Chapter 14
Pachycladon

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14.1 Introduction

The islands of New Zealand are located on the ring of fire in the South Pacific and are the product of intense geological activity including tectonic faulting and volcanic eruptions. The Pliocene epoch, occurring 2.5–5.2 million years ago (Mya), was a time of great geological change in the South Island of New Zealand predominated by the uplift of the southern Alps and Kaikoura Mountain Range (Batt et al. 2000). The southern Alps resulted from trans-current movement of the Alpine Fault that runs along almost the entire length of the South Island, and was formed by the colliding of the Indo-Australian and Pacific tectonic plates. The Pleistocene (0.01–2.5 Mya) was a significant epoch for vascular plants with numerous glacial–interglacial cycles that have shaped both the landscape and distribution of contemporary plants in New Zealand and around the world. The geological and glacial activity of the last 5.2 million years has resulted in a variety of parent-rock substrates, an array of diverse environmental variables, and potential new microhabitats for plants. It is not surprising that this rich and recent geological activity was paralleled by many Late Tertiary/Early Quaternary species radiations to give rise to the incredible diversity of plants found in New Zealand (McGlone et al. 2001).

The genus *Pachycladon* belongs to the Brassicaceae (Cruciferae) family and its member species are found exclusively in New Zealand and Australia (Tasmania). These plants are perennial herbs that grow on rocky substrates and exhibit extensive morphological diversity and a range of habitat preferences. This genus has had a recent allopolyploid origin (<2 Mya) and has undergone rapid speciation and radiation in the last million years. Its unique history, natural diversity, and close relationship to the model plant *Arabidopsis thaliana* have resulted in its development as an emerging model system to study the molecular genetic basis of plant speciation and radiation, and these studies are yielding molecular resources that can be used to study other traits of interest in the Brassicaceae.

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14.2 Basic Botany of the Species

14.2.1 Distribution

There are nine species of *Pachycladon* that are endemic to New Zealand and an additional species, *Pachycladon radicatum* (Hook.f.) Heenan & Mitchell, endemic to Tasmania. The New Zealand species of *Pachycladon* are restricted to the South Island, found predominantly in sparse isolated populations and form three distinct groups based on morphological characters and habitat preference (Heenan and Mitchell 2003). The distribution of New Zealand *Pachycladon* species is illustrated in Fig. 14.1 and their substrate and altitudinal preferences are summarized in Table 14.1.

The first group includes two species. *Pachycladon cheesemanii* Heenan & Mitchell is the most widely distributed species occurring in eastern South Island, having a wide latitudinal and altitudinal range and occurring on a number of different rock types. Its altitudinal range is from near sea level (10 m) to mountaintops (1,600 m), and it is a geological generalist occurring on graywacke, Haast schist, and basaltic

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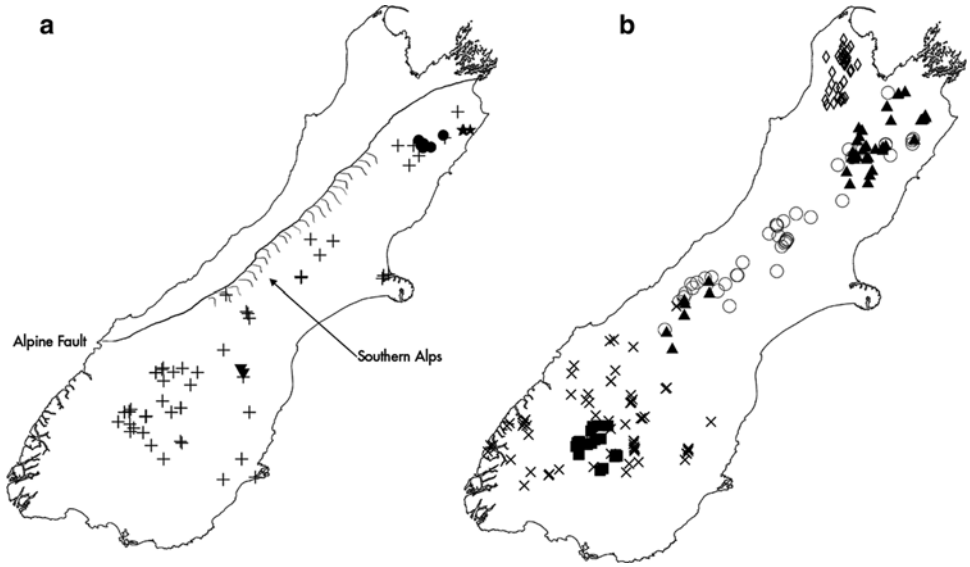

Fig. 14.1 Distribution of *Pachycladon* species. (**a**) *P. cheesemanii* (*plus sign*), *P. exile* (*solid inverted triangle*), *P. stellatum* (*star*), and *P. fasciarium* (*solid circle*) (**b**) *P. enysii* (*open circle*), *P. fastigiatum* (*solid triangle*), *P. latisiliquum* (*open diamond*), *P. novae-zelandiae* (*cross sign*), and *P. wallii* (*solid square*)

and andesitic volcanic rocks (see Fig. 14.1a). The closely related *Pachycladon exile* (Heenan) Heenan & Mitchell has a very restricted distribution in northern Otago and it is known with certainty from only three low-altitude (<500 m) sites comprising calcareous substrates, including limestone and volcanic rock (see Fig. 14.1a).

The remaining species of *Pachycladon* are generally more specialized in their distributional and habitat requirements. The second group is comprised of *Pachycladon* species located in southern South Island that predominantly occur on Haast schist. *Pachycladon novae-zelandiae* (Hook.f.) Hook.f. occurs on rocky outcrops and fell field between 1,080 and 2,031 m (see Fig. 14.1b). *Pachycladon crenatus* Philipson[1] is a western Southland taxon found in the Fiordland area growing on gneiss. *Pachycladon wallii* (Carse) Heenan & Mitchell is found on rocky outcrops and bluffs between 1,100 and 1,875 m (see Fig. 14.1b).

The last group comprises of four alpine species that grow on shaded rocky bluffs and cliffs. Of these, *Pachycladon latisiliquum* (Cheeseman) Heenan & Mitchell, is a Northwest Nelson endemic growing in a narrow latitudinal range and at an altitude of 1,036–1,768 m on a wide variety of rock types including granite, sandstone, marble, limestone, and volcanics (see Fig. 14.1b). The other three species are restricted to greywacke. *Pachycladon enysii* (Cheeseman) Heenan & Mitchell occurs throughout the southern Alps from 975 to 2,492 m above sea level, reaching the highest altitude of all *Pachycladon* species and one of the highest of any vascular plant species in New Zealand (see Fig. 14.1b). Heenan and Mitchell (2003) suggested that *P. enysii* probably survived on nunataks during glacial periods, especially during the last glaciation (~14,000–18,000 years ago). In contrast, *Pachycladon fastigiatum* (Hook.f.) Heenan & Mitchell has a lower altitudinal range (914–2,031 m) and disjunct populations in the northern and southern parts of the southern Alps (see Fig. 14.1b). It is absent from the high mountains of the central southern Alps, and it was suggested that this distribution reflects its extirpation from this area during the last glaciation (Heenan and Mitchell 2003). *Pachycladon stellatum* (Allan) Heenan & Mitchell is restricted to a small geographic area in

[1]The taxonomic standing of this species is questionable as *P. crenatus* appears to grade into *P. novae-zelandiae*, with whom it shares many features. For the purpose of this chapter, therefore, a broad taxonomic concept of *P. novae-zelandiae* will be adopted that includes *P. crenatus*.

Table 14.1 Morphological and distributional characteristics of *Pachycladon* species

Species	Growth habit	Leaves	Inflorescence	Siliques	Seed wings	Altitudinal range (mean ± SD) m	Geological parent material of substrate
P. cheesemanii	Polycarpic, woody caudex, subshrub habit	Leaf heterophylly, ovate to broadly elliptic, lobed to serrate; lamina and petiole with branched hairs	Slender and terminal; narrow petals	Terete; seeds uniseriate	Absent	10–1,600 (811 ± 49)	Greywacke, semi-schist, schist, plutonics
P. enysii	Monocarpic, caudex stout and soft	Ovate to ovate-lanceolate, serrate; lamina and petiole with branched hairs	Terminal and very stout; broad petals	Laterally compressed; seeds biseriate	Present	975–2,492 (1,885 ± 42)	Greywacke
P. exile	Polycarpic, caudex woody	Leaf heterophylly, ovate to broadly elliptic, lobed to serrate; lamina and petiole with branched hairs	Slender and terminal; narrow petals	Terete; seeds uniseriate	Absent	25–500 (276 ± 137)	Limestone, calcareous tuff and basaltic breccia, and alluvium
P. fasciarium	Monocarpic, caudex stout and soft	Narrowly linear to narrowly linear-lanceolate; glabrous	Terminal and very stout; broad petals	Laterally compressed; seeds biseriate	Present	884–1,058 (1,021 ± 59)	Limestone
P. fastigiatum	Monocarpic, caudex stout and soft	Narrowly elliptic to lanceolate, serrate; lamina usually glabrous (rarely with simple hairs), or with simple hairs only on the petiole and leaf margin	Terminal and very stout; broad petals	Laterally compressed; seeds biseriate	Present	914–2,031 (1,485 ± 33)	Greywacke, semi-schist
P. latisiliquum	Monocarpic, caudex stout and soft	Narrowly elliptic to lanceolate, serrate; lamina glabrous, usually with simple hairs on the petiole and leaf margin	Terminal and very stout; broad petals	Laterally compressed; seeds biseriate	Present	1,036–1,768 (1,441 ± 22)	Sandstone, marble, limestone, plutonics, argillite, granite
P. novae-zelandiae	Polycarpic, caudex semi-woody	Oblong to broadly elliptic, usually lobed or crenate; lamina and petiole glabrous or hairy, hairs simple or branched	Lateral, more-or-less slender; narrow petals	Laterally compressed; seeds biseriate	Absent	1,080–2,031 (1,587 ± 26)	Semi-schist, schist, plutonics
P. radicatum	Polycarpic, caudex semi-woody	Obovate to broadly elliptic, serrate; glabrous	Lateral, more-or-less slender; broad petals	Laterally compressed; seeds biseriate	Present	No data available	Dolerite and siliceous rock
P. stellatum	Monocarpic, caudex stout and soft	Narrowly elliptic to lanceolate, serrate; lamina and petiole with branched hairs	Terminal and very stout; broad petals	Laterally compressed; seeds biseriate	Present	900–1,371 (1,021 ± 34)	Greywacke
P. wallii	Polycarpic, caudex semi-woody	Oblong to oval, usually lobed or crenate; glabrous	Lateral, more-or-less slender; broad petals	Laterally compressed; seeds biseriate	Present	1,100–1,875 (1,403 ± 37)	Sandstone, semi-schist, schist

southern Marlborough, where it occurs on greywacke bluffs at a relatively low altitude (900–1,371 m) (see Fig. 14.1a). Finally, a recently identified species, allied to *P. fastigiatum* and named *P. fasciarium* Heenan (see Fig. 14.1a), is found restricted to limestone in eastern Marlborough (Heenan 2009).

14.2.2 Morphology

Pachycladon species are short-lived perennial, rosette-forming herbaceous plants with leaves that are glabrous or bear simple or branched trichomes, terminal or lateral inflorescences, white flowers, and narrow siliques. The growth habit and extent of morphological variation of several species of *Pachycladon* can be seen in Fig. 14.2. Differences in their morphology are further summarized in Table 14.1.

A phylogenetic analysis of morphological characters identified four species groups in *Pachycladon* (Heenan and Mitchell 2003); three of these groups are New Zealand endemics and the fourth represents the single Tasmanian species *P. radicatum*. *P. radicatum* is polycarpic, with decumbent branches, semi-woody caudex, ovate leaves, more or less slender, lateral inflorescences, short, laterally compressed siliques, and seeds that are winged.

Of the New Zealand species, *P. exile* and *P. cheesemanii* (Fig. 14.2a) are polycarpic and have woody caudices, short aerial branches, exhibit heterophylly, have slender, terminal inflorescences, terete siliques with uniseriate seeds, and seeds without wings. These two species are most similar, morphologically, to the closest relatives of *Pachycladon* in the Brassicaceae, viz., *Transberingia* and *Crucihimalaya* (Heenan et al. 2002; Joly et al. 2009). It has been suggested that the ancestral *Pachycladon* may have been the most similar to modern-day *P. cheesemanii* because of this morphological similarity and its generalist nature (Heenan and Mitchell 2003).

A second group includes *Pachycladon novae-zelandiae* (Fig. 14.2d) and *P. wallii* (Fig. 14.2f), which are also polycarpic but have lobed leaves, lateral inflorescences, short and laterally compressed siliques with biseriate seeds, and seeds with or without wings. The siliques of *P. novae-zelandiae* have a unique lanceolate shape.

A final group consists of *P. enysii* (Fig. 14.2b), *P. fastigiatum* (Fig. 14.2c), *P. latisiliquum*, and *P. stellatum* (Fig. 14.2e), and these are monocarpic, have a stout and soft caudex, serrate leaves, stout terminal inflorescences, long and laterally compressed siliques with biseriate seeds, and seeds with wings. *P. fasciarium* is closely related to *P. fastigiatum*, but differs with leaves that are shorter and narrower, linear, glabrous, and having fewer, smaller teeth on the margin.

14.2.3 Reproduction

Pachycladon plants are monocarpic or polycarpic and flower from early to mid-summer between September and January. The monocarpic species develop inflorescences at the end of summer (February to March) and these overwinter (April to August) prior to flowering the next summer. The flowers are usually bisexual and have four white petals, six stamens, and one pistil. *Pachycladon* species are self-compatible (SC) (Heenan unpublished data) and thus are able to self-fertilize. While this mode of reproduction can ensure survival in the absence of other plants to cross-fertilize with, and is therefore a feature that is essential for the success of neopolyploid species (see Sect. 14.4.1), it can also lead to inbreeding depression through fixation of deleterious alleles. Hence, for an established species, outcrossing is a more favorable reproductive strategy.

Mitchell and Heenan (2002) found low pollen:ovule ratios in *P. cheesemanii* (76:1), *P. exile* (74:1) and *P. novae-zelandiae* (181:1) and selfing floral morphology, suggesting an autogamous breeding system for these species. Higher pollen:ovule ratios were determined for *P. fastigiatum* (339:1), *P. stellatum* (525:1), and *P. latisiliquum* (786:1), and examination of their floral morphology suggest that these SC plants are encouraged to outcross. Gender dimorphism (gynodioecy) has also been reported in *P. wallii*, *P. stellatum*, *P. fastigiatum*, and *P. latisiliquum* (Garnock-Jones 1991; Heenan and Garnock-Jones 1999). These findings, and estimates of the percentage of polymorphic loci and among-population genetic diversity (G_{ST}) from AFLP analyses, are consistent with *Pachycladon* species exhibiting predominantly selfing to mixed mating and endemic to regional distribution.

Fig. 14.2 Growth habit and morphological variation of *Pachycladon* species. (**a**) *P. cheesemanii*, (**b**) *P. enysii*, (**c**) *P. fastigiatum*, (**d**) *P. novae-zelandiae*, (**e**) *P. stellatum*, (**f**) *P. wallii*

Pachycladon species also remain interfertile. This was confirmed by the successful production of artificial F_1 hybrids from interspecific crosses under laboratory conditions and the recent discovery of naturally occurring hybrids of greywacke species *P. enysii* × *P. fastigiatum* and *P. fastigiatum* × *P. stellatum* as well as hybrids of *P. novae-zelandiae* × *P. fastigiatum* (Heenan 1999; Bicknell et al. 2009; Heenan unpublished data). Figure 14.4b summarizes the parent plants of both the artificial interspecific hybrids generated, and the naturally occurring hybrids discovered to date.

14.2.4 Cytology and Embryology

Pachycladon species have a somatic chromosomal complement of $2n = 20$ as determined by DAPI staining and chromosomal counting of cells from floral or

root tip tissue (Dawson 1995; Dawson 2000; Lysak et al. 2009). The C-values (1C) of seven species of *Pachycladon* have been estimated recently including *P. exile*[§], *P. cheesemanii**, *P. novae-zelandiae*[§,*], *P. enysii**, *P. fastigiatum*[§,*], *P. wallii**, and *P. stellatum** and were found to range from 0.44 to 0.56 pg ([§]Lysak et al. 2009; *Bicknell and Heenan unpublished data), corresponding to genome sizes of ~430 Mbp for *P. exile*, ~450 Mbp for *P. cheesemanii*, ~460* Mbp for *P. novae-zelandiae*, ~489 Mbp for *P. enysii*, ~499[§]–509* Mbp for *P. fastigiatum*, ~518 Mbp for *P. wallii*, and ~548 Mbp for *P. stellatum* (1 pg = 978 Mbp; Dolezel et al. 2003). This suggests that the genomes of *Pachycladon* species are about three times bigger than the *A. thaliana* genome (1C = 0.16 pg, ~157 Mbp) but smaller than the family average (1C = 0.63 pg; ~616 Mbp; Lysak et al. 2009). They are comparable in size to the genomes of diploid crop species like *Brassica rapa* and *Raphanus sativus* but about half the size of the genomes of polyploid crop species like *Brassica juncea* and *B. napus*. Further, despite recent speciation (see Sect. 14.4.1), significant variation in genome size exists between *Pachycladon* species. To date, there are no published studies that characterize or compare karyotypes of *Pachycladon* species. However, a chromosomal painting study is currently underway to map the genomes of *P. cheesemanii*, *P. enysii*, *P. exile*, and *P. novae-zelandiae* (Mandáková, Lysak and Heenan unpublished data).

Embryological studies of *P. cheesemanii* and *P. exile* (Luo et al. 2003) reveal that their megasporogenesis and embyogenesis are similar to their relative and model plant *A. thaliana* (Mansfield and Briarty 1991). Meiosis was found to occur at Stage 10 and anthesis at Stage 13 (day 9). However, *Pachycladon* showed significant delays in the first cell divisions of the endosperm, occurring at Stage 16 (7 days post-anthesis) and the initiation of embryogenesis, which occurs at Stage 17 (~9–10 days after anthesis) in comparison with *A. thaliana*, where these processes are initiated just hours after fertilization (Mansfield and Briarty 1991). Interestingly, the fruit experienced its most rapid growth during the delay to endosperm formation and embryogenesis, with a rapid decline in growth rate after day 15. Silique dehiscence was observed in *P. exile* 7 days prior to *P. cheesemanii* (day 29 and 36, respectively). It is unknown if this delayed embryogenesis is restricted to these two *Pachycladon* species or if it occurs in all species.

14.2.5 Taxonomy

14.2.5.1 Morphotaxonomy

Pachycladon has a befuddled taxonomic history and this reflects issues of generic circumscription that are common in the Brassicaceae (e.g., Al-Shehbaz et al. 1999). When the species now assigned to *Pachycladon* were first named and described in the late 1800s and early 1900s, the majority were placed in the well-known European and North American genera *Arabis* L., *Braya* Sternb. & Hoppe, *Cardamine* L., *Nasturtium* R.Br., and *Sisymbrium* L., as they appeared morphologically similar to species from these genera. The genus *Pachycladon* was described by Hooker (1867) to accommodate *P. novae-zelandiae*, an alpine species from southern South Island that had been previously placed in *Braya*. Later, a major study of Brassicaceae genera by Schulz (1924, 1936) resulted in the erection of the two new endemic New Zealand genera *Cheesemania* O.E. Schulz (seven taxa) and *Ischnocarpus* O.E.Schulz (two taxa).

In the past, *Cheesemania*, *Ischnocarpus*, and *Pachycladon* have not been considered to have a particularly close relationship as they have different growth habits (monocarpic or polycarpic), fruit (biseriate or uniseriate), and seed types (accumbent or incumbent; winged or not winged). *Cheesemania* was placed in the tribe Arabideae and *Ischnocarpus* and *Pachycladon* in different subtribes of the tribe Sisymbrieae (Schulz 1924, 1936). Much of this confusion has arisen because the Brassicaceae emphasis has been placed on morphological characters that show considerable convergence in the family and therefore are not reliable indicators of true taxonomic relationships (e.g., Mummenhoff et al. 1997; Koch et al. 1999, 2001; Al-Shehbaz et al. 2006).

14.2.5.2 Molecular Taxonomy

Molecular phylogenetic analyses of the nrDNA internal transcribed spacer (ITS) region using PAUP vers. 3.1.1 (Swofford 1993) and the Maximum Likelihood program DNAML, from PHYLIP vers. 3.5c (Felsenstein 1993) showed that species of *Pachycladon*, *Cheesemania*, and *Ischnocarpus* formed a monophyletic clade (Mitchell and Heenan 2000). When species from these genera

were topologically constrained to the tribes they were classified under, the resulting trees were far less parsimonious (requiring up to 75 additional steps) than the original tree, therefore rejecting these traditional taxonomic placements (Mitchell and Heenan 2000).

In order to more accurately infer the taxonomic position of *Pachycladon* within the Brassicaceae, several taxa including those from two studies (Koch et al. 1999; Mitchell and Heenan 2000) were subjected to a more comprehensive molecular phylogenetic analysis of nrDNA ITS data using PAUP vers. 4.0b6 (Swofford 2000; Heenan et al. 2002). The recent origin and close relationship between *Pachycladon*, *Cheesemania*, and *Ischnocarpus* was reflected in their grouping together with high bootstrap support (98%), within a monophyletic clade that lacked well-resolved topology, and showed low sequence divergence (2.6%). Based on the results from these two studies, a reassessment of morphological characters, and the generation of fecund interspecific hybrids between species from each of the three genera (Heenan 1999, Heenan unpublished data), *Pachycladon* was recircumscribed to include species placed in *Cheesemania* and *Ischnocarpus*, and *I. novae-zelandiae* was renamed as *P. cheesemanii* (Heenan et al. 2002).

The *Pachycladon* complex was found to belong to the Arabidopsoid clade of the Brassicaceae family and forms a distinct and relatively young New Zealand lineage estimated to be ~1.0–3.5 million years old based on molecular clock estimates for the nrDNA ITS region (Heenan et al. 2002). It is sister to *Crucihimalaya* from Asia and *Transberingia bursifolia* found in eastern Russia and North America. The ITS region of *Pachycladon* species and the model plant *A. thaliana* were estimated to have diverged ~9–11 Mya, indicating that they are closely related (Heenan et al. 2002).

Since these efforts were made to understand the phylogenetic relationships of *Pachycladon* species and their closest relatives, several *Pachycladon* species have been included in more recent and comprehensive studies of the taxonomy and phylogeny of the Brassicaceae family. Al-Shehbaz et al. (2006) critically reviewed the taxonomy of the Brassicaceae family in terms of morphological traits, generic circumscription, and major clades and proposed a new tribal alignment of 25 tribes to the family, placing *Pachycladon* along with other members of the Arabidopsoid clade in the tribe Camelineae. Bailey et al. (2006), with the aim of determining a global phylogeny for the family, performed a parsimony ratchet analysis in WinClada (Nixon 1999) of the nrITS region for 461 species representing 24 of the 25 Brassicaceae tribes and a super-matrix parsimony ratchet analysis in WinClada of five nuclear genes (nrITS, *PI*, *CHS*, *Adh*, *LFY*) and five chloroplast genes (*rbc*L, *mat*K, *atpb*, *ndh*F, *trn*L-*trn*F). Even though only nrITS and *rbc*L sequences were available for the five species of *Pachycladon* included in these analyses, the phylogenetic position of *Pachycladon* within the Camelineae was confirmed with high bootstrap support (100%). The sister clade of *Pachycladon* consisted of the genera *Transberingia* and *Crucihimalaya*, though in this super-tree, there was only poor bootstrap support for this relationship (37%).

14.2.5.3 Interspecific Relationships

Molecular support for interspecific relationships hypothesized based on habitat preference, morphology, and amplified fragment length polymorphism (AFLP) data (Mitchell and Heenan 2002; see Sect. 14.2.2) was sought using DNA sequence data from three nuclear markers (ITS, *FRIGIDA*, and *gap*C) and one chloroplast marker (*trn*L-*trn*F) that had parsimony informative nucleotides (McBreen and Heenan 2006). Marker sequence was recoverable from most species and subjected to NeighborNet analyses (Huson and Bryant 2006). Overall, the splits graphs generated are characterized by star-like patterns of radiation with little structure in the internal branches suggesting that the onset of diversification occurred very rapidly or simultaneously. In this multigene study, some interspecific relationships within the *Pachycladon* genus were well supported. *P. exile* and *P. cheesemanii* were the most frequently retrieved *Pachycladon* subgroup, occurring in the *FRIGIDA*, ITS, and *trn*L-*trn*F networks. The monocarpic species *P. enysii*, *P. fastigiatum*, *P. latisiliquum*, and *P. stellatum* were only partially retrieved in the *trn*L-*trn*F, ITS, *FRIGIDA*, and *gap*C-copy B networks. Interestingly, the species *P. enysii*, *P. fastigiatum*, and *P. stellatum* clustered together for all genes investigated to date (McBreen and Heenan 2006; Joly, Heenan and Lockhart unpublished data). Finally, the third morphological group consisting of *P. novae-zelandiae* and *P. wallii* was not retrieved in any of the networks.

14.2.5.4 Chemotaxonomy

Current research work reveals that *P. fastigiatum* and *P. enysii* demonstrate variable profiles of major secondary metabolites like glucosinolate compounds (GLS), glucosinolate breakdown products, and flavonoids (Voelckel et al. 2008; Voelckel and Reichelt unpublished data). Both *Pachycladon* species appear to have GLS chemotypes that reflect their innate glucosinolate composition. Instead of two species-specific GLS profiles, five GLS profiles (= chemotypes) were found, two specific to *P. fastigiatum* (chemotypes two and five) and three specific to *P. enysii* (chemotypes one, three, and four). In terms of major GLS compounds, chemotype one produced mainly 4-methylsulfinylbutyl GLS (4MSOB) and 3-butenyl GLS, chemotype two produced mostly 3-methylsulfinylpropyl GLS (3MSOP), chemotype three produced predominantly allyl GLS and 3-butenyl GLS, and chemotypes four and five produced mostly 3MSOP and allyl GLS. Chemotype four and five differed in the minor compound 3-methylthiopropyl GLS (3MTP), which was only produced in chemotype four. All chemotypes produced 7-methylsulfinylheptyl GLS (7MSOH) as a major compound, albeit to different degrees. Interestingly, these chemotypes were not randomly distributed across populations. Instead, individuals of one chemotype were found to dominate each site (except for one *P. fastigiatum* site). It remains to be determined if this is due to local adaptation or neutral processes such as drift and inbreeding. The five *Pachycladon* chemotypes can be matched with three *A. thaliana* ecotypes for which the allelic configuration at two major glucosinolate biosynthetic loci is known (Kliebenstein et al. 2001). Therefore, it is predicted that the chemotypes observed in *P. enysii* and *P. fastigiatum* can be explained by segregation of the *GLS-elong* locus and the *GLS-AOP* locus. These chemotypic differences may be useful in distinguishing, at the interspecific and intraspecific levels, populations that should be targeted for conservation efforts. Glucosinolate profiles are currently being obtained for additional *Pachycladon* species (see Sect. 14.5.2.2).

14.2.6 Agricultural Status and Research Use

Pachycladon species are considered to be important endemic New Zealand plants, some of which are considered nationally threatened and therefore subject to preservation efforts by the New Zealand Department of Conservation to protect them from invasive species. There are no documented uses of these plants by the Maori (indigenous people of New Zealand). The very recent origin and radiation of *Pachycladon* make it an excellent candidate for development as a model system to determine the genetic and molecular basis underlying plant speciation, adaptive radiation, and evolution.

Studies of the model plant *A. thaliana* and its close relatives are leading to a much greater understanding of the genetic processes involved in plant development and evolution. It is now accepted that major changes in plant form can be accomplished by changes in a few key genes (Doebley 1992; Doebley and Lukens 1998). However, there is still much to learn about the drivers and mechanisms most important to morphological and ecological diversification during plant species radiation (Mitchell-Olds 2001; Shepard and Purugganan 2002; Remington and Purugganan 2003). For example, is diversifying selection on a small number of key genes sufficient for plant radiation? Testing this hypothesis is becoming increasingly tractable due to our improved understanding of the genetic processes operating within model plants, and comparative studies with species that have undergone recent morphological and ecological diversification in the wild (Falconer and Mackay 1996; Schmidt 2000; Weinig et al. 2003).

The close relationship between *Pachycladon* and *Arabidopsis* enables the use of available genomic resources. By taking advantage of the vast amount of resources that are available for *Arabidopsis*, and the natural diversity within *Pachycladon*, important advances in understanding the genetic processes underlying plant speciation and adaptive radiation will be made. In particular, *Pachycladon* can be used to understand whether diversifying selection on a small number of key genes is sufficient for plant radiation, and in answering this question, identify some of the genes involved.

14.3 Domestication and Conservation

Currently, *Pachycladon* species are primarily used in research programs to elucidate speciation, adaptive radiation, and evolutionary processes. As they have

no direct agricultural potential per se, domestication in a traditional sense is not an issue being addressed. However, cultivated plants are an essential resource for experimental genetic, molecular, reproductive, and ecophysiological study and *Pachycladon* plants have been found to be amenable to growth and maintenance in growth chambers and greenhouses.

14.3.1 Growth and Maintenance of Pachycladon *Plants*

14.3.1.1 Cultivation and Propagation

Plants are generally readily grown from seed, and if the seed is only a few days old, rapid germination occurs. Fresh seed will often spontaneously germinate in damp conditions around potted plants. Seeds older than 2–3 weeks benefit from stratification at 4°C for 7 days. Germination occurs about 7–12 days after stratification. Young seedlings are most easily potted up when they are 10–15 mm in diameter, as smaller and younger plants are sensitive to disturbance and can be difficult to transplant.

Propagation can also be achieved by cuttings, and these will root in 3–5 weeks when placed in a mist unit with bottom heat. Plants are easily cultivated under greenhouse conditions, but do require overhead shading (prefer about 350 μmol s^{-1} m^{-2}), temperatures below 20–22°C, and good airflow. Cultivated plants respond to long day conditions and during the winter months can be induced to flower if subjected to 16 h light and 8 h dark. Monocarpic species need to be regularly replaced after flowering and fruiting, but the polycarpic species can be maintained for 3–4 years.

14.3.1.2 Pests and Diseases

The main pests and diseases of cultivated plants include root mealy bug (*Rhizoecus* species), white rust (*Albugo candida*) that can disfigure leaves and inflorescences in warm and humid conditions, gray cabbage aphids (*Brevicoryne brassicae*) that occur throughout the summer growing season, and white butterfly larvae (*Pieris rapae*). However, the most serious issue is the recent identification of turnip mosaic virus in *Pachycladon* (Fletcher et al. 2010).

14.3.2 Conservation Initiatives

Three species of *Pachycladon* are listed as nationally threatened according to the New Zealand threat classification system (de Lange et al. 2009). *P. exile* and *P. stellatum* are assessed as Nationally Critical. Today, *P. exile* is known from a single site on a limestone outcrop in northern Otago, although historically it was known from other sites in the area. It has undergone a significant decline, and at its single known location, the population is estimated to be about 50 individuals. Regular weeding of the naturalized *Hieracium pilosella*, *Dactylis glomerata*, and *Sedum acre* is undertaken to prevent smothering of *P. exile* and to provide an open habitat for its establishment. Fencing has also been required to prevent rabbits from accessing the site and damaging the plants by browsing. The site is under private ownership but has been preserved into perpetuity after being designated a National Queen Elizabeth II Covenant.

P. stellatum has a very restricted, naturally sparse distribution and specific, shaded, habitat requirements, and is known from a few scattered and very small populations in Marlborough. Competition from weeds, such as *Hieracium* spp. and *Echium vulgare*, which occupy similar habitats, are a threat to *P. stellatum*.

P. cheesemanii is considered to be Nationally Vulnerable. It is a widespread but sparsely distributed species that generally has small-sized populations (< 50 plants). A study of the impact of exotic weed competition on *P. cheesemanii* suggests that competition with invading weeds threatens current *P. cheesemanii* populations, seedling survival and plant establishment can be enhanced by weed removal, and considerable potential exists for artificially expanding populations by sowing seed into appropriate weed-free habitat (Miller and Duncan 2004).

P. fasciarium is found in a small population of less than 50 plants restricted to limestone cliffs in Marlborough, and has been the subject of a cryopreservation study, whereby successful plant regeneration was achieved from cryopreserved meristems (Hargreaves et al. 1997). These techniques should be applicable to all species of *Pachycladon*. In contrast, seed germplasm is likely to be of limited use in the conservation of species of *Pachycladon*. Hargreaves et al. (1997) reported that after 4 years of storage at 4°C, seed germination reduced from 81 to 43%. These data are

also supported by anecdotal observations that *Pachycladon* seed stored for over 5 years has little or no germination (Heenan unpublished data), which is in contrast to the ex situ longevity reported for seeds of many Brassicaceae species that were found to remain viable even after 20 years of storage in the Millennium Seed Bank (Probert et al. 2009).

14.4 Origin and Evolution of *Pachycladon* Species

14.4.1 Allopolyploid Origin

14.4.1.1 Evidence for Allopolyploid Origin

A study by McBreen and Heenan (2006) revealed a duplication at the glyceraldehyde 3-phosphate dehydrogenase (*gap*C) gene, a nuclear marker, present in all species of *Pachycladon* with greater sequence divergence between the copies (7.9%) than among species, suggesting a possible polyploid origin for *Pachycladon*. To confirm that *Pachycladon* had a polyploid origin, Joly et al. (2009) sequenced several *Pachycladon* and Brassicaceae species for five single-copy nuclear gene markers (*CHS*, *PRK*, *MS*, *CAD5*, and *MtN21*) that map to distinct regions of the *A. thaliana* genome and different blocks of the reconstructed ancestral Brassicaceae karyotype (Schranz et al. 2006). These sequences were subjected to maximum likelihood and Bayesian phylogenetic analyses using PhyML vers. 2.4.4 (Guidon and Gascuel 2003) and BEAST vers. 1.4.7 (Drummond and Rambaut 2007), respectively. *Pachycladon* species were found to have two copies of all five of these genes and gene copies for every gene were distantly related and had the same phylogenetic position within the Brassicaceae, thus lending strong support that this genus has an allopolyploid origin. A Bayesian analysis of the *CHS* gene, which has been sequenced for many Brassicaceae species, was reproduced here using denser taxon sampling in the deeper parts of the Brassicaceae phylogeny (see Fig. 14.3). This *CHS* phylogeny shows that one genome copy of *Pachycladon* (genome-A) is in a derived position in the Arabidopsoid lineage and is closely associated with *Crucihimalaya*, *Transberingia*, and *Boechera*. This position was also found for other nuclear genes (Joly et al. 2009) and corresponds to the phylogenetic position of *Pachycladon* determined using the nrITS region (see Sect. 14.2.5.2). Yet, the other genome copy present in *Pachycladon* (genome-B) has a much deeper position in the Brassicaceae phylogeny, likely at the base of the Arabidopsoid clade. No species sequenced for the *CHS* gene have high affinities with this B-genome copy. Molecular clock estimates infer that the two genome copies diverged about 8.18 Mya, but that the hybridization event that led to the formation of *Pachycladon* occurred between 0.8 and 1.61 Mya (see Fig. 14.3b; Joly et al. 2009). The two genome copies of *CHS* present in the *Pachycladon* genome share a most recent common ancestor with *A. thaliana* approximately 7 and 10 Mya, respectively.

14.4.1.2 Maternal History

To investigate the maternal origin of the genus, Joly et al. (2009) used the chloroplast gene *rbc*L, which is maternally inherited, to construct a phylogeny that included *Pachycladon* and Brassicaceae sequences available in GenBank. This analysis showed that the *Pachycladon rbc*L sequences were relatively basal in the *Arabidopsoid* clade, although the species sampling in this analysis was limited. More recently, four chloroplast genes (*rbc*L, *nad4*, *mat*K, *ndh*F; Couvreur et al. 2010) were sequenced from some *Pachycladon* species and analyzed with 55 representative Brassicaceae species. A Bayesian analysis in BEAST 1.4.8 showed that *Pachylcadon* fell within a well-supported clade (posterior probability = 1.0) that consisted of the tribe Boechereae and some genera of the tribe Camelineae, including *Crucihimalaya* and *Transberingia* (Mandáková et al. 2010). In light of this more comprehensive and better-supported analysis, it is likely that the A-genome was the maternal parent in the allopolyploid event that gave rise to *Pachycladon*.

14.4.1.3 Hybridization and Adaptive Radiation

While the question of whether hybridization acted as a trigger for the adaptive radiation of *Pachycladon* is still the subject of ongoing research, it is clear that an allopolyploid event preceded the radiation of the genus (Joly et al. 2009). Because the allopolyploidization

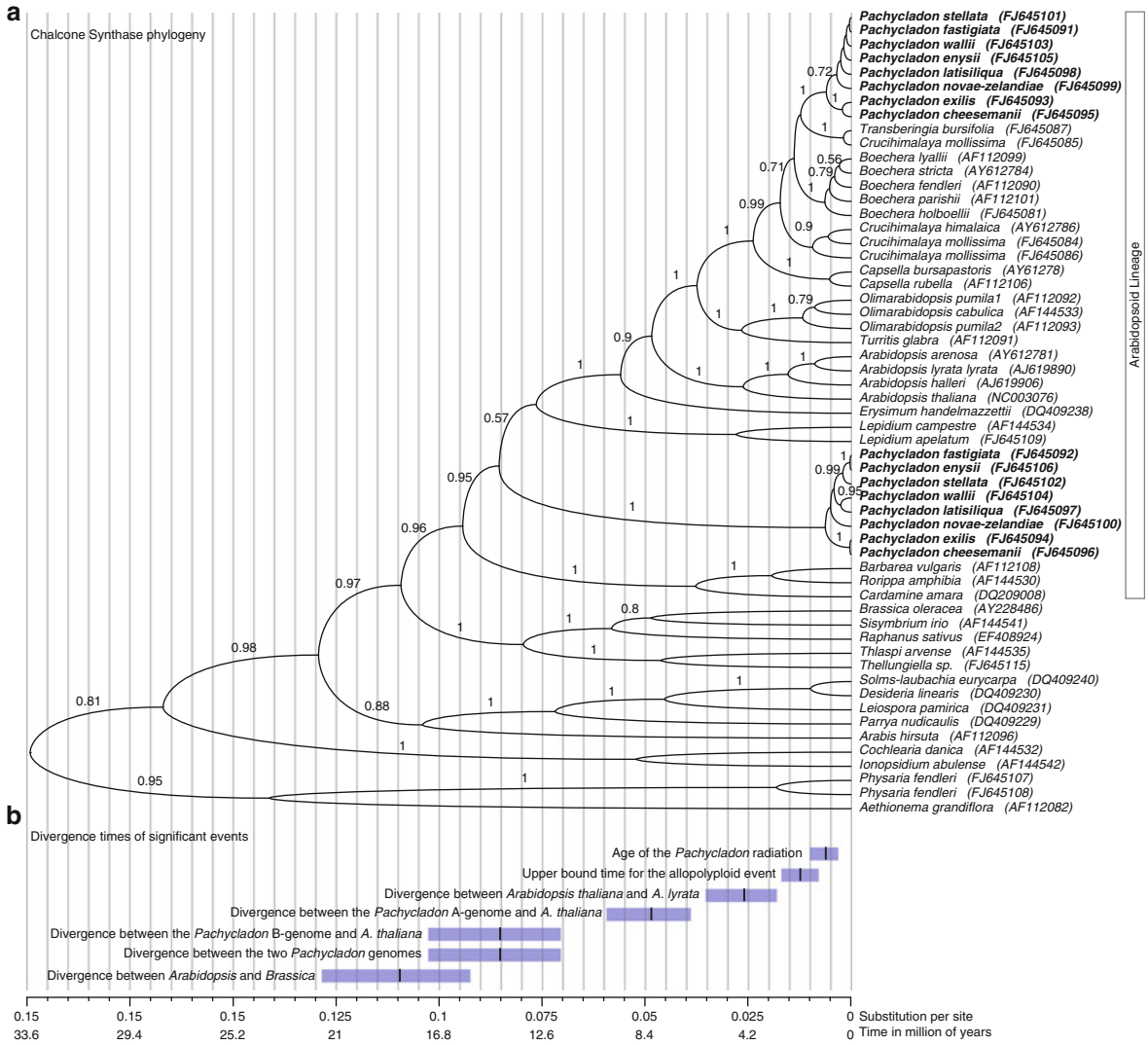

Fig. 14.3 Chalcone synthase (*CHS*) phylogeny for *Pachycladon* species and other Brassicaceae species and estimates of important divergence times. (**a**) Chronogram obtained from a Bayesian phylogenetic analysis in BEAST vers. 1.4.8 (Drummond and Rambaut 2007) using a sample of the accessions available in GenBank (*accession numbers in brackets*). The analysis used a GTR + Γ + I substitution model (selected using Model test 3.7; Posada and Crandall 1998) and a lognormal relaxed molecular clock with the mean substitution rate fixed to 1. Two independent runs of 4×10^7 were run and trees were sampled every 1,000 generations for the second half of the run. Trees sampled from these two runs were combined to obtain a maximum clade probability tree. Posterior probabilities are shown above nodes when these were greater than 0.5. (**b**) Median and 95% credible intervals for important divergence times. The time scale has been obtained using a divergence time of 18.51 million of years between *Arabidopsis* and *Brassica* (see Joly et al. 2009)

event occurred during the early Pleistocene (Early Quaternary), it is possible that the major geological and ecological changes in the Late Tertiary (see Sect. 14.1) have played a major role in driving hybridization, but also speciation and rapid radiation of the neo-species in a new environment of diverse geological substrates and altitudinal range. It is also likely that trans-oceanic dispersal of modern-day *P. radicatum* from New Zealand to Tasmania occurred during this radiation as well.

14.4.2 Pachycladon *as a Model System for Polyploid Evolution in the Brassicaceae*

Polyploidy is known to be a major influential force in the evolution of higher plant genomes (Otto and Whitton 2000; Wendel 2000) with repeated cycles of polyploidization, followed by diploidization appearing to have occurred throughout the evolutionary history of flowering plants (Vision et al. 2000). Polyploids have the inherent advantage of possessing more than one copy of the genome, which allows the plants to tolerate major changes, at both the gene and genomic levels, and this may confer selective evolutionary advantages. In theory, the presence of two copies (homeologous copies) of a gene in an allopolyploid could allow subfunctionalization (Lynch and Conery 2000) and lead to adaptation. Indeed, previous studies on homeologous gene expression have provided examples of gene subfunctionalization in *Gossypium* (Adams et al. 2003) and have demonstrated that variation in expression levels of homeologous genes can affect the phenotype of allopolyploids (Wang et al. 2006) and potentially offer adaptive benefits (Finnegan 2001). These mechanisms, if demonstrated to have been important in the evolution of a group by connecting differential gene expression to selectable changes driving the origin of species (Kellogg 2003), could lend support to the hypothesis that hybridization can act as a trigger for adaptive radiation (Seehausen 2004). The genus *Pachycladon* represents a perfect model to investigate these hypotheses given that a recent allopolyploid event preceded its radiation and that its ecophysiology is currently under in-depth study.

Understanding the molecular basis of evolutionary processes responsible for the speciation of *Pachycladon* might also help to add to our understanding of the impact of polyploidy on the whole family. The "paradox" of the small and narrow range of genome sizes for most Brassicaceae (Lysak et al. 2009), given the preponderance of polyploid taxa in the family, suggests that Brassicaceae genomes are dynamic and that polyploidization (specifically, hybridization in the case of allopolyploids) is crucial to genome evolution in the Brassicaceae. For instance, diploid *A. thaliana* appears to be an ancient polyploid that may have experienced at least three whole-genome duplication events that predate the origin of the Brassicaceae (Vision et al. 2000; Blanc et al. 2003; de Bodt et al. 2005) but nevertheless possesses a very small genome ($n = 5$; 1C ~157 Mbp). Recent evidence from studies of *A. thaliana* and its close relatives suggests that in the last ~5 million years, the ancestral Brassicaceae karyotype [$n = 8$; 1C ~490 Mbp] (Dolezel et al. 2003; Schranz et al. 2006; Lysak et al. 2009) underwent rapid chromosomal rearrangement and genome reduction to give this $n = 5$ karyotype of modern-day *A. thaliana* (Kuittinen et al. 2004; Koch and Kiefer 2005; Yogeeswaran et al. 2005; Lysak et al. 2006). Ancestral genome-wide and partial duplication events may have enabled its genome to tolerate the concomitant loss of chromosomal content (1C ~ 490 Mbp to 157 Mbp) (Yogeeswaran et al. 2005; Lysak et al. 2006). Similar evidence of lineage-specific changes in genome structure and content have been suggested for genome reduction in other Brassicaceae species following polyploidy (Lysak and Lester 2006; Lysak et al. 2006; Schranz et al. 2006; Lysak et al. 2007) and is likely one of the crucial mechanisms of diploidization in the Brassicaceae family. This ability to tolerate such drastic changes to the genome (genomic plasticity), of Brassicaceae species appears to be a direct consequence of polyploidization, and has been suggested to enhance their ability to survive and adapt to environmental change during alternating glacial–interglacial cycles in Eurasia, and possibly drive speciation (McClintock 1984; Kianin and Quiros 1992; Kowalski et al. 1994). It is conceivable that similar comparative genome studies between *Pachycladon* and its close relatives, and between species of *Pachycladon*, may add to our growing knowledge of the evolutionary history of modern-day Brassicaceae, but with special reference to allopolyploid evolution. The latter is clearly crucial as many economically important Brassicaceae species are allopolyploids.

14.4.3 Allied Crop Species

The *Brassica* lineage within the Brassicaceae, the origin of which the B-genome of *Pachycladon* is more closely ascribed, contains many economically important crop plants. These include diploid crops like radish (*R. sativus*), turnip and Chinese cabbage (*B. rapa*), cabbage, kale, broccoli, cauliflower, kohl rabi,

and Brussels sprouts (all subspecies of *B. oleracea*), and black mustard (*B. nigra*) as well as polyploid crops like white mustard (*Sinapis alba*), Indian mustard (*B. juncea*), Ethiopean mustard (*B. carinata*), and canola (oilseed rape) (*B. napus*) (UN 1935).

14.5 Role in Classical and Molecular Genetic Studies

14.5.1 Genetic Studies

14.5.1.1 Forward Genetics: Mapping of Genes and Polygenic Clusters

A number of classical genetic studies are being envisaged for *Pachycladon*. Given the young age of the radiation, species remain largely interfertile. This has enabled the successful generation of hybrids between several *Pachycladon* species tested (see Fig. 14.4b) (Heenan unpublished data). These F_1 hybrids are fertile, so there is the potential to generate F_2 mapping populations segregating for traits of interest.

One such F_2 mapping population derived from parents *P. cheesemanii* and *P. fastigiatum* has been generated in order to help identify the genetic basis of morphological, physiological, and biochemical traits of interest in *Pachycladon*. These two species differ in a number of traits including trichome density (dense, sparse, or absent), trichome complexity (simple or branched), altitudinal preference (generalist vs. alpine), substrate preference (generalist vs. greywacke specialized), number of times they flower before death (polycarpic vs. monocarpic), seed production levels (high vs. low), and dispersability of seeds (wingless vs. winged). They also differ in their profile of glucosinolates, compounds that discourage herbivory, and flavonoids, compounds that help protect against UV damage that can occur at high altitudes (see Sect. 14.5.2 for more details). These traits are all likely to confer adaptive advantage by helping these plants cope with biotic or abiotic stress in their habitats or by improving reproductive success. Many of these traits are also of great interest for crop improvement. Therefore, molecular characterization and improved understanding of any of these traits in the *Pachycladon* system may potentially be useful for the improvement of allied Brassicaceae crops.

Trichome density, geological substrate tolerance, and secondary metabolism are traits that will be the main focus of the mapping project in progress, in order to complement various other studies that are currently underway or are being planned for the near future. Single nucleotide polymorphism (SNP) markers are to be developed for *P. cheesemanii* and *P. fastigiatum* from expressed sequence tag (EST) libraries that are currently under construction (see Sect. 14.7.1 for more details) to facilitate linkage mapping. A quantitative trait loci (QTL)-based approach will be taken to map polygenic clusters of interest (Symonds personal communication). Through comparative profiling (see Sect. 14.5.2) and QTL studies, it is hoped that candidate adaptive gene sets will be identified. These will be subjected to studies of molecular evolution and population genetics.

14.5.1.2 Reverse Genetics: Candidate Gene Approach

The availability of the full genome sequence of the closely related model plant, *A. thaliana*, and the fact that a great deal of functional information is available about its genes, allows a candidate gene approach to be used to look for *Pachycladon* homologs that can then be subject to reverse genetic strategies to determine whether they are responsible for variation in morphological traits of interest. A number of genes have been characterized in *A. thaliana* that have been implicated in trichome development, glucosinolate biosynthesis, glucosinolate hydrolysis, and flavonoid biosynthesis, and their homologs in *Pachycladon* represent candidate genes that might be responsible for the observed variation in these traits. Direct PCR-based approaches or probing of *Pachycladon* EST libraries with *A. thaliana* probes might allow sequencing of *Pachycladon* orthologs and paralogs of these genes. Preliminary evidence suggests that the former approach is tractable in *Pachycladon* (Symonds personal communication). Application of the latter approach was effective in determining the sequence of a candidate rapidly evolving gene from an EST library derived from close relative *A. lyrata* (Yogeeswaran unpublished data) and therefore may be effective for this system as well, at least for more conserved genes. Sequence alignment may reveal differences in the genotype that may be responsible for phenotypic variation between species and can be tested

for associations with phenotypic traits of interest. Ultimately, knock-out or knock-down experiments will be needed to confirm functional roles for candidate genes in *Pachycladon*.

14.5.2 Comparative Transcript and Metabolite Profiling in Pachycladon

Since speciation and adaptive radiation are predicted to have occurred very recently in *Pachycladon*, it is possible that the observed differences in traits of interest between species may not have been fixed in the genotype. In this case, mapping and sequencing efforts may not reveal stably inherited differences at the gene level (true allelic variants) between species that can be exploited for population genetic studies or that explain the observed differences in morphology, physiology, and substrate preference. Instead epigenetic mechanisms may be responsible for differential expression of genes or gene silencing. Therefore, comparative transcript and metabolite profiling have been undertaken in parallel to mapping efforts, to help elucidate the genetic basis of the observed differences in phenotype. Signatures of phenotypic divergence predicted by such comparisons may be indicative of forces driving speciation or secondary adaptation following speciation. Given the young age of the *Pachycladon* radiation, secondary adaptation may be less likely to account for species-specific adaptive phenotypes. Two such comparative profiling studies have been performed (see Sects. 14.5.2.1 and 14.5.2.2) that have already yielded interesting insights into the evolution of this alpine genus. Ecophysiological, morphological, and ecological studies to complement these studies are also being planned or are in progress.

14.5.2.1 Native Population Study

Gene expression differences between the two greywacke specialists, *P. enysii* and *P. fastigiatum*, were investigated using heterologous microarrays from *A. thaliana* and sampling wild populations from each species (Voelckel et al. 2008). The microarray platform was produced by spotting the *A. thaliana* AROS version 1.0 genome set (Operon Biotechnologies, operon.com) to glass slides (Plant and Food Research, Auckland, New Zealand). Each of the 26,282 70-mer oligonucleotides was spotted once. In total, there were 26,880 probes on the array including controls. In this study, results could be obtained for 76% of all probes (Voelckel et al. 2008). Two biological processes found to be differentially expressed between *P. enysii* and *P. fastigiatum*, viz., glucosinolate and flavonoid biosynthesis, were further investigated through biochemical assays. These investigations led to the characterization of twelve glucosinolate (GLS) compounds in both species (Voelckel et al. 2008).

Glucosinolate Biosynthesis

P. enysii produces C4 and C3 GLS, whereas *P. fastigiatum* only produces C3 GLS and *P. enysii* and *P. fastigiatum* predominantly produces alkenyl GLS and methylsulfinylalkyl GLS, respectively. These patterns were in accordance with expression patterns of glucosinolate biosynthetic loci such as methylthioalkylmalate synthase 1 (*MAM1*) and 2-oxoglutarate-dependent dioxygenase 2 (*AOP2*). Moreover, glucosinolate profiles from wild *P. enysii* and *P. fastigiatum* individuals did not group into two species-specific profiles, but rather into five distinct profiles referred to as chemotypes, two specific to *P. fastigiatum* and three specific to *P. enysii* (see Sect. 14.2.5.4 for more on *Pachycladon* chemotypes).

Glucosinolate Breakdown

The differential expression of two myrosinase-associated genes, the epithiospecifier protein gene (*ESP, At1g54040*) and the epithiospecifier modifier 1 gene (*ESM1, At3g14210*), predict that *P. enysii* and *P. fastigiatum* would markedly differ in the formation of glucosinolate breakdown products. In line with the up-regulation of *ESP* and *ESM1* in *P. enysii* and *P. fastigiatum*, respectively, the former was found to produce nitriles, whereas the latter produced isothiocyanates following myrosinase-mediated glucosinolate hydrolysis. In *A. thaliana,* it has been unambiguously demonstrated that ecotypes producing isothiocyanates are more toxic to generalist herbivores than ecotypes producing nitriles (Lambrix et al. 2001; Burow et al. 2006; Zhang et al. 2006). Given the differences in gene expression and hydrolysis product formation, it has

been suggested that *P. enysii* and *P. fastigiatum* resemble *Arabidopsis* ecotypes Ler and Col-0 with *P. fastigiatum* being better protected against generalist herbivores than *P. enysii*. Further studies are necessary to determine whether this presumed difference in toxicity can be explained by greater herbivory pressures in *P. fastigiatum* environments, some unknown selective agent for nitrile formation in *P. enysii* environments, or a combination of both.

Flavonoid Biosynthesis

P. enysii is assumed to have adapted to its high-altitude alpine habitat with the evolution of trichomes and UV-protective compounds. The differential expression of three flavonoid synthesis genes (flavonol synthase, flavonoid 3′-hydroxylase, and ferulate-5-hydroxylase) led to the prediction of increased levels of quercetin and sinapic acid esters in *P. enysii* leaves. Both types of compounds have been implicated in UV-B defense in *Arabidopsis* and other plant species (Landry et al. 1995; Bharti and Khurana 1997; Ryan et al. 2002). Quercetin-glycoside levels were not found to be significantly different between species, but *P. enysii* leaves contained significantly more cinnamic acid derivatives than *P. fastigiatum* leaves. Detailed analyses of individual cinnamic acid derivatives are needed to test if this difference is caused by sinapic acid esters, a subclass of cinnamic acid derivatives.

14.5.2.2 Common Garden Study

Differential expression and metabolite profiling studies suggest that interactions with herbivores and pathogens were important in the differentiation of *P. enysii* and *P. fastigiatum* (Voelckel et al. 2008). A common garden comparison involving *P. cheesemanii*, *P. exile*, and *P. novae-zelandiae* was performed to test if this inference can be extended to additional species within the *Pachycladon* radiation (Voelckel et al. 2010).

Transcript and Protein Profiling

Evidence from heterologous microarrays and shotgun proteomics revealed differential expression of genes not only involved in glucosinolate hydrolysis and biosynthesis but also involved in the interconversion of carbon dioxide and bicarbonate, water use efficiency, and other stress-related genes in *P. cheesemanii*, *P. exile*, and *P. novae-zelandiae* (Voelckel et al. 2010). Thus, it was suggested that the three species diverged in physiological processes that affect carbon and water balance in addition to divergence in glucosinolate metabolism. Experiments to test these predictions are currently underway. Predicted differences in glucosinolate hydrolysis products were directly confirmed and resembled those found in *P. enysii* and *P. fastigiatum* (see metabolite profiling). Given the differential expression of *ESP* and *ESM1* and other myrosinase-associated loci across several *Pachycladon* species (Voelckel et al. 2008, 2010), a characterization of the genetic architecture of the *ESP* gene cluster (*At1g54000* to *At1g54040*) and the *ESM1* gene (*At3g14210*) in *Pachylcadon* has been initiated.

Metabolite Profiling

When comparing glucosinolate profiles in the leaves of *P. cheesemanii, P. exile*, and *P. novae-zelandiae*, patterns did not reflect the phylogenetic relationships of the three species (Voelckel et al. 2010). *P. cheesemanii* and *P. novae-zelandiae*, despite being less closely related than *P. cheesemanii* and *P. exile*, had more similar glucosinolate profiles than *P. cheesemanii* and *P. exile*. They shared their main two compounds allyl and *S*-2-hydroxy-3-butenyl glucosinolate. *P. exile* produced neither alkenyl nor C4 glucosinolates, a profile most similar to the one described for *P. fastigiatum* chemotype 2 (Voelckel et al. 2008). Hence, chemotypes are likely to be the product of convergent evolution and are, therefore, not useful as taxonomic markers. Overall, 14 GLS of different classes were identified suggesting the segregation of various GLS biosynthetic loci across the three *Pachycladon* species. For example, between-species differences in chain length of methionine-derived glucosinolates, methylsulfinylalkyl GLS, alkenyl GLS, and hydroxyalkenyl GLS suggest segregation at the *GLS-elong*, *GLS-AOP*, and the *GLS-OH* loci, respectively. In contrast to *P. cheesemanii* and *P. exile*, *P. novae-zelandiae* produced a complex blend of glucosinolates. One of its major leaf GLS was found to be glucoraphanin (4MSOB). Glucoraphanin is the GLS precursor of the

isothiocyanate, sulforaphane, which is an anticancer compound. Glucosinolates in *P. cheesemanii* were hydrolyzed into isothiocyanates, whereas the two major glucosinolates of *P. novae-zelandiae* were converted into their corresponding nitriles and epithionitriles. Similar to *P. enysii* (nitrile producing) and *P. fastigiatum* (isothiocyanate producing), this polymorphism in glucosinolate hydrolysis was associated with the expression of the ESP protein in *P. novae-zelandiae* but not *P. cheesemanii* (Voelckel et al. 2010). However, *P. enysii* and *P. fastigiatum*'*s* glucosinolate profiles were different from those of *P. cheesemanii* and *P. novae-zelandiae* in that both of the latter produced S-2-hydroxy-3-butenyl, a compound neither present in *P. enysii* nor present in *P. fastigiatum*.

In addition to GLS, 18 flavanoid compounds were identified with distinct patterns in each species. *P. exile* and *P. novae-zelandiae* both produced six different flavonoids, whereas *P. cheesemanii* produced eight different compounds. Similar to glucosinolate profiles, flavonoid profiles of *P. cheesemanii* and *P. novae-zelandiae* were more similar than those of *P. cheesemanii* and *P. exile* as the former shared two of the flavonoid compounds, whereas the latter shared none (Voelckel and Reichelt unpublished data).

14.5.3 Ecophysiological Studies

Initial comparative studies on physiological parameters such as photosynthetic carbon and light-response curves, stomatal and mesophyll conductance, water use efficiency, and carbon isotope discrimination found *P. enysii*, *P. fastigiatum*, and *P. cheesemanii* to differ markedly in these parameters (Bickford and Barbour unpublished data). In future work, the common garden physiological comparisons will be extended to more species and complemented by physiological studies in natural *Pachycladon* populations. Monitoring of environmental conditions such as photosynthetic active radiation, soil and air temperature, and humidity has begun at *P. cheesemanii*, *P. enysii*, *P. stellatum*, and *P. fastigiatum* sites. Collection of these environmental data is expected to lead to a better understanding of the differences in ecophysiology and microclimate in the alpine habitats of these species.

14.6 Potential for a Role in Crop Improvement

14.6.1 Interspecific Hybridization

Heenan (1999) successfully produced interspecific F_1 hybrids between *P. novae-zelandiae* (female parent) × *P. cheesemanii* (male parent) and *P. novae-zelandiae* (female parent) × *P. exile* (male parent) that produced moderate to high levels of viable pollen and could be selfed to generate F_2 populations. Morphological traits of the F_1 hybrids were predominantly intermediate to the parental types. Pollen viability appeared to segregate in a Mendelian fashion in the F_2 population where approximately 1/4th of F_2 plants were male sterile. Interspecific crosses between many species of *Pachycladon* have since been generated and in some cases, natural hybrids have also been found in the wild, and these are summarized in Fig. 14.4b (Bicknell et al. 2009; Heenan unpublished data). Morphological traits of these artificial and natural hybrids were also found to be intermediate to the parent plants as seen in Fig. 14.4a, in the example of leaf size and morphology of hybrids between *P. enysii* × *P. fastigiatum* and *P. novae-zelandiae* × *P. fastigiatum*. These F_1 hybrids are fertile; hence, there is potential to create mapping populations segregating for traits of interest between members of this genus. One such F_2 population derived from a cross between *P. cheesemanii* and *P. fastigiatum* is currently being used for QTL mapping (see Sect. 14.5.1).

14.6.1.1 Apomixis and Matromorphy

While making interspecific crosses using either *P. cheesemanii* or *P. exile* as the female parent and any other *Pachycladon* species as the male parent, it was noted that progeny seedlings frequently resembled the maternal parent rather than showing the usual intermediate phenotype expected of *Pachycladon* F_1 hybrids or segregation for parental traits in the F_2 generation (Heenan unpublished data). This suggested that these seedlings might be a product of apomixis. Apomixis is the production clonal embryos without fertilization of egg cells or in essence, embryo production by asexual reproduction (Asker and Jerling 1992). Many

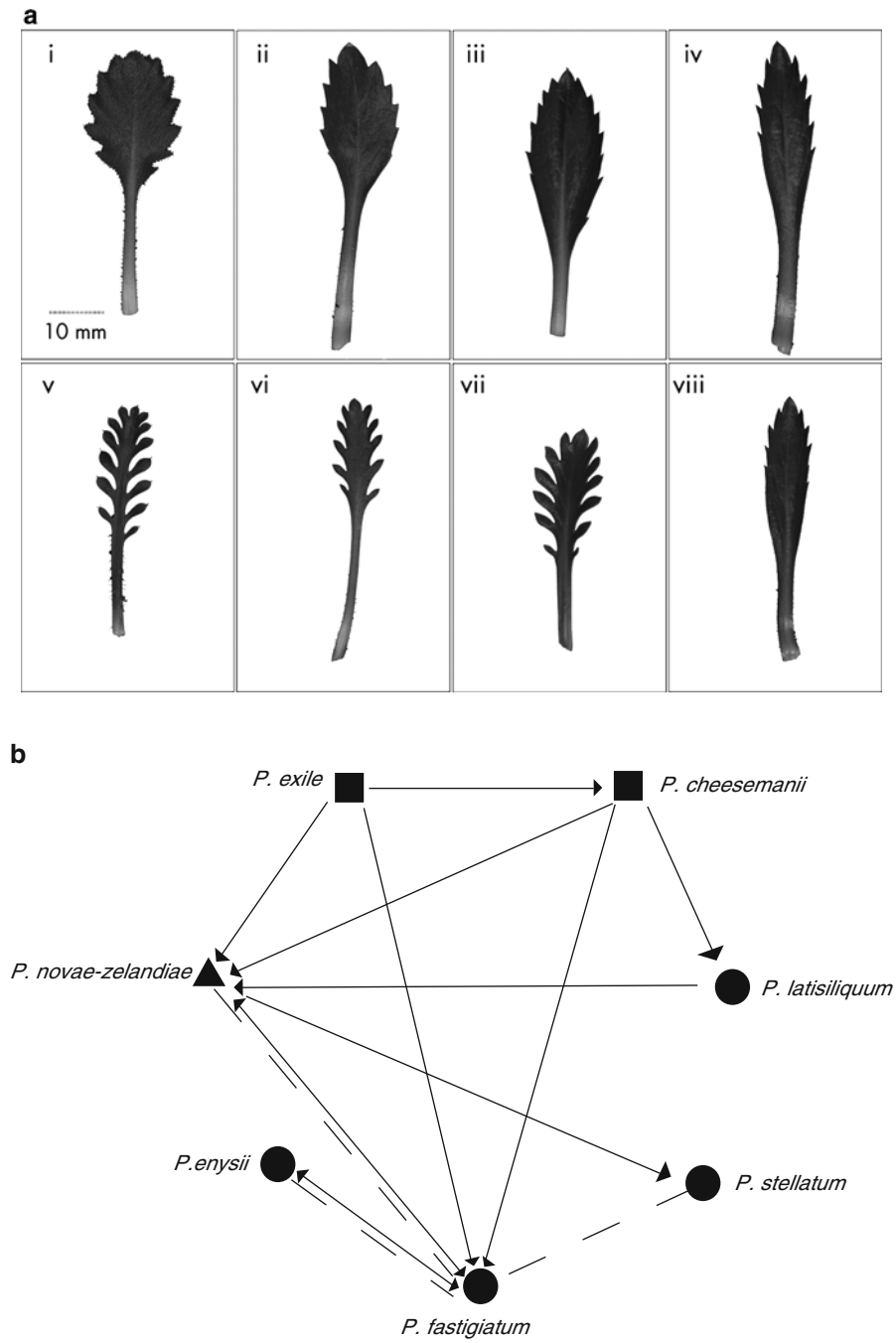

Fig. 14.4 Wild and artificial interspecific hybrids between *Pachycladon* species. (**a**) Leaf morphology (i) *P. enysii*, (ii) *P. enysii* × *P. fastigiatum* (artificial hybrid), (iii) *P. enysii* × *P. fastigiatum* (wild hybrid), (iv) *P. fastigiatum*, (v) *P. novae-zelandiae*, (vi) *P. novae-zelandiae* × *P. fastigiatum* (artificial hybrid), (vii) *P. novae-zelandiae* × *P. fastigiatum* (wild hybrid), and (viii) *P. fastigiatum*. (**b**) Interspecific hybrid combinations between *Pachycladon* species. Species assigned to *Pachycladon* in earlier and current taxanomic assignments (*solid triangle*) and *Pachycladon* species earlier assigned to segregate genera *Cheesemania* (*solid circle*) and *Ischnocarpus* (*solid square*). *Solid lines* represent artificial hybrids with the *arrow head* indicating the female parent, *two headed arrows* indicate that reciprocal crosses were possible and *dash lines* represent wild hybrids

crop plants are products of F_1 hybrid seed that demonstrate desirable heterotic effects, but F_2 seed derived from these are less favorable as they segregate for traits of interest. Apomixis is a highly desirable agricultural trait, as such an F_1 hybrid or indeed any plant with favorable traits, however complex and unstable, could be fixed through the production of clonal seed (Hanna and Bashaw 1987; Savidan 2000). Apomixis and other related processes such as androgenesis, matrocliny, and matromorphy have been previously reported in other Brassicaceae members (e.g., Barabas and Redei 1971; Eenick 1973, 1974; Chen and Heneen 1989; Schranz et al. 2005).

P. cheesemanii and *P. exile* transformants, with single copies of the green fluorescent protein gene (*GFP*), were emasculated and not pollinated, self-pollinated, or crossed with other species of *Pachycladon*, *A. thaliana,* and *B. rapa* to determine the nature of the earlier apomictic observation (Bicknell et al. 2009). *P. exile*, the maternal parent in the crosses with *Arabidopsis* and *Brassica*, could not cross-fertilize with these distant species, indicating that it is capable of recognizing "self" at the genus vs. non-genus level. It was determined that several F_1 generation plants derived from crosses with *Pachycladon* species showed maternal phenotype and these, and all F_2 generation plants derived from them, scored positive for the GFP marker and retained the maternal phenotype (no segregation in the F_2 generation). However, maternal-like seedlings were not produced in the absence of pollination or when the maternal plants were selfed; hence, autonomous apomixis did not occur. The type of apomictic response observed in these *Pachycladon* species is called matromorphy and is triggered by wide hybridization, resulting in seedlings expressing the maternal phenotype, derived asexually from maternal reproductive tissue (Bicknell et al. 2009). Matromorphic plants and natural hybrids derived from *P. exile* or *P. cheesemanii* have not been observed in the wild to date and matromorphy has not been observed in the laboratory or in the wild for other species of *Pachycladon*.

14.6.2 Intergeneric Hybridization

Heenan et al. (2008) successfully generated an artificial intergeneric hybrid by crossing a tetraploid *A. thaliana* accession (female parent, $2n = 4x = 20$) with *P. cheesemanii* (male parent, $2n = 2x = 10$). The hybrid status of this plant, formally named × *Pachydopsis hortorum*, was confirmed by flow cytometry, chromosomal counting, and AFLP analysis and was found to be $2n = 15$. The ×*Pachydopsis* hybrid is a robust perennial herb (see Fig. 14.5a, b) showing profuse vegetative growth, large leaves (see Fig. 14.5c), and floral traits intermediate to both parents (see Fig. 14.5d) but does not produce viable pollen. As a female parent, it allowed pollen hydration, pollen tube germination, possibly fertilization and some embryogenesis, by both *A. thaliana* and *Pachycladon* species. However, embryo development was arrested at the torpedo stage upon which the seeds were aborted. When inflorescences were treated with colchicine, polyploidy was successfully induced ($2n = 30$) and flowers from these inflorescences were backcrossed to *A. thaliana* Ler ($2n = 10$) and *P. cheesemanii* and crossed to *P. novae-zelandiae*. The former resulted in seed that produced backcross plants with the expected genotype ($2n = 20$), while the crosses to *Pachycladon* species failed to produce any seed.

Nasrallah et al. (2000) were able to produce a viable hybrid by ovule rescue from a wide interspecific cross between *A. thaliana* Col and *A. lyrata* ssp. *lyrata*, but these parent plants are only about 5 million years diverged. *A. thaliana* pistils permit hydration and pollen tube germination, even by distantly related pollen like *Brassica* (~20 million years diverged); however, there are no reports of this leading to successful fertilization and embryogenesis outside the *Arabidopsis* genus. *A. thaliana* and *Pachycladon* are more distantly related than *A. thaliana* and *A. lyrata*, with *CHS* genes sharing a most recent common ancestor between ~7 and 10 MYA. The fact that they could produce a viable F_1 hybrid by sexual hybridization is rather remarkable given the distance between these species. It also suggests that their mechanisms of fertilization, embryo development, and endosperm formation are highly conserved and can function in the hybrid background.

In ×*Pachydopsis*, many aborted seeds in both the backcrosses with the F_1 plant and the polyploid were arrested during advanced stages of embryogenesis. In *A. thaliana*, embryogenesis commences just hours after fertilization, and by about 9–10 days after anthesis, the embryos are in the torpedo stage (Mansfield and Briarty 1991; Faure et al. 2002). In contrast,

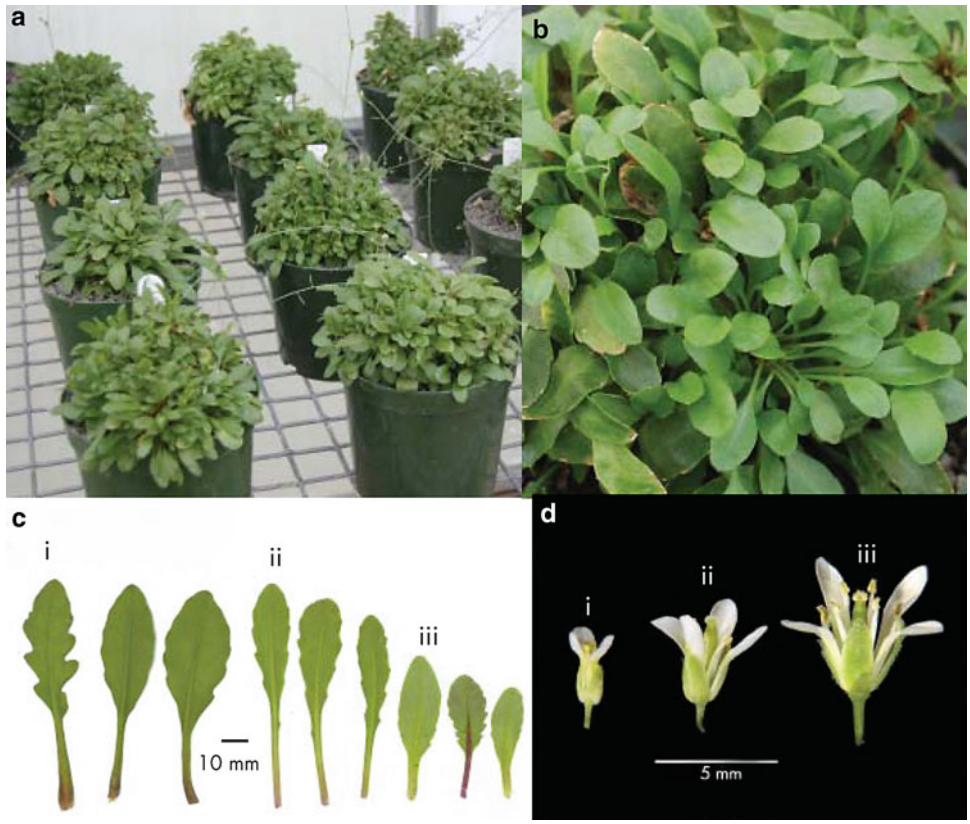

Fig. 14.5 × *Pachydopsis hortorum* intergeneric hybrid. (**a**) Cultivated plants 18 months old. (**b**) Vigorous rosette production in the hybrid (**c**) Leaf morphology of the intergeneric hybrid and parents (i) *Pachycladon cheesemanii*, (ii) × *Pachydopsis*, and (iii) *Arabidopsis thaliana* (**d**) Floral morphology of the intergeneric hybrid and parents (i) *Pachycladon cheesemanii*, (ii) × *Pachydopsis*, and (iii) *Arabidopsis thaliana*

embryogenesis in *P. cheeesemanii* is delayed by about 9 or 10 days post-anthesis (Luo et al. 2003). Otherwise, the processes in both species are very similar. It is possible that during the first 10 days post-anthesis, in the hybrid background, embryogenesis proceeds under the direction of the *A. thaliana*–derived genes. When the *Pachycladon*-derived genes become activated after the delay, the developmental program might become confounded by conflicting signals, thereby leading to arrested development. In the case of successful seed production, some epigenetic mechanism may operate to silence either the *A. thaliana* or the *Pachycladon*-derived embryogenesis genes. This intergeneric hybrid system may be useful for molecular characterization of genes responsible for initiation of embryogenesis and for understanding formation of allopolyploids from divergent genomes, such as those that gave rise to *Pachycladon*.

Finally, evidence for both independent chromosomal assortment (Nasrallah et al. 2000) and intergenomic recombination (Yogeeswaran 2005), despite divergence and differences in chromosomal and genomic structure, were obtained by molecular analysis of two backcross populations derived from the *A. thaliana–A. lyrata* hybrid and linkage mapping of *A. lyrata* (Yogeeswaran et al. 2005). If independent chromosomal assortment and/or intergenomic recombination occurred in the × *Pachydopsis* background, it might be possible to introgress genes from *Pachycladon* into the *A. thaliana* background or vice versa.

14.6.3 Genetic Transformation

Since *Pachycladon* species are relatively interfertile, somatic hybridization has not been attempted for this genus. However, some effort has been made in the arena of genetic transformation. Floral dipping, a transformation strategy developed for *A. thaliana* (Clough and Bent 1998) and successfully used in several other Brassicaceae members, was found to be ineffective for *Pachycladon* species. Instead, a transformation protocol using hypocotyl explants from 14-day-old seedlings resulted in transgenic plants, by regeneration through tissue culture, following cocultivation with *Agrobacterium tumefaciens*. This protocol was used to successfully transform *P. cheesemanii* and *P. exile* with *GFP* (Bicknell et al. 2009).

14.7 Genomics Resources Being Developed for *Pachycladon*

In the course of current and future projects planned for *Pachycladon*, several genomic resources will be created at the Allan Wilson Center for Molecular Ecology and Evolution, Massey University (Lockhart personal communication), that will facilitate future study of these plants. These resources can also be used for comparative genome studies with other Brassicaceae species, which are already sequenced, or are currently being sequenced, including *A. thaliana*, *A. lyrata*, *Capsella rubella*, *Brassica oleracea*, and *B. rapa* (Jackson et al. 2006) and therefore will be of great use to the research community at large. A few of the major resources are briefly outlined below.

14.7.1 EST Libraries, SNPs, and Microsatellites

EST libraries are being developed as a source for generating microarray probes, SNP, and microsatellite markers, for designing primers specific to candidate genes and providing a reference transcriptome for the annotation of short-sequence tags generated by gene expression tag profiling studies. A preliminary library of *P. enysii* roots and leaves has been developed using 36-base pair (bp) single-end Solexa reads, an approach to transcriptome analysis that could be applied to other non-model genomes (Collins et al. 2008). From a total of 40 million reads, 22,631 *Pachycladon* contigs were assembled de novo under the optimal assembly parameter k-mer = 19 (Collins et al. 2008). BLAST results with the assembled contigs identified 4,283 potential *Pachycladon* genes that matched *A. thaliana* ESTs. For 1,155 of these genes, mapped contigs showed a varying degree of overlap amongst themselves enabling the analysis of SNPs between both *Pachycladon* copies and between *Pachycladon* and *Arabidopsis*. There were 141 *Pachycladon* genes with a >100-nucleotide (nt) overlap, and amongst these, there were nine genes with a >300-nt overlap. Subsequently, 36-bp Solexa transcriptome reads were obtained for *P. cheesemanii* and 454 reads (40–320 bp) were obtained for *P. fastigiatum*. The recent development of paired-end sequencing (Fullwood et al. 2009) also enabled another Solexa run with 75-bp reads from both ends of an EST for *P. fastigiatum*. Assembly and annotation parameters for the EST libraries are currently being explored.

14.7.2 Establishment of Sequencing-Based Gene Expression Profiling *in* Pachycladon

A pilot digital gene expression study performed on the Solexa Genome Analyzer is currently underway to explore the potential of tag-based profiling in *Pachycladon*. As a temporary measure, tag annotation is realized by mapping tags to the most recent *A. thaliana* transcriptome. After a successful build of *Pachycladon* EST libraries, the *Pachycladon* transcriptome itself is then intended to serve as the annotation template. In the pilot study, which involved RNA from *P. enysii* and *P. fastigiatum*, over 44 million tags of 18 bp in length were sequenced in total. A quarter of all tags mapped uniquely with either no, or one, or two mismatches to the *Arabidopsis* transcriptome, whereas 19% did not map (because of more than two mismatches) and 56% mapped repeatedly (at more than one location) to the *Arabidopsis* transcriptome. A statistical analysis of uniquely mapped tags is currently underway (Voelckel, Biggs and Lockhart unpublished data). However, since tag profiling only

captures transcripts with particular restriction sites and unambiguous mapping of 18 bp tags may be problematic, mRNA sequencing is also being explored as an alternative to tag profiling for genome-wide gene expression profiling in *Pachycladon*.

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