... ... ... ..A ... ... ...
... ..T
Scleranthus biflorus      ... ... ... ... ... ..C ... ... ... ... ... ... ..T

                          666 666 666 666 666 666 666 666 666 666 666 666 666
                          222 223 333 333 333 444 444 444 455 555 555 556 666
                          567 890 123 456 789 012 345 678 901 234 567 890 123
Mollugo verticillata      CCC ATT TCA GCC CTC ATA CAT GCT GCT ACT ATG GTA GCC
Scopulophila rixtordii    ..T ... ... ..T ... ... ... ... ..A ..C ... .GC ..A
Loeflingia squarrosa      ..T ... ... ..T ... ... ... ??? ??? ..A ... ..C ..A
Spergularia marina        ..G ... ... ..T ..T ..C ... ... ..G ..C ... ..C ..A
Polycarpon tetraphyllum   ..T ... ... ..T ... ... ... ... ..G ..C ... ..C ..A
Hernaria glabra           ..T ... ..C ..T ..G ... ... ... ... ... ... ..C ..A
Drymaria laxiflora        ..T ... ... ..T ... ... ... ... ..G ..C ... ..T ..A
Silene antirrhina         ..G ... ... ..T ..T ... ... ... ..G ..C ... ..C ..A
Dianthus caryophyllus     ..T ... ... ..T ... ... ... ... ..G ..C ... ..C ..A
Dicleranthus plocamoides  ... ... ..C ..T ... ... ... ... ..G ... ... ..C ..A
Paronychia drummondii     ..T ..C ... ..T ... ... ..C ... ... ..C ... ..T ..G
Arenaria benthamii        ..T ... ... ..T ... ... ... ..G ..G ..C ... ..C ...
Cerastium glomeratum      ..T ... ... ..T ... ... ... ... ..G ..C ... ..C ..A
Stellaria media           ..T ..G ... ..T ... ... ... ... ..G ..C ... ..G ..A
Atriplex canescens        ..T ... ... ..T ... ..C ... ... ... ..C ... ..C ...
Colobanthus brevisepalus  ..T ... ... ..T ..G ..C ... ..C ..G ..C ... ..C ..G
Scleranthus biflorus      ..T ... ... ..T ... ... ... ..C ..G ... ... ..C ..A

                          666 666 666 666 666 666 666 666 666 666 666 666 777
                          666 666 777 777 777 788 888 888 889 999 999 999 000
                          456 789 012 345 678 901 234 567 890 123 456 789 012
Mollugo verticillata      GCG GGG ATT TTT CTC GTC GCT CGA CTT CTT CCT CTT TTC
Scopulophila rixtordii    ... ..T ... ... ..A ... ... ... ... ... ... ... ...
Loeflingia squarrosa      .?. ... ... ... ..A ... ... ... ... A.. ... ... ...
Spergularia marina        ... ..? ... ... ..A ... ... ... ... T.. ... ... ...
Polycarpon tetraphyllum   ... ... ... ... ..A ... ... ... ... ... ... ... ...
Hernaria glabra           ... ... ... ... ... ..T ..G A.. ... ... ..C A.. ..T
Drymaria laxiflora        ... ..T ... ... ..A ... ..C ... ... ... ..G ... ...
Silene antirrhina         ..T ... ... ... ..A ..T ... ..T ... T.. ... ... ...
Dianthus caryophyllus     ... ... ... ... ..A .CT ... ... ... T.. ... ... ...
Dicleranthus plocamoides  ... ... ... ... ..A ... ... ... ... ... ... ... ...
Paronychia drummondii     ..A ... ... ... ..A ..G ... ... ... ... ... A.. ...
Arenaria benthamii        ... ... ... ... ..G ... T.. ... ... T.. ... ... ...
Cerastium glomeratum      ... ... ... ... ..A ... ... ... ... T.. ... ... ..T
Stellaria media           ... ... ... ... ..A ... ... ... ... T.. ... ... ...
Atriplex canescens        ..A ..A ... ... T.A ..T ... ... ..G ..G ... ... ...
Colobanthus brevisepalus  ... ... ... ... ..A ..T ... ... ... T.. ... G.. ...
Scleranthus biflorus      ... ... ... ... ..A ... ..C ..T ... T.. ... ... ...
```

	777	777	777	777	777	777	777	777	777	777	777	777	777
	000	000	011	111	111	112	222	222	222	333	333	333	344
	345	678	901	234	567	890	123	456	789	012	345	678	901
Mollugo verticillata	ATA	GTT	ATA	CCG	TAT	ATA	ATG	AGT	GGT	ATA	TCT	TTT	ATA
Scopulophila rixtordii	.G.	...	...	..T	...	...	...	CA.	.TA	...	G..	...	...
Loeflingia squarrosa	.G.	...	...	..T	...	...	...	TA.	..A	..C	G.C	...	...
Spergularia marina	...	A..	...	..T	...	...	...	TA.	.TA	..C	G..	...	...
Polycarpon tetraphyllum	.A.	...	...	..T	...	...	...	TA.	.TA	.C.	G..	...	...
Hernaria glabra	...	...	...	..T	...	...	...	TA.	.TA	...	G..	...	...
Drymaria laxiflora	.G.	...	...	..T	...	...	...	TA.	.TA	...	G..	...	...
Silene antirrhina	...	...	...	..C	...	...	...	TA.	.TA	..C	G..	...	...
Dianthus caryophyllus	...	...	...	..T	...	...	...	TA.	..A	..C	G..	...	...
Dicleranthus plocamoides	.G.	...	..T	..T	C..	...	...	TA.	.TA	..C	G.C	...	...
Paronychia drummondii	G..	...	G..	..T	...	...	...	TA.	.TA	...	G..	...	...
Arenaria benthamii	..G	...	...	..T	...	...	...	TA.	.TA	...	G..	...	...
Cerastium glomeratum	...	...	...	..T	...	...	...	TA.	.TA	...	A..	A..	...
Stellaria media	...	...	...	..T	...	...	...	TA.	.TA	...	A..	A..	...
Atriplex canescens	T..	A..	C..	..T	..C	...	...	TA.	..A	..T	...	...	...
Colobanthus brevisepalus	G..	.GG	..C	..T	...	C..	...	TA.	.TA	..C	G..	...	...
Scleranthus biflorus	...	A..	...	..T	...	...	...	TA.	.TA	..C	G..	..G	..T

	777	777	777	777	777	777	777	777	777	777	777	777	777
	444	444	445	555	555	555	666	666	666	677	777	777	778
	234	567	890	123	456	789	012	345	678	901	234	567	890
Mollugo verticillata	GGT	ATA	ATA	ACA	GTA	CTA	TTA	GGG	GCG	ACT	TTA	GCT	CTT
Scopulophila rixtordii	...	...	..C	...	..C	T..	...	..A	..T	...	...	...	...
Loeflingia squarrosa	...	...	...	...	...	T..	...	..T	..T	..C	...	...	...
Spergularia marina	...	...	...	..G	...	T.T	...	..A	..T	..C	...	...	...
Polycarpon tetraphyllum	...	...	...	...	...	T..	...	..A	..T	..C	...	...	...
Hernaria glabra	...	...	...	...	...	T.T	...	..A	..T	..C	C.G	...	...
Drymaria laxiflora	...	...	...	...	A..	T..	...	..A	..T	..C	...	...	...
Silene antirrhina	...	...	...	..T	...	T.T	...	..A	..T	..C	...	...	...
Dianthus caryophyllus	...	...	...	..T	T..	T.T	...	..A	..T	...	...	...	...
Dicleranthus plocamoides	...	...	...	...	...	T..	...	...	..T	...	...	...	...
Paronychia drummondii	...	...	...	...	...	T..	...	..A	..T	G..	...	...	...
Arenaria benthamii	...	...	...	..T	..C	T.T	...	...	..T	...	...	..C	...
Cerastium glomeratum	...	...	...	..T	..T	T.T	...	..A	..T	..C	...	...	...
Stellaria media	...	...	...	..T	..T	T.T	...	..A	..T	..C	...	...	...
Atriplex canescens	...	...	...	...	...	...	C..	..A	..A	..C	...	...	...
Colobanthus brevisepalus	...	G..	...	..T	T.G	T.T	...	...	..T	..A	...	...	...
Scleranthus biflorus	...	...	...	..T	...	T.T	...	..A	..T	..C	...	...	...

	777	777	777	777	777	777	788	888	888	888	888	888	888
	888	888	888	999	999	999	900	000	000	001	111	111	111
	123	456	789	012	345	678	901	234	567	890	123	456	789
Mollugo verticillata	GCT	CAA	AAA	GAC	--T	TCG	TAT	CGA	GCT	GCG	TTA	TTT	CAT
Scopulophila rixtordii	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Loeflingia squarrosa	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Spergularia marina	...	...	...	..T	...	..T	...	...	.T.	..T	...	...	...
Polycarpon tetraphyllum	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Hernaria glabra	...	...	...	...	...	..T	...	...	A..	..T	...	...	...
Drymaria laxiflora	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Silene antirrhina	...	...	...	..T	...	..T	...	...	...	..T	...	...	...
Dianthus caryophyllus	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Dicleranthus plocamoides	...	...	...	...	...	..?	..T	...	...	..T	...	...	...
Paronychia drummondii	...	...	...	...	...	..T	...	AA.	...	..T	...	...	...
Arenaria benthamii	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Cerastium glomeratum	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Stellaria media	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Atriplex canescens	...	...	...	...	...	..T	...	...	...	..T	...	...	...
Colobanthus brevisepalus	...	...	..G	..T	...	..T	...	...	...	..T	...	...	...
Scleranthus biflorus	...	...	...	..T	...	..T	...	.AT	...	..T	...	...	...

	888	888	888	888	888	888	888	888	888	888	888	888	888
	222	222	222	233	333	333	334	444	444	444	555	555	555
	012	345	678	901	234	567	890	123	456	789	012	345	678
Mollugo verticillata	TTG	ATT	ACT	CAT	GCT	TAT	TCA	AAA	GCA	TTA	TTG	TTT	TTA
Scopulophila rixtordii	...	...	...	...	...	...	...	...	...	...	...	...	...
Loeflingia squarrosa	...	...	..A	.TA	...	...	...	...	...	...	...	...	...
Spergularia marina	...	...	...	...	...	...	...	...	...	..G	...	...	...
Polycarpon tetraphyllum	...	...	...	...	...	...	...	...	...	...	...	...	...
Hernaria glabra	...	...	...	...	...	...	...	...	...	...	...	...	...
Drymaria laxiflora	...	...	...	...	...	...	...	...	...	...	...	...	...
Silene antirrhina	...	...	...	...	...	...	...	...	..T	..G	...	...	...
Dianthus caryophyllus	...	...	...	...	...	...	...	...	...	...	...	...	...
Dicleranthus plocamoides	...	...	...	...	...	...	...	...	...	...	...	...	..G
Paronychia drummondii	...	...	...	...	...	...	...	...	...	...	...	...	...
Arenaria benthamii	...	...	...	...	...	...	...	...	...	...	...	...	...
Cerastium glomeratum	..T	...	...	...	...	...	...	...	...	...	...	...	...
Stellaria media	...	...	...	...	...	...	...	...	...	...	...	...	...
Atriplex canescens	...	...	...	...	...	...	...	...	...	...	...	...	...
Colobanthus brevisepalus	...	...	..C	...	...	...	...	...	...	...	...	...	...
Scleranthus biflorus	...	...	...	...	...	...	...	...	...	..G	...	...	...

	888	888	888	888	888	888	888	888	888	888	888	888	888
	566	666	666	667	777	777	777	888	888	888	899	999	999
	901	234	567	890	123	456	789	012	345	678	901	234	567
Mollugo verticillata	GGA	TCC	GGA	TCT	GTT	ATT	CAT	TCA	ATG	GAA	GCT	ATT	GTT
Scopulophila rixtordii	..T	...	...	..C	A..	...	...	...	...	...	AT.	C..	...
Loeflingia squarrosa	..G	...	..C	..A	A..	...	...	...	...	...	AT.	C..	...
Spergularia marina	...	..T	..G	..C	C..	...	...	...	...	...	A.A	C.C	...
Polycarpon tetraphyllum	..G	...	...	..C	A..	...	...	...	...	...	AT.	C..	...
Hernaria glabra	...	...	...	..C	C.G	...	...	..C	...	...	A..	C..	...
Drymaria laxiflora	...	...	...	..A	A..	...	...	...	...	...	AT.	C..	...
Silene antirrhina	...	...	...	..C	C..	...	...	...	...	...	A.G	C..	...
Dianthus caryophyllus	...	...	..G	...	C..	...	...	...	...	...	A.G	C..	...
Dicleranthus plocamoides	..G	...	...	..A	A..	...	...	...	...	...	AT.	C..	...
Paronychia drummondii	...	...	...	..C	C..	...	...	..C	...	...	A..	C..	...
Arenaria benthamii	..G	...	...	..C	C..	...	...	...	...	...	A.G	C..	...
Cerastium glomeratum	...	...	...	...	A..	...	...	...	...	...	A.G	C..	...
Stellaria media	...	...	...	...	A..	...	...	...	.C.	...	A.G	C..	...
Atriplex canescens	...	..T	...	...	C..	...	...	...	...	...	A..	G..	...
Colobanthus brevisepalus	..G	..G	...	..C	C..	...	T..	...	...	...	ATG	C..	...
Scleranthus biflorus	...	...	...	..A	C..	...	...	...	...	...	A.A	C..	...

	889	999	999	999	999	999	999	999	999	999	999	999	999
	990	000	000	000	111	111	111	122	222	222	223	333	333
	890	123	456	789	012	345	678	901	234	567	890	123	456
Mollugo verticillata	GGA	TAT	TCT	CCA	GAT	AAA	AGT	CAA	AAT	ATG	GTT	CTT	ATG
Scopulophila rixtordii	...	...	...	...	...	...	..C	...	...	...	...	...	...
Loeflingia squarrosa	...	...	...	..C	A..	...	..C	...	...	...	...	A..	...
Spergularia marina	...	...	..G	...	A..	...	...	..G	...	...	...	...	...
Polycarpon tetraphyllum	...	...	...	...	...	...	..C	...	...	...	...	...	...
Hernaria glabra	...	...	...	...	...	...	..C	..G	...	...	...	T..	...
Drymaria laxiflora	...	...	...	...	...	...	..C	...	...	...	...	...	...
Silene antirrhina	...	...	...	...	...	...	.A.	..G	...	...	...	...	...
Dianthus caryophyllus	...	...	...	...	...	...	...	..G	...	...	...	...	...
Dicleranthus plocamoides	...	...	...	...	...	...	...	...	..C	...	...	...	...
Paronychia drummondii	...	...	...	...	...	...	T..	..G	...	...	...	...	...
Arenaria benthamii	..C	...	..A	...	...	...	...	..G	...	...	...	A..	...
Cerastium glomeratum	..G	...	...	...	..A	...	...	..G	...	...	...	...	...
Stellaria media	...	...	...	...	..A	...	...	..G	...	...	...	...	...
Atriplex canescens	...	...	...	..C	A..	...	...	..G	...	...	...	...	...
Colobanthus brevisepalus	...	...	...	...	...	...	...	..G	...	...	...	...	...
Scleranthus biflorus	...	...	...	...	...	...	...	..G	...	...	...	...	...

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	333	444	444	444	455	555	555	556	666	666	666	777	777
	789	012	345	678	901	234	567	890	123	456	789	012	345
Mollugo verticillata	GGG	GGT	TTA	ACA	AAA	CAT	GTG	CCA	ATT	ACA	AAA	ATT	GCT
Scopulophila rixtordii	...	..G	...	...	...	...	..T	...	...	...	...	.C.	...
Loeflingia squarrosa	...	..G	...	...	...	..C	..T	...	...	...	...	.C.	...
Spergularia marina	...	..A	...	...	...	...	..T	...	...	...	...	.C?	?..
Polycarpon tetraphyllum	...	..G	...	...	...	...	..T	...	...	...	...	.CC	...
Hernaria glabra	...	..G	C..	...	...	...	..T	...	...	...	...	.C.	...
Drymaria laxiflora	..A	..G	...	...	...	...	..T	..G	...	...	...	.C.	...
Silene antirrhina	..A	..A	...	...	...	...	..T	...	...	...	...	.C.	...
Dianthus caryophyllus	...	..A	...	...	...	...	C.T	...	...	...	...	.C.	...
Dicleranthus plocamoides	..A	..G	C..	...	...	...	..T	...	...	...	...	.C.	...
Paronychia drummondii	...	..G	C..	...	...	...	..T	...	...	...	...	.CA	...
Arenaria benthamii	...	..A	...	...	..G	...	..T	...	...	...	...	.C.	...
Cerastium glomeratum	...	..A	...	...	...	...	A.T	...	...	...	...	.A.	...
Stellaria media	...	.?A	...	...	...	..C	..T	...	...	...	...	.A.	...
Atriplex canescens	..A	..G	...	...	...	...	..A	...	...	...	...	.C.	T.G
Colobanthus brevisepalus	..T	..C	...	...	...	...	..T	...	...	...	...	.C.	...
Scleranthus biflorus	..A	..A	...	...	...	...	..T	...	...	...	...	..G	..A

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	678	901	234	567	890	123	456	789	012	345	678	901	234
Mollugo verticillata	TTT	TTA	ATA	GGT	ACA	CTT	TCT	CTT	TGT	GGT	ATT	CCG	CCT
Scopulophila rixtordii	...	...	...	...	..T	...	..G	...	...	...	...	...	...
Loeflingia squarrosa	...	...	...	...	..T	...	...	...	...	...	...	...	...
Spergularia marina	...	...	...	..G	..T	...	...	...	...	...	...	...	..A
Polycarpon tetraphyllum	...	...	...	...	..T	...	...	...	...	...	...	...	...
Hernaria glabra	...	..T	...	...	..C	...	...	...	...	...	...	...	...
Drymaria laxiflora	...	...	...	...	..T	...	...	...	..C	...	...	...	...
Silene antirrhina	...	...	...	...	..G	...	...	...	...	...	...	..T	...
Dianthus caryophyllus	...	...	...	...	..G	A.C	...	...	...	...	...	..A	...
Dicleranthus plocamoides	...	..T	...	...	..T	...	...	...	...	...	...	..T	...
Paronychia drummondii	...	...	...	...	..G	...	..G	...	...	...	...	...	...
Arenaria benthamii	...	...	..C	...	...	...	...	...	...	...	...	...	...
Cerastium glomeratum	...	...	...	..C	..C	...	...	...	...	...	...	...	...
Stellaria media	...	...	...	...	..C	...	...	...	...	..G	...	...	...
Atriplex canescens	...	...	...	...	...	...	..G	...	...	...	...	...	...
Colobanthus brevisepalus	...	...	..T	...	T.G	...	...	...	...	...	...	..A	...
Scleranthus biflorus	...	...	...	...	..G	...	...	...	...	...	...	..A	..C

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	111	112	222	222	222	333	333	333	344	444	444	445	555
	567	890	123	456	789	012	345	678	901	234	567	890	123
Mollugo verticillata	CTT	GCT	TGT	TTT	TGG	TCC	AAA	GAT	GAA	ATT	CTT	AAT	GAT
Scopulophila rixtordii	...	..G	..C	...	...	...	...	...	...	...	...	...	...
Loeflingia squarrosa	...	..G	...	...	...	...	...	...	...	...	...	...	...
Spergularia marina	...	...	...	...	...	...	...	...	...	...	...	.G.	..C
Polycarpon tetraphyllum	...	..G	...	...	...	...	...	...	...	...	...	...	...
Hernaria glabra	...	...	...	...	...	...	...	...	...	..C	...	...	...
Drymaria laxiflora	...	..A	...	...	...	...	...	...	...	...	...	...	...
Silene antirrhina	...	...	...	...	...	...	...	...	...	...	...	...	...
Dianthus caryophyllus	T.G	...	..?	...	...	...	...	...	...	...	...	...	...
Dicleranthus plocamoides	...	..G	...	...	...	...	...	...	...	...	...	...	...
Paronychia drummondii	...	...	.CG	...	...	..T	...	..C	...	..C	...	...	...
Arenaria benthamii	...	...	...	...	...	..A	...	...	...	...	...	...	...
Cerastium glomeratum	...	...	...	...	...	...	...	...	...	...	...	...	...
Stellaria media	...	...	...	...	...	...	...	...	...	...	...	..C	...
Atriplex canescens	...	...	...	...	...	...	...	...	...	...	...	...	...
Colobanthus brevisepalus	...	...	...	...	...	...	...	...	...	...	...	...	.C.
Scleranthus biflorus	...	..G	...	...	...	...	..G	...	...	...	...	...	..A

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	000	000	000	000	000
	555	555	666	666	666
	456	789	012	345	678
Mollugo verticillata	AGT	TGG	TTA	TAT	TCA
Scopulophila rixtordii	...	...	G.G	...	..G
Loeflingia squarrosa	...	...	GCG	...	..G
Spergularia marina	...	...	..G	...	...
Polycarpon tetraphyllum	...	...	G.G	...	...
Hernaria glabra	...	...	..G	...	...
Drymaria laxiflora	...	...	GCG	...	..G
Silene antirrhina	...	...	..G	...	...
Dianthus caryophyllus	...	...	...	...	..G
Dicleranthus plocamoides	...	...	..C	...	..G
Paronychia drummondii	...	...	..G	...	..G
Arenaria benthamii	T..	G..	..C	...	..T
Cerastium glomeratum	...	...	..G	...	...
Stellaria media	...	...	..G	...	...
Atriplex canescens	...	...	A..	...	...
Colobanthus brevisepalus	...	...	...	...	..G
Scleranthus biflorus	...	...	..T	G..	..T

Appendix 9: Glossary of some terms used in the thesis

Acetolysis. Treatment of pollen grains with acidic solution to remove non-exine material (Cellular contents, intine etc).

Alternisepalous. Used in this thesis to mean on a floral radius midway between radii on which sepals are located.

Antisepalous. Used in this thesis to mean on the same floral radius as a sepal.

Asymmetric PCR. A PCR reaction carried out with an unequal ratio of two primers so that an excess of one strand of the target DNA accumulates.

Autogamous breeding system. Mode of reprduction in which habitual self-pollination (pollination flowers by pollen from the same flower) occurs.

Biotinylated primer. An oligonucleotide with the chemical Biotin bonded to the 5' end.

Caryophyllad embryogeny. A pattern of embryo development in which the basal cell of the two celled pro-embryo does not divide and the apical cell contributes to both the embryo proper and the suspensor.

Commissurally. Along a line or seam where two parts meet.

Disjunct. (-distribution) Distribution of an organism over two or more non-contiguous regions.

Concerted evolution. The phenomena observed for gene families in which sequence uniformity of paralogous copies is maintained.

Consistency index. A measure of homoplasy obtained by dividing the minimum possible number of steps required for the characters in a data set by the actual number of steps over a given tree.

Decay value. A measure of support for specific edges of a shortest parsimony tree calculated by subtracting the length of the shortest tree from the length of the shortest tree in which the clade in question is not found.

Deletion. A class of mutation events leading to a decrease in length of a gene through removal of a number of nucleotides.

Dichasium. An inflorescence in which terminal flowers are produced above pairs of bracts and further branches of the inflorescence arise in the axils of the bracts.

Dichogamy. Temporal separation of male and female functions in a hermaphrodite flower (i.e., release of pollen before or after stigmas are receptive)

Dideoxy chain termination. The truncation of a synthesized DNA strand on

addition of a dideoxy-nucleotide (rather than deoxy-nucleotide) by a polymerizing enzyme. Dideoxy and deoxy-nucleotides are used together in a reaction mixture to produce partial copies of a DNA molecule that vary in length during enzymic DNA sequencing procedures.

Dideoxy sequencing. DNA sequencing procedure utilizing the property of DNA polymerases to stop synthesis of a copy strand on addition of a dideoxy nucleotide.

Druses. Stellate crystals found in plant tissue.

Ektexine. The outer (sculptured) part of the exine. More precise terminology in the thesis would be sexine.

Embryology. In flowering plants, the study of male and female gametophyte development as well as development of the embryo and other seed structures.

Epitropous. Positioning of an ovule so that its micropyle points "upward" toward the style.

Exine. The outer part of the pollen wall containing sporopollenin.

Facultative autogamy. Breeding system where self-pollination is most common but some opportunity for cross pollination occurs.

Gene conversion. The "correction" of mismatched DNA strands that occur after crossing over where allelic differences exist or unequal crossing over has occurred.

Gynophore. A stalk at the base of a gynoecium.

Herkogamy. Spatial separation of male and female functions of a hermaphrodite flower.

Hypostase. A region of differentiated cells at the base of the embryo sac. Often with suberized or sclerenchymous walls or secretory. Tissues designated as hypostase have been implicated in nutrition of the embryo sac and in water conservation.

Indel. An insertion or deletion (where direction of change is not established).

Insertion. A class of mutation events leading to an increase in length of a gene through addition of a number of nucleotides.

ITS. Internal Transcribed Spacer. Most commonly of the nuclear ribosomal DNA tandem repeat.

Mesoporal. Around and between the pores of a pollen grain

***ndh*F**. A putative NADH dehydrogenase subunit gene of the chloroplast.

Obdurator. A structure for guiding or supporting pollen tubes in the ovary.

Ontogeny. The process by which an organism or organ develops from a simple (homogenous) undifferentiated state to a complex (heterogeneous) differentiated state.

Pantoporate. Pollen with many roughly circular pores distributed over the whole grain.

Peduncle. A stalk bearing an inflorescence.

Perforate. A pollen grain with holes in its tectum

Perigynum. "Floral cup". A cup-like structure surrounding the ovary and bearing the outer floral organs above the ovary of a flower.

Perisperm. A food reserve tissue in some seeds derived from the nucellus.

Polymerase chain reaction. A laboratory procedure for the synthesis of large quantities of a specific DNA species by use of thermally stable DNA Polymerase and sequence-specific oligonucleotide primers.

Primordium. An organ in its earliest stage of differentiation.

Prisms. Polyhedral crystals found in plant tissue.

Punctate. Perforate pollen grain with perforations typically about 1 μm diameter

Pyriform. Pear-shaped.

Rescaled consistency index. The product of Retention Index and Consistency Index, where retention index is equal to 1 minus the actual homoplasy divided by maximum homoplasy for the character set (i.e., on bush or star phylogeny where all shared states are due to homoplasy).

Reticulate evolution. Evolution within an interbreeding population, or among populations where hybridization or introgression are taking place, such that individuals or groups may have more than one lineage of ancestors (or ancestral populations).

Solanad embryogeny. A pattern of embryo development in which the basal cell of the two celled pro-embryo divides with one of the resulting daughter cells contributing to the embryo proper.

Spinules. Extratectal processes which are pointed and less than 3 μm long.

Stele. Central cylinder (especially of stem or root structure) containing primary vascular tissue and associated tissues.

Transmission tissue. Hair-like tissue which conducts or supports the pollen tube in its growth from the style to the ovules.

Unequal crossing over. Crossing over events that occur between non-homologous parts of chromosomes.

Unrooted tree. A phylogenetic tree in which ancestral states/taxa (or direction of evolution) are not designated.

Vicariance. The separation of biota by geological, climatological or any other disrupting factor.

Xenogamy. Breeding system in which habitual cross-pollination (pollination of a plant's flowers by pollen from flowers of other plants) occurs.