Final Report to Australian Flora Foundation

Implications of reproductive biology and morphology on the conservation of *Grevillea williamsonii* (Proteaceae)

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SUMMARY

The ex-situ population of *G. williamsonii* contains approximately 150 plants representing five genotypes. Only seven genotypes remain in the wild population. It is unlikely that any individuals will remain in the wild in five years because plant health is poor and a number of plants have died since monitoring began in 1995? This could be due to an inability to compete with other vegetation but could also be due to *Phytophthora cinnamomi*.

Microscopy has revealed that *G. williamsonii* does not have anthers and cannot produce pollen. Therefore any pollinated flowers must have received pollen from another source. The most likely candidate is *G. aquifolium* which is very common and grows amongst *G. williamsonii*.

The flowers of *G. williamsonii* are smaller than those of *G. aquifolium* and *G. ilicifolia*. The stigma appears to be deformed with no stigmatic disk surrounding the receptive area and the latter quite irregular compared to other *Grevillea* species. The papillae are swollen at the base and have a smaller swollen tip. In the related species *G. aquifolium* and *G. ilicifolia,* anthers were present in every flower examined and large amounts of pollen were present. The stigma was typical of the holly-leaf grevilleas with a large disk surrounding the receptive area in the centre. The papillae are slender, finger-like projections without any swelling. It is not known whether or not the stigmas of *G. williamsonii* become receptive but papillae do become more pronounced and an exudate is secreted as the flowers mature. Reports of seed production suggest that pollen germination and subsequent fertilisation of ovules does occur. However, there is no doubt that any seed produced is not a result of pollination by *G. williamsonii*.

The hairs on the underside of the leaves were indistinguishable in G. williamsonii and G. aquifolium. Those two species could be differentiated from G. ilicifolia, G. infecunda, G. floripendula and G. dimorpha.

Allozyme assays could not distinguish between *G. williamsonii* and *G. aquifolium* but *G. ilicifolia* and *G. dimorpha* were different. Only a small number of enzymes were resolved and there was little polymorphism so a different analytical method, such as DNA analysis, is required to establish whether there are genetic differences between *G. williamsonii* and *G. aquifolium*. However, the allozyme analysis has confirmed that the *G. williamsonii* plants are from seedlings rather than a group of genetically identical plants resulting from vegetative propagation such as root suckering.

The most likely explanation is that *G. williamsonii* is an unusual mutation originating in *G. aquifolium*. The population discovered in 1993 is probably the result of a single pollination event with the same mutation occurring in all siblings. It is possible but less likely that *G. williamsonii* is a species at the end of its evolutionary potential and has become reproductively isolated.

Appropriate management for the species depends on its taxonomic position and is reliant on further genetic analysis. Sufficient ex-situ material is in cultivation to re-introduce *G. williamsonii* if it is shown to be of specific rank but this option will not produce a self-sustaining species in the longterm. If *G. williamsonii* is, instead, a rare variant of *G. aquifolium* then management practices, including fire, should be designed for the general habitat type rather than the remaining in-situ plants.

1. INTRODUCTION

Grevillea williamsonii *is* an example of many Australian species that are poorly understood. It is an extremely rare grevillea that occupies an area of approximately 0. 1 ha of open *Eucalyptus baxteri* woodland near Cassidy's Gap in the Grampians National Park. It is classified as Endangered under the Commonwealth Endangered Species Protection Act 1992 and the Department of Natural Resources and Environment (unpublished list 1997). It is also considered endangered by Briggs and Leigh (1995).

The taxon has been recorded on two occasions. The original collection (MEL75250) was made by H.B. Williamson in November 1895 from a plant discovered in October 1893 in the Mt. Abrupt area. The plant was the only known specimen and was destroyed by fire in December 1897.

McGillivray (1993) considers that *G. williamsonii* is either a hybrid with *G. aquifolium* as one of its parents or an unusual variant of that species. This was prior to its rediscovery at Cassidy's Gap in November 1992. With the discovery of 12 plants in 1992, its status as a species was considered to be justified (Olde and Marriott 1995).

The single location and small number of individuals led to the species being classified as nationally endangered. A Recovery Plan (James et al. 1999) was prepared, with reservations because of the lack of knowledge of the biology of the species. However, a Recovery Plan was required before the federal government would consider funding any actions to conserve the species.

A major difficulty in the preparation of a management strategy for *G. williamsonii* is an apparent anomaly in its breeding system. Plants appear to be incapable of producing pollen, yet fruit and seed have been found on plants in the wild. Seed is thought to be viable but there are no records of its successful germination. This raises questions about the taxonomic status of any offspring of *G. williamsonii* and also the origin of *G. williamsonii* itself. It may be a relict species that has become reproductively incapable, it could be a variant of *G. aquifolium* or a hybrid between unknown parents. The taxonomic status has a direct bearing on which management actions are appropriate.

There is evidence of reproductive anomalies in rare plant species (Falk and Holsinger 1991 and references therein). Other *Grevillea* species that do not set fruit are known. *G. infecunda* produces pollen which is deformed and pollen viability is extremely low. Follicle development in *G. infecunda* has not been recorded in the wild and although follicles have developed under cultivation, all aborted before maturity (Olde and Marriott 1995). Vegetative reproduction via root suckering occurs in *G. infecunda* providing a mechanism for the species to perpetuate itself in the absence of sexual reproduction. On the other hand, *G. williamsonii* appears to be of seedling origin with no alternative to sexual reproduction.

Grevillea williamsonii grows well under cultivation (Fig. 1) and displays a dense branching habit (Fig. 2). In its natural habitat, competition from other species appears to restrict its growth. *G. aquifolium* is very common at the site and grows in close proximity to *G. williamsonii* (Fig. 3).

The aim of this project was to provide information on the breeding system of *G. williamsonii* and its relatedness to other local holly-leafed grevilleas in order to have a biological basis on which to develop a conservation strategy.

2. MATERIALS AND METHODS

2.1 Ex-situ collection

Cuttings were taken from G. *williamsonii* plants in the field in September, 1999. Not all plants could be sampled because some were too small. Propagation from the initial collection of material from Cassidy's Gap has continued as cutting material on cultivated plants has become available.

Cuttings were prepared from semi-hardwood and the cut end dipped in Blue Clonex (3g/L IBA) rooting hormone. Cuttings were inserted into a propagation mix comprising perlite, sand and peat (3:2:1) and kept in a foghouse until roots formed. After hardening off, cuttings were potted into pinebark-based media with no-P slow release fertiliser and kept in a glasshouse, shadehouse or on a concrete standing out area in sun.

2.2 Floral morphology and reproductive phases

Fresh flowers were collected from cultivated and wild plants and examined at various stages of development under both a dissecting microscope and a scanning electron microscope (SEM). SEM work was done on a JEOL 6340F scanning electron microscope. A cryostage was used because it is not necessary to fix the material prior to examination and the living appearance of tissue is preserved. Any secretions are retained for observation whereas the process of fixation and dehydration removes them.

2.3 Leaf surface

Leaves were collected from three populations of *G. aquifolium* (Cassidy's Gap, Roses's Gap and Black Range), two populations of *G. ilicifolia* (Black Range and Cooak), two populations of *G. infecunda* (Love's Track and Breakfast Creek), single populations of *G. floripendula* (Musical Gully) and *G. dimorpha* (Cassidy's Gap) and one putative hybrid between G. *williamsonii* and *G. aquifolium* (Cassidy's Gap).

The underside of both fresh and dried leaves was examined under a dissecting microscope and a scanning electron microscope using a cryostage and an ambient temperature stage (without fixation).

2.3 Enzyme assay

The enzyme assay was used to detect taxonomically informative differences between *G. williamsonii, G. aquifolium, G. ilicifolia* and *G. dimorpha,* and to look for evidence of hybridisation in *G. williamsonii. G. dimorpha* was included because it occurs within a kilometre of *G. williamsonii* but unlike *G. williamsonii, G. aquifolium* and *G. ificifolia* it does not belong to the holly-leafed grevillea group. Fresh leaf tissue from individuals of each species was ground in borate buffer and the extracts were run on a Titan III cellulose acetate gel electrophoretic system (Helena Laboratories) (Warburton et al. 2000). Gels were stained for a number of enzymes but only four, *Gpi, Mdh, Pgm* and *Lap,* were sufficiently resolved to be useful.

2.5 Seed viability

No seed was produced on cultivated plants or plants at Cassidy's Gap during the study period so seed viability could not be tested.

3. RESULTS

3.1 Ex-situ collection

Approximately 150 plants representing five genotypes are currently held in the ex-situ collection at the Royal Botanic Gardens Melbourne and include two genotypes that are no longer present at the Grampians site. Duplicate collections will be transferred to other locations after approval from Parks Victoria.

A number of plants flowered in spring 2000. More flowers were present on plants that had been grown outside in winter rather than in the glasshouse where minimum temperature rarely fell below ten degrees Celsius.

3.2 Floral morphology and reproductive phases

3.2.1 Female phase

Pistils of all flowers of *G. williamsonii* were small compared to *G. aquifolium* and *G. ilicifolia* and did not have a flat disc surrounding the stigmatic surface. Almost half the flowers examined (47%) had extra pistils (Fig. 4). Most were quite small but a number were at least 2/3rds the size of the large pistil and sometimes a stigmatic area was visible. The ovary appeared to be normal and the ovules did not show any gross abnormalities.

Under the dissecting microscope, a small area corresponding to the pre-receptive stigmatic zone was visible in immature flowers. When the pistil is released from the corolla tube, the stigma is immature and not receptive. The pre-receptive zone in *G. williamsonii* was visible at the lower edge of the stigma and the stigmatic area was no wider than the diameter of the style. In contrast, *G. aquifolium* and *G. ilicifolia* stigmas were considerably wider than the style and the pre-receptive zone was located in the centre of the disk. At this stage the papillae had not extended beyond the surface of the disk (Fig. 5). As the flowers matured, the stigma became receptive as the papillae emerged from the disk and a secretion was visible. In *G. williamsonii* (Fig. 6a,b) the receptive area was not as discrete as in *G. aquifolium* (Fig 6c,d) and *G. ilicifolia* (Fig. 6e,f).

Under electron microscopy, the stigmatic papillae of *G. williamsonii* appeared to be deformed (Fig. 7a,b,c) with bulging rather than fingerlike projections seen in *G. aquifolium* (Fig. 8a,b) and *G. ilicifolia* (Fig. 9a,b) that are typical of stigmatic papillae in *Grevillea*.

This pattern of development is consistent with other *Grevillea* species because the stigma acts as a pollen presenter when immature. Under natural conditions, by the time the stigma becomes receptive, pollen has been removed by pollinators.

3.2.2 Male phase

None of the *G. williamsonii* flowers examined showed any evidence of stamen development inside the corolla. No anther development was seen on any *G. williamsonii* buds or flowers examined. A total of 250 individual flowers were examined. These comprised flowers sampled from the field, from cultivated plants grown in the ground (Pomonal Nursery) and in pots (RBGM).

The flowers of *G. williamsonii* were smaller than those of *G. aquifolium* and *G. ilicifolia* (Fig. 10) where the stigma folded into the end of the corolla tube in bud because no anthers were present. When flowers were dissected, anthers containing pollen were clearly visible pressed up against the stigma in *G. aquifolium* and *G. ilicifolia* but neither pollen nor anthers were present in *G. williamsonii* under the light microscope (Fig. 11) or SEM (Fig. 12).

3.3 Leaf surface

The gross morphology of *G. williamsonii* leaves differs markedly from other species (Fig 13) but the hairs on the underside were indistinguishable from those of *G. aquifolium*. The leaf shape of *G. aquifolium* is very variable and some individuals in a population at Serra Road had some entire leaves but they were not easily confused with *G. williamsonii*.

The general appearance of *G. williamsonii* was the same as *G. aquifolium* and their putative hybrid. Both *G. williamsonii* and *G. aquifolium* could be distinguished from *G. ilicifolia* because the hairs were curly rather than straight (Fig. 14). When the leaves were examined under SEM, the differences remained whether leaves were dried first or used fresh. No individuals of *G. aquifolium* (Fig. 15a,b,c) and *G. williamsoni*i (Fig. 15g,h) and their putative hybrid (Fig. 15i) could be differentiated from each other. The hairs on *G. ilicifolia* leaves were always straight (Fig. 15d,e). *G. infecunda* (Fig. 15j,k) leaves also had straight hairs very similar in appearance to *G. ilicifolia*. *G. dimorpha* (Fig. 15f) also had straight hairs but they were much larger than those in *G. aquifolium* and *G. ilicifolia*. *G. floripendula* (Fig. 15l) was easily distinguished due to its low hair density.

3.4 Enzyme assay

Allozyme analysis showed that the *G. williamsonii* plants were not identical. The enzyme, *Lap*, was variable within *G. williamsonii* confirming that the plants were of seedling origin and not a result of vegetative reproduction, for example via root suckers.

Insufficient enzymes were resolved to provide an estimate of genetic diversity in *G. williamsonii* compared to the more widespread *G. aquifolium* and *G. ilicifolia*. Genotypes could only be assigned for *Lap* and *Pgm*.

Although *Mdh* could not be resolved satisfactorily for genotypes to be assigned, all *G. williamsonii* individuals showed a uniform pattern and appeared to be homozygous for *Mdh*. Three areas of activity, assumed to correspond to loci, were observed for *G. dimorpha*. Two areas did not overlap with those for *G. williamsonii*, *G. aquifolium* or *G. ilicifolia*. On this basis, it is unlikely that *G. williamsonii* is a result of recent hybridisation between *G. dimorpha* and *G. aquifolium* or *G. ilicifolia*

G. williamsonii was indistinguishable from *G. aquifolium* with all four enzymes. *G. ilicifolia*, while showing some alleles in common could be distinguished from *G. williamsonii* and *G. aquifolium* as it did not share any alleles at the *Pgm-2* locus or *Lap*. *G. dimorpha* did not share any alleles for *Pgm* or *Lap* with either *G. williamsonii* or *G. aquifolium* and only one with *G. ilicifolia* at the *Pgm-1* locus (Table 1).

Table 1. Allele frequencies for G. *williamsonii, G. aquifolium, G. ilicifolia* and *G. dimorpha.*

	Species			
Locus	***G. williamsonii***	***G. aquifolium***	***G. ilicifolia***	***G. dimorpha***
Pgm-1				
(N)	**11**	**8**	**7**	**8**
1	.000	.000	.000	.313
2	.000	.000	.286	.688
3	1.000	1.000	.714	.000
Pgm-2				
(N)	**11**	**8**	**7**	**8**
1	.000	.000	.000	1.000
2	.000	.063	.000	.000
3	1.000	.875	.000	.000
4	.000	.063	.000	.000
5	.000	.000	.929	.000
6	.000	.000	.714	.000
Pgm-3				
(N)	**11**	**8**	**7**	**8**
1	.000	.000	.000	.000
2	1.000	1.000	.000	.000
3	.000	.000	1.000	.000
Lap				
(N)	**11**	**8**	**7**	**8**
1	.000	.313	.571	.000
2	.773	.625	.429	.000
3	.000	.000	.000	.188
4	.227	.630	.000	.000
5	.000	.000	.000	.813

Table 2. Matrix of genetic similarity and distance coefficients.

Below diagonal: Nei (1978) unbiased genetic identity
Above diagonal: Nei (1978) unbiased genetic distance

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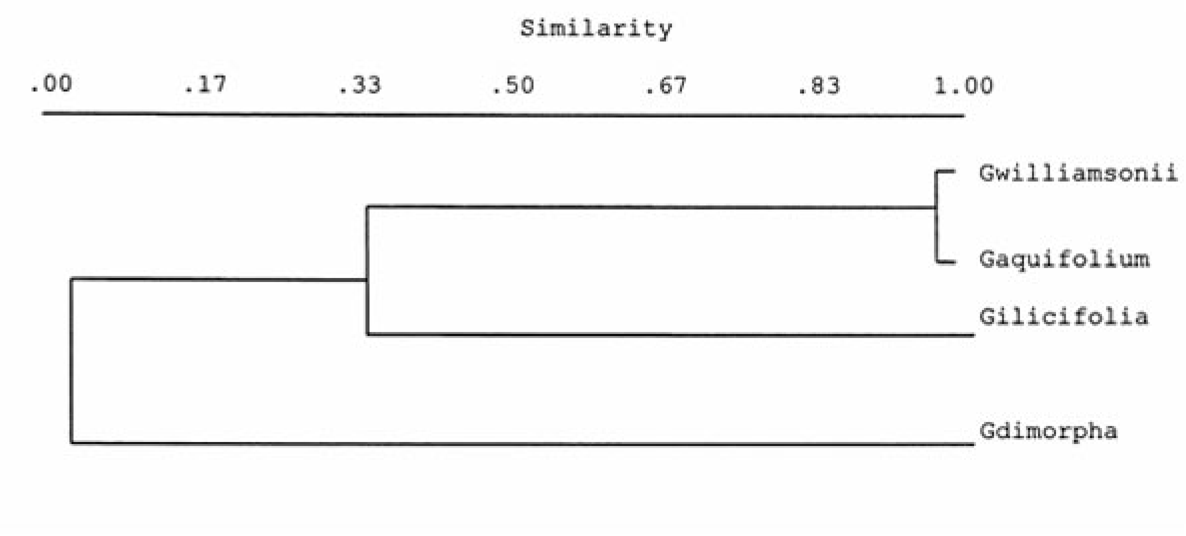
Species	**G. *williamsonii***	**G. *aquifolium***	**G. *ilicifolia***	***G. dimorpha***
G. williamsonii	-	*.014*	1.131	
G. aquifolium	.987	-	.966	-
G. ilicifolia	.323	.381	-	2.742
G. dimorpha	.000	.000	.064	-

On the basis of allele frequencies, genetic similarity (Table 2) and clustering on similarity (Fig. 16), *G. williamsonii* and G. *aquifolium* cannot be confirmed as separate species.

Figure 16. Clustering of *G. williamsonii*, *G. aquifolium*, *G. ilicifolia* and G. *dimorpha* based on allozyme data.



4. DISCUSSION

Survival of *G. williamsonii*

In May 2000, a survey was undertaken based on the plan by Miller (1998) but no additional plants or populations were located (Cropper & Cropper 2000). Only 7 plants remained at Cassidy's Gap in October 2000.

The ex-situ population of *G. williamsonii* contains approximately 150 plants representing five genotypes. Under cultivation, *G. williamsonii* is a vigorous, floriferous and bushy plant that is easily grown from cuttings. In contrast, plants in the field are kept in check by competition from other species. No flowers were recorded in 1999 and very few were seen in 2000 despite prolific flowering in other years (Marriott, pers. comm.). The time since the site was last burnt in not known. Vegetation is quite dense and estimates of time since last fire range from 17 years (Molyneaux, MEL herb. spec.) to "long unburnt... potentially several decades" (Miller 1998). The plants at Cassidy's Gap presumably became established when the other vegetation was not as dense. Location of a seedling in 1997 shows that inter-fire recruitment is possible but it is of note that the seedling has not grown or produced more leaves than it had when first observed.

Plants of both G. *aquifolium* and G. *williamsonii* have died at Cassidy's Gap since 1992 and the few remaining specimens look diseased. Whilst P. *cinnamomi* (cinnamon fungus) has not been isolated from the site, the death of a number of plants belonging to the Proteaceae family look suspiciously like die-back due to *Phytophthora cinnamomi* (pers. obs.) It is unlikely that any individuals will remain in the wild in five years due to a combination of an inability to compete with other vegetation, senescence of individuals and the effects of *P. cinnamomi*.

Reproductive structures in *G. williamsonii* compared to *G. aquifolium* and *G. ilicifolia*

The microscopy results suggest that both the male and female reproductive structures are abnormal. There is no mention of the lack of anthers in the original description (von Mueller 1893) but examination of specimens from the original collection and there is no evidence that pollen is ever produced. It is feasible that the pistil is partly functional because stigmatic surfaces developed as the flowers aged suggesting that they could become receptive after anthesis despite the abnormal appearance of the papillae. The species, therefore, appears to be totally male sterile and female function is quite likely to be impaired.

If pollen is transferred to *G. williamsonii* flowers it will almost certainly originate from nearby plants of *G. aquifolium*. Follicle development might be stimulated by pollen germination on the stigma but pollen tube penetration could be impaired because of the deformed papillae. Although there were no gross abnormalities in the ovules, the observation that few seeds are set despite prolific follicle development (Olde & Marriott 1995) may be an indication of suboptimal fertilisation.

Unfortunately, the absence of flowers in the field and in cultivated plants at the Royal Botanic Gardens Melbourne in 1999 has meant that the study of female function in *G. williamsonii* with pollen from different species could not be completed.

Comparison of G. *williamsonii* with related species and nearby G. *dimorpha*

In von Mueller's (1893) description of the species, *G. williamsonii* is thought to have an affinity to *G. aquifolium* and *G. ilicifolia* and that has been broadly supported in this study. Allozyme assays could not distinguish between *G. williamsonii* and *G. aquifolium* but *G. ilicifolia* and *G. dimorpha* were different. The inability of the allozyme data to differentiate *G. williamsonii* and *G. aquifolium* strongly suggests that they belong to the same species but is not conclusive because of the small number of enzymes resolved. A different analytical method based on DNA analysis is required to establish whether there are genetic differences between *G. williamsonii* and *G. aquifolium* that are taxonomically informative and that could also be used to confirm their separation from *G. ilicifolia*.

There was not sufficient enzyme data to provide an assessment of genetic diversity. However, the analysis has confirmed that the *G. williamsonii* plants are from seedlings rather than a group of genetically identical plants resulting from vegetative propagation such as root suckering.

There is no evidence of recent hybridisation between *G. williamsonii* and either *G. dimorpha* or *G. ilicifolia*. *G. williamsonii* does not show any morphological characteristics in common with *G. dimorpha* so despite its proximity, it does not interbreed with *G. williamsonii*. The enzyme differences do not support recent hybridisation between *G. williamsonii* and *G. ilicifolia* and align *G. williamsonii* with *G. aquifolium* rather than *G.ilicifolia* in contrast to Olde and Marriott (1995). McGillivray (1993), prior to the discovery of plants in 1992, considers *G. williamsonii* to be either a hybrid with *G. aquifolium* as one of the parents or an unusual variant of that species. Cropper and Cropper (2000) also suggest that the plants of *G. williamsonii* are a result of a random mutation in *G. aquifolium*. They suggest that a mutation in a gene controlling plant development such as the structure of a growth hormone could explain the 'mutated' or partially developed flowers and the juvenile-like *G. aquifolium* leaves seen in these plants. They also say that "the recent discovery of a seedling despite no seed being seen on *G. williamsonii* plants indicates that, if we assume this hypothesis to be correct, the parent *G. aquifolium* plant is still present" Seed has been produced by plants in the wild (N. Marriott, pers. comm. 1999) and was thought to be viable but none is known to have germinated.

Implications for management of *G. williamsonii*

The taxonomic uncertainty of *G. williamsonii* has implications for the management of the area in which it occurs. There are three possibilities for the origin of *G. williamsonii* and the appropriate management actions differ for each possibility.

1. Hybrid- The population of *G. williamsonii* may be the result of a chance pollination event between *G. aquifolium* and another, unidentified species. Reproductive abnormalities such as sterility are common consequences of hybridisation. The population does not produce pollen so cannot be self-sustaining in the longterm. In evolutionary terms, the occasional production of hybrids can be important if they are able to reproduce. If *G. williamsonii* is a hybrid, the appropriate management action would be to manage the area to enable hybridisation to occur but the preservation of individual hybrid populations should not be a goal of management.

2. Variant of *G. aquifolium*- The population of *G. williamsonii* is a sibling cohort of a variant of *G. aquifolium* that occurs sporadically. A mutation which produces abnormalities in male and female function as well as leaf morphology is undoubtedly rare but cannot be discounted. Any seeds produced on *G. williamsonii* will actually be *G. aquifolium*. The occasional production of a variant may be the result of sexual reproduction where rare genetic variants are combined. Such combinations can provide plants that are better suited to a particular area and so provide the species with more chance of having individuals that can thrive under changing conditions. In the case of *G. williamsonii*, there is no obvious evolutionary advantage in having plants with reduced reproductive capacity. Management of areas containing *G. aquifolium* need to take into account the patterns of genetic diversity in the species so that it is retained giving the species the best chance of survival in the longterm.

3. Species- The population is a relict species that has become reproductively isolated. Site conditions suggest that the extant individuals will not be present within five years. New populations could be established from cultivated material but the inability of the species to reproduce means that the species will not be self-sustaining in the longterm.

Conclusion

Grevillea williamsonii remains somewhat of a taxonomic enigma but evidence from this study points to its position as an unusual variant of *G. aquifolium* rather than a distinct species. The results emphasise the need to conduct appropriate research prior to the development of Recovery Plans so that the actions are developed and implemented with the benefit of information on the biology and taxonomy of the conservation target.

The identity of *G. williamsonii* as a true species, a hybrid or a variant of another species impacts on way the extant population can be managed. Whilst the recovery plan can certainly be implemented, the decisions made on the management of the population could be financially wasteful if made on the basis of incorrect identity and also detrimental when based on a lack of specific knowledge of the biology of *G. williamsonii*.

Appropriate management for the "species" depends on clarification of its taxonomic position and is reliant on further genetic analysis. Sufficient ex-situ material is in cultivation to re-introduce *G. williamsonii* if it is shown to be of specific rank but this option will not produce a self-sustaining species in the longterm and must be considered to be a low priority. If *G. williamsonii* is, instead, a rare variant of *G. aquifolium* then management practices, including fire, should be designed for the general habitat type rather than to preserve the remaining in-situ plants.

Acknowledgments

1 would like to thank Jill Read and other Parks Victoria staff for their help during this project. Neil Marriott generously provided plant material and details of locations of different populations of *G. aquifolium*, *G. ilicifolia* and *G. floripendula*. He and Bob Makinson, provided information and opinions about the holly-leafed grevilleas and various species. Thank you, also, to the nursery staff at the Royal Botanic Gardens Melbourne who have propagated and maintained the ex-situ collection.

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Fig. 1. *G. williamsonii* in cultivation showing vigorous growth and densely branching habit.

Fig. 2. Close up of foliage and branching of *G. williamsonii*. This plant has only entire leaves.

Fig. 3. One of the larger plants of *G. williamsonii* showing close proximity to other species. *G. aquifolium* is very common at Cassidy's Gap and is frequently found growing next to *G. williamsonii*.

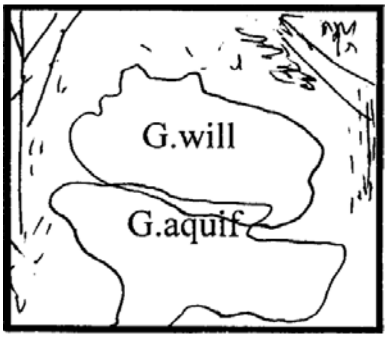

Fig.4. An example of a flower of *G. williamsonii* with an extra pistil (arrowed).
Bar = 0.1 mm

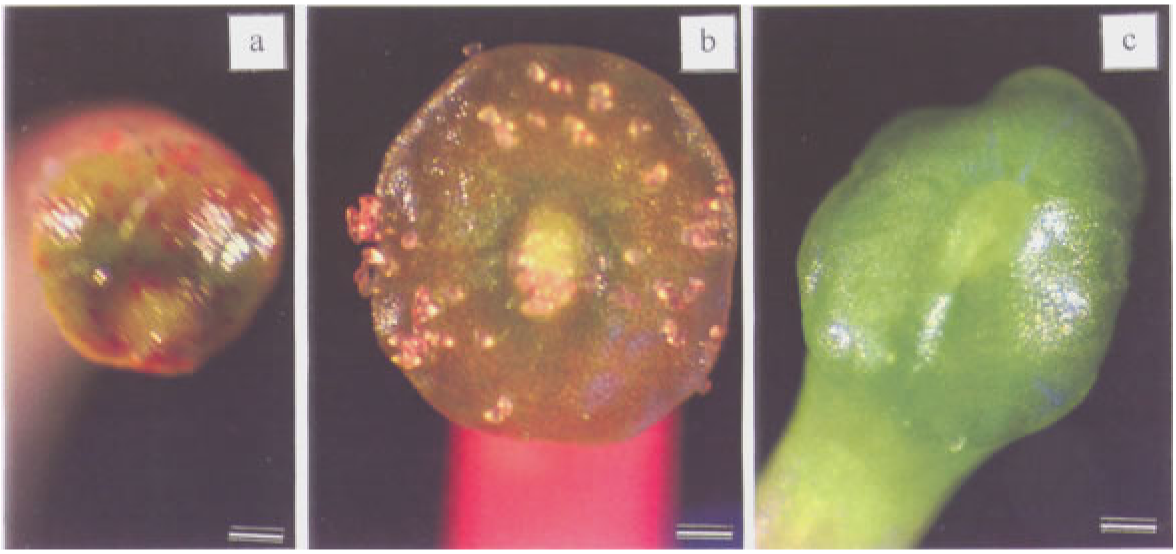

Fig. 5. Immature stigmas before papillae have protruded from surface of disk. **a.** *G. williamsonii*.

b. *G. aquifolium* with pollen showing its role as a pollen presenter.

c. *G. ilicifolia* with pollen removed. Bar = 1 mm

Fig. 6. Raised, receptive area can be seen protruding from the surface of the stigmatic disk. Bar = 0. 1 mm
G. williamsonii **a**. side view. **b**. front view.
G. aquifolium **c**. side view. **d**. front view.
G. ilicifolia **c**. side view. **f**. front view.

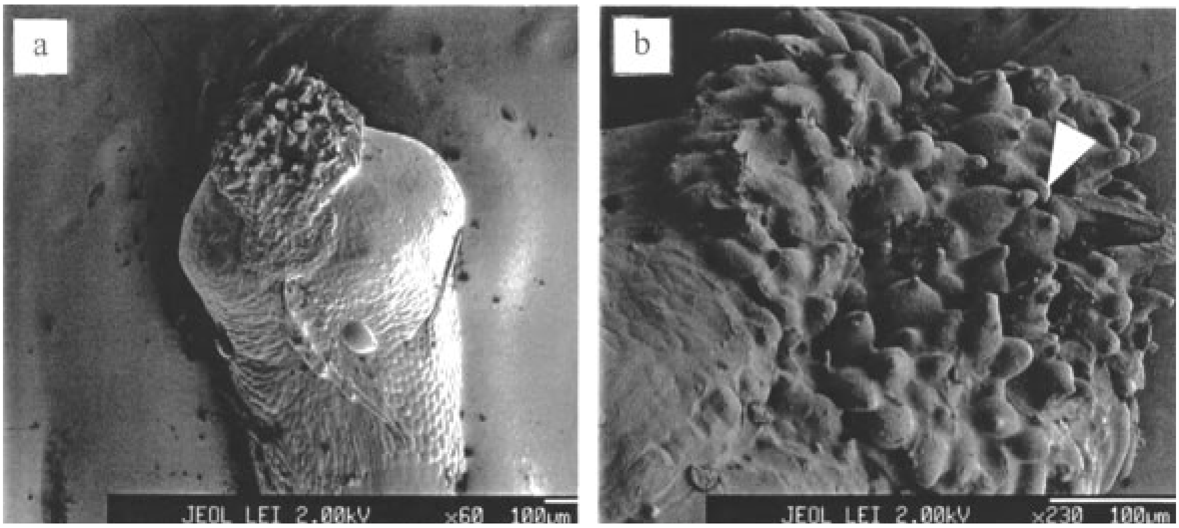

Fig. 7. SEM of *G. williamsonii* **a**. raised stigmatic area with papillae and lack of disk. **b**. papillae are swollen at the base and have abnormal points at the ends. Stigmatic secretion visible on papillae.

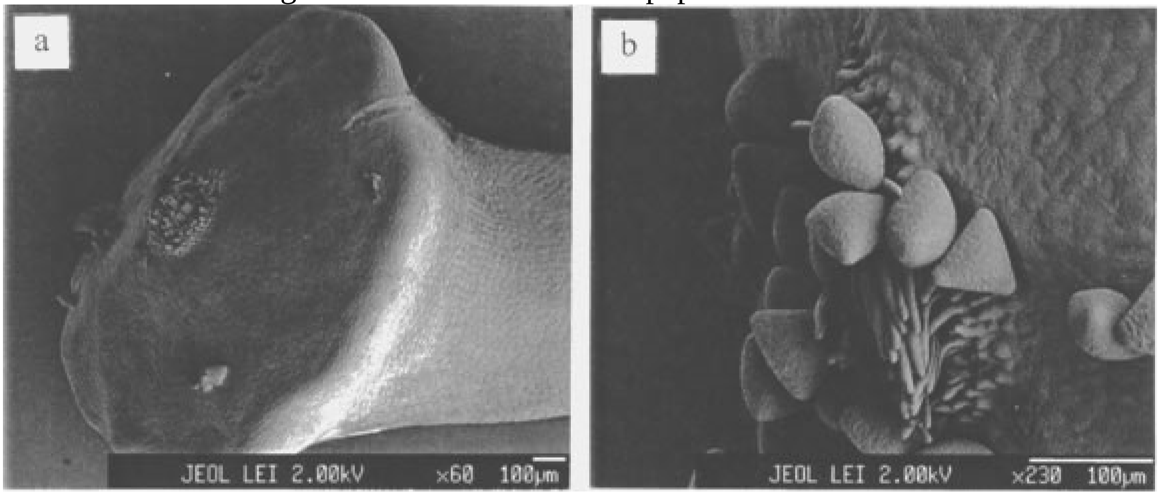

Fig. 8. SEM of *G. aquifolium*. **a.** young stigma with (broken) papillae in the centre of the stigmatic disk. **b**. pollen grains can be seen near the slender, fingerlike papillae on this immature stigma.

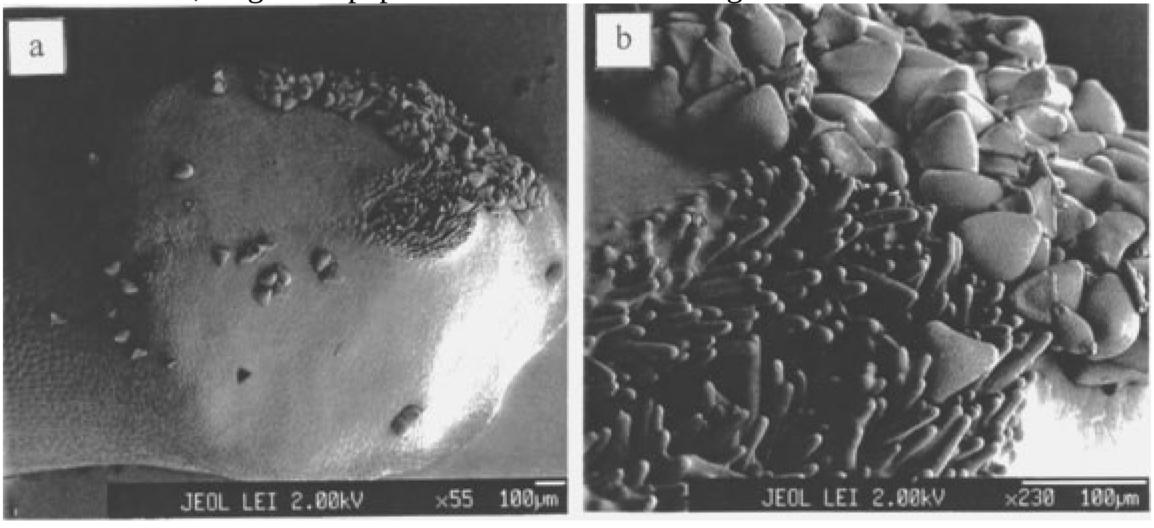

Fig. 9. SEM of *G. ilicifolia*. **a**. young stigma with emerging papillae and pollen grains. *b*. slender, fingerlike papillae.

Fig. 10. Buds of **a**. *G. williamsonii*, **b.** *G. aquifolium* **c.** *G. ilicifolia* showing the difference in size of buds and particularly the swollen corolla tube in *G. aquifolium* and *G. ilicifolia* containing anthers. Bar = 1 mm

Fig. 11. Dissected flowers showing **a**. absence of anthers in *G. williamsonii* and their presence in **b**. *G. aquifolium* and **c**. *G. ilicifolia*. Bar = 1 mm

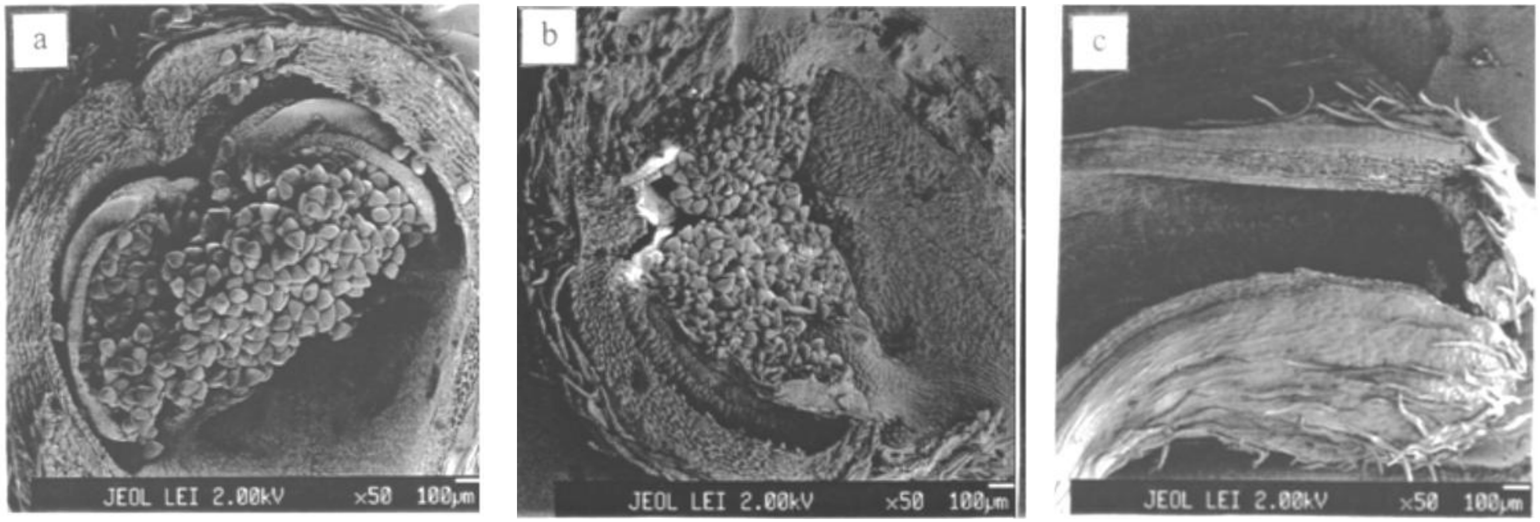

Fig. 12. SEM of anthers showing pollen in anthers of **a**. *G. aquifolium* and **b**. *G. ilicifolia* but not **c**. *G. williamsonii*.

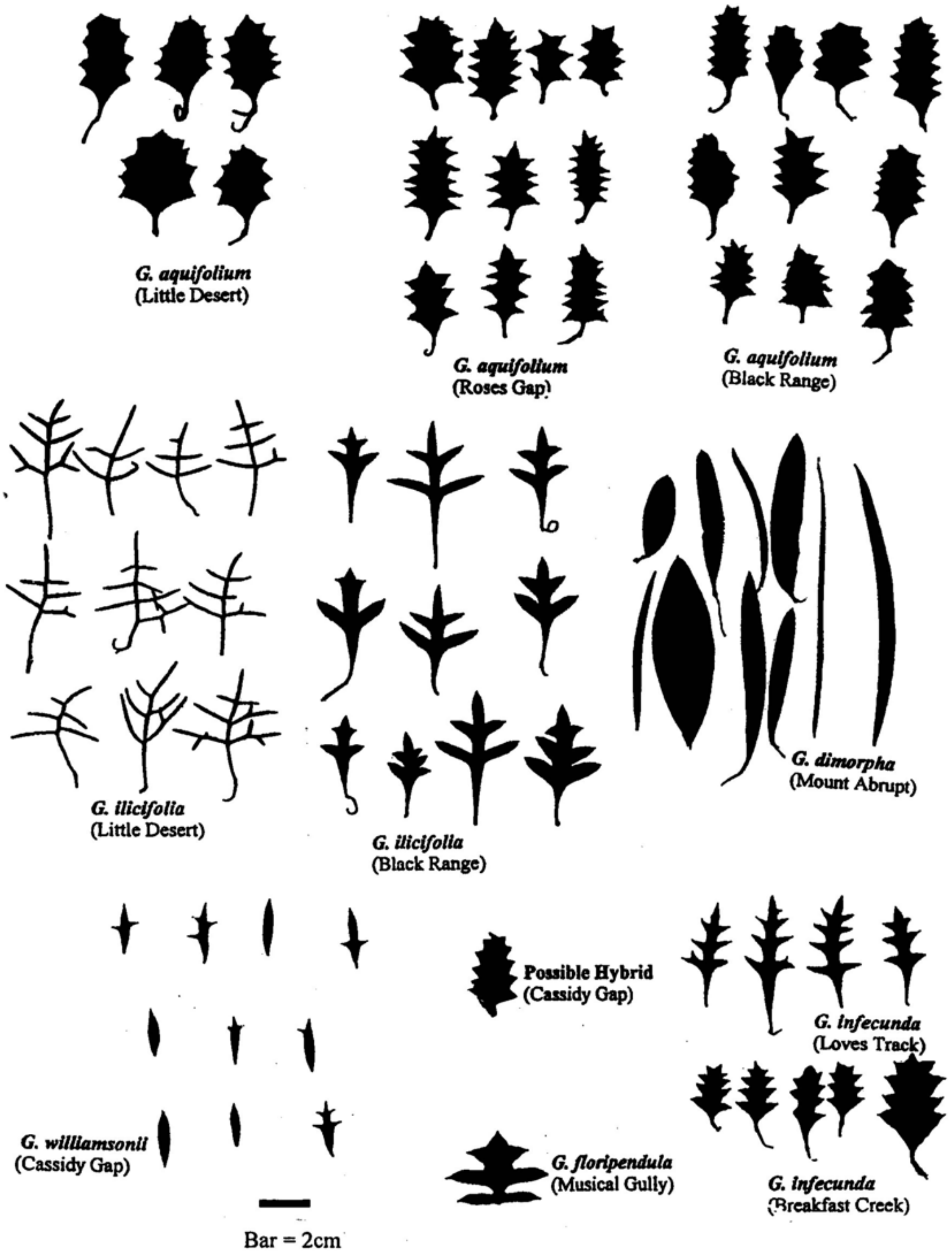

Fig. 13. A comparison of leaf types typical of populations and species sampled for this study.

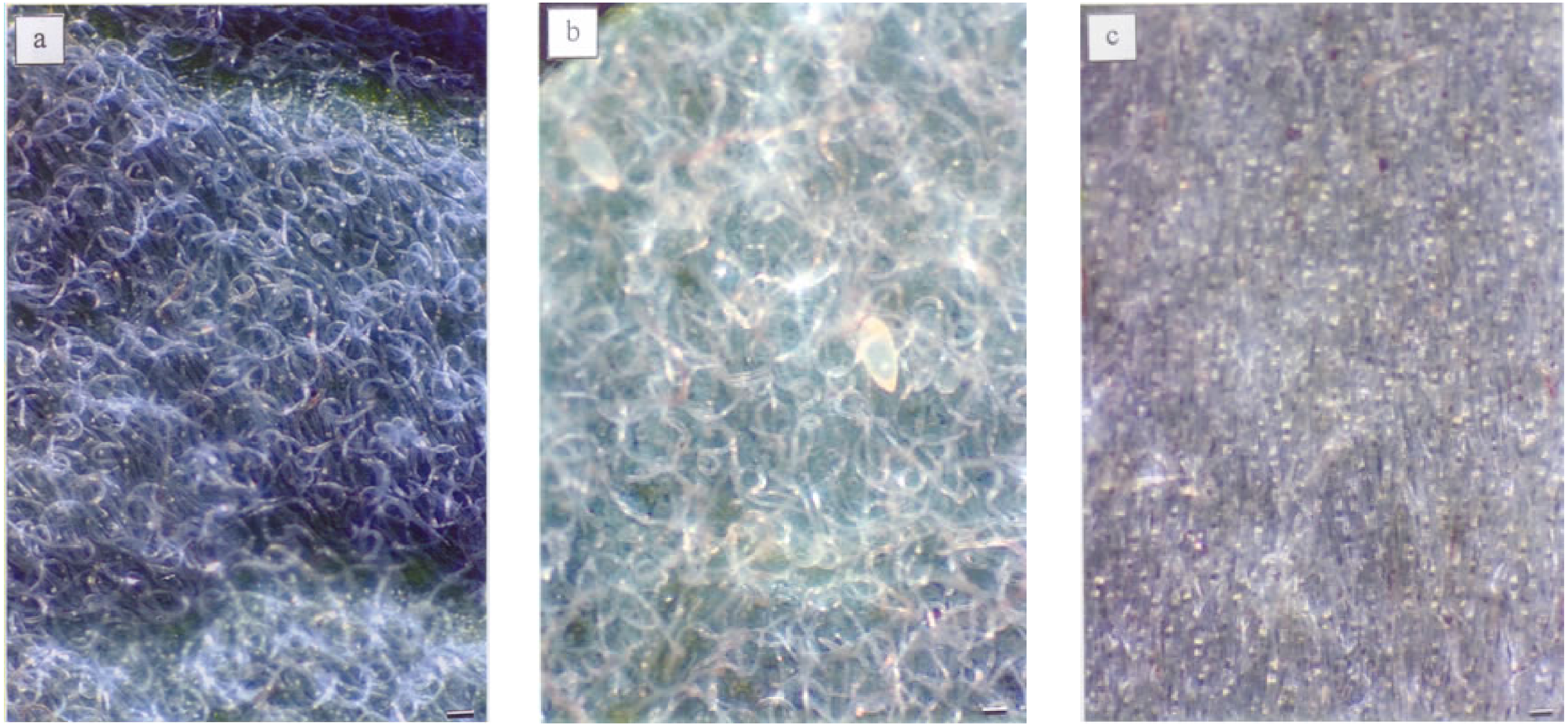

Fig. 14. Hairs on leaves of **a**. *G. williamsonii* **b**. *G. aquifolium* and **c.** *G. ilicifolia* under the light microscope. Bar = 0. 1 mm

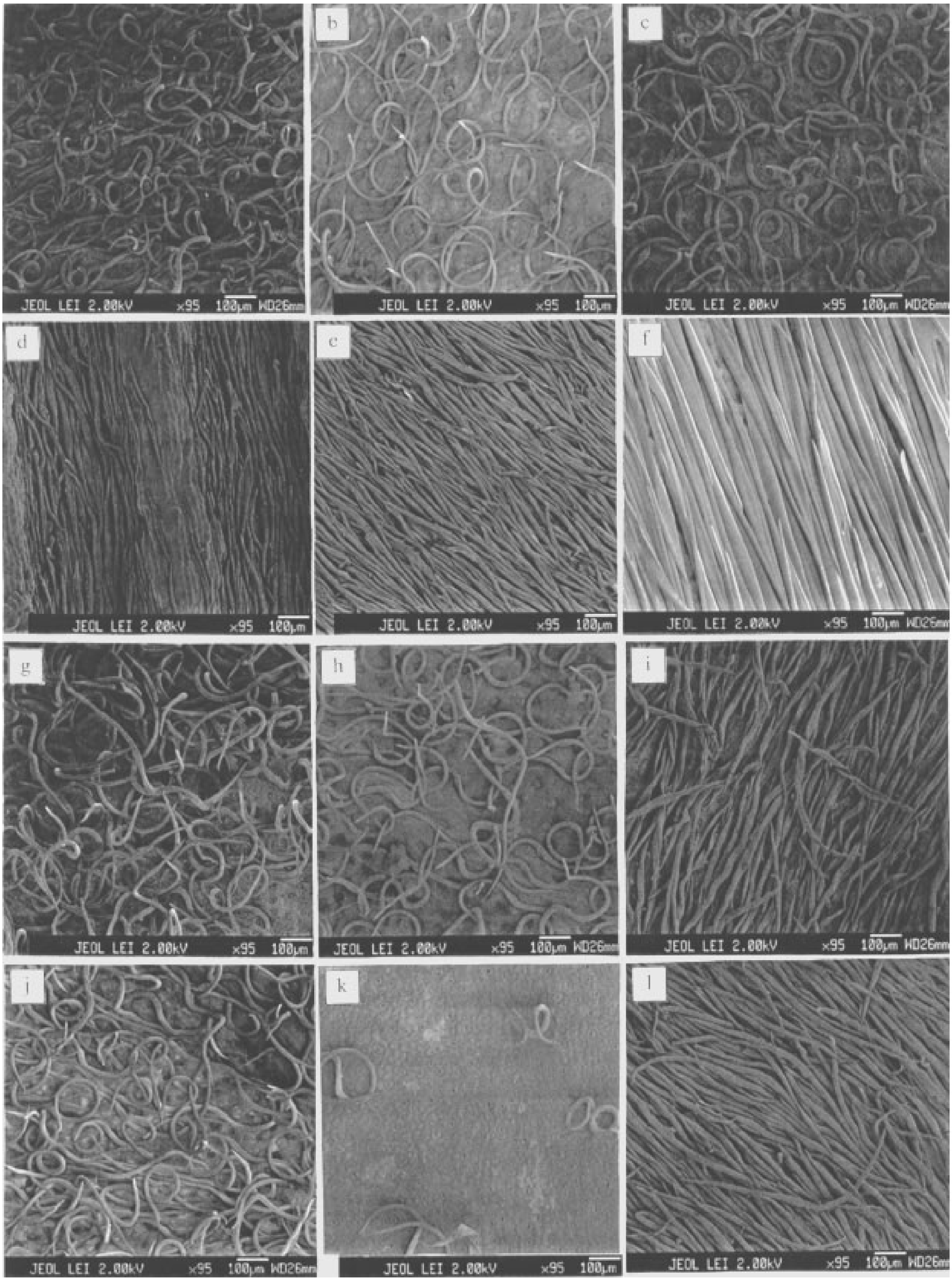

Fig. 15. Hairs on leaves of **a**, **b**, **c**. *G. aquifolium* **d**, **e**. *G. ilicifolia* **f**. *G. dimorpha* **g**. *G. williamsonii* **h**. putative hybrid between *G. aquifolium* and G. *williamsonii* **i** *G. infecunda* **j**. *G. williamsonii* **k**. G. *floripendula* **1**. *G. infecunda* under the scanning electron microscope.