Evolution of the genera *Vitex* (Lamiaceae) and *Zygogynum* (Winteraceae) on New Caledonia

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Steven John Pratt

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ABSTRACT

The New Caledonia archipelago is home to a richly diverse flora including a disproportionate number of lineages whose heritage can be traced to ancient Gondwana. Much of this species richness is owed to the complex geologic history, including an extensive period of submersion, of the region which actively shaped the flora over millennia. Given such complexity there is always unknowns both with regards to the circumscription of such a diverse flora and to understanding aspects of the archipelagos geologic past, which in many cases prove extremely difficult to resolve. This thesis investigates two aspects of this notion using modern day molecular techniques.

The first aspect that we investigate is the question of species circumscription with regards to *Vitex* (Lamiaceae). Here we scrutinize the monophyly of the morphologically variable species *Vitex collina,* as described by Mabberley (1990), previously suggested by Mabberley to comprise a minimum of three distinct morphotypes. Additionally we consider the genetic uniqueness of a recently discovered species, tentatively named *Vitex* sp. *"unifolia",* from currently accepted New Caledonian *Vitex collina s.l.* Maximum likelihood (ML) analyses using the internal transcribed spacer (ITS) genetic loci revealed that New Caledonian *Vitex collina* is paraphyletic with New Zealand *V. lucens* and Australian *V. lignum-vitae* nested within a well-supported *V. collina s.l.* clade. Our results suggest a minimum of two genetically distinct entities within *V. collina,* though the new species *Vitex* sp. *"unifolia"* was not distinguished genetically from *Vitex* cf. *"collina"*. The additional morphological analysis in light of our molecular analyses revealed further distinction of taxa within our sample group. This revealed a potential for three separate morphological entities

within *Vitex collina s.l.* specimens, with *Vitex* sp. *"unifolia"* representing a fourth. Further research will result in the formal recognition of *Vitex* sp. *"unifolia"* upon publication, as well as further delimitation of the distinct entities within *V. collina s.l.* These revisions will have implications for the conservation status of these revised species, especially with regards to the rare *Vitex* sp. *"unifolia"*.

The second aspect investigated New Caledonian Winteraceae focussing on two research aims. The first aim scrutinised the New Caledonian *Zygogynum* s.l. in light of revisions made in Vink (1988; 1993; 2003), where four previously recognised genera (*Belliolum, Bubbia, Exospermum*, and *Zygogynum*) were dismantled into a single broadly circumscribed genus. The second aim was to assess any major morphological trends within *Zygogynum s.l.* and biogeographic patterns within the Winteraceae. Maximum Likelihood and Bayesian analyses using the internal transcribed spacer (ITS) and *psb*A-*trn*H genetic loci confirmed the monophyly and position of *Takhtajania, Tasmannia, Drimys,* and *Pseudowintera* within the Winteraceae. New Caledonian *Zygogynum s.l.* was shown to be paraphyletic with *Z. schlechteri* nested within a distinct Australian *Bubbia* clade separate from remaining *Zygogynum*. Our analyses showed no support for the distinction of species previously belonging to *Belliolum* and *Exospermum* with all remaining *Zygogynum* forming a monophyletic clade. The monophyly of species within the *Zygogynum* were for the most part resolved, with only few species left unresolved or paraphyletic relationships. Further investigation of biogeography within the Winteraceae revealed that Zealandic Winteraceae share their common ancestor with South American taxa, reflecting the Gondwanic roots of this family, with Australian *Bubbia* having originated from New Caledonian *Zygogynum*. From this we suggest, upon further

investigation, that the retention of *Bubbia* within *Zygogynum s.l.* was supported and should be maintained. Alternately, if *Bubbia* is maintained as distinct from *Zygogynum s.l., Z. schlechteri* will be revised to *Bubbia schlechteri*. Further research including specimens of all described taxa will improve the resolution of our analyses, likely identifying further inconsistences that require revision or attention. Our investigation into morphological trends within the *Zygogynum* uncovered a trend of carpel evolution, in which a single well supported clade of *Zygogynum* exhibit fused carpels with all others within the family exhibiting unfused gynoecia. The significance of this is that it is a derived trait recognised as a reoccurring trend within the Angiosperm lineage, though this is the first instance where it has been identified occurring within a single genus.

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THESIS INTRODUCTION

New Caledonia is an archipelago situated between 20-22°S in eastern Melanesia within the southwest Pacific Ocean. New Caledonia sits in close proximity to the tectonic margin of the Australian and Pacific plates. The archipelago is 18,576km2 in area consisting of the main island of Grande Terre, the Bélep islands to the north-west, Isle de Pines to the south, and the Loyalty Islands to the east (Heads, 2008). The main island of Grande Terre is the largest of these islands being 16,890 km^2 in area and is the focal point of the archipelago. Along with the Isle of Pines and the Bélep Islands, Grande Terre represents the northernmost emergent portion of the submerged landmass of Zealandia and a fragmented piece of the Gondwanan super continent (Figure 1). Originating from the ancient supercontinent of Gondwana (Schellart *et al.,* 2006), Grande Terre has endured an extensive period of isolation from other major landmasses, although it is debated whether connections to New Zealand via the Norfolk ridge existed (Ladiges & Cantrill, 2007). Its proximity to other landmasses within the south-west pacific show the island's relative isolation with Australia 1220km to the west, New Zealand 1700km to the south-southeast, Papua New Guinea 1800km to the northwest, and the islands of Vanuatu 400km to the northeast. Grande Terre represents an emerged portion of the New Caledonian ridge Grand Terre and is distinctively elongate in shape being 500km long and only 50km wide and roughly strikes a northwest-southeast bearing. The central mountain chain runs parallel to this axis, reaching a maximum height of 1628m at Mount Panié.

The archipelago has a tropical climate and a predominantly south-easterly wind. This, in addition to the largely uninterrupted central mountain chains

(Figure 2), causes high annual precipitation to occur on the eastern margin of Grande Terre with orographic rain shadowing causing drastically lower precipitation on the western and northern margins.

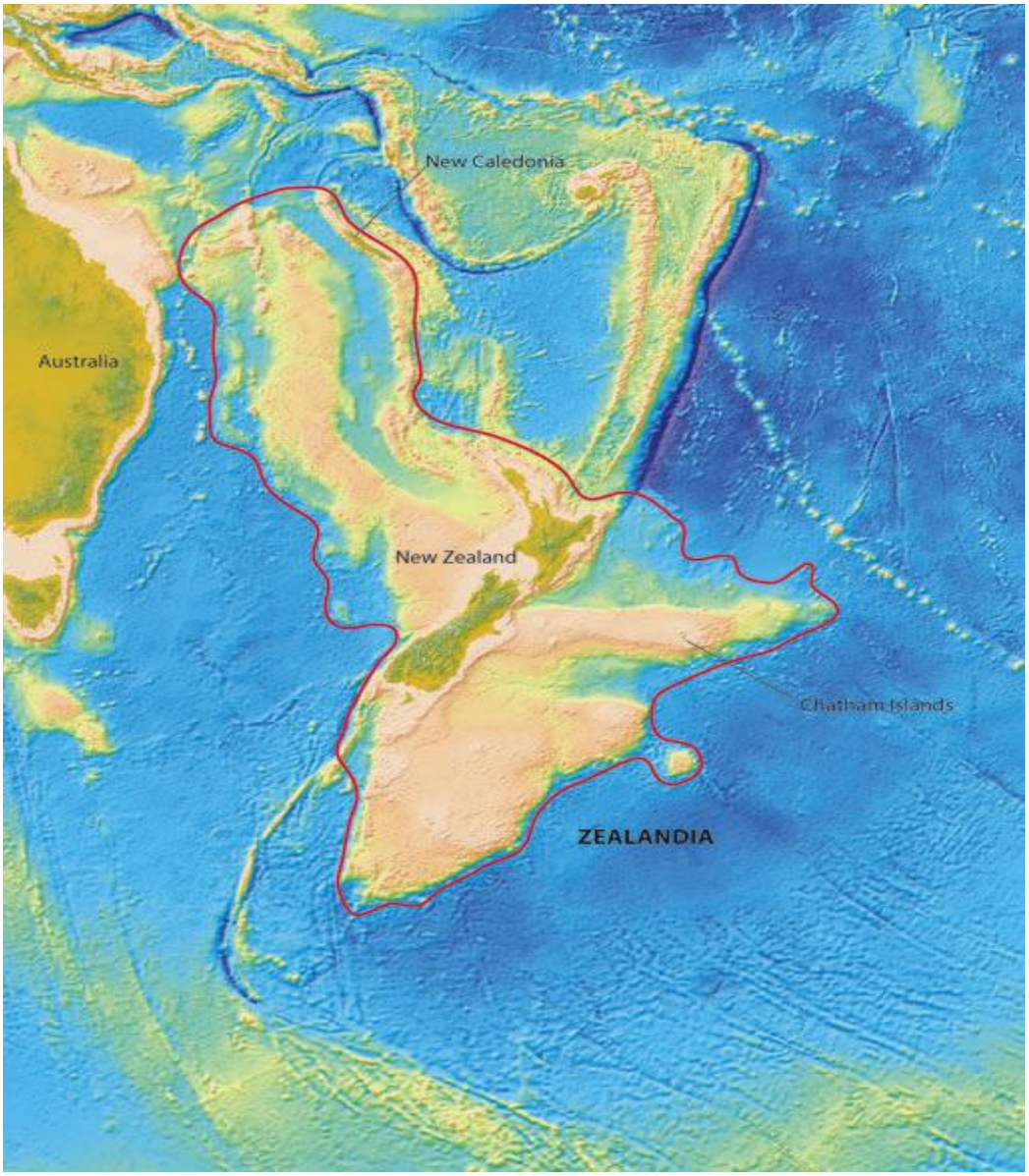

Figure 1: Map depicting the emerged and submerged extent of the continent Zealandia with New Caledonia as its northern-most emergent landmass (Stagpoole, 2002).

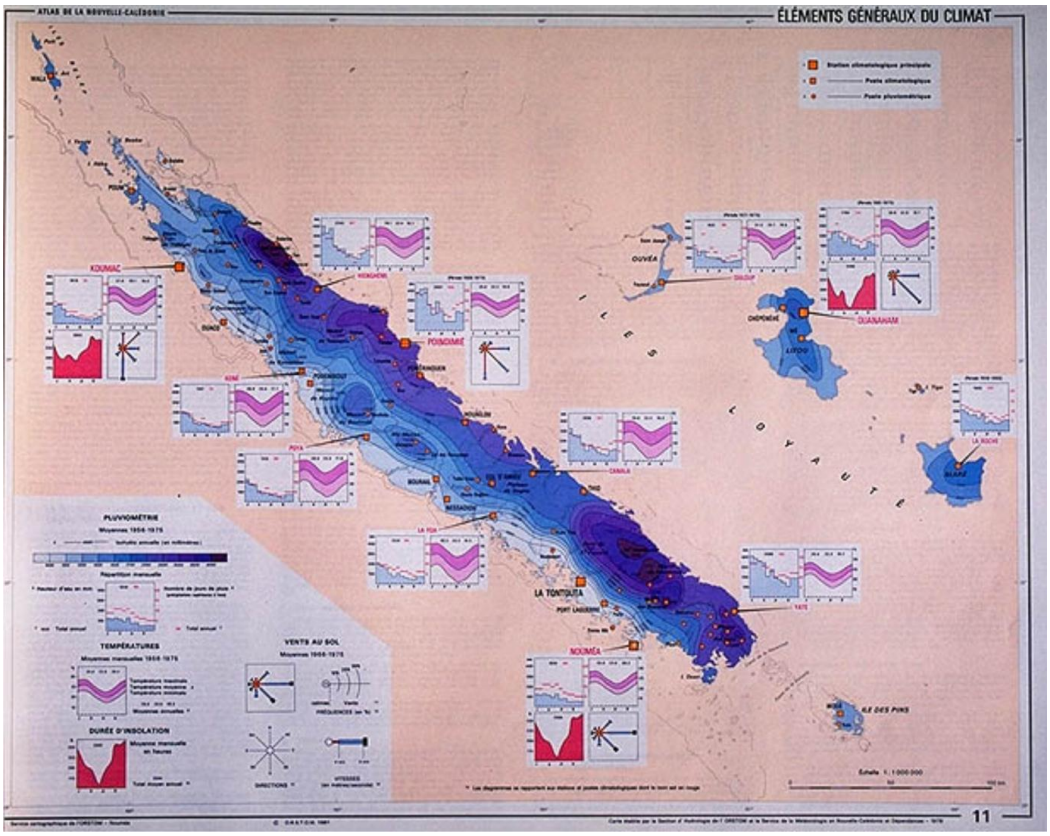

Figure 2: Map depicting topographic elevation on New Caledonia (Lapouille, 1981). Dark purple indicates high elevation and light blue indicates low elevation.

This Gondwanan fragment subsequently endured a complex and dynamic geologic history including submergence, only re-emerging 37mya (Grandcolas *et al.*, 2008). This geological template has provided a diverse and unique substrate upon which the modern day flora and fauna have evolved. Grande Terre is a composite mosaic of rock and soil types that have resulted from the island's complex geological history. The major soil type present is ultramafic (peridotite and serpentinite) that is rich in iron and magnesium including a number of heavy metals such as nickel, chromium, cobalt, and manganese. Ancient sedimentary soils resulting from the islands Gondwanan heritage, and volcano-sedimentary soils, the result of more recent volcanism in close proximity of the islands, are also present over large areas on Grande Terre.

The archipelago is renowned for the sheer diversity of biota. This is exemplified by the high level of endemism, with 77.8% endemism at the species-level for angiosperms, as well as endemic genera (Morat *et al.*, 2012). Another notable feature is the presence of many basal groups of both plants and animals which have intrigued researchers interested in the origin and possible connection to the original Gondwanan biota. Perhaps the most famous of the basal groups, most importantly the basal angiosperms, being *Amborella trichopoda* Baill., within the monotypic family Amborellaceae Pichon., recently identified as the basal most extant angiosperm (Matthews & Donoghue, 1999; Parkinson *et al*., 1999; Qiu *et al.*, 1999; Graham & Olmstead 2000; Qiu *et al.*, 2000; Soltis *et al*., 2000). In light of so many basal groups being present within New Caledonia early research suggested that the flora was of Gondwanic origin, an array of unbroken lineages that have survived to the present day, presenting a unique opportunity to study evolution within the region (Murienne *et al.*, 2005). This theory was also supported due to New Caledonia remaining within a stable tropical climate since its separation from Gondwana when other fragments, such as New Zealand which drifted south, experienced significant climate changes. Opposing this is an early theory suggested by Jeannel (1942), Faivre *et al.* (1955), and Darlington (1957) which identified a flaw in this Gondwanan theory due to the absence of mammals and continental beetles indicating that much of the terrestrial fauna was absent (Grandcolas *et al.*, 2008). This opposing theory has gained a large amount of support in recent (2000's) literature due to extensive work in both the geology of the region and application of molecular research. A new model for New Caledonia is now being suggested in which much of the flora is the result of multiple introductions via long distance dispersal, which is evident in the western margins

of the archipelago showing affinities to Australian/Malesian flora and the eastern margins showing affinities to the flora of the pacific island groups (Morat, 1993).

The islands have been praised as a model system for the study of evolution given its high endemic biodiversity, isolated position, and complex geological history (Murienne *et al.*, 2005; Grandcolas *et al.*, 2008). At present a number of vascular plant groups are threatened due to increased anthropogenic encroachment largely due to the demand for farmland and the pursuit of the islands rich nickel deposits. Given high biodiversity and rapid habitat loss, New Caledonia was identified as one of the original 25 eco-regions to be given the status of "biological hotspot" and recognition as a conservation priority (Myers, 1988; Myers, 2000). Given the need to comprehend and recognise the huge diversity of biota within New Caledonia as well as the need to protect it there has been steady growth in the number of ecological and phylogenetic studies done in New Caledonia (Grandcolas *et al.*, 2008). Also the discovery and description of new species continues due to increased exploration of remote regions of New Caledonia as well as formal revision of a number of families and genera.

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CHAPTER I

LITERATURE REVIEW

Geological history of New Caledonia

Origin and formation of New Caledonia

New Caledonia's geologic origin can be traced back to the continental landmass of Gondwana. The Eastern Gondwanan margin has endured a very complex geological history which has seen the region drastically transformed to its present day configuration within the South-West pacific (Figure 3). Prior to and in conjunction with separation of the eastern Gondwanan margin, the region experienced regional volcanism which acted to alter and add to the original composition of the regions geology. This occurred during the late Jurassic to early Cretaceous (130-200Ma) when a mosaic of volcanic and tectonic (in this case metamorphic) activity resulting from plate convergence and subduction along the eastern Gondwanan margin resulted in the emplacement of volcanic material throughout the area that would form the basement terranes of the region (Figure 4a) (Pelletier, 2006; Heads, 2008; Nicholson *et al.*, 2011). These geologic units form the core units of modern Day New Caledonia (Cluzel *et al.*, 2001). This period has similar timing and indeed can be correlated to the Rangitata orogeny, a period of uplift and collisions in New Zealand (Figure 4a) (Cluzel *et al.*, 2001; Pelletier, 2006).

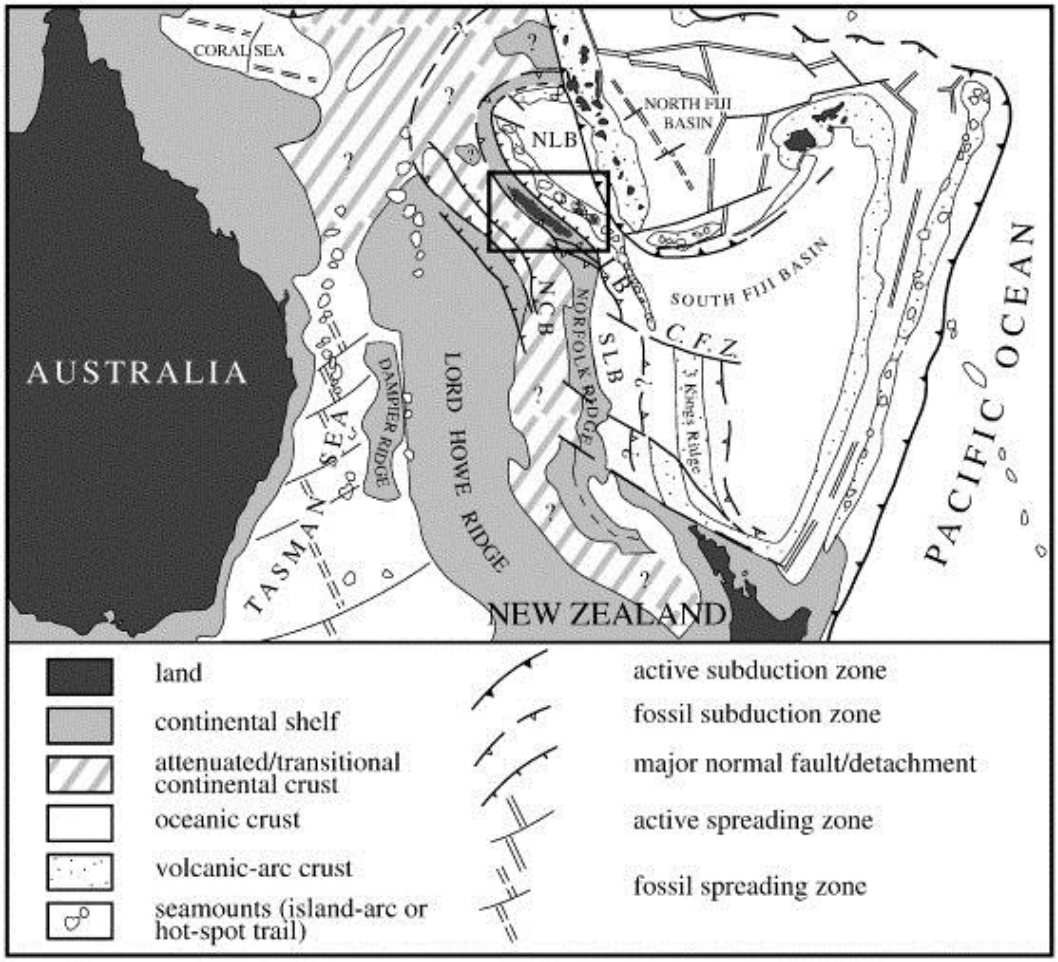

Figure 3: Sketch map of the southwest pacific depicting the complex nature of the regions geology (Cluzel *et al.*, 2001). Highlighted by the black box is the position of New Caledonia.

During the early Cretaceous (100-120Ma) a fragment of the east Gondwanan landmass began to separate and migrate in an easterly direction in response to marginal rifting (Figure 4b) (Pelletier, 2006; Cluzel *et al.*, 2001). This early fragment consisted of what would eventually be the modern day landmasses of New Caledonia, New Zealand, and Australia. The Norfolk (at this stage includes New Caledonian ridge) and Lord Howe ridges subsequently began to separate from the landmass of Australia as well as from one another around 83Ma representing the early phases of the formation of the northern Tasman Sea and New Caledonian basin via extensional stresses initiating the separation of the

Zealandic continent from the Australian continent (Figure 4c) (Grandcolas *et al.*, 2008). Complete separation and rifting of the Norfolk ridge from the Australian mainland and Lord Howe ridge had occurred c. 65Ma during the late cretaceous. Throughout this period a series of submersion events affecting the New Caledonian and Norfolk ridge systems occurred in response to tectonic activity, including the extensive 20my submersion event during the Paleocene-Eocene (35-65Ma). These events allowed for the emplacement of a number of marine sedimentary rock units onto the New Caledonian ridge and also facilitated a number of erosional cycles for previously emplaced geologic units (Grandcolas *et al.*, 2008). During the early stages of the Eocene, subduction had begun along an eastern fault boundary within the South-Loyalty basin leading to convergence of the Norfolk ridge with the, then distant, Loyalty ridge (Figure 4d). By the late Eocene (c. 34 Ma) convergence of the two ridge systems was near complete and collision between the two ridge systems resulted in obduction of the Loyalty basin and ridge system over the New Caledonian ridge (now distinguished from the Norfolk ridge) (Figure 4e). The obduction of the Loyalty ridge over the adjacent New Caledonian ridge resulted in ultramafic material being emplaced atop continental crust of the New Caledonian ridge (Pelletier, 2006; Grandcolas *et al.*, 2008). The two ridge systems of the region subsequently underwent an extensional phase during the Oligocene (23-35Ma) associated primarily with lithosphere extension which allowed for the emergence of the portion of the New Caledonian ridge that is Grande Terre (Crawford *et al.*, 2003; Pelletier, 2006; Schellart *et al.*, 2006; Grandcolas *et al.*, 2008). This also coincided with the most extensive erosional period during the Oligocene, giving rise to the extensive

weathering of the emplaced ultramafic material resulting in the present day highly weathered lateritic material and rock rich in nickel (Pelletier, 2006).

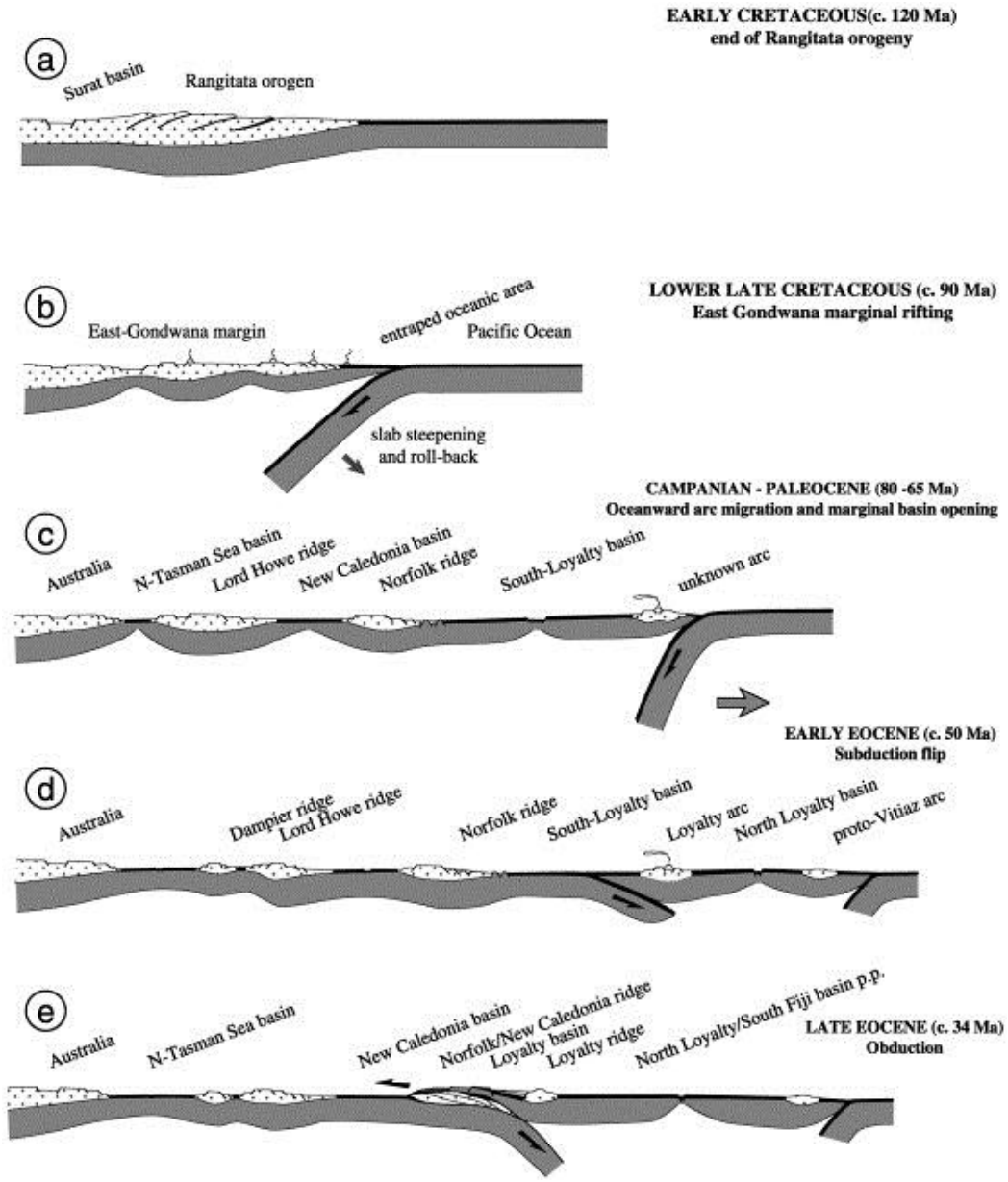

Figure 4: Successional development of the south-west Pacific during the Late Cretaceous-Late Eocene depicting the separation of the Norfolk Ridge along the eastern Gondwanan margin and later convergence of the Norfolk Ridge with the Loyalty Ridge (Cluzel *et al.*, 2001).

The Loyalty Islands, situated 100km to the east of Grande Terre, consist of six small islands Lifou Island, Maré Island, Tiga Island, Ouvéa Island, Mouli Island, and Faiava Island, as well as a number of uninhabited small islands and islets. This group is considered to be of volcanic origin associated with the Loyalty ridge volcanic arc. The six islands are largely composed of uplifted calcareous reef formations overtopping basement rock of the loyalty ridge (Pelletier, 2006). The formation of the Loyalty Islands and the Loyalty Ridge is less understood and much of the current knowledge of its origin and nature are based on inferences from other structures of the region. The Loyalty ridge is generally considered to be an Eocene (24-56Ma) island arc in many of the regions geological reconstructions (Pelletier, 2006). The ridge could have potentially supported sub-aerial islands during the past though at this stage this is almost completely speculative. At present the ridge supports the emerged Loyalty Islands which represent very young late Miocene-Pleistocene (23-0.1Ma) uplifted reef formations (Pelletier, 2006; Grandcolas *et al*., 2011); the forces that brought about this emergence are thought to be lithosphere flexure (Dubois *et al.*, 1974).

Present day Grande Terre

The modern day substrates present on Grande Terre can be classified as large undifferentiated basement terranes exposed through the central axis of the island, with a number of more recent geologic units emplaced upon this (Figure 5). Clarke *et al.* (1997) differentiates this basement terrane into three major basement terranes known as the Koh, Boghen, and Téremba terranes. Overlying this is a number of geologic units and groups. The first of these is the Ophiolitic (Clarke *et al.*, 1997; Cluzel *et al.*, 2001) or Peridotite nappe (Cluzel *et al.*, 2001), originating from obduction of the ridge, found predominantly to the south with a

number of smaller outcrops further north. The “formation des basalts” nappe (Poya terrane) is present predominantly along the western coast margin resulting from localised volcanism. The metamorphic Pouebo terrane is presented in the north-eastern margin of Grande Terre consisting of Eocene eclogites, Clarke *et al.* (1997) also identifies an Eocene blueshist metamorphic unit in the same region known as the Diahot terrane. Collisional foredeep sedimentary units and Gondwana breakup and rift drift sedimentary units are also identified in Clarke *et al.* (1997) occurring in the south-western margin of Grande Terre (Figure 5). Cluzel *et al.* (2001) also includes the Loyalty Islands and some coastal fringe units representing calcareous sedimentary units (Neogene-Recent).

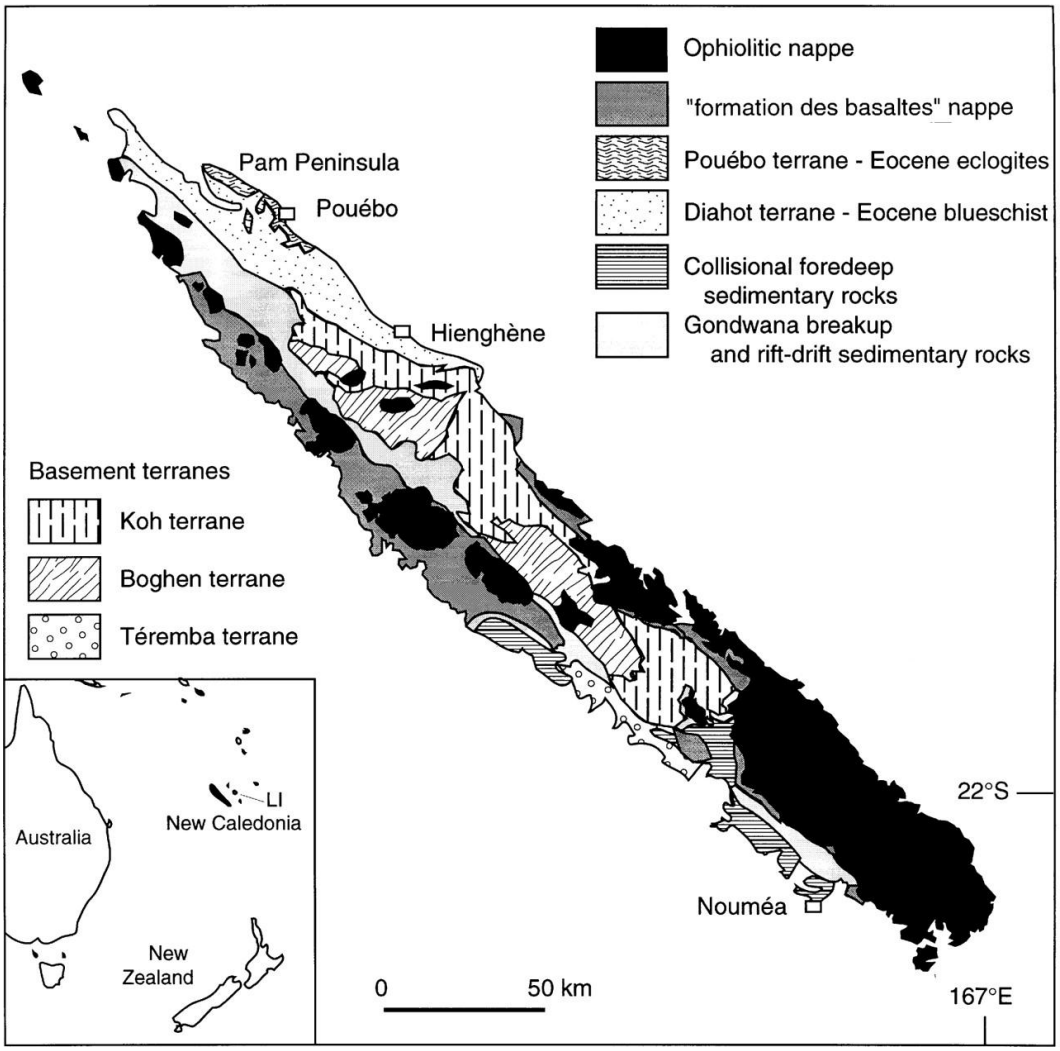

Figure 5: Simplified map of Grande Terre depicting the major exposed geologic units (Clarke *et al.*, 1997). Note. Ultramafic rock units represented here as Ophiolitic nappe.

Origin of New Caledonia's angiosperm flora

The origin debate

The presence of a large number of ancient putatively relictual angiosperm families such as Amborellaceae, Annonaceae (Juss.), Atherospermataceae (R.Br.), Chloranthaceae (R.Br. ex Sims), Hernandiaceae (Bercht. & J.Presl), Lauraceae (Juss.), Monimiaceae (Juss.), Nothofagaceae (Kuprian.), Piperaceae (Giseke, nom. Cons.), Proteaceae (Juss.), and Trimeniaceae (Gibbs) within the region has suggested that the biota of New Caledonia is a subset of the early Gondwanan flora that has survived since the regions separation from Gondwana (Lowry, 1996; Parkinson *et al*., 1999; Lowry *et al.*, 2005; Murienne *et al*., 2005; Heads, 2008; Ladiges, 2008). This view of the flora's origin gained support over some previous alternative theories suggesting the flora was of a more recent origin (Jeannel, 1942; Faivre *et al.*, 1955; Darlington, 1957). Given that the main island Grand Terre has remained in a similarly tropical climate since its separation 80Ma it was plausible that the early Gondwanan flora could have persisted within the region since its separation (Heads, 2005; Ladiges & Cantrill, 2007). Heads (2005) and Ladiges (2008) support the theory of a Gonwanic origin of the biota on the basis that previously existing islands could have provided refugia during this period for the biota of New Caledonia. One of the major arguments extensively covered by Heads (2005; 2008) is the limitations and flaws involved with modern day molecular dating and the use of evolutionary clock models. Chief among these is the selection criteria for molecular clock models and their reliance on, often incomplete, fossil records for calibration. Heads (2005; 2008) warns researchers

not to simple rely on these methods and estimated divergence dates to interpret New Caledonia's history, in turn warning against discarding a vicariant origin.

Perhaps one of the most enticing notions presented by the presence of these families, together with their purported continental origin, is that New Caledonia may potentially represent a remnant portion of early Gondwanan biota and represent a "key to the past". This has gained support from those arguing that the archipelagos species richness is the result of cladogenesis over an extensive time period rather than the result of more recent radiation (Morat, 1993).

One point of debate is the role that the regions submergence events played in the evolution and survival of taxa. If the submergence of the local landmasses was entire, no terrestrial life would have survived in situ from its Gondwanan origin, thereby dismissing vicariance as the source of New Caledonia's flora. Alternately if the submergence was only partial then pockets of refugia or even short lived volcanic arcs could have maintained unbroken lineages that could be traced to Gondwana. The extent and duration of the submersion of Grande Terre during the Paleocene and Eocene continues to be hotly debated in the literature and is indeed crucial to the interpretation of the origin and evolution of taxa on New Caledonia.

It was not until extensive geologic research into the geology of the New Caledonian region occurred that the full appreciation of submergence during the Paleocene and Eocene (33.9-65.5Ma) was comprehended. Recent interpretations of the geologic evidence (Pelletier, 2006; Grandcolas *et al.*, 2008) reveal that the region experienced total submersion for 20my during the Paleocene-Eocene due to the presence of emplaced limestones and cherts fining upward, indicating deep

water low energy deposition. From the work by Pelletier, the theory of an unbroken Gondwanan lineage for many families was strongly challenged. Though Pelletier (2006) speculates that it is possible, given the close proximity of an active subduction zone to the west, the region could have supported short-lived emerged seamounts which could have served as refugia.

Following these conclusive findings within the geology of Grande Terre researchers are now suggesting that the flora of Grande Terre is the result of multiple post-Eocene colonization events. The Gondwanan "ark" hypothesis has been challenged further in the recent literature as to whether these putatively relictual taxa have truly persisted on Grande Terre since its separation from Gondwana or if they are the result of multiple recent introductions via long distance dispersal post submergence (Swenson *et al*., 2001).

Recent studies, such as Bartish *et al*, (2005), into the origin of the flora have relied heavily on molecular dating techniques which look to date the divergence age of taxa. This has been used in conjunction with bio-geographical and geological evidence to elucidate the flora's true origins. Studies focusing on the basal angiosperm taxa, and hence purportedly of Gondwanan origin, are crucial in providing data to rigorously test these alternative hypotheses. Expanding on this, it is fundamental for research investigating these floristic origins to use as many basal taxa groups as possible to ensure that a holistic view of New Caledonia's history is attained. Should all basal taxa investigated provide similar divergence dates for their arrival onto New Caledonia then more robust conclusions can be drawn with regard to the region's history.

This notion of an unbroken Gondwanan lineage, although intriguing, has since been challenged with more recent geologic and phylogenetic evidence suggesting re-colonisation of biota to New Caledonia during the mid-late cretaceous after extensive and prolonged submersion of Grande Terre and surrounding landmasses. Grandcolas *et al.* (2008) contests Pelletier (2006) suggestion of sub aerial sea mounts potentially being present as there are no identified eroded seamounts within the area, therefore no refugia existed. Major evidence includes the absence of mammals and continental beetles as well as geological emplacement of sediments identifying marine transgression (Grandcolas *et al*., 2008). Grandcolas *et al.* (2008) suggests that the taxa could be as old as 37Ma given current molecular research, diversifying and adapting for a far longer period than many other oceanic islands. New Caledonia is currently referred to as being a composite biota dominated by neoendemism and disharmonic colonisation via localised and long distance dispersal. In this sense New Caledonia is more akin to an oceanic island situation than a true fragment of continental origin bearing original flora. To begin to elucidate the history of this archipelago many suggest that molecular studies will be the most suitable tool in combination with a well-established understanding of the region's geological history**.**

Floral affinities of New Caledonia

Regardless of the true origin of the flora it is widely accepted that New Caledonia shares many floral affinities to nearby and distant landmasses, sharing both species and genera. A number of these affinities are facilitated by the orographic rain shadowing that New Caledonia experiences, creating a dry

western ecotype dominated by sclerophyll forest and wet eastern ecotype dominated by moist evergreen forest both of which show a number of distinct affinities (Table 1). The Marquis Forest type also appears to have a high affinity towards landmasses to the west. The most common affinities are shared with Australia both due to its close proximity, similar climate, and closely linked geologic history. This is evident given Australia's strong representation in all of New Caledonia's major forest types (Table 1**)**. Similarly the flora of New Guinea and the greater Malesia biogeographic region are strongly represented within New Caledonian flora in all forest types. These major representations are from landmasses located to the west and north-west of New Caledonia indicating that there is a strong force driving connectivity. In this regard there is support for New Caledonia's characterization as an oceanic island given such a disharmonic flora; this is also in keeping with the islands historical submergence events. This point of view is argued similarly for New Zealand in which Trewick *et al.* (2006) suggest, given New Zealand's submergence and subsequent emergence free of terrestrial biota, it should be considered as oceanic in process regardless of its continental origin.

New Caledonia's affinity and connection with New Zealand's flora, or vice versa, has been a point of interest within the literature given their somewhat disjunct positions in regards to one another. Early research by Oliver (1925) suggests that a land bridge between New Zealand and New Caledonia existed, including Lord Howe Island and extending to the New Hebrides (Vanuatu Islands), connecting the two continental fragments biotas. This theory is still current within the literature having once again been suggested by Swenson *et al.* (2001) and supported by extensive work in Meffre *et al.* (1996; 2006) who infer

that during the Oligocene the ridge systems between New Caledonia and New Zealand were emergent as an arc, Lee *et al.* (2001) suggest that this connection potentially could have continued to exist as recently as 10ma coinciding with a reduction in volcanism and extinction of many sub-aerial seamounts. The idea of a continuous land bridge has been refuted in research by Pole (1994) given sub-aerial islands between New Caledonia and New Zealand are oceanic seamounts of volcanic origin, therefore more recent and short-lived. Instead Pole suggests that the flora of these oceanic islands indicates that New Zealand's flora is entirely long distance dispersed with such islands representing stepping stones rather than a continuous land bridge. Patterns that support such a connection are highlighted in Lee *et al.* (2001) where extant New Caledonian *Nothofagus* Blume. share distinct characters with extinct species present in the New Zealand fossil record, similarly many extant New Caledonia genera are also represented within New Zealand's fossil record, and finally a number of shared taxa unique to the two archipelagos. The true mechanism for connection between these large emergent portions of Zealandia still needs to be consolidated. Though it is clear that connection has existed between New Zealand and New Caledonia given similarities in biota as well as subsets of these floras found on both the Lord Howe and Norfolk Islands, distinct from Australian floras (Green, 1994).

Table 1: Table detailing the phytogeographic affinities of New Caledonian flora towards nearby landmasses (values given are percentages). (Adapted from Lowry, 1996)

REGION	Moist Evergreen Forest	Marquis Forest	Sclerophyllous Forest
Australia	27.2	31.3	23.6
New Guinea	20.2	16.8	14.3
Malesia	11.7	13.3	15.2
Fiji	9.8	5.5	3.6
Vanuatu	7.6	6.9	7.5
Solomon Islands	6.8	4.2	5.2
New Zealand	4.3	5.6	2.5
Asia	3.2	5.8	10.4
Africa	2.8	4.3	6.3
Lord Howe Island	1.6	1.6	3.6
America	1.6	1.0	3.5
Samoa-Tonga	1.4	0.6	0.0
Norfolk Island	1.0	1.0	0.9
Polynesia	0.5	1.2	1.0

Characteristics of the flora of New Caledonia

The flora of New Caledonia has been shaped and moulded by a unique geologic and geographic template. High species endemism has resulted from the huge variation in habitat and numerous disturbance events, including submergence and tectonic activities. For a long time the New Caledonian archipelago has been recognised for having high degree of species richness and endemism which, given the relatively small land area, is exceptional (Myers *et al.*, 2000). Smith *et al*. (2007) suggest the isolation of the island in combination with the complex geological history and landscape has allowed for multiple adaptive radiations. After an extensive period of submergence, the re-emerged landmass presented a mosaic of unoccupied niche's which early colonising groups responded to by rapidly diversifying. Diversity also appears to be strongly influenced by soil and climatic variation, with ultramafic peridotite soils and the central mountain range playing crucial roles in habitat division.

New Caledonia's current flora

The most recent collation of New Caledonia's flora is in the recently published Morat *et al.* (2012) which draw on a number of sources to present up to date figures on the archipelagos lycophytes, ferns, gymnosperms, and angiosperm flora (Table 2). At present 272 species of ferns and lycophytes are present on the archipelago, these represent four genera across two families for the lycophytes and 91 genera across 27 families for the ferns. The level of endemism for the ferns contrasts sharply between genera and species, at the generic level just one of the 91 genera are endemic compared with 94 (37.5%) endemic species. The gymnosperms are often considered the most distinct group within New Caledonia,

totalling 46 species the islands flora represents a total of 7% of the world's gymnosperm flora diversity. The species *Cycas seemanii* Braun. although a native species it is the only non-endemic gymnosperm of the 46 species, giving an extremely high endemic level of 97.8%.

The monocotyledons of New Caledonia are an interesting group as there are few families and genera which account for the majority of richness with others comparable depauperate. In conjunction with this New Caledonia also have a number of other well-known pacific groups which are either few in number, such as Commelinaceae (Mirb), Dioscoreaceae (R. Br.), and Araceae (Juss.), or absent all together in the case of Restionaceae (R. Br.) and Marantaceae (Peterson). This aside, the monocotyledons of New Caledonia include 30 families comprising 199 genera and 560 species, of these 17 (8.5%) genera and 264 (47.1%) species are endemic. The last major group covered in Morat *et al.* (2012) are the dicotyledons which account for the vast majority of species richness. The dicotyledons number 2493 species within 491 genera within 128 families (116 eudicotyledons families). Endemism is also high for this group with 2108 (84.5%) endemic species and 77 (15.7%) endemic genera. Included within the dicotyledons described by Morat *et al.* (2012) there are eight families classified as Magnoliidae, as well as the orders Amborellales (Melikyan, Bobrov, and Zaytzeva), Austrobaileyales (Takht. ex. Reveal), Chloranthales (R. Br. ex. Sims), and Ceratophyllales (Link) represented by a single family respectively. New Caledonia is also of special interest and significance to botanists given the presence of such a large number of these basal plant family lineages including Amborellaceae, Annonaceae, Atherospermataceae, Chloranthaceae, Hernandiaceae, Lauraceae (Juss.), Menispermaceae (Juss.), Monimiaceae,

Piperaceae, Trimeniaceae, and Winteraceae (R. BR. ex. Lindl.) which all exhibit a range of archaic or primitive characters. Some of these, such as the Winteraceae, also have fossil records which make them ideal candidates for modern day phylogenetic analysis. This group is similar to the monocotyledons in that there is an imbalance of species distribution between families with some families over-represented and other common families under-represented or absent.

Table 2: Table detailing the family, genera, and species number for major plant groups in New Caledonia. Number of endemics is also detailed for both genera and species. Modified from Morat *et al.* 2012.

	Total family	Total genera	Total endemic genera	Total species	Total endemic species
Lycophytes	2	4	0	21	8
Ferns	27	91	1	251	94
Gymnosperms	5	15	0	46	45
Monocotyledons	30	199	17	560	264
Dicotyledons	128	491	77	2493	2108

Biological hotspot

Extensive floral endemism is a major contributor to the archipelago's status as a biodiversity hotspot and a conservation priority (Myers *et al.*, 2000). According to the definition of Myers *et* al. (2000) there are a number of general requirements that a region must exhibit to gain hotspot status. The first of these is of course high levels of endemism, typically ≥ 1500 species or ≥0.5% of all plants globally must be endemic. In addition the region must have experienced significant (>70%) habitat loss of primary vegetation. One of the major threats to the flora species of New Caledonia is the excavation of the huge nickel, chromium reserves present on the islands which, given the world demand for these raw materials, are highly valuable exports for New Caledonia accounting for 7-10% of the country's GDP, and 97% of total exports value (excludes tourism) (Bouchet *et al.*, 1998). Much of the mining of this material is done through opencast mining in which entire mountains are cleared to gain access to the nickel reserves. Another threat to biodiversity is mass clearance of forest using fire to clear land for farm expansion and new pastures (Bouchet *et al.*, 1998). Outside of these major threats urban encroachment is increasingly taking its toll on the New Caledonian forests. An assessment by Myers *et al.* (2000) indicated that only 5200km2 (28%) of the original 18972km^2 of primary vegetation remains on New Caledonia. In 2009 a total of 26 terrestrial protected areas existed totalling 62634 hectares (626.3km^2) in area, around 3.4% of the total terrestrial landmass (Morat *et al.*, 2012). Morat *et al.* (2012) also comment on this figure being "very insufficient" given that two of the archipelagos provinces lack protected areas. This level of endemic diversity is comparable to that of New Zealand, the

Hawaiian Islands, and Madagascar (Jaffré *et al.*, 2001; Lowry *et al*., 2005) and is a unique botanical resource worth protecting.

Microendemism

In addition to the large number of endemic species found on the archipelago there are also a number of species that are also limited in distribution to a specific habitat, altitudinal range, and/or soil type/s, resulting in a high degree of microendemism. Grandcolas *et al.* (2008) highlights the fact that microendemism on Grande Terre is often understated and overshadowed by emphasis of New Caledonia's overall endemic biota. Microendemism has traditionally been explained as a direct result of the mosaic nature of habitat on the island. This is suggested to be the result of habitat diversification in response to a combination of orography, soil type, and climatic variation (Morat, 1993). Grandcolas *et al.* (2008) also inferred aspects of historical factors and speciation processes to explain aspects of the microendemism. Furthermore Grandcolas *et al.* (2008) identified niche conservatism (Wiens, 2004) and population divergence as major influencing factors, in particular when environments (e.g. mountains) become/became isolated in the aftermath of climatic, geologic, or geographic change. Attesting to this concept of niche conservatism are the species *Dracophyllum alticola* Däniker., *Scaevola racemigera* Däniker., and a recently discovered species of Iridaceae Juss. which are found only on the peak of Mt. Humboldt, New Caledonia's highest mountain.

Molecular phylogenetic studies on the biota of New Caledonia

New Caledonia's sheer diversity and phyletic radiation of taxa has often made it difficult for researchers to circumscribe taxa. The remote locality of New Caledonia has also meant that much of the flora has historically been under-studied and much of the archipelago under-explored until more recent times Sharma and Giribet (2009). The traditional use of morphological traits and characters to delimit and classify taxa has created the foundations for many of today's studies. These morphological studies have combined many of New Caledonia's taxa into broadly defined groups in an attempt to encompass such rich variation. More recently phylogenetic analysis in conjunction with morphological analysis in modern day taxonomic and phylogenetic work has made major advancements into recognising and understanding the relation between taxa. Often refining and improving upon previous classifications of New Caledonia taxa and indeed their relation in the worldwide context. More recent research investigating taxonomic relationships is relying increasingly more on molecular studies to augment traditional approaches, this is especially so for investigations into taxonomic groups on New Caledonia (Table 3). It is also a significant and important area of study given New Caledonia's status as a biodiversity hotspot (Myers *et al.,* 2000), as the true extent of diversity within the archipelago is still being discovered.

Molecular phylogenetics is still a young scientific field that has only recently been developed into an everyday tool for scientific research (within the last 20 years). With continued development, molecular phylogenetics has become appreciated as a field highly relevant to investigating many biological questions.

A relatively new area in which molecular studies are proving very useful is in the field of biogeography and the inference of the origin of taxa, as well as landmasses. Molecular phylogenetic studies have been undertaken on many New Caledonian taxa in an attempt to understand the island's biotic history. The recent development of molecular dating techniques to estimate divergence of taxa has been indicated as a crucial tool in testing the Gondwanan ark hypothesis in light of recent contrasting geologic evidence and uncertainty as to the effect of submergence. For this vicariance hypothesis to hold true we would predict divergence times older than 70 Ma between New Caledonian taxa and their closest sister taxa (Ladiges *et al.,* 2003).Investigation utilising this method and reasoning have largely suggested that flora of the islands are indeed the result of post-Oligocene long distance dispersal events and subsequent radiations (Table 3). Resulting in a current shift towards rejection of the Gondwanan ark hypothesis and support for the hypothesis of long distance dispersal.

A firm grasp of the origin of New Caledonia's flora and the impact of Eocene submergence is essential in order to begin to understand the evolutionary history of biota on New Caledonia and importantly it will broaden our overall understanding of plant biogeography in the south-west pacific.

Table 3: Recent molecular studies providing estimated divergence dating of selected New Caledonia taxa

TAXON	**LOCI USED**	**ESTIMATED LINEAGE AGE**	**REFERENCE**
Troglosironidae (Harvestman family)	16S rRNA 18S rRNA 28S rRNA COI	Pre-submersion	Sharma & Giribet, 2009
Paratya (Fresh water shrimp genus)	16S rRNA	12-19 Ma	Page *et al.*, 2005
Dytiscidae (Water beetle family)	*Cox*1 *Cob* *rrnL-tRNA Leu-Nad1*. 18S rRNA H3	9 Ma	Balke *et al.*, 2007
Scincidae (Skink family)	NADH ND2 Rag-1 c-mos	12.7 Ma	Smith *et al.*, 2007
Lanceocercata (Clade within the stick insect order Phasmatodea)	COI COII H3 28S rRNA	41.06 Ma	Buckley *et al.*, 2010
Hydropsychidae (Caddisflie family)	EF-1α RP2 CAD COI	5-13 Ma	Espeland & Johanson, 2010
Eneopterinae (Cricket subfamily)	cyt *b* 12S rRNA 16S rRNA 18S rRNA	10.6 Ma	Nattier *et al*., 2011
Sapotaceae (Angiosperm family)	5.8S rRNA 18S rRNA 26S rRNA	32.4 Ma	Bartish *et al.*, 2005
Metrosideros (Myrtaceae family)		16.6Ma 21.7Ma 29.9Ma (Dates dependant on sub-genera analysed)	Papadopulos *et al.*, 2011 Wright *et al.*, 2000 Pillon, 2012
Dracophyllum (Ericaceae family)	*matK* *rbcL*	6.7Ma	Wagstaff *et al.*, 2010
Santalum (Tree genus inc. Sandalwood)	18S–26S nrDNA 3' trnK intron	1-1.5 Ma	Harbaugh & Baldwin, 2007
Rutaceae (Angiosperm family) Aurantioideae (Citrus subfamily)	rbcL atpB	Post submersion	Pfeil & Crisp, 2008
Ebenaceae (Angiosperm family) Diospyros (Genus within the Ebenaceae)	atpB matK ndhF trnK intron trnL intron trnL-trnF spacer	Both pre and post submersion	Duangjai *et al.*, 2006

Research focus

The New Caledonia archipelago presents a distinct suite of characters which have acted in unison to create an unparalleled level of biodiversity and hence an important natural laboratory providing a unique opportunity to study insular evolutionary processes. In my research I have addressed two questions related to taxonomy and phylogenetics. The first question looks at the specific level where I assessed the genetic distinctiveness of a putative new species of *Vitex* L., a genus currently represented by two species within New Caledonia (Mabberley & de Kok, 2004).The second was focussed at the specific-generic level in which I investigated the relationships of taxa within the genus *Zygogynum* Baill. within the Winteraceae, testing recent revisions performed by Wim Vink. Within this question I have also investigated the evolution of morphological characters in light of my phylogenetic tree to assess if more appropriate characters exist for treatment of the genus.

***Vitex* (Lamiaceae)**

The mint family Lamiaceae Marty nov. nom. Cons. (Labiatae Jussieu *nom. Cons*. et nom alt.) has a cosmopolitan distribution with representatives from the tropics to arctic tundra (except Antarctica), and high altitude to sea level (Figure 6), only being excluded from extremely arid regions. The family was traditionally recognised as having close links to the Verbenaceae, in light of this Lamiaceae was recently revised to envelope a number of genera from the Verbenaceae and now includes more than 7000 species (Bramley *et al.*, 2009) and 236 genera. The family is noted to have a main centre in South-East Asia where species diversification is exceedingly high in comparison to other regions (de Kok, 2008). The family includes a number of economically important taxa, including a large number of culinary or aromatic herbs (*Thymus* L., *Lavandula* L., *Rosmarinus* L., *Salvia* L., *Mentha* L.), and some important timber-producing species such as *Tectona* L. (teak).

Figure 6: Cosmopolitan distribution of the Lamiaceae, except areas of extreme drought/aridity and cold (Stevens, 2001 onwards).

The Lamiaceae is currently divided into seven subfamilies: Viticoideae, Symphorematoideae, Ajugoideae, Prostantheroideae, Nepetoideae, Scutellarioideae, and Lamioideae (Harley *et al.*, 2004; Bramley *et al.*, 2009). The Viticoideae Briquet consists of ten genera which have a largely pan-tropical distribution, with rare representation in temperate regions (Figure 7). Relationships within the Viticoideae are not well resolved (Harley *et al.*, 2004), and many currently recognized genera are non- monophyletic (Bramley *et al.*, 2009). Bramley *et al.* (2009) used ITS (Internal Transcribed Spacer) and ndhF sequence data to perform DNA analyses on Viticoideae within south east Asia to elucidate the position of a number of groups and taxa, which resulted in SE Asian *Paravitex* (Fletcher), *Viticipremna* (Lam) and *Tsoongia* (Merrill) being synonymized with *Vitex*.

Figure 7: Distribution of Viticoideae, absent from greater Eurasia, northern Africa, north-west United States, and Canada. (Stevens, 2001 onwards).

The genus *Vitex* consists of c. 250-300 species (de Kok, 2008; Bramley *et al.*, 2009) that are distributed primarily within old and new world tropics with some representation in temperate regions. *Vitex* tend to be common in a range of habitats including primary rainforest and swamp habitat (de Kok, 2008) and are prevalent in secondary growth of disturbed vegetation largely associated with coastal habitat or those influenced by a fire regime (de Kok, 2008). The following morphological description of *Vitex* was adapted from the New Caledonia endemia website (http://www.endemia.nc). *Vitex* species are generally trees or shrubs often exhibiting white-grey furrowed bark. The leaves are typically opposite palmately compound comprising 1-7 leaflets forming whorls. The inflorescence can be terminal or axillary; with the Calyx either truncated or toothed. The corolla ranges from 1-2-lobed, typically five in number though this can vary between four and six. Stamens are four in number and form into pairs, the style ends in two equal segments. Fruits are drupes, each dividing into 4 segments; seed number is low and ranges between 1-4 seeded. Members of the genus are traditionally used throughout the south pacific for their timber, which is used in building houses and boats as well as being crafted into tools (de Kok, 2007). The type species for the genus *V. agnus-castus* L. has been used for centuries as an anti-fertility and anti-aphrodisiac, and is still in use in modern times within Melanesia (de Kok, 2007).

Vitex require further taxonomic delimitation, as the genus has been considered to be taxonomically problematic given a number of unresolved relationships. In fact, the genus has yet to be revised in full and de Kok (2007) emphasised the enormity of such a task. In spite of this, some regionally-focused revisions of *Vitex* represent steps towards a full revision of the genus. Within the

Asia-Pacific region revisions include those of Lam (1919), Lam & Bakhuizen van den Brink (1921) for South-East Asian taxa, Munir (1987) for Australian taxa, and Rajendran & Daniel (2002) for Indian taxa. Although thorough, some revisions are likely out of date due to the discovery of new species in centres of high diversity that are yet to be fully explored. More recent attempts to revise the genus in the region have been produced by de Kok (2007; 2008) and Bramley *et al.* (2009) focussing primarily on SE Asia, New Guinea, Malesian region, and the South Pacific islands.

Currently Mabberley & de Kok (2004) recognize two species of *Vitex* in New Caledonia, *V. trifolia* L. with two subspecies and *V. collina* L.. *V. trifolia* is a pantropical species typically found on coastal margins on calcareous soils (subsp. *trifolia*) and coastal sclerophyll forest (subsp. *littoralis*). *V.trifolia* is a small herbaceous shrub that is typically only found along coastal forest margins, subsp. *littoralis* is distinct in that it has a creeping habit. The leaves are compound with 1-6 leaflets, 3-10 x 2.5-4 cm, with petioles 3-5cm in length. The leaflets are oblong or elliptic with an entire margin, *Vitex trifolia* subsp. *littoralis* is distinct with unifoliate leaves with a very short petiole and sometimes sessile. Leaf margins are entire for both subspecies. The flowers are purple to blue in colour upon branched inflorescences which are terminal or axillary. The Calyx has five teeth. The fruit is an oblong drupe typically 5mm in length. *Vitex collina* is a species, native to New Caledonia and likely Vanuatu, which is typically found in rainforest, sclerophyll, and maquis forest. It is a small tree 0.6-20m in height (de Kok 2007) with light grey bark that peels in an irregular fashion. The leaves are palmately-compound with 1-7 leaflets, 3-16 x 1.5-8cm, which are lanceolate or obovate with petioles 2.5-5cm in length. The leaf apex is acuminate with a

cuneate base. Leaf margin varies with maturity; juveniles have a crenulated margin whilst adults have an entire margin. The flowers are bright pink or purple forming axillary branched inflorescences. The Calyx is campanulate and truncated, with stamens barely exposed. The fruit is a round drupe.

Mabberley (1998) acknowledges that *V. collina* is a highly variable species with respect to its morphology with at least three dominant morphotypes. Mabberley further suggested that revision of this species was premature given the need for a revision of the entire genus. The first of these morphotypes are small shrublets with small inflorescences, often with only a single flower, largely restricted to maquis forest on serpentine substrates. The second of these morphotypes is a tree, exemplified by herbarium sample MacKee 20521, which has both unifoliate and trifoliate leaves with inflorescences bearing many flowers. This morphotype was also noted as being restricted to rainforest habitat. The last was a distinct population restricted to north-west New Caledonia which is distinguished as having leaves with seven leaflets. Recently a potential new species has been discovered on the western central coast of New Caledonia and has been given the tentative name *Vitex* sp. *"unifolia"* owing to its unifoliate leaf shape. This new discovery could represent a new species or alternately an additional morphotype of *V. collina s.l.*

My research will investigate the molecular distinctness of this recently discovered *Vitex* sp. *"unifolia"* tree taxon from currently recognised New Caledonian taxa using molecular phylogenetics. In particular we will use sequence information from the ITS (Internal Transcribed Spacer) nuclear ribosomal gene region. From this work I hope to assess whether this taxon is a

new species or one of the multiple morphotypes contained within *V. collina s.l.* I will also in turn further investigate the morphotypes described in Mabberley (1998) to assess whether *V. collina s.l.* is a single species or alternately multiple species.

Winteraceae (Canellales)

The angiosperm plant family Winteraceae, first described in 1836, is one of two members of the order Canellales Cronquist within the Magnoliids along with Canellaceae Martius (APGII, 2003; Cai *et al.,* 2006). The Winteraceae is almost entirely restricted to the southern hemisphere with species representation in Australia, Borneo, Celebes, Moluccas, New Caledonia, New Guinea, New Zealand, Philippines, Madagascar, Central America, and South America (Vink, 1993; Vink, 2003; Stevens, 2001 onwards). The number of genera within the Winteraceae varies according to floristic treatments. Vink (1993; 2003) includes 79 species distributed across five genera: *Drimys* J.R.Forst. & G. Forst, *Pseudowintera* Dandy, *Takhtajania* M. Baranova & J.-F. Leroy, *Tasmannia* R.Br., and broad *Zygogynum s.l.* (Vink, 1993; 2003). Other authors, such as Guymer (2007) recognise 130 species distributed across eight genera; recognising *Belliolum* Tiegh., *Bubbia* Tiegh., and *Exospermum* Tiegh. as separate from *Zygogynum s.s.*

Three genera (*Pseudowintera*, *Takhtajania*, and *Zygogynum*) within Winteraceae are currently classified as endemic, with the remaining two genera (*Drimys* and *Tasmannia*) having a broader distribution (Figure 8). *Pseudowintera* consists of four species, two of which are widespread in distribution, all of which are endemic to the New Zealand archipelagos. *Takhtajania* is a monotypic genus endemic to Madagascar represented by the single species *T. perrieri* (Capuron) Baranova & J.-F. Leroy. *Drimys* is a new world genus consisting of seven species (Marquínez *et al.*, 2009) restricted to Equatorial and South America. The genus *Tasmannia* is an old world genus which consists of approximately 40 species

spread between Australia, New Guinea, Malesia, and the Philippines. The last genus *Zygogynum* is restricted to and endemic to New Caledonia.

Figure 8: Distribution of Winteraceae (adapted from Stevens 2001 onwards, base map sourced from www.outline-world.com). Colour indicates the distribution of the genera *Drimys* (orange), *Pseudowintera* (yellow), *Takhtajania* (red), *Tasmannia* (blue), and *Zygogynum* (purple).

The family is also well known for being among the first angiosperm lineages to be recognized in the fossil record. The ‘‘winteroid’’ pollen, characteristic of the family and its relatives, extends to the Early Cretaceous Barremian-Aptian 112-130 Ma (Walker *et al*., 1983; Doyle & Jarzen, 1990; Doyle, 2000). The Winteraceae have historically had a far greater geographic range than that of present day Winteraceae as shown by fossil records.

Morphological characters of the Winteraceae

A formal and comprehensive morphological description of the Winteraceae was given Guymer (2007) in his treatment of the Australian Winteraceae. The Winteraceae are trees or shrubs that are dioecious, polygamo-dioecious or bisexual. The wood is vesselless and hence composed of tracheids. The stems branch sympodially (lateral growth in which the apical meristem is terminated) or monopodially (vertical growth occurring from the apical meristem). The leaves are generally glabrous with rare exceptions having hair-like papillae. The leaves are exstipulate and can be alternate, in pseudo-whorls or sub-opposite in arrangement on a stem. The leaves are simple with entire margins, often covered in fine transparent or translucent glands. Flowers are solitary occurring terminally at the end of the stem or occur as a simple axillary determinate inflorescence. Flowers are radially symmetrical and are complete in form with all four whorls present, rarely incomplete. The gynoecium is superior and the flower is hypogynous. The calyx is calyptrate or generally two to four lobed, with rare exceptions of six, and generally has a valvate orientation. Petals range from two to many or may be entirely absent. Petals are generally arranged in one, two, or more whorls and are generally free, though some have connate outer whorls. Stamens range from three in number to being numerous and are free. In shape the stamens can be inflated, obovoid or ribbon-like with anthers adnate or anthers distinct from the filament. Anthers are tetrasporangiate and commonly have terminal or subterminal bisporangiate pollen sacs. The pollen is usually tetrad in form. The carpels range from one to several in number and are either in a single whorl and free, or, slightly basally connate in arrangement with a single terminal style and stigma. The ovary contains two placenta usually with one row of ovules which are

linear in shape, opposite in orientation, and either parallel to the stigma or ventral. The ovules can number from one to many, being either anatropous, descending or apotropous. Seeds are copiously endospermic, in this the endosperm is not ruminate, with a well differentiated embryo. The fruit can be apocarps, berry-like, and follicles, or are connate forming either multilocular capsules or syncarps.

Guymer (2007) uses a small number of key morphological features to differentiate Australian Bubbia and *Tasmannia* from one another at the generic level. The first morphological difference noted is that Bubbia have bisexual flowers with twigs elongating sympodially after flowering contrasting with *Tasmannia* which have functionally unisexual flowers with twigs elongating monopodially after flowering. The second point of difference Guymer (2007) uses is the calyx development. In this Bubbia have a calyx soon rupturing and persisting under the developing fruit alternately *Tasmannia* have a calyx rupturing post anthesis and subsequently falling away from developing fruit. Vink (1988) produced a comprehensive table noting key features differentiating the genera of Winteraceae. In this table *Takhtajania* is distinguished from other genera by possessing an ovary that is multicarpellate unilocular and having a cap shaped stigma. Vink identifies *Drimys* as having a number of characters in common with *Tasmannia* (then *Drimys* sect. *Tasmannia*) includes monopodial growth, terminal inflorescence subsequent replacement by a vegetative bud, calyx that encloses petals until anthesis. Species within *Tasmannia* also possess seeds with a curved axis. The single character used to differentiate *Drimys* from *Tasmannia* is that *Drimys* exhibits stigmas of free carpels that are strongly restricted. *Pseudowintera* is distinguished by having inflorescence that are lateral (in leaf axil) and flower several seasons. Lastly *Zygogynum* is noted as having species with examples of

connate outer petals, bioculate anthers, and multicarpellate ovaries which are multilocular.

Evolutionary trend within carpels

Armbruster *et al*. (2002) identified that the evolution of fused carpels is a repeating feature of angiosperm macroevolution and suggests that it has occurred independently at least 17 times in angiosperms and must therefore be highly adaptive. Early studies such as Carr & Carr (1961) observed and commented on an unequal distribution of pollen on stigmatic surfaces of flowers with multiple carpels. Armbruster *et al*. (2002) argue for two more subtle advantages associated with the transition that could potentially be of greater significance. Expanding on Carr & Carr (1961); Armbruster *et al*. (2002) suggested that excess pollen that accumulates on a syncarpus stigma could cross carpel boundaries and in turn fertilize multiple carpels which would theoretically reduce the chance that some carpels would go unfertilised which would otherwise reduce seed quantity.

Alternately another early study by Stebbins (1974), inferred that the main advantage of syncarpy was that it provided a structural defence for developing seeds from seed predators, and from this it was hypothesised that investment into a single outer fruit wall holding multiple seeds is energy efficient and more effective than multiple individual seeds each requiring protection. Similarly Endress (1982) suggested that this transition from apocarpus gynoecia to syncarpus gynoecia evolved with adaptations for dispersal, as it potentially provided extended protection and/or life during and post dispersal. Endress (1982) also commented that the ability to cross pollen between carpel tubes could

also act as a means of intensifying the competition between pollen spores. Carpel fusion or syncarpy is a common feature in a number of taxa within the Winteraceae. Though studies into carpel evolution within the Winteraceae are limited to one study by Frame-Purguy (1996) who investigated carpel development within *Tasmannia insipida* in which it was identified that fusion of the carpel structure occurred late in development and was postgenital. Within *Zygogynum* there are representatives of both apocarpus gynoecia (*Z. amplexicaule*) and syncarpus gynoecia (*Z. mackeei*), with *Z. stipitatum* appearing as a transitional stage between the two states (Figure 9).

Figure 9: Variation in Zygoynum from unfused to fused carpels. From left to right; *Z. mackeei* (syncarpus gynoecia), *Z. stipitatum* (transitional/intermediate state), *Z. amplexicaule* (apocarpus gynoecia).

Chemistry of Winteraceae members

Sesquiterpene lactones and dialdehydes are present within members of the *Drimys, Pseudowintera,* and *Zygogynum s.l.* These chemical groups are also shared with a number of other groups including a range of plants, fungi, and marine organisms (Jansen & de Groot, 2004). The Winteraceae possess unique sesquitpene dialdehydes, differing from other groups due to the sesquitpene dialdehydes bearing cinnamate or coumarate groups (Cechinel *et al*., 1998; Ferreto *et al*., 1988; Malheiros *et al*., 2001). The leaves of New Zealand *Pseudowintera colorata* (Raoul) are of particular interest to researchers as they contain sesquiterpene dialdehydes polygodial 1 and 9-deoxymuzigadial 2 with anti-*Candida,* insect antifeedant and anthelmintic properties (Gerard *et al*., 1993; Lorimer *et al*., 1996; McCallion *et al*., 1982). *Pseudowintera colorata* leaves also contain the sesquiterpene cyclocolorenone 6 (Corbett & Speden, 1958) which are common flavonols and flavones. Antioxidant anthocyanins and other flavonoid glycosides are found at higher levels in the red portions of leaves around wounds (Gould *et al*., 2002).

Physiology within Winteraceae

The Winteraceae have been traditionally considered one of the basal-most families within the angiosperms due to their retention of primitive characters, though its exact position was tentative. Lack of vessels, a condition that has long been considered primitive within angiosperms given the hydraulic advantage of xylem over tracheid based vascular systems, is a primary character used to support this view. Central to this view is that vessel bearing plants possess greater hydraulic capacity in contrast to tracheids largely based on the idea that vessels provide a more open path for water (Becker *et al*., 1999; Brodribb & Field, 2000).

Taylor & Hickey (1996) suggested that it is this potential for greater hydraulic capacity and transport that has led to the ecological dominance of angiosperms, being able to better utilise available water. This is supported by the suggestion that vesselless angiosperms have large hydraulic constraints that influence the ecological range of these groups (Feild & Holbrook, 2000; Feild *et al*., 2002). Field *et al.* (2002) indicate that the portrayal of Winteraceae as relicts with declining ecological range is largely fuelled by their restriction to cool to cold low evaporative-demand environments which include environments such as montane cloud forest, alpine shrublands, etc. In a recent study by Field *et al*. (2002) the functional advantage of this state was investigated and it was shown that this reversion to a tracheid based vascular system is advantageous in habitats prone to regular freeze-thaw events. The position of Winteraceae within the basal angiosperms has since been questioned further by recent molecular studies that have shown the Winteraceae to be several nodes above the angiosperm root within the Canellales (Doyle & Endress 2000). The presence of Winteraceae among a large number of vessel bearing taxa indicates that, although still a basal group, the lack of vessels is not a retained ancestral morphological state but rather a derived state.

Problematic taxonomy within the Winteraceae

Since being first described by Lindley in 1836 the taxonomy within the Winteraceae has been reviewed and revised a number of times, including the addition of a number of new taxa. The Winteraceae are a problematic group with reference to *Zygogynum s.l.* as its taxonomy and treatment varies widely among authors and additionally the relationship between the genera *Tasmannia* and *Drimys*. Arguably, the most comprehensive and controversial revision for

Winteraceae of New Caledonia was in Vink (1985; 1988), where he reduced the number genera within New Caledonia to a broadly circumscribed *Zygogynum* comprising the previously recognised genera *Bubbia*, and *Exospermum*, as Vink did not see sufficient morphological variation existed to justify separate genera. Subsequently Vink (1988) concluded that there was not sufficient evidence to distinguish *Belliolum* from *Bubbia* and thus combined the two together. This resulted in a reduction from eight genera recognised within the family (*Belliolum, Bubbia, Drimys, Exospermum, Pseudowintera, Takhtajania, Tasmannia,* and *Zygogynum s.s.*) to only five recognised genera. In his study Vink considered *Takhtajania* to be basal within the Winteraceae, based on a comparably low chromosome number compared with other genera in this family, followed by *Tasmannia, Drimys, Pseudowintera, and Zygogynum* s.l.. Three independent analyses using molecular data by Suh *et al.* (1993); Karol *et al.* (2000); and Doust & Drinnan (2004) all infer the same relationship found in Vink's study, with the most basal genus being *Takhtajania* followed by *Tasmannia*/*Drimys*, with *Pseudowintera* and *Zygogynum s.l.* being the most derived from the basal lineage. Another troubled relationship within this family identified in Endress *et al.* (2000) is that between *Drimys* and *Tasmannia* (Previously *Drimys sect. Tasmannia*). Some of the earliest work on this group was performed by Smith (1943) in which the original split within *Drimys* was presented. Here Smith recognised two sects within the *Drimys*, these were sect. *Tasmannia* (R. BR.) F.Muell. and sect. *Wintera* DC. (sect. Wintera synonymous to sect. *Drimys*) (Smith, 1969). This division of *Drimys* corresponded to a split of Old and New World species. Chromosome studies performed by Ehrendorfer *et al.* (1968) concluded that the two sects were in fact separate genera leading Smith (1969) to distinguish

Tasmannia as a separate entity from *Drimys.* This division was further supported by Suh *et al.* (1993) using ITS sequence information. More recent research (Endress *et al.,* 2000; Marquínez *et al.,* 2009) indicates that despite having highly similar floral morphology, *Drimys* and *Tasmannia* do not form a monophyletic clade in light of these studies. Throughout the family's taxonomic history and indeed to the present day the genera of *Zygogynum s.l.* and *Drimys* remain particularly problematic and a focus for current research. *Zygogynum s.l.* presents a problem with reference to its delimitation due to the lumping of taxa in light of limited collections and limited knowledge of the group. Whilst *Drimys* is still considered a taxonomic issue largely due to the poor sampling and representation of this genus within phylogenetic studies (Marquínez *et al.,* 2009)

Ehrendorfer & Lambrou (2000) investigation of the chromosome number variation within the Winteraceae produced a comprehensive account. Their work shows three clear groups within the Winteraceae; the first clade consisted of *Takhtajania* (n=18); the second consisted of *Tasmannia* (n=13); the third includes *Drimys, Pseudowintera,* and *Zygogynum s.l.,* and is distinct from the other clades as it had stabilised on a higher polyploidy level (n=43) with *Z. balansae* being autopolyploidy (n=86).

Zygogynum s.l.

According to Vink (1993; 2003) *Zygogynum* consists of 19 endemic species, 14 subspecies, and 5 varieties within the New Caledonian islands. Its distribution throughout New Caledonia is extensive with a number of species within the genus found on soils derived from ultramafic or non-ultramafic substrates and in some cases both.

Morphological characters within the *Zygogynum*

The following morphological description of the genus was derived from Vink's 1993 description. *Zygogynum* is composed of trees or shrubs that are always evergreen, rarely epiphytic. The wood is without vessels, a feature of the family. Species are entirely glabrous and rarely found with papillae (miniscule) or uniseriate hairs. The leaves are alternate, sometimes subvertisilate, entire exstipulate, and can sometimes possess cells that have oleanenes. The abaxial leaf surface is often glaucous with a covering of alveolar material or wax. The stomata are most often filled with a waxy stomatal plug. Individuals are dioecious to protogynous with an inflorescence that is typically terminal, but sometimes axillary. Inflorescences are subtended by a series of cataphylles. Bracts are small to miniscule, and rarely absent. The calyx is calyptracle. The petals are two to numerous in number, being free or externally partially fused. Stamens are three to numerous in number and free. Anthers are lateral to apical, often opening longitudinally. The pollen is free and is shed either as tetrahedronal tetrads or as monads. Carpels are free or fused into an ovary that is either unilocular or possess two to numerous locules. Stigmas are usually sessile. Ovules are two to numerous in number and uniseriate, rarely in more than one series along two placentas that

are generally partial to the stigmas. Flower colour is variable within the genus and is often useful for distinguishing taxa (Figure 9). The fruit is a berry, in the case of free carpels each ones becomes an individual fruitule. Seeds are usually more or less obovoid, the testa hard and brittle, with copious endosperm, embryo miniscule.

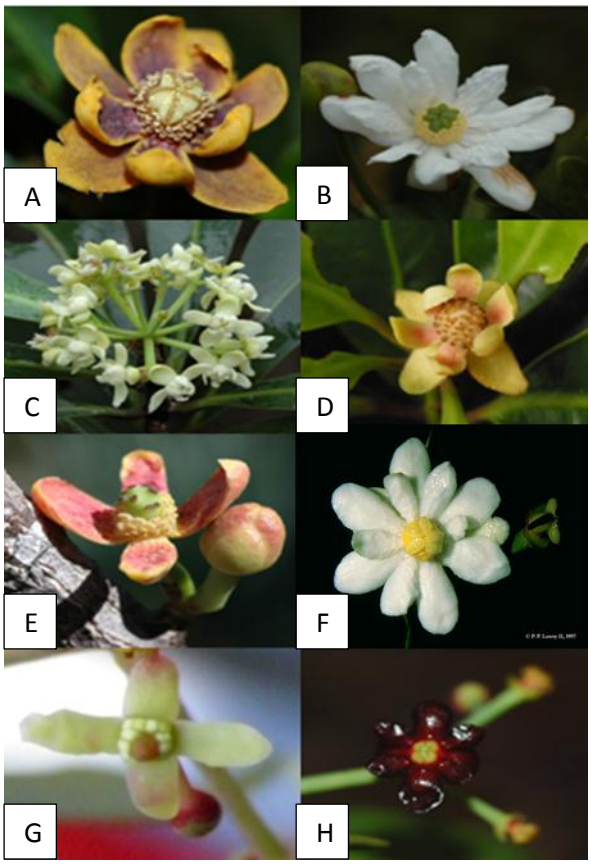

Figure 10: Variation in flower morphology in *Zygogynum*. A) *Z. stipitatum*; B) *Z. fraterculum*; C) *Z. pancheri*; D) *Z. oligostigma*; E) *Z. viellardii*; F) *Z. cristatum* (Lowry 1997); G) *Z. schlechteri*; H) *Z. amplexicaule.* Photo credit: photos A, B, C, E, H: Dr J. Munzinger; photo F: Dr P. Lowry.

Taxonomy within the *Zygogynum*

Since its first description by Baillon (1867) *Zygogynum* has been subject to a number of revisions. Baillon (1867) described a single species, *Z. vieillardii* in his original work. This was followed by a revision by Baillon in which he described a further two species *Z. stipitatum* and *Z. pomiferum.* Van Tiegham (1900) described two additional species *Z. baillonii* and *Z. bicolour.* These earlier descriptions of species within the genus brought the total number of species to five, representing the extent of *Zygogynum* before Vink's 1991 treatment.

Vink has played a large role in the more recent taxonomy of Winteraceae and more importantly in *Zygogynum* describing a further 13 species as well as defining a number of varieties and subspecies within the genus.

Vink (1977) described the species *Z. acsmithii, Z. mackeei, and Z. pomiferum* subsp. *balansae*.

Sampson (1983) added and described the species *Z. vinkii.*

Vink's 1985 revision was the most comprehensive, in which he included and delimited the species *Z. amplexicaule, Z. comptonii, Z. crassifolium, Z. cristatum, Z.pancheri, Z pauciflorum, Z. schlechteri, Z. tieghemii,* as well as *Z. amplexicaule* var. *amplexicaule, Z. amplexicaule* var. *isoneurum, Z. comptonii* var. *taracticum, Z. tieghemii* subsp. *synchronanthum,* and *Z. tieghemii* subsp. *tieghemii.*

Vink (1990) described the species *Z. oligostigma, Z. tanyostigma* as well as the subspecies and varieties *Z. amplexicaule* subsp. *luteum, Z. pancheri* subsp. *deplanchei, Z. pancheri* subsp. *rivulare, Z. tieghemii* subsp. *thulium.*

Vink (2003) added and described a further species *Z. fraterculum*.

Pollination of *Zygogynum*

One of the earliest studies into the pollination of *Zygogynum* was performed by Thien *et al.* (1985) in which he observed pollination of *Z. baillonii* by a moth within the *Sabatinca* Walker (Micropterigidae). In this paper it was also identified that both the moth and *Zygogynum* are found together in the fossil record dating back to the early stages of the Cretaceous period (65.5-145.5 Ma) suggesting a long association between the plant genus and its pollinating vector. Thien *et al.* (1985) also suggested that the life cycles of the plant and pollinator are linked, given that the moths aggregate during flowering and utilise the flowers as platforms for mating as well as feeding stations. The pollination of *Zygogynum* within New Caledonia was investigated further by Pellmyr *et al*. (1990) in which they identified two species of ancestral moths, three species of weevils (*Palontus* within the Curculionidae), and a single species of thrips, which were observed as frequent visitors to 12 species of New Caledonian Winteraceae, indicating them as potential pollinators.

Conservation

Conservation within the *Zygogynum* and Winteraceae is difficult because delimitation of some species within the genus is still a work in progress. At present three species of *Zygogynum* are recognised on the IUCN redlist as being of conservation interest based on assessments made by Jaffré *et al.* (1998a). *Z. cristatum* and *Z. tanyostigma* have both been identified as "vulnerable" due to their limited distribution. In the case of *Z. cristatum* it is restricted to ultramafic soil in the Kouaoua region, this region is not a protected site and therefore the

species is exposed to threats from mining activities, fires, and habitat clearance (Jaffré *et al*., 1998b). *Zygogynum tanyostigma* on the other hand is found on Mt. Panié which is a protected botanical reserve, though it is thought that these populations require increased protection (Jaffré *et al*., 1998d). *Zygogynum oligostigma* has been classified as "endangered" based on its distribution in relict lowland forest and ultramafic maquis forest (Jaffré *et al.*, 1998c). The area where it is found is unprotected and similarly to *Z. tanyostigma* exposed to threats from mining activities, fires, and habitat clearance (Jaffré *et al.*, 1998c). Though these assessments are in dire need of updating given that their assessment is based on surveys done over 10 years ago (IUCN 2012).

RESEARCH AIM

The focus of this research will be to use molecular techniques to try and elucidate the relationships within *Zygogynum s.l.* , with a large focus on the earlier generic revisions performed by Vink. To test Vink's taxonomic hypotheses and resolve relationships among these taxa, we comprehensively sampled Zealandic taxa using both nuclear (ITS) and cpDNA (*psb*A-*trn*H) sequence variation. Once we have robust phylogenetic hypotheses we will then evaluate species delimitations within the context of geographic variation. We will be particularly interested in assessing the position of taxa previously classified as *Belliolum, Bubbia,* and *Exospermum* in light of Vink's earlier revisions. Furthermore, we will investigate the origin and divergence of *Zygogynum s.l.* on New Caledonia using modern methods of molecular dating.

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CHAPTER II

Molecular confirmation of a new species of New Caledonian *Vitex* L. (Lamiaceae) and assessment of *Vitex collina s.l.*

This chapter has been prepared in manuscript form, representing the molecular contribution of a manuscript to be combined with a formal taxonomic treatment and species description in preparation for submission to the international journal of Systematic Botany. Referencing follows journal of Systematic Botany guidelines.

Steven Pratt conducted DNA extraction and PCR amplification, sequence preparation, editing, and alignment. Molecular analyses were performed by SP under the supervision of CG. The review of current and topical literature was performed exclusively by SP. The manuscript was drafted by SP with direction and contribution from CG.

Dr Chrissen Gemmill is the primary supervisor for this research project. CG facilitated the research via laboratory supervision during the early stages of research, funding acquisition, and collected numerous plant tissue samples from NOU herbarium that were used in this research. CG contributed to analyses and revisions of the draft manuscripts.

Dr Jérôme Munzinger is the secondary supervisor for this research. JM initiated this project based on his field observations and collected samples within New Caledonia. JM also hosted and mentored SP with respect to fieldwork in New Caledonia. JM will lead the formal taxonomic revision and species description.

Abstract

An assessment of the current circumscription of the genus *Vitex* (Lamiaceae), with special emphasis on the New Caledonian *V. collina s.l.*, is provided. Our assessment is based on analysis of morphological and DNA sequence data of the internal transcribed spacer (ITS) region. Relationships among non-New Caledonian species of *Vitex* are congruent with a previous study on the genus by Bramley *et al.* (2009). We found strong support for patterns of biogeographic separation and clustering of *Vitex* taxa. The New Caledonian *V. collina s.l.* is further resolved in this study indicating two major New Caledonian clades, with New Zealand and Australian *Vitex* nested within the clade. The larger of the two *V. collina s.l.* entities indicates patterns supporting further division of the complex into at least two species or subspecies pending further investigation. *Vitex* sp. *"unifolia"* specimens share unique ITS sequences distinct from others within *V. collina s.l.* and are moderately supported as distinct within our ML analyses, likely reflecting its recent divergence. In light of its morphological distinctness, there is sufficient argument for formal recognition to the level of species.

Keywords- Native, Internal Transcribed Spacer (ITS), Taxonomic Revision, Conservation.

Introduction

The archipelago of New Caledonia is located within eastern Melanesia in the southwest Pacific Ocean. The islands are renowned for their high level of endemic flora and fauna, as well as high levels of specific and generic diversity (Morat *et al.* 2012). In light of this it was designated as one of the original eight biodiversity hotspots (Myers *et al.*, 2000) highlighting the archipelago's taxa as a conservation priority, especially given mining and agricultural pressures (Morat *et al.* 2012). Myers *et al.* (2000) suggest a number of criteria to be met when considering areas to be protected in order to safeguard unique biotic diversity. Foremost of these is that designated conservation areas are both extensive in size, relative to the country's landmass, and encompass a number of vegetation ecotypes. Currently 62634 hectares of land is divided into 26 terrestrial protected areas (Morat *et al.* 2012). Morat *et al.* (2012) highlights that a significant portion of this protected land consists of secondary regrowth that contributes poorly to protecting vulnerable plant species. The need to protect New Caledonia's unique biota is unquestionable though given the high level of endemic taxa and the number of taxa in need of taxonomic work and revision, species circumscription is still incomplete.

Furthermore, new plant species are regularly being discovered, many of which are still awaiting formal description and publication. For example Swenson *et al.* (2007) recently described eight new species of Sapotaceae and Pillon *et al.* (2009) identified a number of cryptic species within *Spiraeanthemum* (Cunoniaceae) that are awaiting formal revision. Morat (1993) suggests, based on herbarium and field discoveries, that 5-10% of vascular plant species remain

undescribed. In respect to this, it is impossible to protect any vulnerable species if they are not yet formally recognised through valid publication.

Recent field work and assessment of herbarium materials suggest that New Caledonian *Vitex* L. (Lamiaceae) is a vulnerable genus in need of revision. *Vitex*, placed within the subfamily Viticoideae, consists of c. 250-300 species (de Kok, 2008, Bramley *et al.*, 2009) distributed largely throughout the tropics, with few species found in warm temperate regions e.g. Mediterranean, New Zealand. Most *Vitex* species have palmately compound leaves that are often variable in leaflet number within and between species, though a few species have unifoliate compound leaves e.g. *Paravitex*, which is nested within *Vitex sensu* Bramley *et al*. 2009. In the most recent comprehensive treatment of New Caledonian *Vitex*, Mabberley & de Kok (2004) recognised only two species, *V. trifolia* L. and *V. collina* (Montrouz.) Beauvis. In an earlier treatment Mabberley (1998) synonymised *Rapinia collina* Mountrouz., *Vitex rapinii* Beauvis., *V. rapinioides* Guillaumin, *Neorapinia collina* Montr. *V. rapinii* f. *dentata* Moldenke, *V. rapinii* var. *nana* Moldenke (syn *Rapinia triphylla* Montrouz ex Beauvis into a morphologically variable *V. collina s.l.* As treated by Mabberley & de Kok (2004), *Vitex collina s.l.* is typically found in dry to mesic forests along the eastern margin of the main island, Grande Terre. They are small trees 0.6-20m in height with palmately-compound leaves and branched inflorescences (Figure 1).

Figure 1: Copy of specimen plate used in Flore de la Nouvelle-Calédonie et Dépendances 25 (Mabberley & de Kok, 2004) depicting New Caledonian *V. collina s.l.* A: flowering branch; B: branch with flower buds exhibiting 5 leaflet form; C: 7 leaflet form; D and E: depiction and cross-section of *V. collina* flower; F: branch exhibiting maturing fruit.

Mabberley (1998) acknowledged that at least three distinct morphotypes (Figure 1 and 2) were present within this assemblage and that the type material of *V. collina* was a small tree with unifoliate leaves that was restricted to maquis on serpentine substrates. The second group was similar to *V. rapinii*, having a large tree habit exhibiting 3-5-foliate compound leaves, and a third group comprised a distinct local population on the southeast coast exhibiting compound leaves with up to seven leaflets. However, Mabberley considered that formal recognition of these entities was premature in light of the need for a complete revised treatment of the entire genus. Additionally there also appears to be a rare, morphologically distinct species found only along the dry west coast at Presauíle de Pindaï exhibiting the unique characters of unifoliate leaves and single axillary flowers, similar to that described in Mabberley (1998) (Figure 3). As such, this taxon may represent a distinct undescribed species of *Vitex* in New Caledonia.

The goal of this study was to use nrDNA sequence variation of the internal transcribed spacer regions to test Mabberley's hypothesis that three distinct taxa exist within *V. collina s.l.* and specifically to determine whether the unifoliate taxon is also genetically distinct from the other species of *Vitex* within New Caledonia; this would lend further support to this taxon being recognized at the species-level.

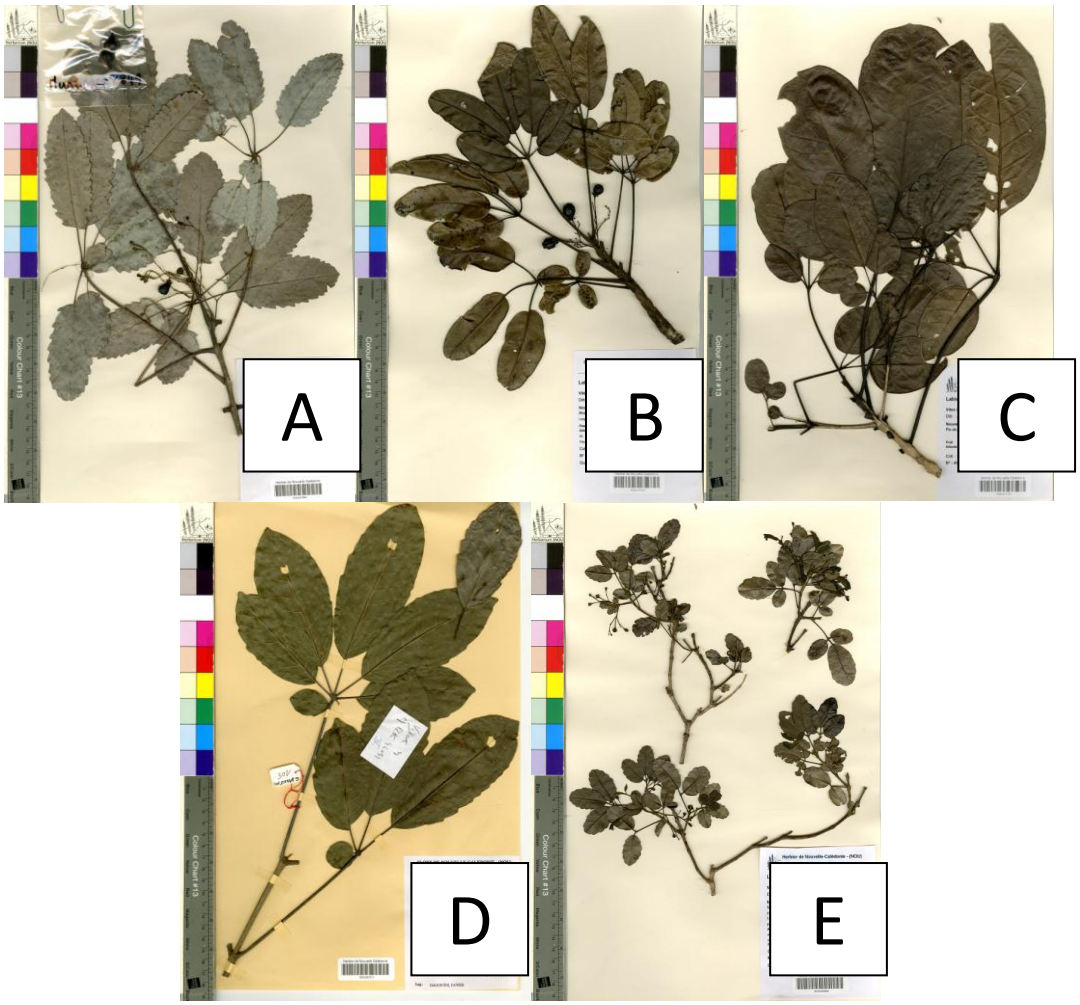

Figure 2: Herbarium specimens of *Vitex* cf. "*collina" s.l.* showing the morphological variability observed within *Vitex collina s.l.* A and B, JM2849 and JM3573 are specimens from northern New Caledonia distinguished by having comparatively long petioles and petiolules; C, JM4143 is a specimen of southern New Caledonia having moderate petiole and petiolule lengths; D and E, Dagostini 105 and JM4394 are specimens representing the central south-western coast of New Caledonia distinguished by occurrences of specimens with 7 leaflets.

Figure 3: Photo of the unidentified *Vitex* sp. *"unifolia"* highlighting its simple opposite leaves with entire margins.

Materials and methods

Taxon sampling: Field collections were undertaken on Grande Terre, New Caledonia. Fresh leaf materials were collected in the field and dried in silica gel. Leaves were also sampled from herbarium specimens held at L'Institut de recherche pour le développement (IRD), Nouméa (NOU). A total of 36 sequences (Electronic appendix 1 and 2, Appendix 1; note ######## denotes sequences yet to be uploaded and/or given NCBI accession numbers) were used in the phylogenetic analysis, composed of 12 new sequences (8 *Vitex* sp. *"collina",* 3 *Vitex* sp. *"unifolia"* and 1 *V. trifolia* subsp. *trifolia* from New Caledonia; Table 1) and 24 obtained from GenBank (Appendix 1) representing species from the south west Pacific through to mainland Africa. The use of multiple accessions within New Caledonia in addition to broad sampling of outgroup taxa was an essential criteria for assessment of the monophyly of *V. collina s.l.*

DNA extraction, amplification, and sequencing: Total DNA was extracted from 0.2-0.3 g of dried leaf tissue using an Isolate DNA extraction plant mini-kit (Bioline, Alexandria, NSW, Australia) according to the manufacturer's directions. Except that the duration of the lysis was extended from 30min to 3hr; this extended lysis improved the yield of DNA from both field-collected silica dried materials and herbarium specimens.

The ITS region was selected for this study given its successful application in previous studies within *Vitex* as well as its extensive use in resolving relationships between closely related island species (Baldwin *et al.* 1995, Gemmill *et al.* 2002). The ITS region was amplified using a combination of standard and degenerative *Vitex*-specific primers (Table 2). We developed *Vitex* specific degenerative

primers to avoid amplification of fungal endosymbionts which appears to be a common problem within this group (e.g. Bramley *et al.* 2009). The entire ITS region (ITS-1, 5.8s, ITS-2) was amplified using a combination of the standard ITS4 reverse + *Vitex* specific forward primer (*Vitex* ITS5) and ITSHP5 forward + *Vitex* specific reverse primer (*Vitex* ITS4). The primer combination of ITS4 with Vitex ITS5 resulted in the strongest and most specific amplification of the *Vitex* DNA. Full sequencing of the ITS region proved inconsistent and difficult, we therefore utilised the primer ITS2 (reverse) and ITS3 (forward) to sequence the two variable sub-units of the region.

Table 1: Specimens of New Caledonian *Vitex collina s.l.* sequenced in this study, including some morphological characters observed on herbarium specimens.

Sample	Location	Forest type	No. of leaflets	Petiole: terminal leaflet length / Petiolule: leaflet length	No. of flowers (Multiple or Single)	Inflorescence colour
Munzinger 2849	Far northwest coast, Paagoumène, creek à Paul	Low elevation forêt sur sol brun	(7)-8-(9)	1.5:1 long / 1:2 moderate	Multiple	Orange
Munzinger 3573	Northwest coast, Koné,Riviére Pandanus	Rivular maquis	5	1.25:1 long / 1:3 moderately short	Multiple	Yellow
Munzinger et al. 5791	Northwest coast, Koné, Vallée du Pandanus	Rivular maquis	(4)-5-(6)	1.5:1 long / 1:2 moderate	Multiple	Orange
Munzinger 5781	Mid-west coast, Nepoui, Pindaï	High open maquis on brown soil	1	Not observed	Single	Orange
Munzinger 3570	Mid west coast, Pouembout, Rivière Encaissée	High open maquis on brown soil	1	Not observed	Single	Unknown
Dagostini 1177	Mid-west coast, Boulinda, base of Boulinda	Maquis on brown soil	1	Not observed	Single	Unknown
Dagostini 105	Central East coast, Canala, sud de la presqu'île de Bogota	Forest on ferralitic soil	7	1:1 Equal / 1:4 short	Multiple	Unknown
Munzinger et al. 4206	Central south east, Tontouta, Vallée de la Tontouta, forêt à Kaori	Unknown	3	0.75:1 short / 1: 8 very short	Multiple	Pink-red
Munzinger 4394	Tontouta, Vallée des Kaoris	Unknown	7	0.75:1 short / 1:4 short	Multiple	Unknown
Munzinger 4143	Far south, Pic du Grand Kaori	Unknown	5	1.5:1 to 1:1 long-equal / 1:2 moderate	Multiple	Unknown
Gemmill 713	Far south east, Yaté, Port Boisé	Unknown	5	0.75:1 short / 1:5 short	unknown	Unknown

Table 2: Primers used in this study on New Caledonian *Vitex*.

PRIMER	SEQUENCE	AUTHOR
ITSHP5	5′-GGA AGG AGA AGT CGT AAC AAG G-3′	Gemmill et al. 2002
Vitex ITS5	5′-GCA AAG CAG ACC GCG AAC ACG -3′	This study
ITS4	5′-TCC TCC GCT TAT TGA TAT GC-3′	White et al., 1990
Vitex ITS4	5′-AAT CCC GCC CTC ACC TGG G -3′	This study
ITS3	5′-GCA TCG ATG AAG AAC GTA CG-3′	White et al., 1990
ITS2	5′-GCT GCG TTC ATC GAT GC-3′	White et al., 1990
*psb*A	5′-GTT ATG CAT GAA CGT AAT GCT C-3′	Sang et al., 1997
*trn*H^{GUG}	5′-CGC GCA TGG TGG ATT CAC AAT CC -3′	Tate and Simpson, 2003

PCR was performed in a total reaction volume of 20 ul consisting of 12.6 ul MQH_2O, 0.25 µM of each primer, 1.25X My Taq Reaction buffer (Bioline), 0.1% bovine serum albumin (BSA), 0.05U of MyTaq™ polymerase, and 1.0 ul stock total genomic DNA. PCR was performed on an Eppendorf Mastercycler® pro thermalcycler as follows: initial denature of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. We were unable to obtain complete high quality bidirectional reads for all samples. In these cases multiple PCR products were sequenced at least twice using the internal ITS primers ITS2 and ITS3 producing sequences of the internal ITS-1 and ITS-2 subunits, respectively (Table 2). By performing PCR amplifications in this way we were able to generate independent sequences over the desired region.

To verify amplification, 3ul of PCR product was mixed with 2ul loading buffer and loaded into a 1% 1× TBE agarose gel with 0.001% volume of ethidium bromide (EtBr 5mg/ml) for 50 min at 54 V and was then visualised using an Innotech Alphaimager™. A 100bp DNA ladder (Invitrogen™) was used as a size standard.

PCR products were purified of unincorporated reagents and prepared for sequencing using a standard ExoSAP protocol with 2.7ul MQH_2O, 0.2 ul Exo (ExonucleaseI; Global Science & Tech Ltd.), 0.1 ul SAP (Shrimp Alkaline Phosphate; Global Science & Tech Ltd.), and 10.0ul of PCR product. This was heated at 37 °C for 30 min then 80 °C for 15 min using an Eppendorf Mastercycler® pro thermalcycler.

Initial sequence alignment and editing was performed using Geneious pro ver.5.4.2. (Drummond *et al.* 2010). Sequences were confirmed as target DNA, and

not fungal endophyte, through sequence comparison using the NCBI database BLASTn search algorithm (Altschul *et al.* 1990).

Sequencing was conducted at the University of Waikato DNA Sequencing Unit using a 3130XL Genetic Analyzer System fitted with 50 cm capillary arrays (Life Technologies Corporation). DNA templates were prepared using Big Dye v3.1 terminator chemistry (Life Technologies Corporation).

Twenty-four additional sequences were sourced from GenBank ® and represent a broad geographic sample of species within the genus. Given that our samples were determined in the field and have not been formally determined as *V. collina s.l.,* all samples have been referred to as either *Vitex* cf. *"collina* or *Vitex* sp. *"unifolia".* Two sequence alignment matrices were generated with the first comprising all 36 taxa (Electronic appendix 1) and the second comprising New Caledonian taxa and their nearest sister taxa (Electronic appendix 2) as estimated from the full analysis (Table 4). In reducing the matrix to the target New Caledonian *Vitex* and their nearest sister taxa we hoped to further resolve relationships. Optimal alignment of both matrices was performed using MUSCLE alignment algorithm (Edgar 2004) as implemented in Seaview 4.0 (Galtier *et al.*1996, Gouy *et al.*2010).

Phylogenetic analyses

jModel test 2.1.1(Darriba *et al.* 2012, Guindon and Gascuel 2003, Posada 2008) was used to select the optimal model of evolution for maximum likelihood (ML) heuristic searches. The appropriate model of evolution selected for the full and reduced matrices were TIM1 + G (–lnL 2516.8798) and TIM3 + I (–lnL

941.8334), respectively. The best likelihood tree produced from these initial runs was selected as the "best" tree for visualisation of bootstrap scores. ML analyses were conducted using GARLI ver. 0.951 (Genetic Algorithm for Rapid Likelihood Inference; Zwickl 2006). Parameters used are given in Table 3; all other parameters used the default settings. 1024 bootstrap replicates (Felsenstein 1985) were performed for both datasets using GARLI to evaluate the robustness the clades produced in our ML analyses. The strict and 50% majority consensus bootstrap trees were visualised using PAUP* ver.4.0b10 (Swofford 2002).

Table 3: Parameters used maximum likelihood analyses using GARLI (Zwickl, 2006) based on results of j.modeltest model selection for the full and reduced matrices. f = frequency, r = rate, pinv (I) = proportion of invariable sites, and alpha (G) = gamma distribution.

Matrix	fA	fC	fG	fT	rAC	rAG	rAT	rCG	rCT	rGT	pinv (I)	alpha (G)
Full	0.1803	0.3598	0.3082	0.1516	1.2933	3.6388	0.6582	0.4321	6.8408	1.000	0.4596	0.5435
Reduced	0.1479	0.3976	0.3330	0.1216	142.9520	246.3388	0.6980	143.0154	283.3351	1.000	0.8234	0.0300

Results

The ML analyses of the full data matrix resolved a number of strongly supported (>90 BS) clades among the *Vitex* specimens included in this study. The addition of the reduced matrix analysis produced only minor increases in support for some nodes. This analysis produced a topology in which six major clades are readily recognised. The clade of *V. agnus-castus, V. negundo, V. parviflora,* and *V. trifolia* (subsp. *trifolia* and subsp. *litoralis*) formed a strongly supported clade (87 BS). Remaining species form a strongly supported clade (99 BS) and are divided into clades A-E. Clade A is strongly supported (100 BS) comprising African species and is sister to a single northwestern Australian species *V. glabrata*. The remaining taxa are resolved into a trichotomy with weak support (57 BS). The backbone of clades B, C, and D are unresolved. Indo-Malesian and southeast Asian species are resolved in the strongly supported clade B (100 BS). Clade C is composed again of Indo-Malesian species forming a strongly supported clade (84 BS) with a single northeastern Australian species *V. queenslandia* that is strongly supported (100 BS) as being sister to *V. turczaninowii* (syn *V. phillipinensis*). The two Madagascan species *V. lanigera* and *V. leandrii* form the strongly supported clade E (100 BS).

The remaining taxa form a strongly supported clade D (figure 5) consisting of predominantly New Caledonian with New Zealand and Australian *Vitex* (93 BS). The backbone of this clade is unresolved. Three specimens of New Caledonian *Vitex* cf. *"collina"* form a strongly supported sub-clade (97/92 BS) (Figure 5). The remaining species comprise the Australian *V. lignum-vitae* and *V. lucens,* the sole New Zealand species, forming a weakly supported (62/ BS) sub-

clade. The last is a moderately-well supported (/74 BS) sub-clade of *Vitex* cf. *"collina"* and *Vitex* sp. *"unifolia"*.

Within first clade of *Vitex* cf. *"collina"* is strongly supported (97/94 BS) and represents specimens restricted to the southeast coast of Grande Terre. Within the second moderately supported (/74 BS) *Vitex* cf. *"collina"* clade a further two weakly supported (55/70 and 55/ BS) subclades are found, the first corresponds to specimens restricted to the northwest coast of Grande Terre and the second to specimens restricted to the southeast coast of Grande Terre grouped with specimens of *Vitex* sp. *"unifolia"*. Using the reduced dataset *Vitex* sp. *"unifolia"* is further resolved, although weakly supported (67 BS), as distinct from *Vitex* cf. *"collina"*.

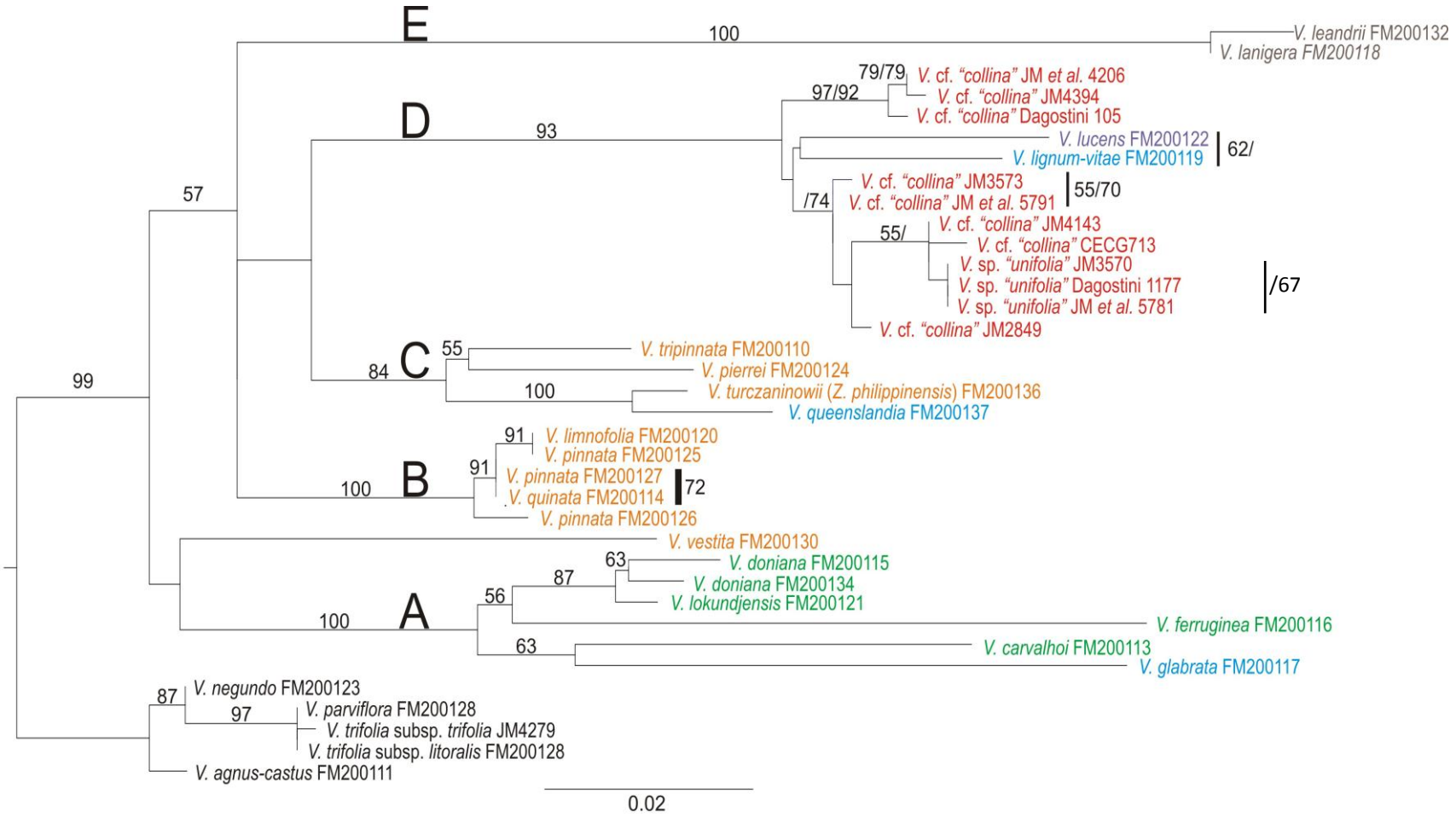

Figure 4: The maximum likelihood tree (-lnL 2663.14571) is of *Vitex* nrITS sequences with bootstrap values given above the branches, solid vertical bars indicate support if not indicated in best tree but indicated in the bootstrap consensus tree. (1024 replicates). A /# indicates a bootstrap value added from reduced dataset ML analyses. Refer to the text for detailed descriptions of clades A-E. Geographic regions are indicated as follows: Africa, green; Australia, blue; Indo-Malesia and Southeast Asia, orange; Madagascar, brown; New Caledonia, red; and New Zealand, purple.

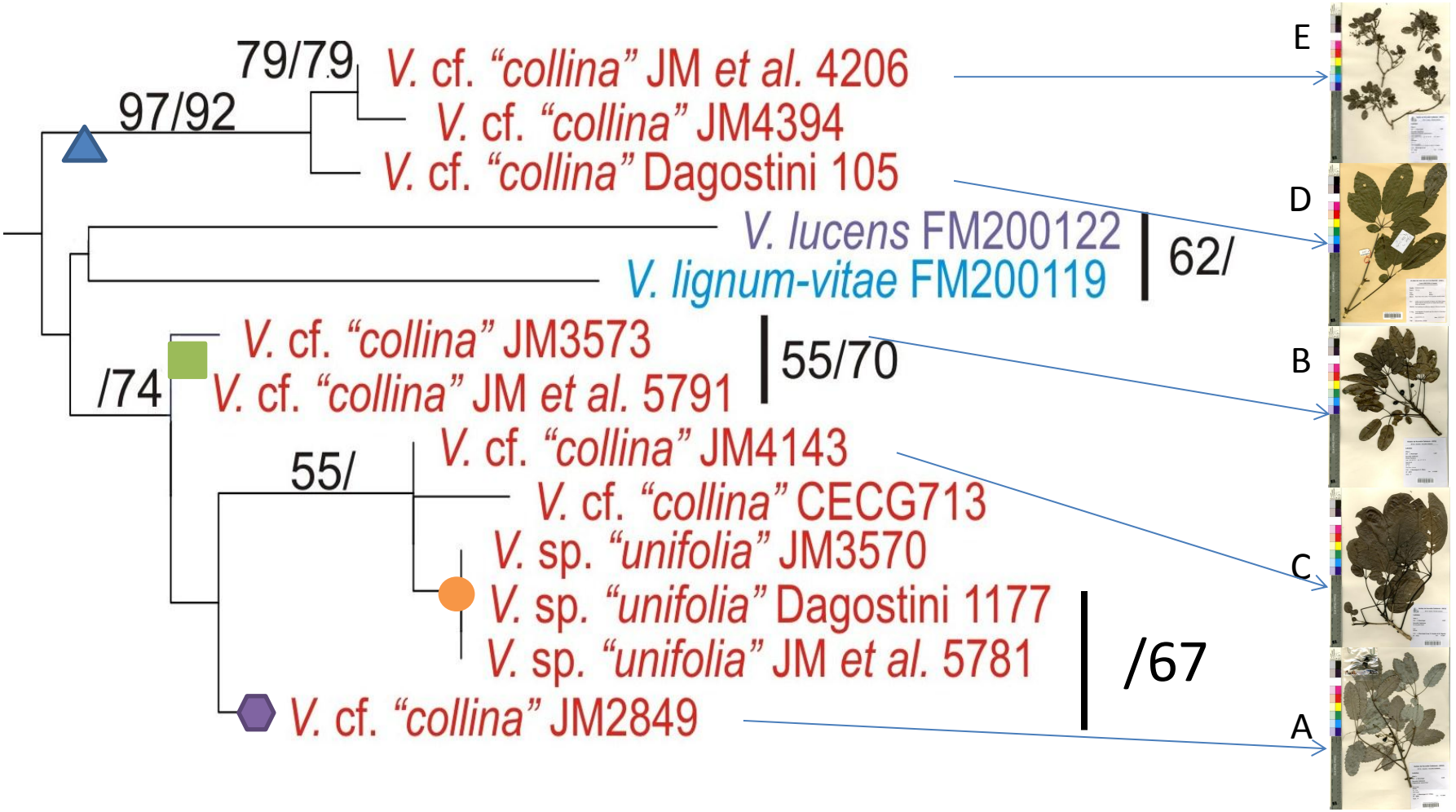

Figure 5: Enlarged view of clade D with select representative specimens (Letters comparable in Figure 1). Colour coding of names as in Figure 4. Shapes indicate unique morphological character states; green square= Long petiole/long petiolules, blue triangle= short petiole/short petiolules, orange circle= unifoliate leaves, and purple hexagon= very long pedicle/peduncle. A /# indicates a bootstrap value added from reduced dataset ML analyses.

Table 4: Summary statistics of the entire and reduced matrices ML analyses of *Vitex* nrITS data.

Matrix	Total number of sequences (new + sourced)	Number of base pairs	Nucleotide pairwise distances	Substitution model -lnL	Best tree -lnL	Number of resolved relations (50-74 BS)	Number of resolved relations (75-94 BS)	Number of resolved relations (>95 BS)
Entire	36 (12 + 24)	545	89.8%	TIM1 + G -2516.8798	-2663.1	11/26	8/26	7/26
Reduced	13 (11 + 2)	532	97.6%	TIM3 + I -941.8334	-972.25	3/5	2/5	0/5

Discussion

Amplification of quality sequences from the *Vitex* samples proved troublesome given the presence of an endosymbiotic fungi *aff. Arthrinium* sp. as identified via BLASTn search (confidence of 95%). Amplification of cpDNA *psb*A-*trn*H loci for *Vitex* DNA extracts confirmed the presence of target plant DNA. All samples were confirmed to contain target plant DNA, though the *psb*A-*trn*H loci was invariable and therefore un-informative for analyses. In order to amplify target *Vitex* DNA we designed the degenerative primers V. ITS5 and V. ITS4. In addition we used the internal ITS primers, ITS2 and ITS3, for sequencing which proved to resolve the issue of non-target DNA amplification, producing high quality target DNA sequences which were replicated for the purposes of nucleotide call confirmation.

The ML trees produced from our analyses indicate a strongly supported division between *Vitex* outgroup taxa with a shrub habit and those with a tree habit (clades A-E). This relationship was previously observed in the study of Bramley *et al.* (2009) on tropical mints. In our analyses the outgroup clade includes taxa that are quite removed from one another geographically; this likely is an artefact of limited sampling. A major trend we can see from our ML trees within the remaining taxa (exhibiting tree habit), clades A-E, is that the distinct and well supported clades depicted appear to have a strong affinity towards notable bio-geographical regions. In total we see five strongly supported clades individually sharing a common distribution and restriction to a biogeographic region.

Clade A is strongly supported (100% BS) consisting almost exclusively of species from the African continent, with a single northwestern Australian species

V. glabrata nested within the clade. Clade B and C are strongly supported (100% and 84% BS respectively), consisting of species distributed throughout the SE Asia and Indo-Malesian floristic provinces. A single Australian species, *V. queenslandia*, is nested within clade C, though is restricted in its distribution to the near tropical climate of Queensland. Clade D is a strongly supported (93% BS) clade comprising New Caledonian *Vitex* cf. *"collina"* and *Vitex* sp. *"unifolia"* with New Zealand *V. lucens* and Eastern Australian *V. lignum-vitae*. Clade E is a strongly supported clade (100% BS) of two species, *V. lanigera* and *V. leandrii,* which are restricted to the island of Madagascar situated off the Eastern coast of mainland Africa. A point of interest from the biogeographic affinities we have shown in this analysis is the disjunct relationship of the species of the African continent with the two species of Madagascar, which may be investigated further in future research.

Bramley *et al.* (2009) investigated the generic limits within the Vitocoideae using ITS sequence information, revising a number of specie's position within the Viticoideae. Bramley commented on an unusual relationship found in their analyses where *V. leandrii* and *V. lanigera* (Madagascar) were found to be sister to *V. lucens* and *V. lignum-vitae*. Bramley comments on it being a difficult relationship to explain given that there is no morphological similarity between the Madagascan and Australia/New Zealand taxa. Our analyses produced a trichotomy of *V. lucens* and *V. lignum-vitae* and additionally the New Caledonian *Vitex* cf. *"collina"* clade, with the Madagascan *Vitex* weak-moderately supported (61% BS) as sister to this group. It is possible that the New Caledonian *Vitex* may represent morphological intermediates between Madagascan and Australian/New Zealand *Vitex,* though commenting on this morphology is beyond the immediate

scope of this research. The trichotomy produced in our analysis likely reflects in part the lack of broad geographic sampling of the *Vitex,* which at this point is beyond the goals of this research.

The strongly supported clade of New Caledonian *Vitex* cf. *"collina"* with New Zealand *V. lucens* and Australian *V. lignum-vitae* is of great interest to our research, allowing us to assess the treatment of New Caledonian *Vitex* by Mabberley & de Kok (2004). This clade indicates relationships that challenge current taxonomic treatment of New Caledonian *Vitex.* The first relationship that must be considered is that New Caledonian *V. collina* is non-monophyletic given that *V. lucens* and *V. lignum-vitae* are nested within *V. collina s.l.* Although *V. lucens* and *V. lignum-vitae* are nested within this complex they are geographically removed from other taxa within this complex and are morphologically distinct species. In light of this it seems logical that closer investigation of the *V. collina s.l.* is necessary and likely there is plausible argument for the recognition of multiple distinct species, as suggested previously by Mabberley (1998). Furthermore the clade indicates two clear sub-clades of *V. collina s.l.* The first sub-clade consists of three samples of *Vitex* cf. *"collina"* and is very well supported. Alternately the second sub-clade of *Vitex* cf. *"collina"* is poorly supported within the entire dataset and moderately supported in the reduced dataset; relationships within this sub-clade are largely unresolved and poorly supported. This second cluster includes morphologically "typical" *Vitex* cf. *"collina"* taxa with palmately compound leaves as well as the morphologically distinct *Vitex* sp. *"unifolia"* exhibiting unifoliate leaves. The *Vitex* cf. *"collina"* specimens of the second sub-clade are distinct from the other sub-clade of *Vitex* cf. *"collina"* in that their petiolule length is half to a third the length of the

terminal leaflet blade with the first sub-clade exhibiting petiole lengths of one quarter to one eighth the length of the terminal leaflet blade. A similar pattern is seen within the morphological data for petiole length, with petiole length ≤ the terminal leaflet blade length in the first sub-clade and with petiole length ≥ the terminal leaflet blade length in the second sub-clade. Flower colour may also vary between the clades with red flower colour typical of the first sub-clade and orange/yellow flowers typical of the second, though the use of flower colour as a trait is largely speculative given limited sample information and that this trait may be indicative of flower maturity as opposed to differences between taxa. The specimen JM2849 is distinct from other *Vitex* cf. *"collina"* in this study exhibiting exceptionally long pedicle and peduncle lengths, as well as a much larger inflorescence flower number. This specimen, although nested within the second sub-clade, is positioned as an outlier and given further sampling may form another distinct sub-clade typified by its unique morphology. Now that we have molecular evidence for distinct groups within the *Vitex* it is important that we include and consider other lines of evidence (ecology, morphology, etc.) to further investigate New Caledonian *Vitex.*

These distinct *Vitex* cf. *"collina"* sub-clades and morphotypes also correspond to discrete geographic localities within New Caledonia (Figure 6). Many of which, given New Caledonia's mountainous topography, are likely isolated from other regions and therefore likely restricting geneflow leading to divergent evolution between populations of *V. collina s.l.*

The specimens of *Vitex* sp. *"unifolia"* are clearly distinct from *Vitex* cf. *"collina"* morphologically with reference to leaf anatomy giving strong reasoning for formally recognising this as a new species. Our molecular analyses indicate

that there are indels and nucleotide mutations common between sequences of *Vitex* sp. *"unifolia"* distinct from those found in *Vitex* cf. *"collina"* sequences, though these are too few in number to distinguish *Vitex* sp. *"unifolia"* into a supported clade. This likely indicates that divergence of *Vitex* sp. *"unifolia"* from *Vitex* cf. *"collina"* is relatively recent suggesting that additional genetic loci that exhibit more rapid evolution may provide increased resolution with which to assess its distinctiveness.

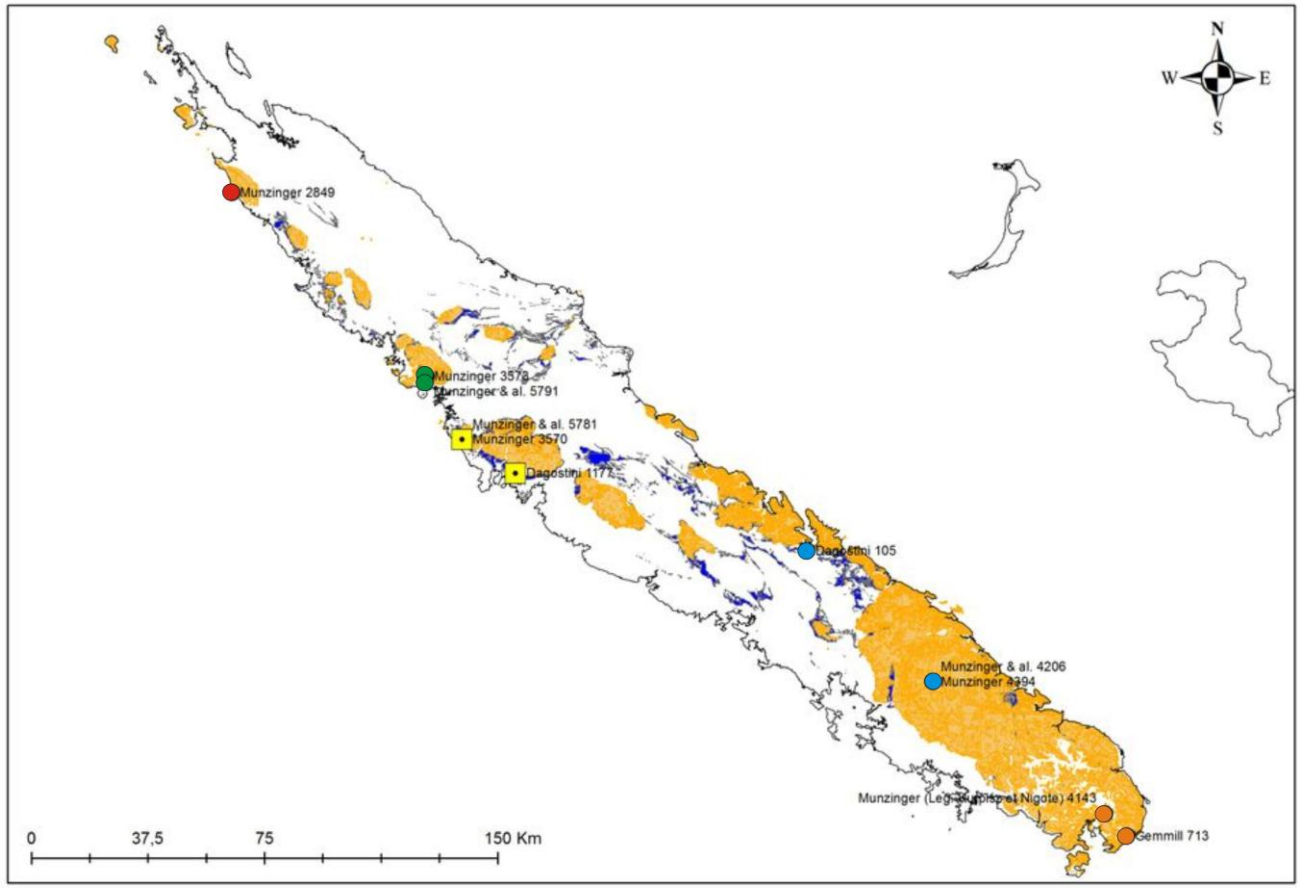

Figure 6: Map of New Caledonia depicting *Vitex* sp. *"unifolia"* sample localities indicated by yellow squares. Coloured circles indicate distinct clades of *Vitex* cf. *"collina"* as indicated from ML analyses.

Implications for conservation

These findings clearly indicate that New Caledonian *Vitex* are a complex group and are in need of thorough taxonomic revision. The New Caledonian *V. collina s.l.* is an understudied species that requires further work to tease apart its true complexity. For the purposes of conservation it is important to further investigate morphological variation, and indeed increase the sampling depth, within the *V. collina s.l.* to identify and appreciate any distinction between the distinct "*collina*" sub-clades represented within this research. If, as our ML analyses indicate, *V. collina s.l.* comprises distinct clades formal recognition of at least three species seems sensible. To assess the potential for further species distinction in light of the apparent morphological distinctness within remaining *V. collina* further investigation using additional samples is required. Given an increase in the number of recognised taxa within this group, it is prudent that further field investigation is done to assess the rarity of these entities and how threatened they are. This data will be useful in future IUCN red list assessments on New Caledonia.

Future directions

Following this research it appears that further delimitation and revised circumscription of the New Caledonia *V. collina s.l.*, including the putative species *Vitex* sp. *"unifolia"*, is recommended. We will also investigate Mabberley's suggestion that Vanuatu's *Vitex* may be conspecific with *V. collina s.l.* which would render it non-endemic to New Caledonia. Potential morphological characters requiring further investigation include presence of domatia, ovule type, fruit division, and the presence of seed endosperm. Further

molecular phylogenetic resolution could be acquired through the use of multiple gene regions increasing the size of the sequence dataset. If these steps increase the resolution of our analyses formal recognition and morphological description of the putative species will likely be required and will be completed in Munzinger *et al.* (in prep.). This work, upon further completion, may present significant changes to the conservation status of *Vitex* in New Caledonia. Given the rare nature of the putative species it would seem appropriate to consider a need for potential protected status until more surveying into its distribution is conducted. Similarly the distinct *Vitex* sp. *"collina"* clades may suggest re-evaluation of the species complex is required potentially resulting in restricted distribution ranges within New Caledonia potentially justifying protected status. One further avenue that we will investigate is Mabberley's suggestion that Vanuatu's *Vitex* may be conspecific with *V. collina s.l.* which would render it non-endemic to New Caledonia.

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Appendices

Vitex cf. "*collina"*, far northwest coast, Paagoumène, creek à Paul, Munzinger J. 2849, ########; *Vitex* cf. "*collina"*, northwest coast, Koné,Riviére Pandanus, Munzinger J. 3573, ########; *Vitex* cf. "*collina"*, northwest coast, Koné, Vallée du Pandanus, Munzinger J. et al. 5791, ########; *Vitex* sp. *"unifolia"*, mid-west coast, Nepoui, Munzinger 5781, ########; *Vitex* sp. *"unifolia"*, Mid west coast, Pouembout, Rivière Encaissée, Munzinger J. 3570, ########; *Vitex* sp. *"unifolia"*, mid-west coast, Boulinda, Dagostini G. 1177, ########; *Vitex* cf. "*collina"*, Central east coast, Canala, sud de la presqu'île de Bogota, Dagostini G. 105, ########; *Vitex* cf. "*collina"*, Central south east, Tontouta, Vallée de la Tontouta, forêt à Kaori, Munzinger J. et al. 4206, ######## ; *Vitex* cf. "*collina"*, Tontouta, Vallée des Kaoris, Munzinger J. 4394, ########; *Vitex* cf. "*collina"*, Far south, Pic du Grand Kaori, Munzinger J. 4143, ########; *Vitex* cf. "*collina"*, Far south east, Yaté, Port Boisé, Gemmill C.E.C. 713, ########; *Vitex leandrii, Ralimanana, H. & al. 120* (CANB, MO), Madagascar, FM200132; *Vitex lanigera, Lowry 5178* (CANB), Madagascar, FM200118; *Vitex lucens, Hind 4433* (NSW), New Zealand (cultivated), FM200122; *Vitex lignum vitae*, cultivated (MEL), FM200119; *Vitex tripinnata, Nguyen van Du & al. HNK884* (K), Vietnam, FM200110; *Vitex pierrei, Maxwell, J.F. 76-537* (K), Thailand, FM200124; *Vitex phillippinensis, de Kok s.n.* (K), Philippines, FM200136; *Vitex queenslandia, de Kok BG2529* (QRS), Australia, FM200137; *Vitex limnofolia, Nguyen van Du & al. HNK968* (K), Vietnam, FM200120; *Vitex pinnata, Nguyen van Du & al. HNK1148* (K), Vietnam, FM200127; *Vitex pinnata, de Kok s.n.* (K), Malaysia, FM200125; *Vitex quinata, Nguyen van Du & al. HNK1008* (K), Vietnam, FM200114; *Vitex vestita, Ambriansayah AA1662* (K), Indonesia, FM200130; *Vitex doniana, van Wyk, P. BSA2765* (K), Zimbabwe, FM200115; *Vitex lokundjensis, Thomas, D.W. 44-3* (K), Cameroon, FM200121; *Vitex ferrunginea, Luke, W.R.Q. & Kibure, O. 9753* (K), Tanzania, FM200116; *Vitex glabrata*, cultivated (QRS), FM200117; *Vitex carvalhoi*, cultivated (BRI), FM200113; *Vitex negundo*, Cultivated (MEL), FM200123; *Vitex parviflora, de Kok s.n.* (K), Philippines, FM200133; *Vitex trifolia* subsp. *trifolia*, New Caledonia, JM4279 (NOU), ######;*Vitex trifolia* subsp. *litoralis*, cultivated (CANB), FM200128;*Vitex agnus-castus*, cultivated RBG Kew, *Chase 22221* (K), FM200111;

Appendix 1. List of specimens investigated: Taxa, voucher, locality information, and Genbank numbers for ITS sequence information.

CHAPTER III

Phylogenetic analysis of *Zygogynum s.l.* (Winteraceae), a basal angiosperm, on a dynamic Gondwanan fragment.

This chapter has been prepared in manuscript form and represents the foundation of a manuscript in preparation for submission to the international journal of Biogeography. Referencing follows journal of Biogeography guidelines.

Steven Pratt, the MSc candidate, produced the review of current and topical literature. SP performed DNA extraction and PCR amplification, sequence preparation, editing of sequences, and making initial alignments. Molecular analyses were conducted by SP under the supervision of CG. The manuscript was drafted by SP with direction and contribution from CG.

Dr Chrissen Gemmill is the primary supervisor for this research project. CG facilitated the research via laboratory supervision of research, funding acquisition, and collected numerous plant tissue samples from the field and herbaria. CG contributed to analyses and revisions of the draft manuscripts.

Dr Jérôme Munzinger is the secondary supervisor for this research. JM performed field collections within New Caledonia, and hosted and mentored SP with respect to field work in New Caledonia. JM contributed to this work through his extensive knowledge of the flora of New Caledonia.

Dr Porter P. Lowry initiated this project in collaboration with CG and JM. PPL performed field collections of Winteraceae. PPL will contribute to future morphological analyses, taxonomic revision of this family, and preparation of IUCN Red List evaluations of extinction risks and conservation needs.

Steven J. Pratt, Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.

Chrissen E. C. Gemmill, Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.

Jérôme Munzinger IRD, UMR AMAP, Laboratoire de Botanique et d'Écologie Appliquées, Herbarium NOU, 98848 Nouvelle-Calédonie; IRD, UMR AMAP, Montpellier, 34000 France

Porter Pete Lowry,

Muséum National d'Histoire Naturelle, Case Postale 39, 57 rue Cuvier, 75231 05 CEDEX, Paris, France and Missouri Botanical Garden, P.O. Box 299, St Louis, MO 63166-0299, USA.

Abstract

Aim To generate a molecular phylogeny of New Caledonian Winteraceae to test the taxonomic treatments of Vink (1993), which collapsed four genera into a broadly defined *Zygogynum s.l.* and that of Guymer (2007), which maintains *Bubbia* as separate to *Zygogynum.*

Location New Caledonia, New Zealand, and Australia.

Methods A total of 82 samples representing all genera of the Winteraceae, with 73 new taxa sampled, were sequenced for nuclear ribosomal internal transcribed spacer (nrITS) and chloroplast intron *psb*A-*trn*H regions. We recovered a total of 1112 bp of aligned sequence. DNA matrices (ITS, cpDNA as well as concatenated ITS + cpDNA ML; partitioned, unlinked and linked tree BI analyses) were analysed phylogenetically using maximum likelihood (ML) and Bayesian inference (BI). ML analyses were conducted using GARLI ver. 0.951 (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006) with 1024 bootstrap replicates (BS; Felsenstein, 1985) replicates. BI analyses were performed using BEAUTI ver.1.6.1 and BEAST ver.1.6.1 (Drummond & Rambaut, 2007)

Results Both BI and ML analyses of the Winteraceae highly congruent topologies between complimentary matrices. The *psb*A-*trn*H matrix was the least informative with the ITS + *psb*A-*trn*H matrix being most informative. Our analyses support the following relationship (*Takhtajania* (*Tasmannia* (*Drimys* (*Pseudowintera* + (*Zygogynum s.l.*)))). Our phylogeny presents a hypothesis where Zealandic and South American Winteraceae share a common ancestor, as well as two well resolved clades one composed of Zygogynum and the other composed of *Z. schlechteri* sister to the three Australian *Bubbia* samples.

Main conclusions Our analyses support Vink's hypothesis of a broadly circumscribed *Zygoynum*, which includes *Bubbia*. An alternative would be to recognize a monophyletic, endemic *Zygogynum* in New Caledonia, and to transfer *Z. schlechteri* back to *Bubbia*, to retain this genus as monophyletic. However further morphological studies and a broader phylogenetic analysis of more Australian taxa are needed before final conclusions can be drawn. While most Winteraceae are apocarpous, a single strongly supported clade united by syncarpy, was observed within *Zygogynum s.l.*

Keywords: New Caledonia, Zealandia, *Zygogynum*, *Bubbia*, *Drimys, Tasmannia*, *Takhtajania*, vicariance, long distance dispersal, ITS, *psb*A-*trn*H.

Introduction

The islands of the south west Pacific Ocean share a complex geologic history driven by active tectonics within the region. Australia, New Zealand, and New Caledonia, share a single continental origin having rifted away from eastern Gondwana and subsequently each other, while others such as Samoa, Vanuatu, and Tonga are of much more recent oceanic volcanic origin. An intimate connection is shared between New Caledonia with the islands of New Zealand via the submerged Zealandic continent (Figure 1), a fragment of continental crust that rifted away from the eastern Gondwanan margin 65 Ma and establishing its present day position 50 Ma. Although connected to New Zealand, New Caledonia has remained in prolonged and distant isolation from neighbouring landmasses since its separation 65 Ma, barring a speculative continuous sub-aerial island chain along the Norfolk ridge with New Zealand (Grandcolas *et al.*, 2008). Sharma and Giribet (2009) highlight that New Caledonia has received far less attention concerning the evolution and origin of taxa compared to New Zealand and Australia, hence, much of the biota of New Caledonia has yet to be studied. Given the shared history it is important to include species from both New Zealand and Australia in biogeographic based studies on New Caledonia and vice versa.

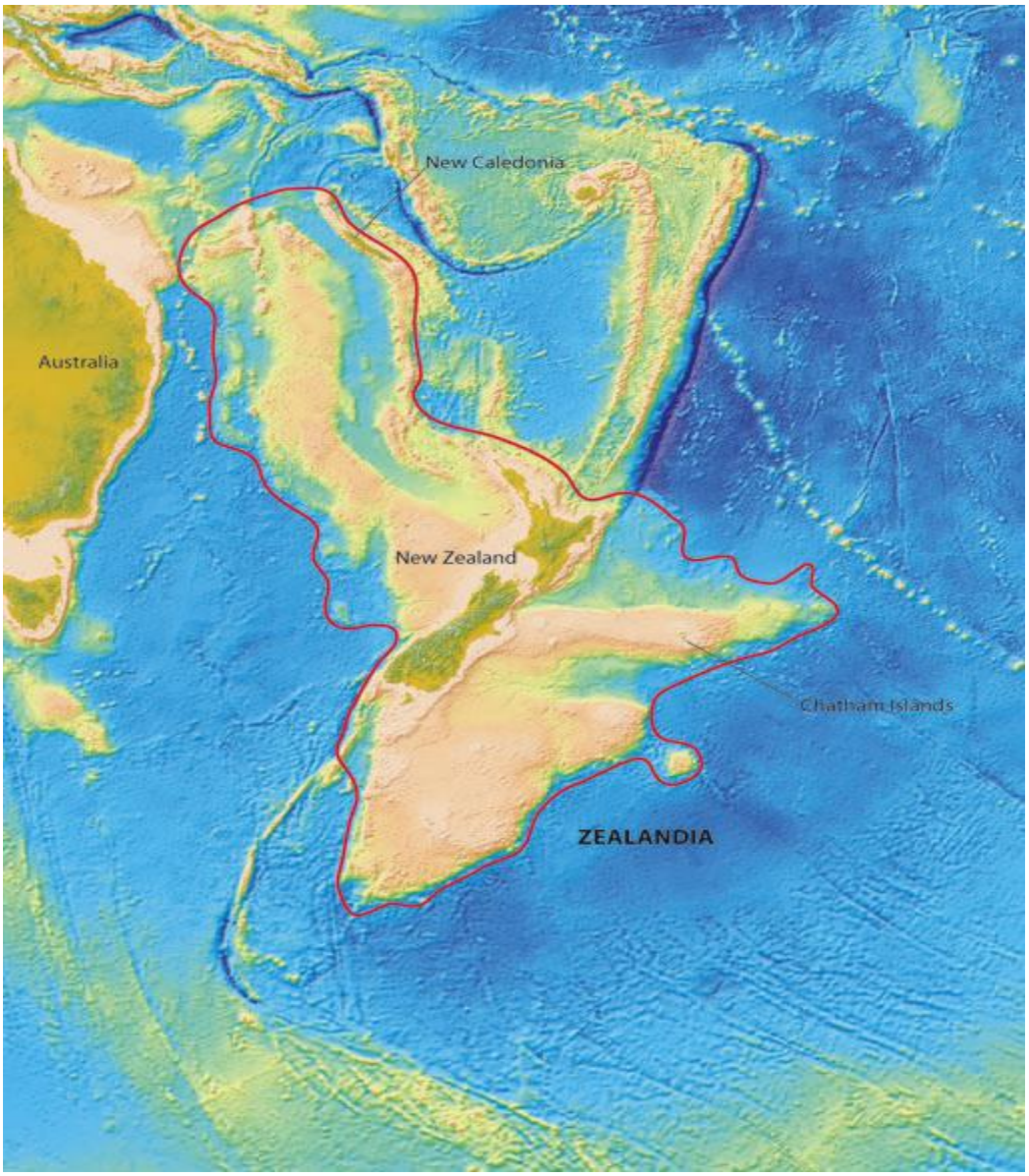

Figure 1: Map depicting the emerged and submerged extent of the continent of Zealandia with New Caledonia as its northern-most emergent landmass, New Zealand as its central emergent landmass, and New Zealand's sub-Antarctic islands representing the southern-most landmasses (Stagpoole, 2002).

During the Mid-Late Eocene (ca. 40-34 Ma) the New Caledonian ridge, including the largest island of New Caledonia, Grande Terre, collided with the adjacent Loyalty Ridge. This event resulted in the New Caledonian ridge being obducted under the crustal slab supporting the Loyalty Ridge. This obduction brought about marine transgression of the New Caledonian ridge and emplacement of ultramafic materials derived from the earth's mantle, which developed into the unique ultramafic substrates found across Grande Terre today. However, the extent of and duration of submersion of Grande Terre remains controversial and is still debated in the literature.

The first hypothesis suggests that submersion of the archipelago was not entire and/or that short-lived island refugia existed hence maintaining the original Gondwanan lineages. This vicariance-based hypothesis cites the extensive extant "primitive" basal seed-plant lineages and suggests that major diversification occurred in response to the emplacement of ultramafic substrate emplaced during the Eocene coupled with long isolation of lineages within a stable climate (Pole, 1994; Murienne *et al.,* 2005; Heads, 2008). Conversely the second hypothesis suggests total submersion of the archipelago occurred and lasted for millions of years; this hypothesis is supported by current geologic evidence and infers that the biota is the result of long-distance dispersal (Pole, 1994; Grandcolas *et al.,* 2008). This blank canvas then provided vast ecological opportunity for subsequent phyletic radiations and evolution within this unique and insular environment.

The relictual nature of the basal angiosperm family the Winteraceae make it a prime candidate for molecular phylogenetic studies as a prerequisite to any dating analyses to help elucidate the history of New Caledonia. Within this context, our study focuses on the New Caledonian members of this family,

Zygogynum Baillon (sensu Vink 1993), in conjunction with other genera from New Zealand (*Pseudowintera* Dandy), Australia (*Bubbia* Tiegh. and *Tasmannia* R.Br.), South America (*Drimys* J.R.Forst. & G. Forst), and Madagascar (*Takhtajania perrieri* M. Baranova & J.-F. Leroy), all of which share a Gondwanan origin.

The genus *Zygogynum* is the sole representative of Winteraceae currently recognised in New Caledonia, and all taxa within New Caledonia are endemic (Vink, 1993; 2003). As currently circumscribed it contains 19 species, 14 subspecies, and five varieties. It is a genus of evergreen trees or shrubs with entire leaves exhibiting characteristic penninerved lateral veins. Vegetative features within *Zygogynum* are typically uninformative in discerning between species, though flower and fruit morphology is typically distinctive and thereby informative (Figure 2). *Zygogynum* species are restricted to Grande Terre and show affinities for specific soils, predominantly of sedimentary or ultramafic origin, with only a small number of taxa found growing on multiple substrate types.

Since Baillon described the genus in 1867, *Zygogynum* has been subject to a number of revisions. Arguably the most comprehensive revision and the source of the majority of additions to the family were done by Vink (1985; 1988; 2003), where he added an additional 13 species as well as a number of subspecies and varieties. Perhaps the most radical aspect of Vink's treatment was subsuming the three other previously recognized genera *Belliolum* Tiegh., *Bubbia* Tiegh., and *Exospermum* Tiegh. into a broadly defined *Zygogynum s.l.* (Vink, 1988).

To test Vink's taxonomic hypotheses and resolve relationships among these taxa, we comprehensively sampled Zealandic Winteraceae (New Caledonian *Zygogynum*, New Zealand *Pseudowintera* and *Bubbia howeanum*, Lord Howe Island) and included Australian taxa *Bubbia* and *Tasmannia*, South American *Drimys* and Madagascan *Takhtajania perrieri* as outgroup taxa. We sampled all specimens using both nuclear ribosomal internal transcribed spacer regions (ITS) and the cpDNA intron *psb*A-*trn*H sequence variation. Of the 82 sequences for each of the ITS and cpDNA regions, 73 were newly generated in this study. This produced a robust phylogenetic assessment of the taxa that we used to evaluate species delimitations and investigate any morphological and biogeographical trends within *Zygogynum*. This unique study is the first to comprehensively sample a basal angiosperm group on New Caledonia for inferring the biogeographic history of the Flora of Zealandia.

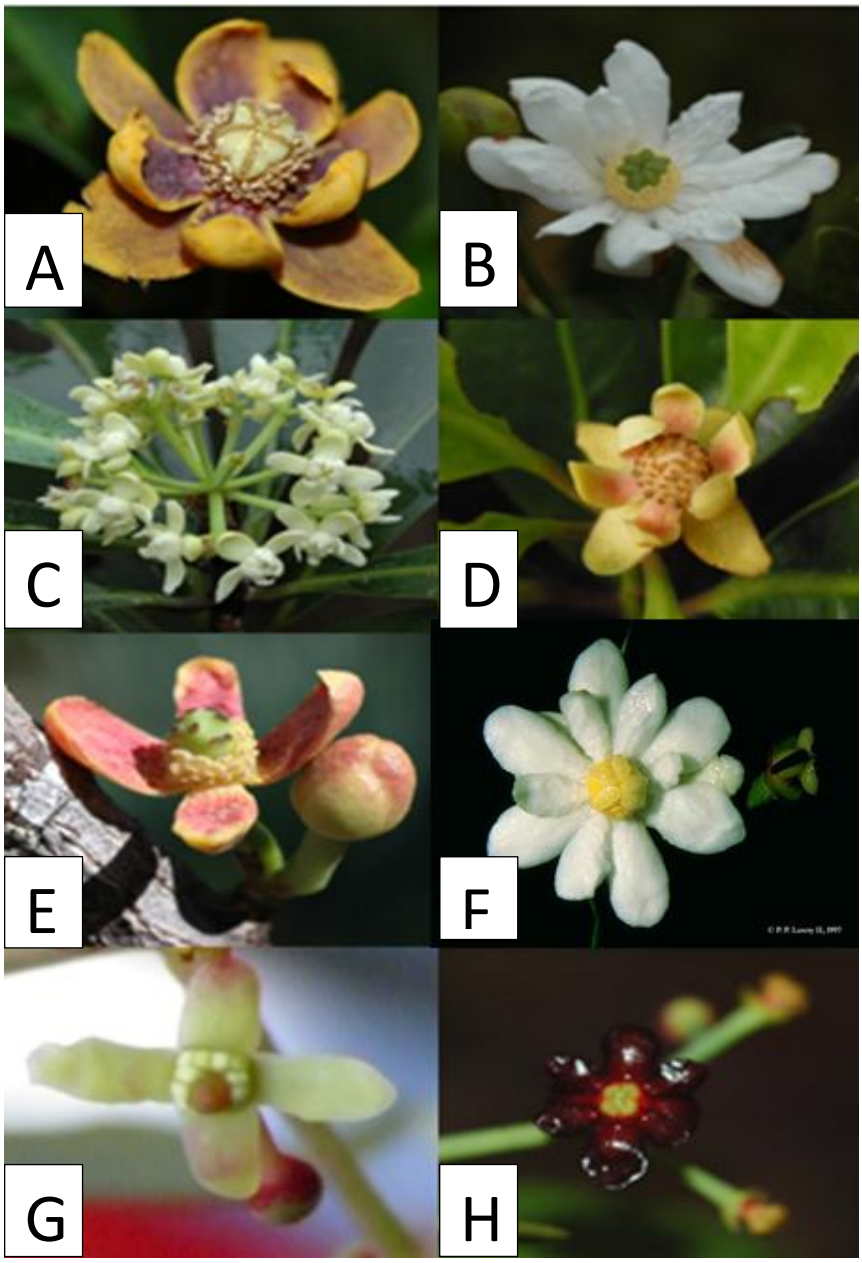

Figure 2: Variation in flower morphology, exhibiting a number of easily observable key diagnostic characters within *Zygogynum*. Some key features include extent of carpel fusion, flower colour, and fusion of petals and sepals. A) *Z. stipitatum*; B) *Z. fraterculum*; C) *Z. pancheri*; D) *Z. oligostigma*; E) *Z. viellardii*; F) *Z. cristatum* (Lowry 1997); G) *Z. schlechteri*; H) *Z. amplexicaule*. Photo credits: A, B, C, E, H by JM; photo from Lowry (1997).

Materials and methods

Taxon sampling

Field collections of *Zygogynum* were undertaken on Grande Terre, New Caledonia. Broad sampling of the genus was a priority to ensure sufficient coverage in order to validly test the current taxonomic treatment and monophyly of *Zygogynum s.l.* Where possible we included multiple samples of species that showed extensive and/or unusual geographic distributions, or with subspecific taxa. Within *Zygogynum s.l.* our samples comprises 17 of the 19 described species, with an additional eight subspecies and four varieties. Fresh leaf materials were collected in the field and dried in silica gel. Leaves were also sampled from herbarium specimens held at L'Institut de recherche pour le développement (IRD), Nouméa (NOU). Samples from outgroup species included South American *Drimys*, New Zealand *Pseudowintera*, Madagascan *Takhtajania*, and Australian *Tasmannia* as well as samples of Australian *Bubbia* (*Zygogynum* sensu Vink). The total number of new samples sequenced was 73, comprising the monotypic *Takhtajania perrieri*, three *Tasmannia*, one *Drimys*, four *Pseudowintera*, and 64 *Zygogynum*. Nine additional DNA sequences were acquired from GenBank ® and added to our matrices representing taxa from all genera except *Pseudowintera*.

Molecular methods

Total DNA was extracted from 0.2-0.3 g of silica dried and/or herbarium specimen leaf tissue using an ISOLATE Plant DNA Mini-kit (Bioline, Alexandria, NSW, Australia) according to the manufacturer's directions. Lysis was extended for dried leaf material from the manufacturer's recommendation of

30min to 3hr, which improved the yield of DNA from both field-collected silica dried materials and herbarium specimens.

Both the ITS and *psb*A-*trn*H regions were selected for this study given their successful application in previous studies of the Winteraceae (Suh *et al.,* 1993; Karol *et al.,* 2000; Doust & Drinnan, 2004; Ruiz *et al.,* 2008; Marquínez *et al.,* 2009). ITS has also performed well in resolving relationships between closely-related island species (Baldwin *et al.,* 1995; Gemmill *et al.,* 2002). Full amplification of the 18-26S nuclear ribosomal ITS region was achieved using the forward primer ITSHP5 and the reverse primer ITS4 and amplification of the *psb*A-*trn*H region was achieved using the forward primer *psb*A and the reverse primer *trn*H$^{(GUG)}$ (Table 1).

Table 1: Primers used in sequencing New Caledonian Winteraceae and outgroups.

PRIMER	SEQUENCE	AUTHOR
ITSHP5	5′-GGA AGG AGA AGT CGT AAC AAG G-3′	Gemmill *et al.*, 2002
ITS4	5′-TCC TCC GCT TAT TGA TAT GC-3′	White *et al.*, 1990
*psb*A	5′-GTT ATG CAT GAA CGT AAT GCT C-3′	Sang *et al.*, 1997
*trn*H^{GUG}	5′-CGC GCA TGG TGG ATT CAC AAT CC -3′	Tate & Simpson, 2003

PCR was performed on an Eppendorf Mastercycler® pro thermalcycler in a total reaction volume of 20 ul consisting of 12.6 ul MQH$_2$O, 0.25 µM of each primer, 1. 25× My Taq Reaction buffer (Bioline), 0.1% bovine serum albumin (BSA), 0.05U of MyTaq^{TM} polymerase, and 1.0 ul stock total genomic DNA. Amplification of the ITS region was performed as follows: initial denature of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplification of the *psb*A-*trn*H region was performed as above with an annealing temperature of 49 °C.

To verify amplification, 3ul of PCR product was mixed with 2ul loading buffer and loaded into a 1% 1× TBE agarose gel with 0.001% volume of ethidium bromide (EtBr 5mg/ml) for 50 min at 54 V and was then visualised using an Innotech Alphaimager™. A 100bp DNA ladder (Invitrogen™) was used as a size standard.

PCR products were purified of unincorporated reagents and prepared for sequencing using a standard ExoSAP protocol with 2.7ul MQH_2O, 0.2 ul Exo (ExonucleaseI; Global Science & Tech Ltd.), 0.1 ul SAP (Shrimp Alkaline Phosphate; Global Science & Tech Ltd.), and 10.0ul of PCR product. This was heated at 37 °C for 30 min then 80 °C for 15min using an Eppendorf Mastercycler® pro thermalcycler.

Cycle sequencing of PCR products used Big Dye® v3.1 terminator chemistry (Life Technologies Corporation) and were sequenced bidirectionally, using the same primers as in the initial amplification, at the University of Waikato DNA Sequencing Facility. Products were then separated using a 3130XL Genetic Analyzer System fitted with 50 cm capillary arrays (Life Technologies Corporation).

Nucleotide call confirmation and initial alignment was performed using Geneious Pro ver. 5.4.2 (Drummond *et al.*, 2010). Nine additional sequences, representing additional outgroup taxa as well as additional *Zygogynum* taxa, were sourced from NCBI GenBank®. Three matrices were generated, the first and second consisted of the full ITS matrix (Electronic appendix 1) and full *psb*A-*trn*H (Electronic appendix2), respectively, with the third being a concatenated matrix of the two genetic loci (Electronic appendix 3). Optimal alignment of all

matrices was performed using the MUSCLE alignment algorithm (Edgar 2004) as implemented in Seaview 4.0 (Galtier *et al.,* 1996; Gouy *et al.,* 2010).

Phylogenetic analyses

Maximum likelihood: jModel test 2.1.1(Guindon & Gascuel, 2003; Posada, 2008) was used to select the optimal model of evolution for Maximum Likelihood (ML) heuristic searches. ML analyses were conducted using GARLI ver. 0.951 (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006). The appropriate models of evolution selected for the ITS, *psb*A-*trn*H, and the concatenated matrices were TIM3 + G (-2482.0647) TPM1uf+G (-1302.3898) and GTR + G (-3976.544), respectively (Table 2). The best likelihood tree produced from a minimum of five initial runs was selected as the "best" tree for visualisation of bootstrap scores. Parameters used are given in Table 3; all other parameters used the default settings. 1024 bootstrap replicates (Felsenstein 1985) were performed for all matrices using GARLI to evaluate the robustness the clades produced in our ML analyses. The strict and 50% majority consensus bootstrap trees were visualised using PAUP* ver.4.0b10 (Swofford, 2002).

Bayesian inference: BEAUti ver.1.6.1 (Drummond & Rambaut, 2007) was used to create a BEAST xml input file. Within BEAUti we fixed the following parameters for all analyses: Partition – ITS, cpDNA; Sites – substitution model GTR, base frequencies estimated, site heterogeneity model, gamma with 4 categories; Clocks – relaxed molecular clock with uncorrelated lognormal distribution with fixed means: Trees – speciation: Yule process and a random

starting tree. We performed two separate analyses resulting in a total of three trees; in the first analysis we used unlinked trees (two trees) and in the second analysis we used linked trees (one tree). This allowed us to compare the individual ITS and *psb*A-*trn*H trees, and account for independent rates of evolution when producing the linked tree. The MCMC chain length was set to 50,000,000 with an echo of 5000 and a log parameter of 5000.

Bayesian analyses of the three matrices were performed using Bayesian Evolutionary Analysis Sampling Trees (BEAST) ver.1.6.1 (Drummond & Rambaut, 2007). Results of the BEAST MCMC analyses were visually inspected in Tracer v1.5 (Rambaut & Drummond, 2007) to ensure the analysis reached convergence. Upon confirmation of a successful run TreeAnnotator v1.7.4. (Drummond & Rambaut, 2007) was used to visualise posterior probabilities (PP) from the resulting sample of trees. TreeAnnotator v1.7.4. used the default setting of 10% (5000000) burn in producing a sample of 10000 trees. The Bayesian majority rule tree was visualised using PAUP* ver.4.0b10 (Swofford, 2002).

Morphological and biogeographic inference: Mapping of synapomorphies was achieved using MacClade ver. 4.0 (Maddison & Maddison, 2000). Additionally, select morphological characters and biogeographic data were investigated and mapped.

Table 2: Summary statistics of Winteraceae sequence matrices for ITS, *psb*A-*trn*H, concatenated ITS + *psb*A-*trn*H for ML analyses, and linked tree partition. Including maximum likelihood and bayesian inference results.

Region	ITS	*psb*A-*trn*H	ML ITS + *psb*A-*trn*H and BI linked partition
No. of new sequences	82	82	82
No. of sourced sequences	9	9	9
No. of aligned base pairs	645	467	1112
Max sequence length	607	410	1010
Min sequence length	591	327	931
No. of variable sites	174	80	254
No. of parsimony uninformative sites	37	38	75
No. of parsimony informative sites	137	42	179
Mean % pair wise identity	95.0%	95.1%	95.0%
Substitution model (-lnL)	TIM3 + G (-2482.0647)	TPM1uf + G (-1302.3898)	GTR + G (-3976.544)
ML best tree (-lnL)	–2449.93221	-1303.04451	-4027.71284
ML No. of resolved clades (50-74 BS)	24	6	18
ML No. of resolved clades (75-94 BS)	12	9	13
ML No. of resolved clades (>95 BS)	16	1	16
BI No. of resolved clades (0.50-0.74 PP)	3	4	5
BI No. of resolved clades (0.75-0.94 PP)	7	5	9
BI No. of resolved clades (>0.95 PP)	32	13	40

Table 3: Parameters used maximum likelihood analyses using GARLI (Zwickl, 2006) based on results of j.modeltest model selection for the full and reduced matrices. f = frequency, r = rate, pinv (I) = proportion of invariable sites, and alpha (G) = gamma distribution.

Matrix	fA	fC	fG	fT	rAC	rAG	rAT	rCG	rCT	rGT	pinv (I)	alpha (G)
ITS	0.2101	0.2516	0.3195	0.2187	0.7849	4.5169	1.2663	0.7208	10.7032	1.000	0.5100	0.4501
*psb*A-*trn*H	0.3380	0.1422	0.1520	0.3679	0.8919	0.4016	0.4158	0.4158	0.4016	1.000	0.7065	0.1750
Concatenated	0.2658	0.2053	0.2506	0.2783	0.9400	1.6429	0.7723	0.5561	3.8535	1.000	0.6270	0.2612

Results

In our analyses we recovered 645bp (max.607; min. 591), 467bp (max. 410; min. 327), and 1112bp (max.1010; min. 931) for the ITS, *psb*A-*trn*H, and ITS + *psb*A-*trn*H matrices respectively (Table 2). The ITS and *psb*A-*trn*H matrices contained 137 and 42 parsimony informative sites respectively (Table 2). The mean pair wise identity for all matrices ranged from 95%-95.1% (Table 2).

The Maximum Likelihood (ML) analyses of 82 taxa for ITS, *psb*A-*trn*H, and ITS + *psb*A-*trn*H (concatenated) datasets produced strongly supported outgroup clades from *Zygogynum s.l.*, as well as support for monophyly in a number of species Table 2). All trees were rooted using the monotypic *Takhtajania perrieri* Schatz. with remaining Winteraceae genera (*Drimys, Pseudowintera,* and *Tasmannia*) forming successive outgroup sister taxa to *Zygogynum.* Both ML ITS (appendix 1c) and ITS + *psb*A-*trn*H (Figure 3) trees were highly congruent with the topology of (*Takhtajania* (*Tasmannia* (*Drimys* (*Pseudowintera* + *Zygogynum s.l.*)))). The *psb*A-*trn*H tree (appendix 1d) was congruent with the other trees with respect to placement and position of *Takhtajania, Tasmannia,* and *Drimys,* though failed to further resolve the *Zygogynum* and sister *Pseudowintera*.

The Bayesian inference (BI) analyses of ITS + *psb*A-*trn*H matrices with unlinked trees and the partitioned ITS and *psb*A-*trn*H matrices with trees linked produced well resolved trees. The Winteraceae genera (*Drimys, Pseudowintera,* and *Tasmannia*) were resolved as monophyletic forming outgroup sister taxa to *Zygogynum s.l.* Both BI ITS unlinked tree (appendix 1a) and ITS + *psb*A-*trn*H linked tree (Figure 4) were highly congruent with (*Takhtajania* (*Tasmannia* (*Drimys* (*Pseudowintera* + *Zygogynum s.l.*)))). The *psb*A-*trn*H unlinked tree

(appendix 1b) was congruent with the other trees with respect to placement and position of *Takhtajania, Tasmannia,* and *Drimys,* though failed to further resolve *Zygogynum s.l.* and *Pseudowintera*.

A similar trend in both ML and BI analyses was that the *psb*A-*trn*H and ITS trees alone produced inferior resolution in comparison to the ML ITS + *psb*A-*trn*H (concatenated) tree and BI partitioned linked tree. In light of this results mentioned within the remainder of this section and within the discussion will refer to relationships and support found in the ML ITS + *psb*A-*trn*H (concatenated) tree and BI partitioned linked tree. When visualising synapomorphies the ITS matrix produced a total of 58 synapomorphies (Figure 7) and the *psb*A-*trn*H matrix produced a total of 13 synapomorphies (Figure 8).

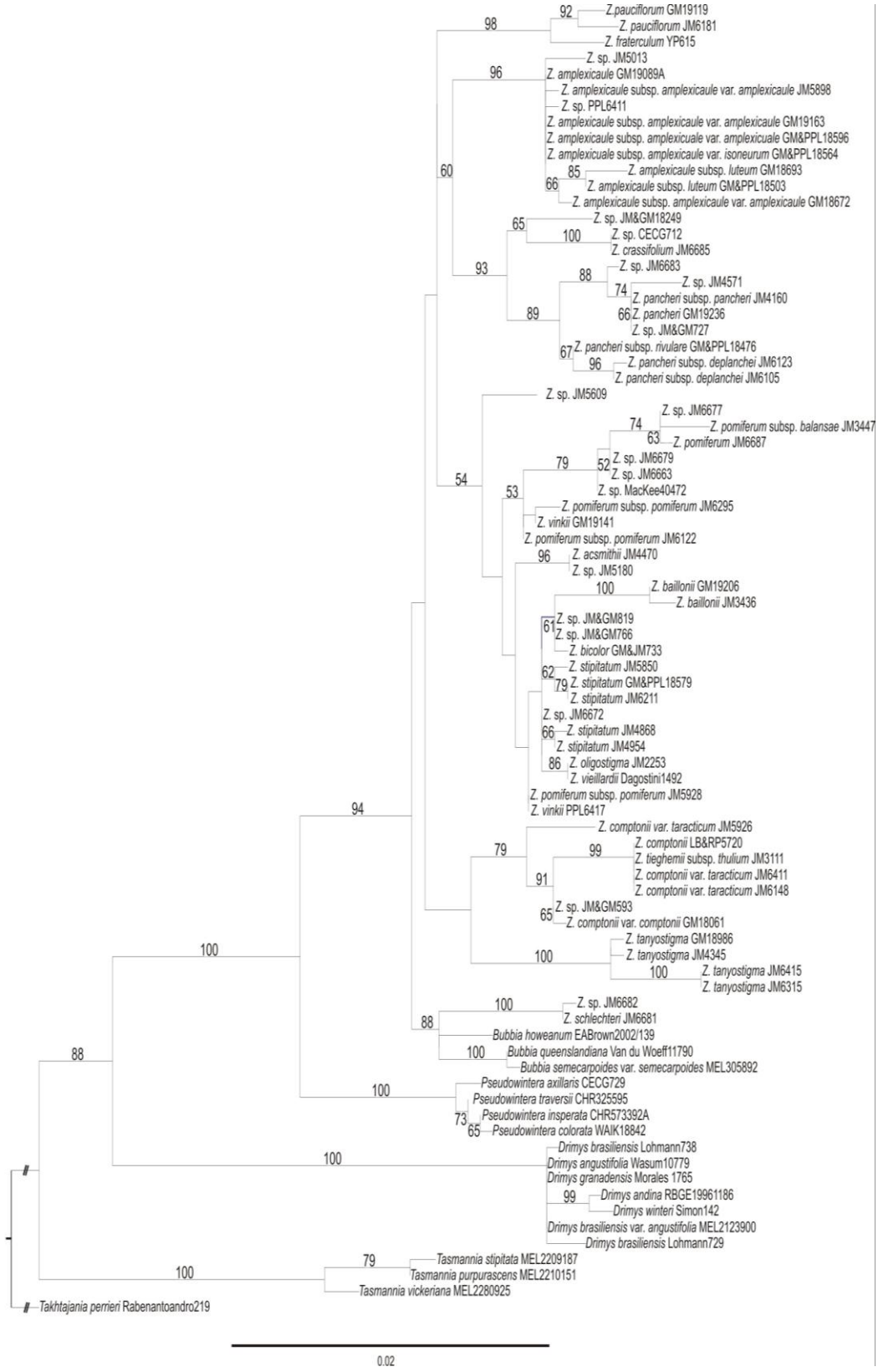

Figure 3: Maximum likelihood tree of the Winteraceae based on concatenated alignment of ITS + *psb*A-*trn*H loci. Bootstrap support values greater than 50% are indicated above branches.

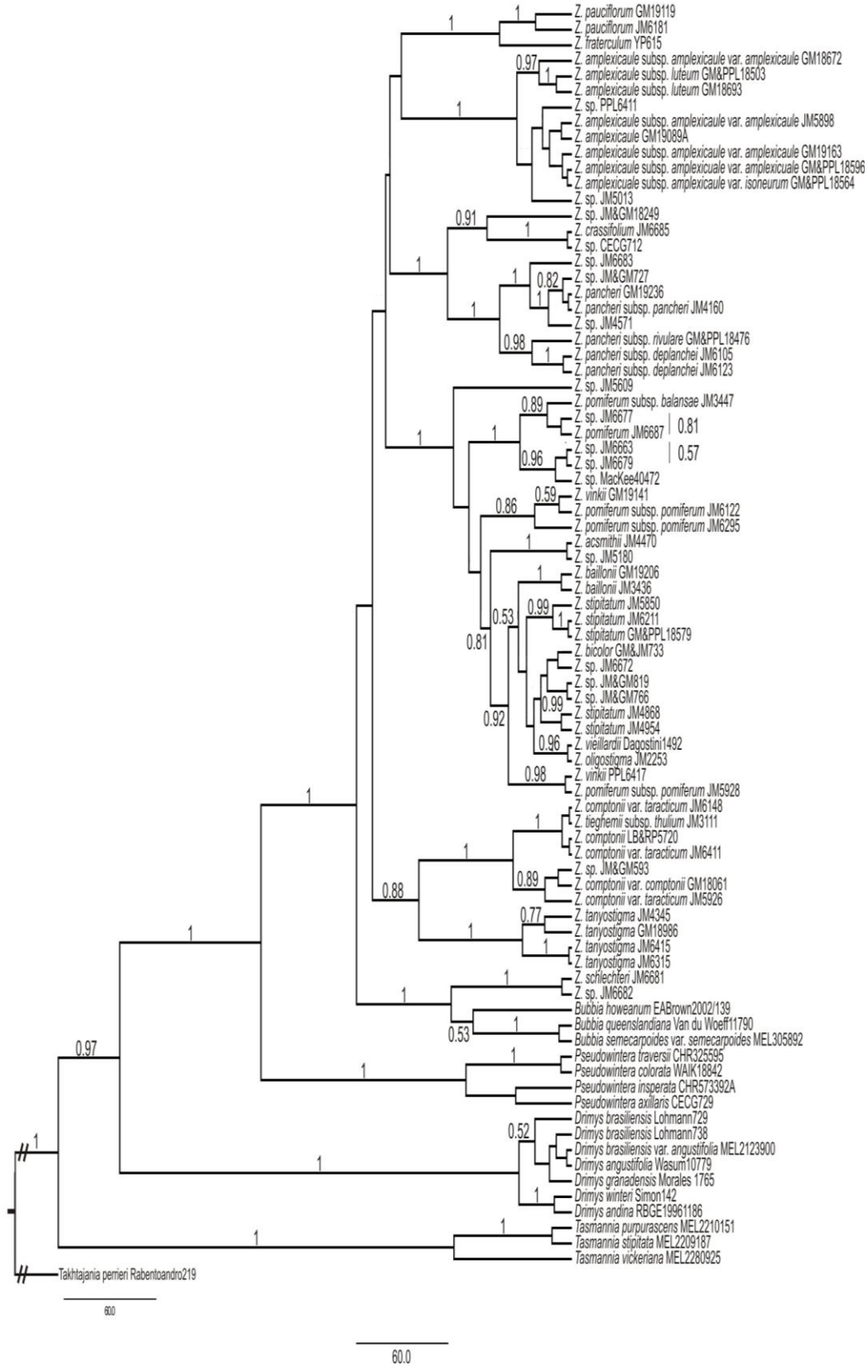

Figure 4**:** Bayesian majority rule consensus probability tree for the partitioned ITS + *psb*A-*trn*H with linked tree analysis of the Winteraceae. Posterior probabilities values (PP) above 0.50 are indicated on branches preceding nodes.

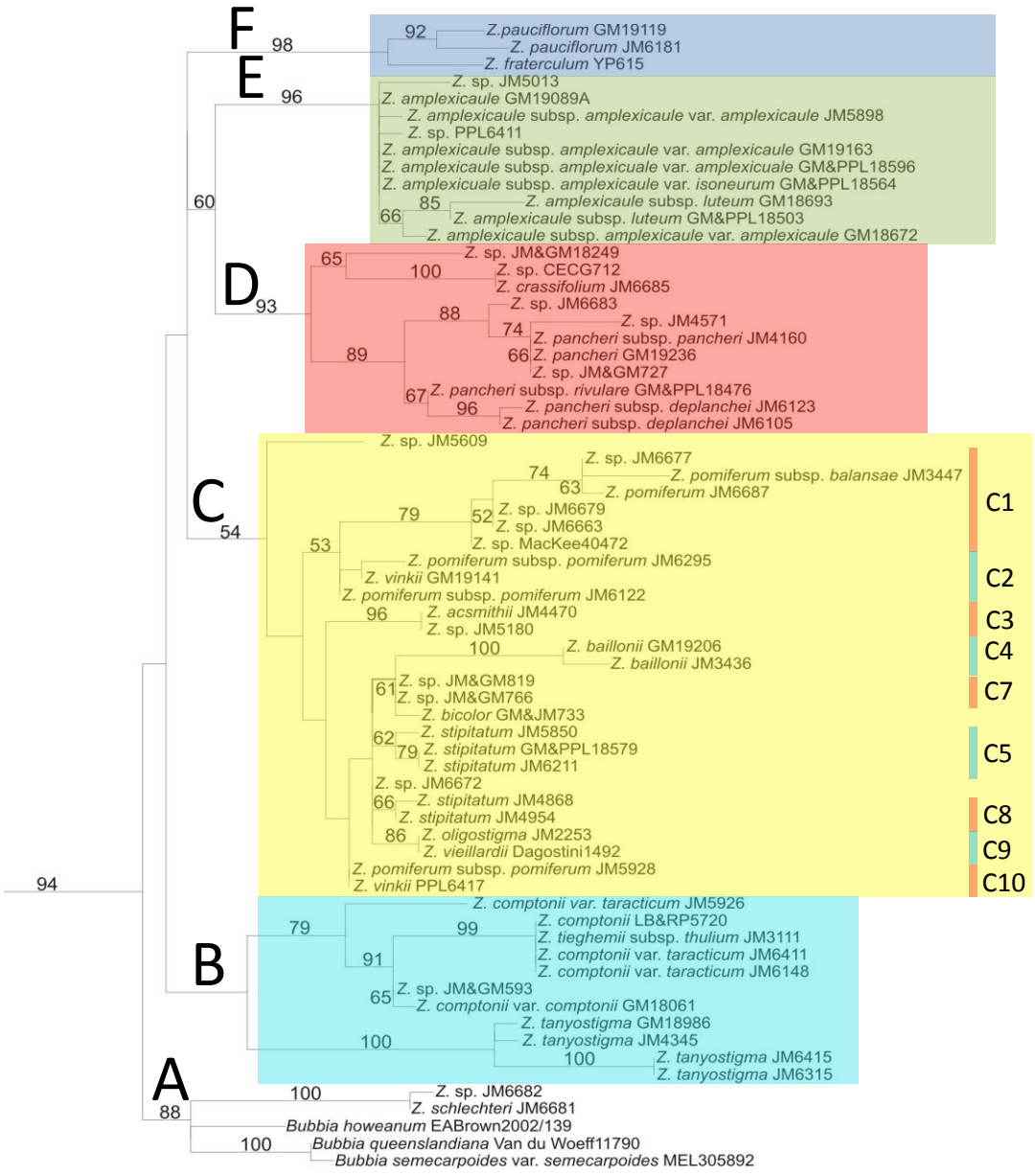

Figure 5: Enlarged portion representing *Zygogynum s.l.* from the maximum likelihood tree f the Winteraceae based on concatenated alignment of ITS + *psb*A-*trn*H loci. Bootstrap support values greater than 50% are indicated on above branches. Clades are indicated by letter and highlighted in colour. Clade C is further differentiated into subclades referred to in text.

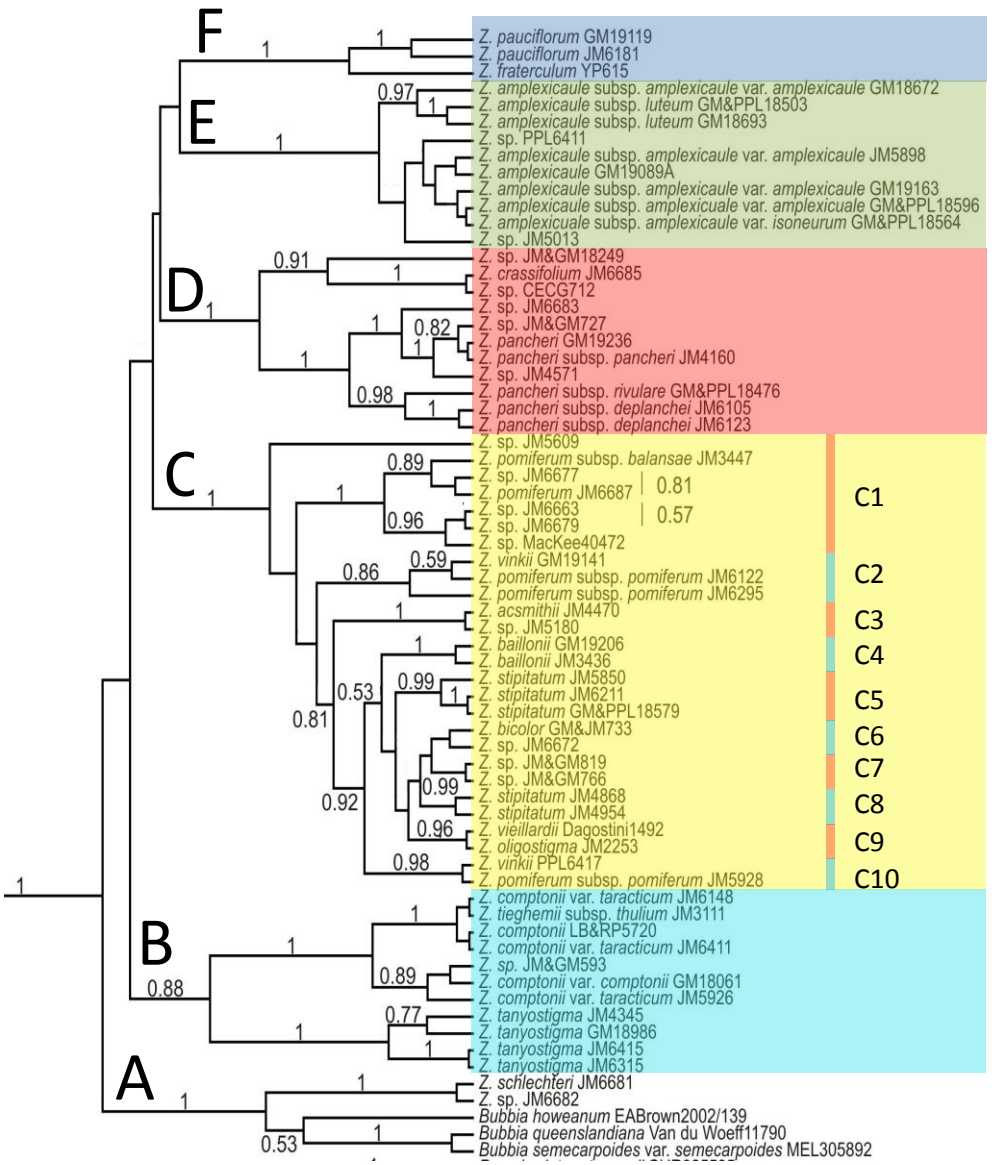

Figure 6: Enlarged portion representing *Zygogynum s.l.* from the Bayesian majority rule probability tree for linked ITS + *psb*A-*trn*H analysis of the Winteraceae. Posterior probabilities values (PP) above 0.50 are indicated on branches preceding nodes. Clades are indicated by letter and highlighted in colour. Clade C is further differentiated into subclades referred to in text.

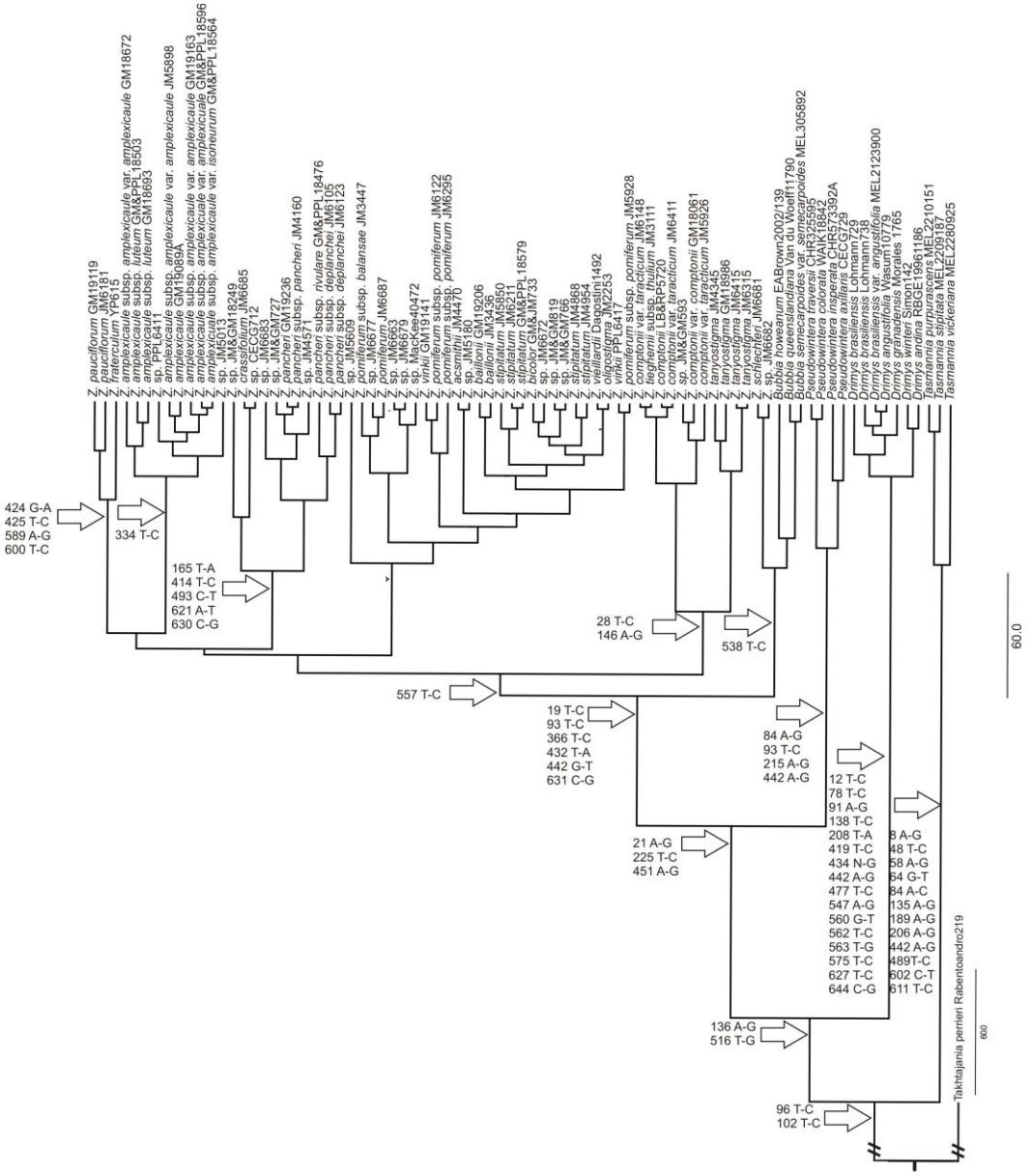

Figure 7: Maximum likelihood best tree tree of the Winteraceae based on concatenated alignment of ITS + *psb*A-*trn*H loci indicating ITS sequence synapomorphys. Synapomorphys are not mapped beyond the level indicated as clades A-F (Figures 4 and 5).

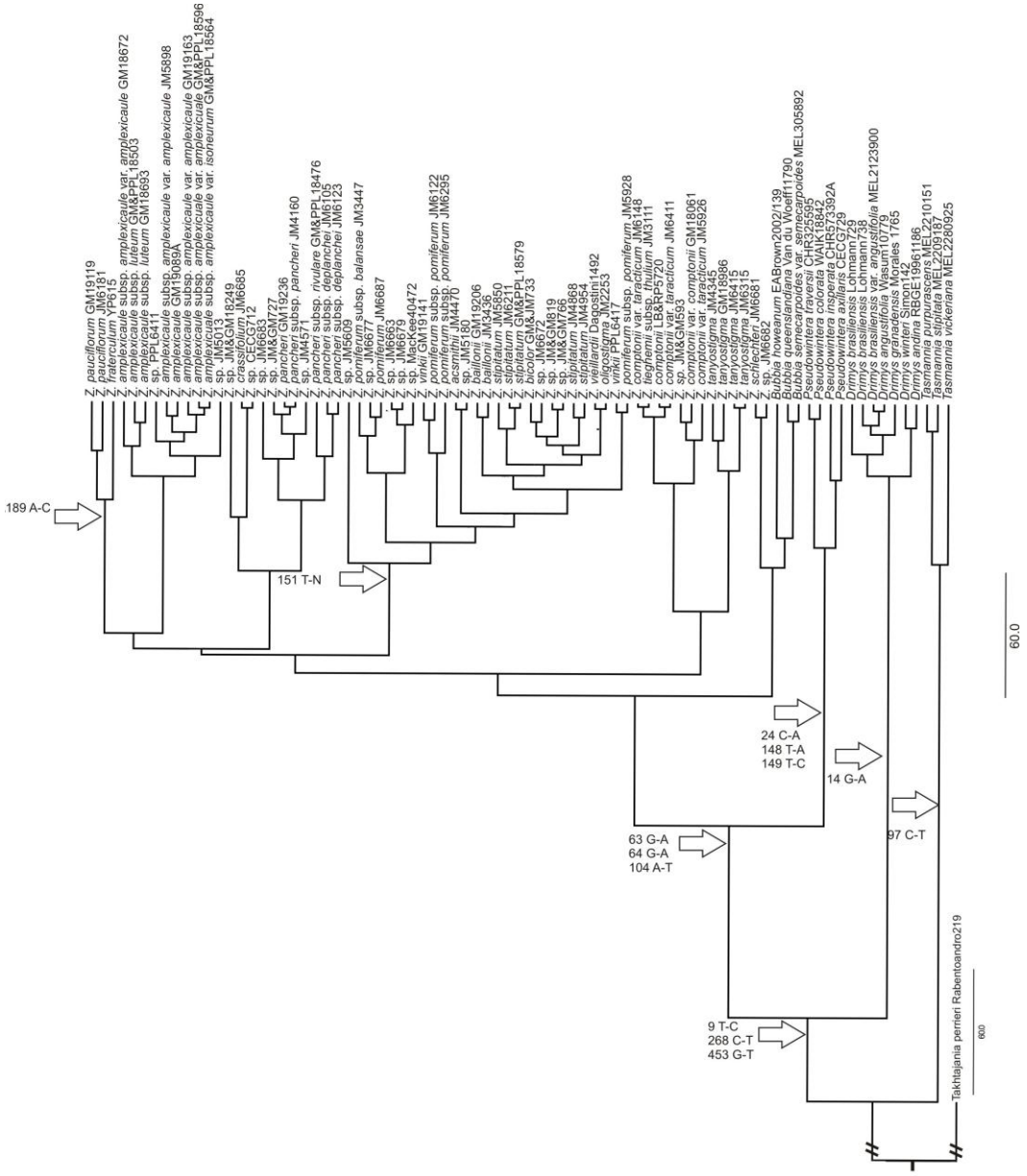

Figure 8: Maximum likelihood best tree tree of the Winteraceae based on concatenated alignment of ITS + *psb*A-*trn*H loci indicating *psb*A-*trn*H sequence synapomorphys. Synapomorphys are not mapped beyond the level indicated as clades A-F (Figures 4 and 5).

Both the ML and BI analyses of the concatenated and partitioned ITS+ *psb*A-*trn*H data matrices provided strong support for the generic relationship of within the Winteraceae (Figure 3 and 4). *Takhtajania* is situated as sister to all other taxa based on previous analyses (Karol *et al.*, 2000; Marquínez *et al.*, 2009). *Tasmannia* form a strongly supported clade (100 BS, 1.0 PP, 13 synapomorphies) and situated as sister to remaining taxa (5 synapomorphies). *Drimys* form a strongly supported clade (100 BS, 1.0 PP, 17 synapomorphies) and is strongly supported (88 BS, 0.97 PP, 6 synapomorphies) as sister to *Pseudowintera* and *Zygogynum s.l*. *Pseudowintera* is strongly supported as sister to *Zygogynum s.l.* (100 BS, 1.0 PP, 7 synapomorphies). Within *Pseudowintera* there is conflicting topologies for the ML and BI analyses. In the MI analysis we find the moderately supported (73 BS, 65 BS) topology (*P. axillaris* (*P. traversii* (*P. insperata, P. colorata*))), alternately in the BI analysis we find the strongly supported (1.0 PP) topology ((*P. insperata, P. axillaris*) (*P. traversii*, *P. colorata*)).The remaining taxa form a large *Zygogynum s.l.* clade, though the backbone of this clade is largely unresolved.

The *Zygogynum* taxa form a large poorly resolved clade. Within this large clade there are six distinct clades which are labelled A, B, C, D, E, and F (Figures 3 and 4). Clade A is strongly supported (88 BS, 1.0 PP, 1 synapomorphy) consisting of the New Caledonian *Z. schlechteri* and an undetermined specimen along with Australian *Bubbia semecarpoides, B. queenslandiana*, and *B. howeanum.* Within this clade *Z. schlechteri* form a strongly supported clade (100 BS, 1.0 PP) is strongly supported (88 BS, 1.0 PP) as sister to Australian *Bubbia* taxa. *B. semecarpoides* and *B. queenslandiana* also form strongly supported (100 BS, 1.0 PP) clade distinct from *B. howeanum*. Clade B represents a large strongly

supported (0.88 PP) clade of *Z. comptonii* and *Z. tanyostigma* with a single *Z. tieghemii* subsp. *thulium* and single undetermined specimen present. Within clade B there are two distinct subclades. The first moderate to strongly supported (79 BS, 1.0 PP) subclade consists of *Z. comptonii* with the single *Z. tieghemii* subsp. *thulium*, within this clade there is moderate-strong support for the distinction of *Z. comptonii* var. *comptonii* from *Z. comptonii* var. *taracticum*. The second subclade is a strongly supported (100 BS, 1.0 PP) monophyletic clade of *Z. tanyostigma*, within which there is clear support for differentiation of two distinct clades of *Z. tanyostigma.* Clade C is poorly supported (51 BS, 1 synapomorphy) within the ML analysis and strongly supported (1.0 PP, 1 synapomorphy) within the BI analysis; furthermore the backbone of this clade is resolved in the BI analysis only. Clade C consists of a number of small, moderately to well supported clades which have been split into subclades C1-C10 (Figures 5 and 6) for the purposes of future discussion. Clade C1is moderate-strongly supported (79 BS, 1.0 PP) consisting of a two specimens determined as *Z. pomiferum* subsp. *balansae* and *Z. pomiferum* with an additional five undetermined specimens. C2 is strongly supported within the BI analysis (0.86 PP) and consists of *Z. pomiferum* subsp. *pomiferum* with a single *Z. vinkii.* C3 is strongly supported (96 BS, 1.0 PP) consisting of *Z. acsmithii* and a single undetermined specimen. C4 is a strongly supported (100 BS, 1.0 PP) monophyletic clade of *Z. baillonii*. C5 is a strongly supported (62 BS, 0.99 PP) clade consisting of three specimens of *Z. stipitatum.* C6 is an unsupported clade of *Z. bicolor* and an undetermined specimen which is evident only in the BI analysis.C7 is a strongly supported (0.99 PP) clade consisting of two undetermined specimens. C8 is a poorly-strongly supported (66 BS, 0.96 PP) clade consisting of two specimens of *Z. stipitatum.* C9 is a strongly

supported (86 BS, 1.0 PP) clade consisting of two taxa representing *Z. vieillardi* and *Z. oligostigma.* C10 is a strongly supported (0.96 PP) clade consisting of *Z. vinkii* and *Z. pomiferum* subsp. *pomiferum.* Outside of these nine clades within clade C there are additional species whose position and relation to other taxa is unresolved. Within the ML analysis there is poor support for (60 BS) for clades D and E being sister taxa. Clade D is a large strongly supported (93 BS, 1.0 PP, 5 synapomorphies) clade consisting largely of *Z. pancheri* with a single *Z. crassifolium* and five undetermined specimens. Within clade D there is support distinction and monophyly of *Z. pancheri* subsp. *pancheri*, subsp. *rivulare*, and subsp. *deplanchei* from each other. Clade E is a strongly supported (96 BS, 1.0 PP, 1 synapomorphy) clade consisting of *Z. amplexicaule* and two undetermined specimens. Within this clade *Z. amplexicaule* subsp. *amplexicaule* is poorly defined and *Z. amplexicaule* subsp. *luteum* forms a strongly supported (85 BS, 1.0 PP) clade. Clade F is strongly supported (98 BS, 1.0 PP, 5 synapomorphies) consisting of *Z. pauciflorum* and *Z. fraterculum*, with the two *Z. pauciflorum* forming a strongly supported (92 BS, 1.0 PP) clade.

Discussion

The use of two distinct analyses, ML analysis of the concatenated alignment (ITS + *psb*A-*trn*H; Figure 3) and BI analysis of the partitioned matrices with linked trees (ITS, *psb*A-*trn*H; Figure 4), allowed us to assess the strength of resolved relationships. The ML analysis used a single model of evolution for the entire ITS + *psb*A-*trn*H data matrix while the BI analysis employed independent models of evolution for the partitioned ITS and *psb*A-*trn*H matrices with linked trees to produce a single consensus majority rule tree. Despite these differences, both analyses produced highly congruent tree topologies providing increased confidence in our results. Similarly the ML analyses of each ITS and *psb*A-*trn*H data matrices and the BI partitioned unlinked tree analyses generated trees with congruent topologies (Appendix 1a-1d).

The higher order generic relationships resulting from our ML and BI analyses are congruent with those found in previous studies by Suh *et al.* (1993), Karol *et al.* (2000), Doust & Drinnan (2004), Ruiz *et al.* (2008), Marquínez *et al.* (2009), and Zimmer *et al.* (2012). The earliest phylogenetic investigation of the Winteraceae using DNA sequence data was performed by Suh *et al.* (1993) in which the eleven specimens, representing *Drimys* (includes two sect. *Tasmannia*), *Pseudowintera,* and *Zygogynum* were analysed. Suh *et al.* (1993) recovered a phylogeny where *Tasmannia* was sister to all other taxa, with *Drimys* sister to *Pseudowintera*, and *Pseudowintera* subsequently sister to *Zygogynum*. Subsequently Karol *et al.* (2000) investigated the phylogenetic position of *Takhtajania perrieri* within the Winteraceae using phylogenetic analysis of the ITS and *trn*L-*trn*F genetic loci. In this Karol *et al.* (2000) recovered *Takhtajania perrieri* as basal within the Winteraceae and sister to all other genera. Doust &

Drinnan (2004) later investigated the evolutionary relationship between *Tasmannia* and *Drimys* using phylogenetic analysis of the ITS region, incorporating additional sequences of ITS and *trn*L-*trn*F from the earlier study of Karol *et al.* (2000). In this study Doust & Drinnan (2004) confirmed earlier conclusions suggesting that *Tasmannia* and *Drimys* are separate monophyletic groups. Further investigation by Doust & Drinnan (2004) of floral morphology showed that, despite being genetically distinct groups, they share a number of inflorescence and floral morphological characteristics. Ruiz *et al.* (2008) investigated the phylogenetic relationships among Chilean *Drimys* using the ITS genetic loci. The results of this study resolved the generic relationship and tree topology of (*Takhtajania* (*Tasmannia* (*Drimys* (*Pseudowintera* + *Zygogynum*). Marquínez *et al.* (2009) further investigated generic relationships within the Winteraceae using phylogenetic analysis of the ITS, *psb*A-*trn*H, and *rp*S16 genetic loci. In this study Marquínez *et al.* (2009) recovered the same relationships as in previous studies. Additionally, Marquínez *et al.* (2009), using limited samples, assessed the monophyly of *Zygogynum s.l.* with respect to the position of *Belliolum*, *Bubbia*, *Exospermum*, and *Zygogynum s.s.* Marquínez *et al.* (2009) recovered support for the monophyly of *Zygogynum s.l.* in agreement with Vink's revisions. The most recent investigation including Winteraceae was performed by Zimmer *et al.* (2012) where the placement of the recently described taxon *Pleodendron* Tiegh. within the Canellaceae was assessed using phylogenetic analysis of the ITS, 18S, *rbc*L, *atp*B, and *trn*LF genetic loci. Here the Winteraceae, used as outgroup taxa, exhibit the same generic relationships, though this is with limited sampling of the Winteraceae given a focus on the Canellaceae. A point of concern with the studies performed after Suh *et al.* (1993)

is that all subsequent studies rely heavily on sequencing performed in Suh *et al.* (1993) adding few new sequences to their investigations. Only Marquínez *et al.* (2009) performed an investigation significantly independent from previous studies. The concern here is that by relying so heavily on large portions of previously published data is that highly similar topologies are likely the result of using the same data and therefore much of the original research is not robustly challenged. Another point of concern is the now out-dated sequencing technology used in Suh *et al.* (1993) whose sequences are being used as recently as the investigation by Ruiz *et al.* (2008) and Zimmer *et al.* (2012) more than 15 years later. Exemplifying this is Ruiz *et al.* (2008) in which only five new sequences are used in a matrix including 12 sequences from Suh *et al.* (1993), six from Karol *et al.* (2000) and five from Doust & Drinnan (2004).

Both our ML and BI analyses, with subsequent mapping of synapomophies (Figures 7 and 8), support the relationship of (*Takhtajania* (*Tasmannia* (*Drimys* (*Pseudowintera* + *Zygogynum s.l.*)))), as well as the monophyly of all outgroup genera, on trees rooted with *Takhtajania* (Figures 3 and 4). This is in agreement with previous work performed on the Winteraceae (Karol *et al.* (2000), Doust & Drinnan (2004), Marquínez *et al.* (2009), and Zimmer *et al.* (2012). Resolution among species within genera was achieved for both ML and BI with strong support in *Tasmannia, Drimys,* and *Pseudowintera,* though within *Drimys* a number of taxa remain unresolved. Resolution among species within *Zygogynum s.l.* was achieved in both ML and BI analyses; however some relationships remain unresolved (Figures 5 and 6).

With this research we aimed to investigate further than generic relationships within the family instead evaluating the current taxonomic hypothesis

circumscribing *Zygogynum s.l.* (sensu Vink), as suggested in Marquínez *et al.* (2009).

Our analyses exceeded those previously done in Marquínez *et al.* (2009) with regards to *Pseudowintera*. Here we have included samples representing all *Pseudowintera* species recognised in New Zealand. Our ML analysis indicates a well-supported division within the genus, with *P. colorata* and *P. insperata* distinct from *P. axillaris* and *P. traversii.* The alternate topology indicated from the BI analysis, with *P. colorata* and *P. traversii* separate from *P. axillaris* and *P. insperata,* is in sharp contrast to that of the ML analysis suggesting that our limited sample size is insufficient for recovering a robust and congruent topology for this group. The analyses do indicate that *P. axillaris* and *P. colorata* are distinct from one another, as expected from their distinct morphology and distribution. We also find similar distinction between *P. insperata* and *P. traversii*.

Zygogynum s.l.

In testing Vink's hypothesis, analyses of Marquínez *et al.* (2009) did not support the monophyly of previously recognised generic segregates *Bubbia, Exospermum,* and *Belliolum* within *Zygogynum s.l.* in agreement with Vink's (1988) treatment, though this is study included a limited sample of *Zygogynum s.l.* In contrast our ML and BI analyses give strong support (Figures 5 and 6) to the clade comprising New Caledonian *Z. schlechteri* (syn. *Bubbia schlechteri*) with Australian *B. howeanum, B. queenslandiana,* and *B. semecarpoides* as distinct from and sister to a large clade comprising all remaining *Zygogynum* taxa. One view would be to maintain Vink's broad circumscription of the genus. This would render *Zygogynum* as indigenous to New Caledonia and Australia. An alternate

view would be to treat the Z. schlechteri clade as a distinct genus *Bubbia*, thereby making *Bubbia* native to New Caledonia, Lord Howe Island, and Australia; we need further information before we can make a formal recommendation. Our analyses provide no support for the re-establishment of *Exospermum* (now *Z. stipitatum*) given that it is nested within a largely unresolved clade C. Alternately; *Belliolum* (now *Z. pauciflorum*) forms a monophyletic clade F with *Z. fraterculum.* If *Z. fraterculum* was revised to *Belliolum,* clade F could potentially represent a monophyletic *Belliolum*. Though without further investigation into the morphology of these taxa this is purely speculative.

Both analyses resolved both generic and specific relationships within the family, in some cases resolving sub-specific relationships. We find strong support for the monophyly and distinctness of a number of *Zygogynum* species based on BS and PP support (Figures 5 and 6), as well as comparative branch lengths from our ML analyses (Figure 5). These include *Z. fraterculum, Z. pauciflorum, Z. tanyostigma, Z. amplexicaule, Z. baillonii, Z. acsmithii, Z. bicolor,* and *Z. pancheri.* Within *Z. amplexicaule, Z. amplexicaule* subsp. *luteum* is well supported by both ML and BI analyses as distinct from *Z. amplexicaule* subsp. *amplexicaule,* though there is no support for distinction between varieties of subsp. *amplexicaule.* Similarly there is support for distinction between subspecies of *Z. pancheri,* though further investigation of this species is required given our analysis only include three of the five subspecies. Within *Z. comptonii* there is little support for the distinction of varieties within the species.

Within clade C a number of species remain largely unresolved likely requiring further investigation through the addition of more samples for molecular analyses and morphological study. These include *Z. pomiferum, Z. vinkii,* and *Z.*

stipitatum. Z. pomiferum subsp. *pomiferum* appears to be closely linked to *Z. vinkii* forming two mixed clades within our analyses. Both species share similar morphological characters and can be hard to distinguish depending on whether flowers or fruits are present. Given its proximity to a number of other *Z. pomiferum* it is likely that the identification of GM19141 (*Z. vinkii*) within clade C2 has been a mistakenly identified. Similarly this scenario of misidentification may explain the pairing of *Z. vinkii* and *Z. pomiferum* subsp. *pomiferum* in clade C10, given its isolation from the major *Z. pomiferum* clades of C1 and C2. Further investigation and re-examination of these specimens will be required, as they may result in both *Z. pomiferum* subspecies and *Z. vinkii* forming monophyletic clades. *Zygogynum stipitatum* forms two distinct clades (Figure 3 and 4), C5 (62 BS, 0.99 PP) and C8 (66 BS, 0.99 PP), which appear correlated to geographic locality. Expanding on this we see that clade C5 consists of samples from the north-eastern margin of Grande Terre while the remaining clade C8 consists of samples located centrally on Grande Terre. This scenario is different to that posed by the *pomiferum/vinkii* scenario in that the species involved are morphologically very distinct, additionally specimens were collected either in flower, fruit, or both making their identification harder to question.

Z. crassifolium is of concern given its positioning nested within the two distinct well supported clades of *Z. comptonii* and *Z. pancheri.* Its morphology is a close match to both of the species it is nested with. To further investigate this issue both voucher specimens will need to be checked to ensure accurate identification and additional *Z. crassifolium* specimens should be included in subsequent analyses.

Our phylogenetic analysis sheds light on the evolution and biogeographic history of the Winteraceae with particular reference to *Zygogynum s.l.* (Figure 9). Further investigation of the biogeography of the Winteraceae given our ML and BI topologies suggests that Zealandic Winteraceae share their common ancestor with South American *Drimys*, reflecting the Gondwanic roots of this family. This hypothesis also suggests that Zealandia was important in the evolution of the New Zealand and New Caledonian. There are only four species of *Pseudowintera* extant in New Zealand, in contrast to the 19 species in New Caledonia; this high rate of phyletic radiation seems to be common among New Caledonian plant lineages (Morat *et al.* 2012). Additionally, this hypothesis also suggests that the Zealandic Lord Howe Island species and subsequently the continental Australian species of *Bubbia sensu* Guymer, are the result of a dispersal event from New Caledonia.

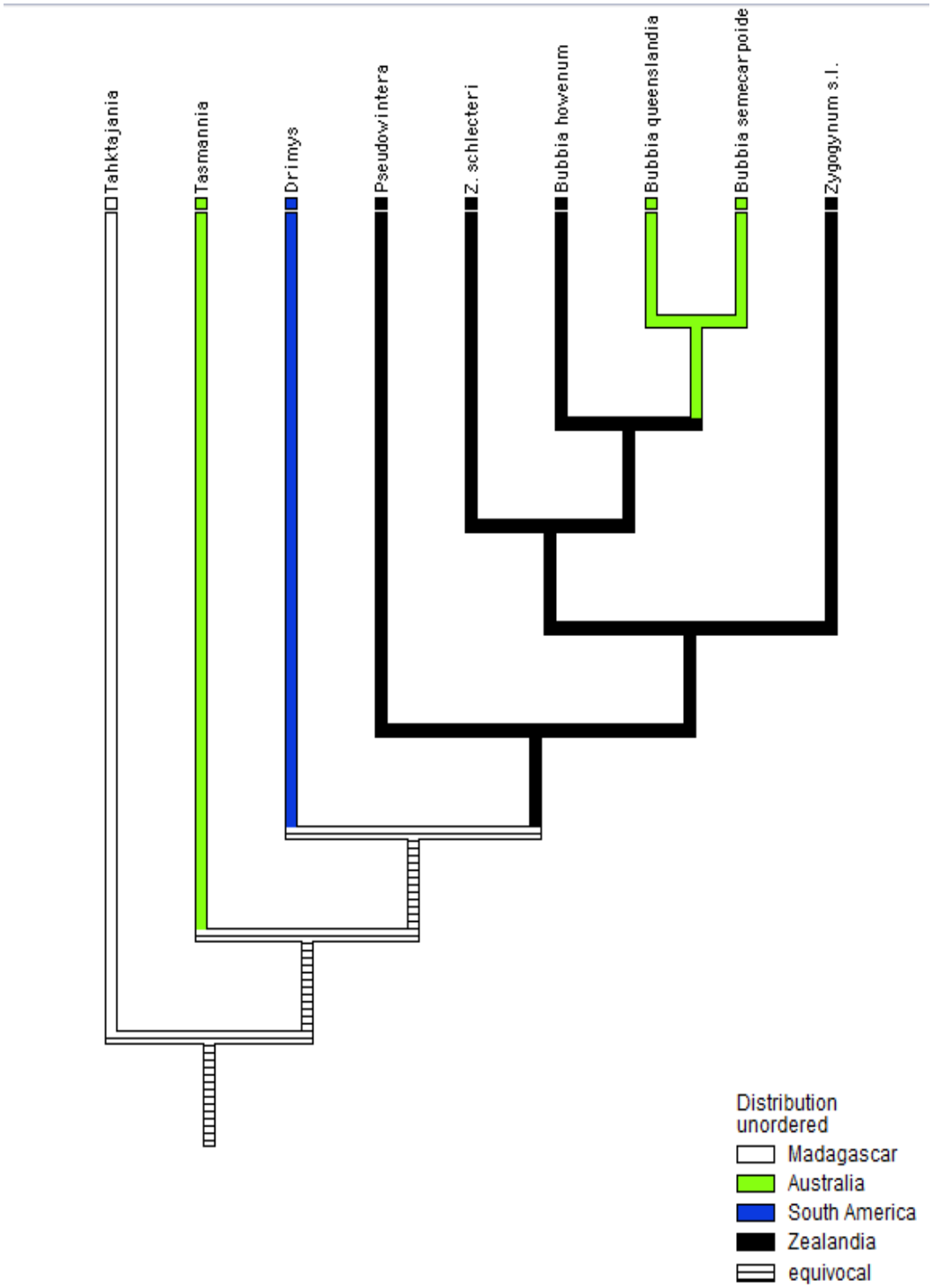

Figure 9: Biogeographic hypothesis of members of the Winteraceae based on tree topologies produced BI and ML analyses. Colours indicate distribution of extant species.

Within the large unresolved, indicated as clades B-F (Figures 5 and 6), clade of *Zygogynum* we see a morphological trend of carpel evolution (Figures 10 and 11). Clades B, D, E, and F all exhibit apocarpous gynoecia, whereas clade C exhibit the derived character state of having syncarpous gynoecia. This is an evolutionary trend that has been suggested as a repeating feature of angiosperm macroevolution (Armbruster *et al.*, 2002), with syncarpous gynoecia representing a major adaptive advantage over apocarpous gynoecia (Endress, 1982; Armbruster *et al.*, 2002). Though the significance of this trend within *Zygogynum* requires further investigation, including investigation into the degree and development of syncarpy within the genus.

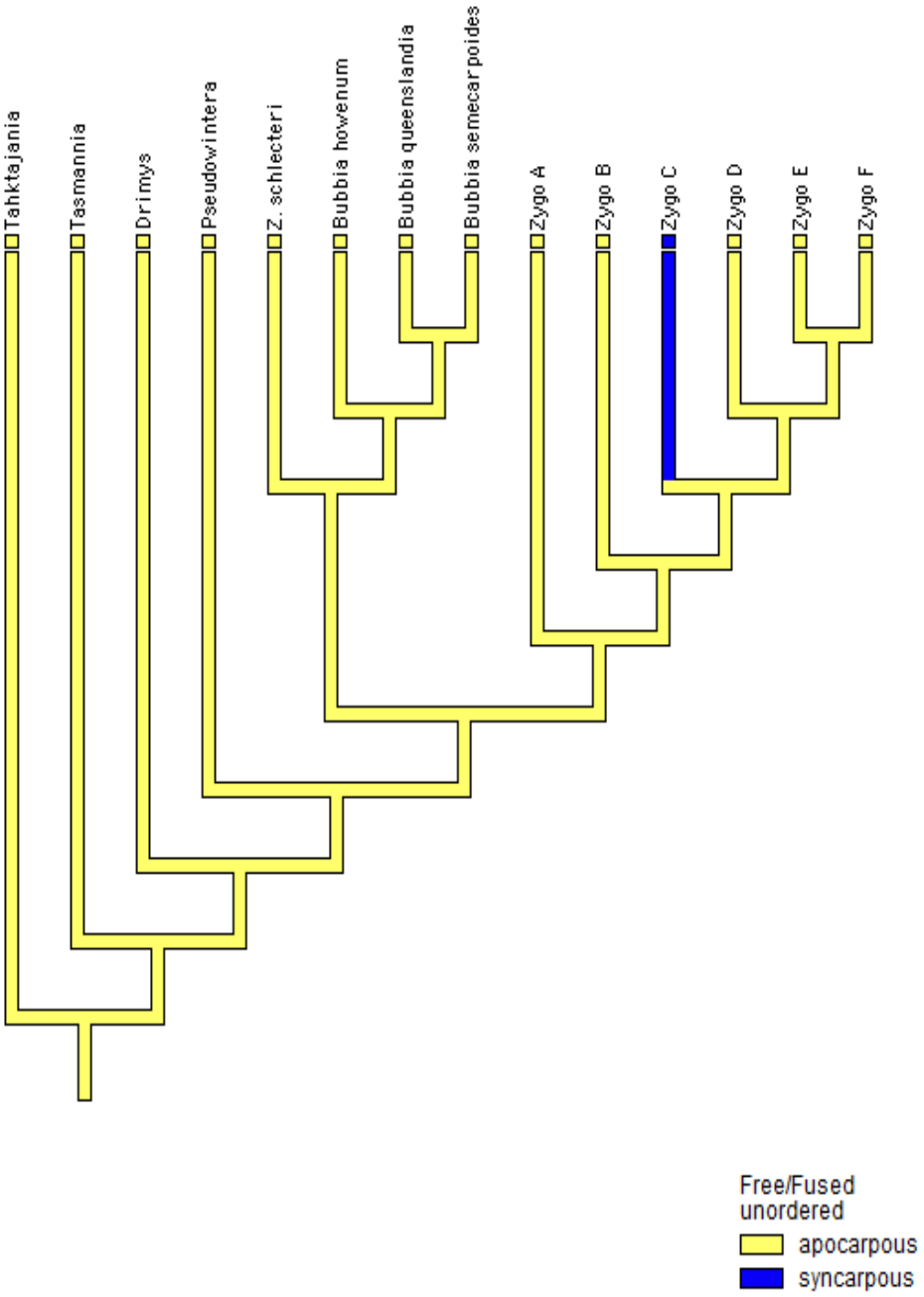

Figure 10: Evolution of carpel fusion within the Winteraceae indicating apocarpous (free) or syncarpous (fused) gynoecia character states.

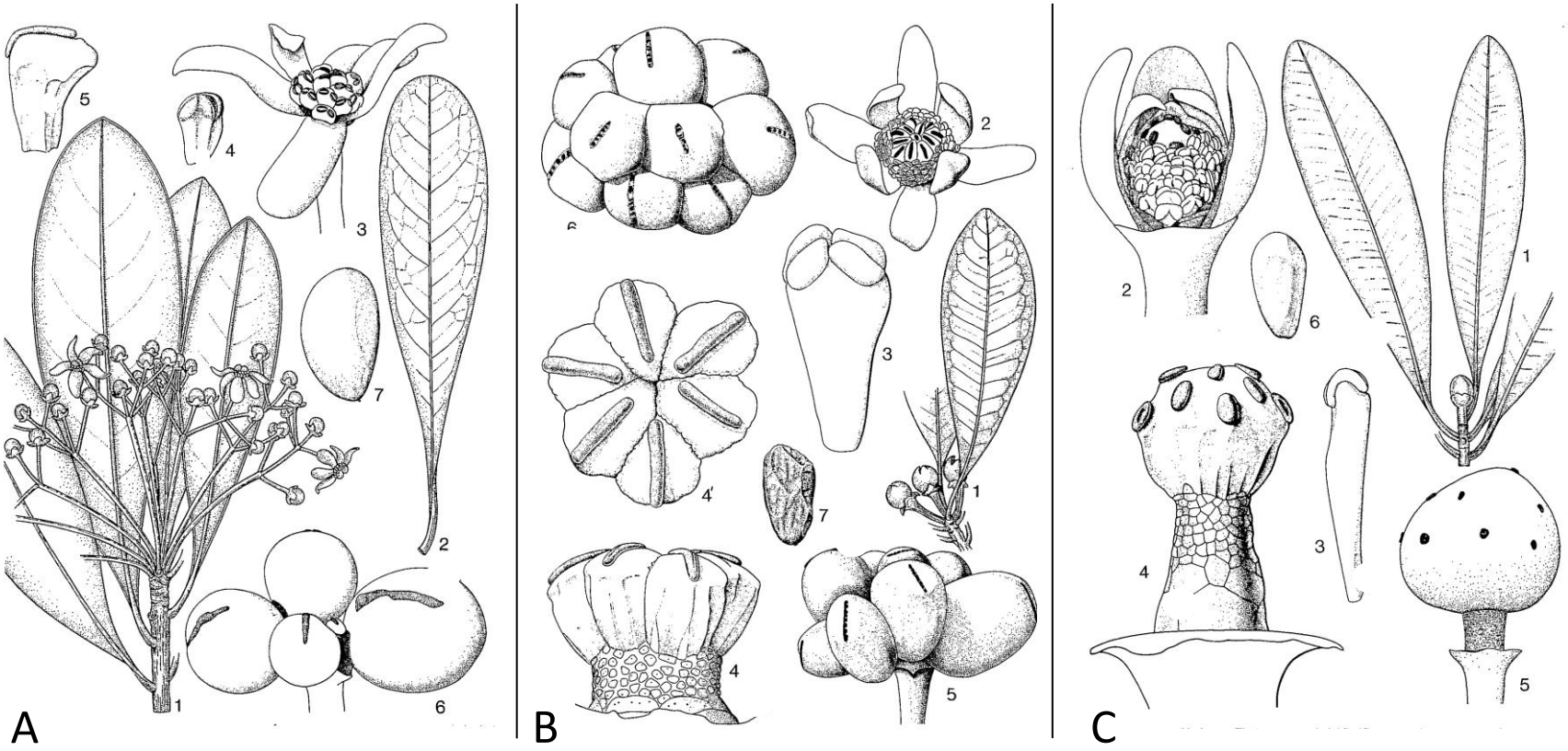

Figure 11: Illustration plate modified from Vink (1993) exhibiting variable state of apocarpy to syncarpy within the *Zygogynum.* A: *Z. crassifolium* with highly branched inflorescences, flowers with few petals, and free fruits; B: *Z. stipitatum* showing increased degree of fusion; C: *Z. bicolor* showing complete fusion.

Future directions

The first priority for future work will be to check determination of problematic specimens, such as *Z. crassifolium, Z. Vinkii,* and *Z. pomiferum* subsp. *pomiferum,* as well as identify and formally determine the multiple undetermined specimens within this study. Second, given our current results concerning specific delimitation within the genus *Zygogynum,* we will increase our sampling of taxa included within clade C to attempt to further resolve these relationships. We will attempt to collect samples of *Z. mackeei* and *Z. cristatum* and any additional subspecific taxa outlined in Vink (1993). Thirdly we will work to produce a complete, holistic, and comprehensive taxonomic revision of the family for the Flora of New Caledonia series. Of interest would be to include further Australian *Bubbia* with the goal of identifying the position and taxonomy of *Z. schlechteri* and sister *Bubbia* taxa, within *Zygogynum s.l.*

Additional highly variable genetic markers (*ycf1*-a, *trn*K, *rpl32-trn*L) are also likely to be added so as to increase our phylogenetic resolution and increase the robustness of our analyses. A particular goal we hope to achieve using additional markers is to further resolve the backbone of *Zygogynum s.l*. To achieve these goals we will be collaborating with an Australian research group to produce a comprehensive account of the entire family. A morphological investigation of our results will also be a priority in order to assess background morphological trends within the genus in light of molecular phylogenetic results. At this time we will undertake a comprehensive analysis to attempt to date the divergences to shed new light on the origins and evolution of these Island species. Once a comprehensive assessment of taxa within the *Zygogynum* has been produced, we

will investigate and revise extinction risks and conservation assessments adding new and current information to the IUCN Red List.

Once a complete and comprehensive molecular sequence matrix has been produced we will perform molecular divergence dating analyses on the Winteraceae in order to estimate the divergence timing of New Caledonia *Zygogynum s.l.* This method is relatively controversial and a conservative approach to assigning fossil lineages should be used in conjunction with multiple lines of evidence such as geology. For support for the vicariance hypothesis we would expect divergence of New Caledonia taxa to be older than the submergence event (ca. 40-34 Ma). Support for the long-distance dispersal hypothesis would come in the way of more recent (<34 Ma) divergence dates of New Caledonian lineages. This data will, in conjunction with estimates of other New Caledonian lineages, contribute towards gaining a holistic view of New Caledonia's biogeographic history and more specifically the extent and duration of Eocene submergence.

Conclusions

This research represents the first comprehensive molecular phylogenetic investigation of the genus *Zygogynum*, and the first comprehensive investigation of a basal angiosperm group on New Caledonia. In our investigation we confirm the results of previous studies regarding the position and relationships of genera within the Winteraceae. We also identify problematic species within *Zygogynum s.l.* that require further investigation in order to further resolve relationships within the genus. We present a new hypothesis regarding the origin and subsequent dispersal of Winteraceae genera, indicating a common Gondwanan

origin between *Drimys* and Zealandic Winteraceae, and subsequent dispersal from New Caledonia to Australia. In light of our analyses and revised origin hypothesis the circumscription of Australian *Bubbia* is questioned, indicating that it likely should be retained within a broadly circumscribed *Zygogynum s.l.* though further investigation will be required to confirm this. Furthermore the generic segregates of *Exospermum* and *Belliolum* are both nested within the greater *Zygogynum s.l.* therefore our results currently support their retention within *Zygogynum s.l.* given an unresolved backbone to this clade. Finally our findings indicate a surprising trend of carpel evolution within the Winteraceae, the significance of which is yet to be determined but will be revisited in future research.

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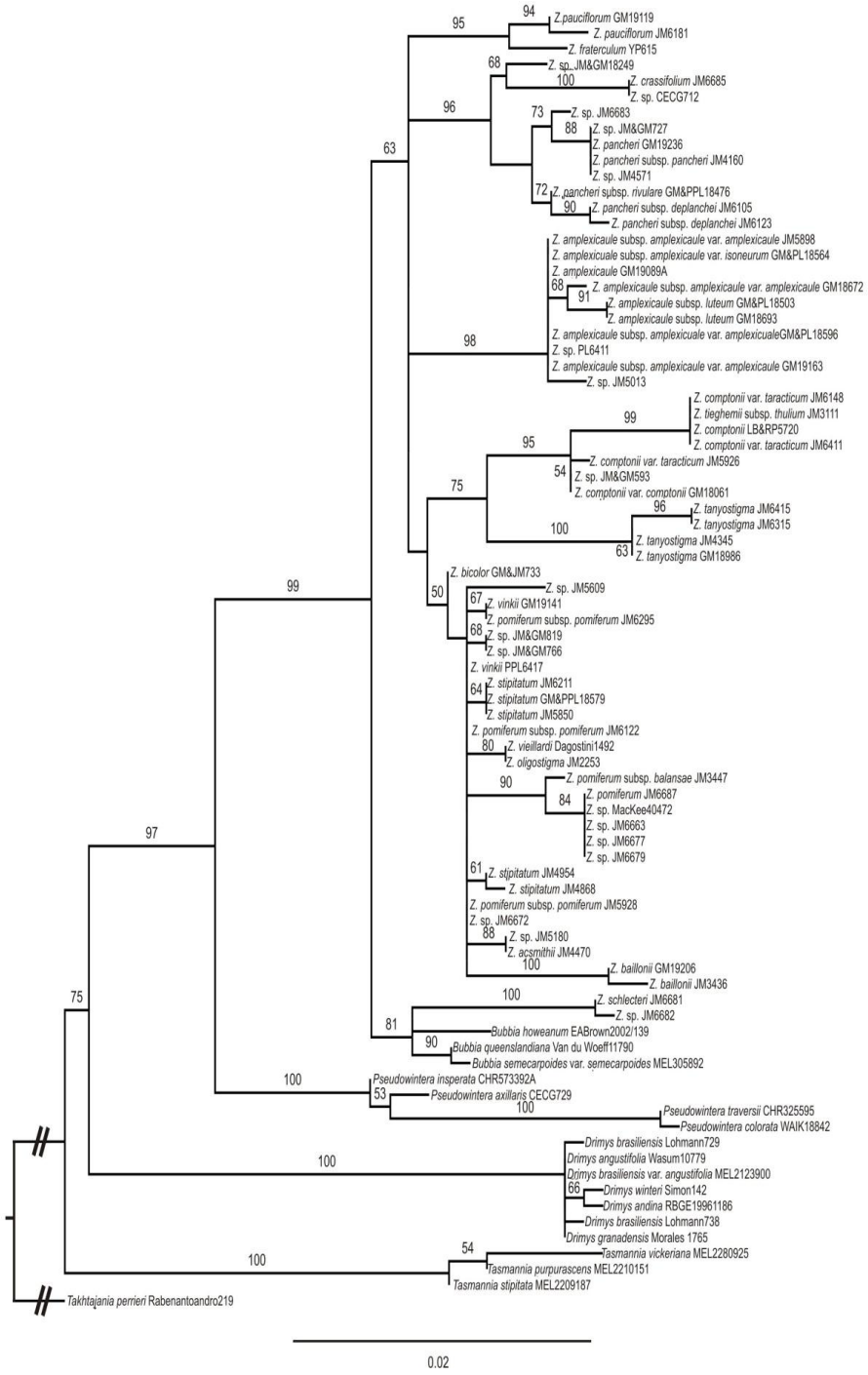
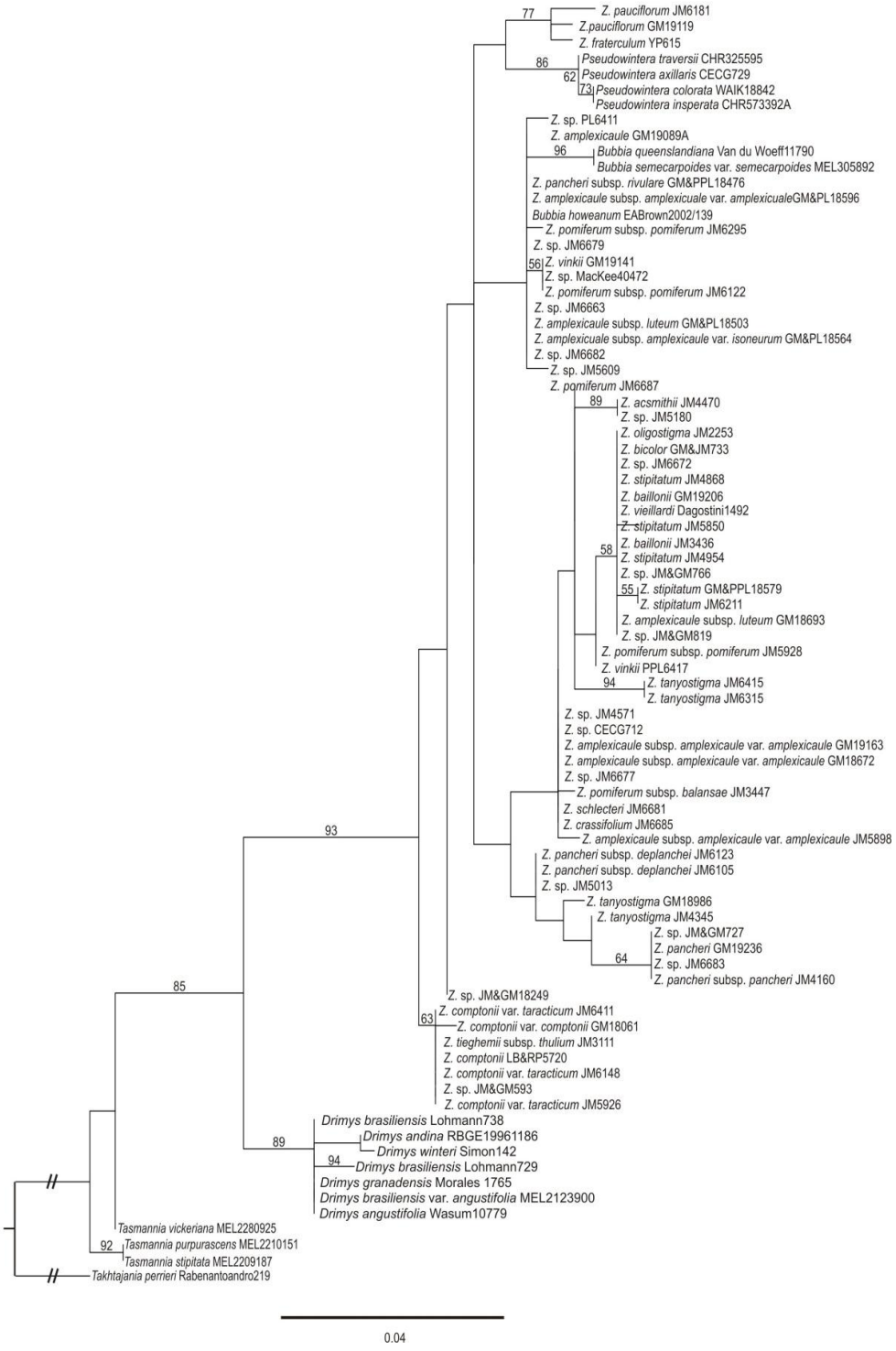
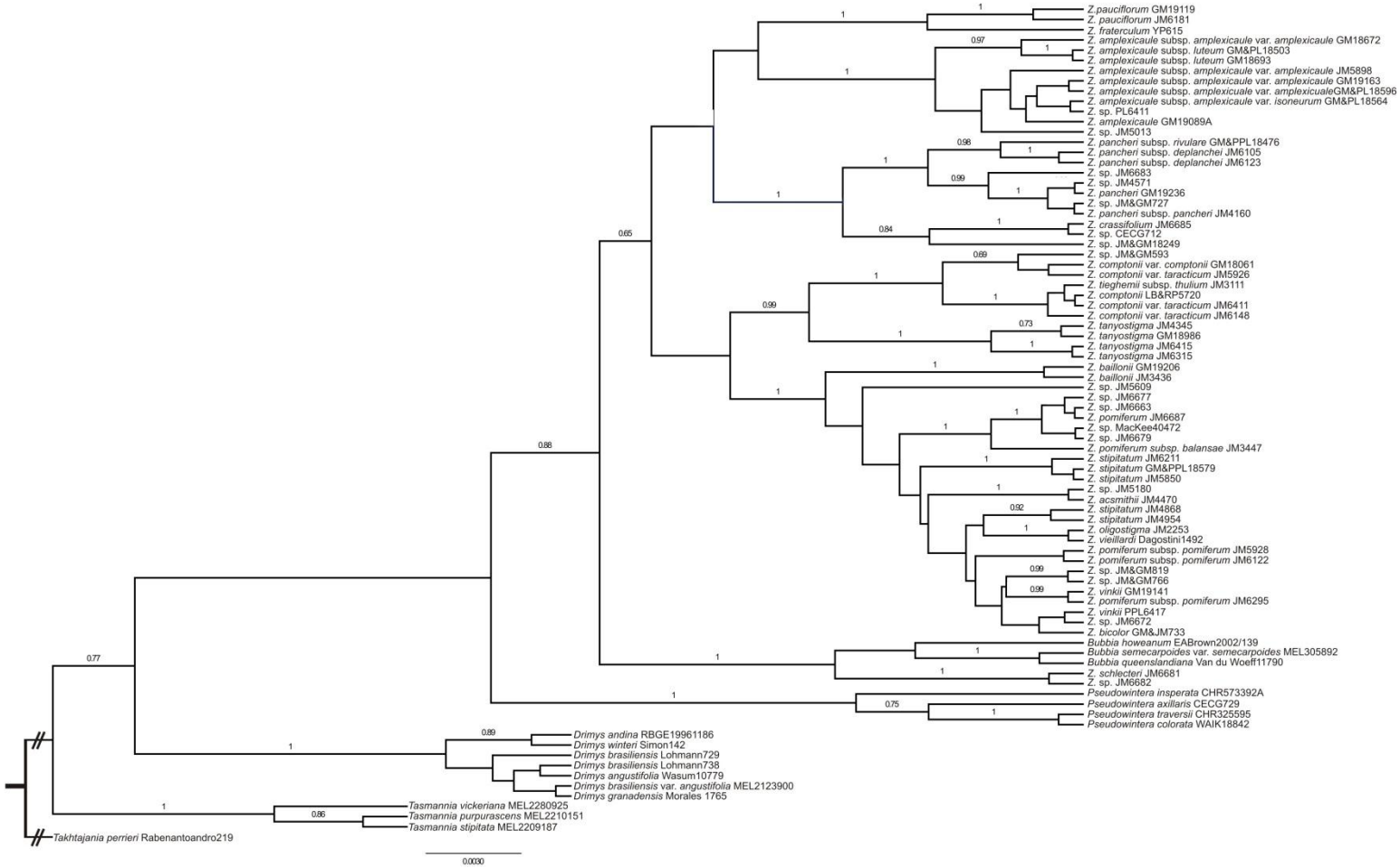
Appendices



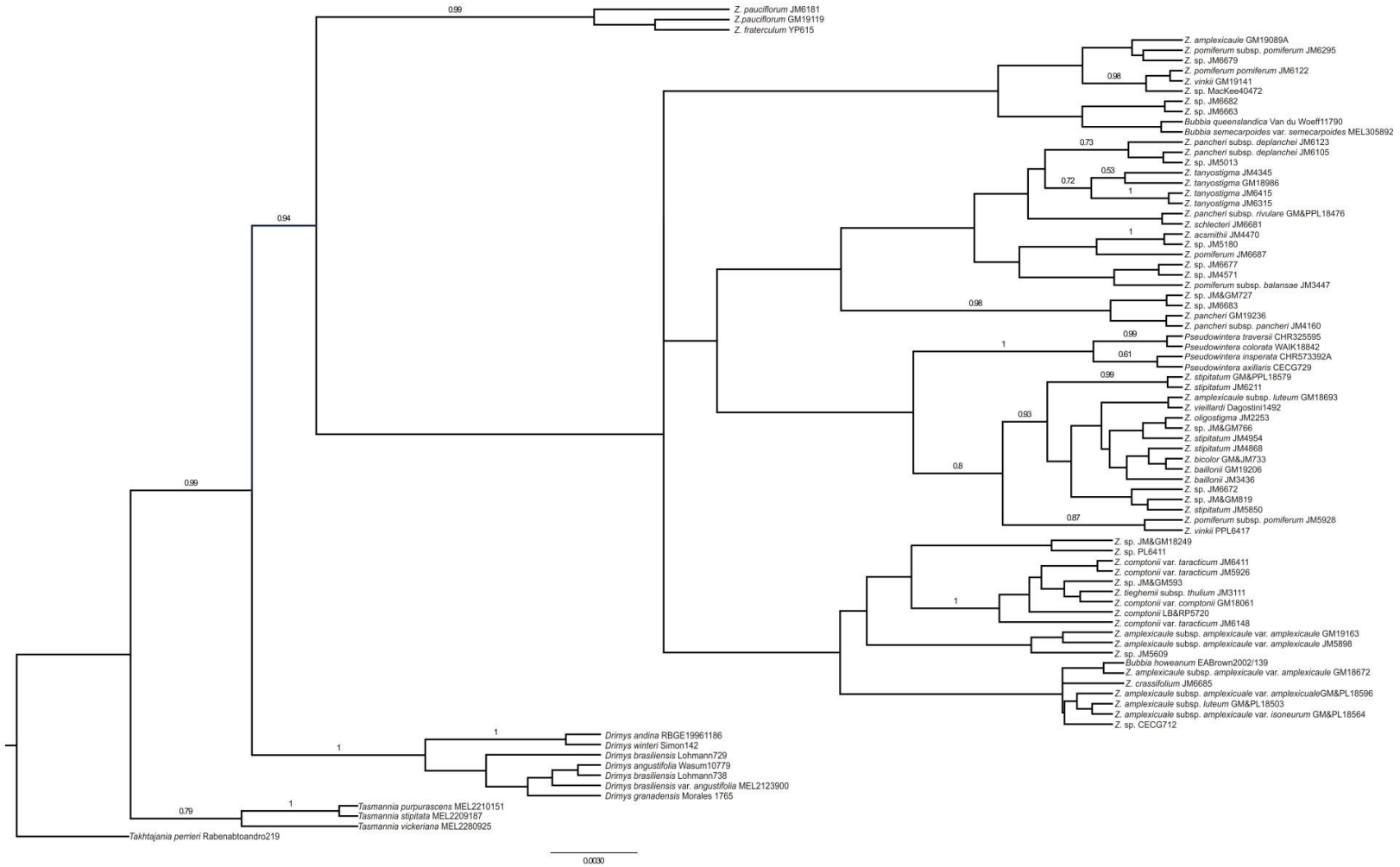
Appendix I.a. Maximum likelihood tree of the Winteraceae based on sequence alignment of the ITS loci. Bootstrap support values greater than 50% are indicated above branches.



Appendix I.b. Maximum likelihood tree of the Winteraceae based on sequence alignment of the *psb*A-*trn*H loci. Bootstrap support values greater than 50% are indicated above branches.



Appendix I.c. Bayesian majority rule probability tree for unlinked ITS analysis. Posterior probabilities values (PP) above 0.50 are indicated on branches preceding nodes.



Appendix I.d. Bayesian majority rule probability tree for unlinked *psb*A-*trn*H analysis. Posterior probabilities values (PP) above 0.50 are indicated on branches preceding nodes.

THESIS CONCLUSION

New Caledonian vascular flora is characterised by its high level of species richness. In contrast to many other tropical island flora's, New Caledonia's flora is unique having at the same time both extremely rich and depauperate floristic groups common throughout the Pacific (Morat *et al.*, 2012). Additionally, New Caledonia is renowned as being home to a number of ancient and purportedly Gondwanic lineages, which continue to intrigue researchers to this day (Heads, 2008; Grandcolas *et al.*, 2008). One factor that is repeated within the literature is that New Caledonia is still vastly under-explored and under-researched, with increased research efforts only recently becoming evident.

This thesis research has contributed important new knowledge to our understanding of two groups of New Caledonian flora. I investigated two genera within New Caledonia using modern molecular techniques to address questions of taxonomy and evolutionary relations. The first investigation (Chapter II) focussed on species level relationships within the genus *Vitex* and addressed the monophyly of the New Caledonian species *V. collina s.l.*, considering evidence for multiple morphotypes within the species, as well as determined the placement and distinction of a newly discovered species *Vitex* sp. "*unifolia*" within *V. collina s.l.* The second investigation (Chapter III) assessed generic relationships within the basal angiosperm family Winteraceae and the monophyly of the genus *Zygogynum s.l.* with special emphasis on the generic segregates *Belliolum, Bubbia, Exospermum,* and *Zygogynum s.l.* This second investigation also addressed species level relationships within *Zygogynum s.l.* and considered both biogeographical and morphological trends in light of our analyses.

Within Chapter II, our analyses supported at least three genetically distinct clades within *V. collina s.l.* Furthermore, our analyses confirmed the placement of *Vitex* sp. *"unifolia"* within the larger *V. collina s.l.* clade, further supported as distinct from other entities based on morphologically distinct characters. Additionally the distinct clades identified in our molecular analyses reflect both biogeographic and morphological support for multiple entities, though more thorough investigation is required before we can publish a revision of this genus in New Caledonia. This work will be combined with the formal morphological description of this new species by Dr. Jérôme Munzinger to formally recognise this new and rare species. In light of this work subsequent research will investigate the distribution, extinction risk and conservation assessment of *Vitex* sp. *"unifolia"* to contribute to the IUCN Red List for this species.

Within Chapter III, our analyses supported the monophyly and position of all genera within the Winteraceae with the following relationship (*Takhtajania* (*Tasmannia* (*Drimys* (*Pseudowintera* + *Zygogynum s.l.*)))). Our results on generic relationships within the family were congruent with all previous molecular studies on the Winteraceae. Concerning the generic segregates within *Zygogynum s.l.*, our analyses support two clades within *Zygogynum*, one composed of *Z. schlechteri* + *Bubbia* and the second composed solely of New Caledonian species of *Zygogynum s.l. Belliolum* potentially forms a monophyletic clade depending on how *Z. fraterculum* could be treated in the instance of a revision i.e. combined with *Z. pauciflorum* though the backbone of this clade is unresolved and therefore at this point *Belliolum* is nested within *Zygogynum s.l. Exospermum* is similarly nested within *Zygogynum s.l.* Our analyses also investigated species level

relationships within *Zygogynum s.l.* where we identified areas in need of further investigation and potentially formal taxonomic revision.

We have proposed a revised hypothesis on the origin of Zealandic Winteraceae. In our hypothesis, both Zealandic (*Zygogynum s.l* and *Pseudowintera*) and South American (*Drimys*) Winteraceae share a common ancestor reflecting their Gondwanan origin. Subsequently Zealandic Winteraceae dispersed to Australia (likely from New Caledonia) and diversified into what is now *Bubbia* sensu Guymer (2007). This has implications for the taxonomy of this clade: either a broadly defined *Zygogynum s.l.* is retained, with *Bubbia* in synonymy, or *Bubbia* be reinstated, including *Z. schlechteri* (syn. *Bubbia schlechteri*). Further research into the morphology of species concerned is required before we will make a recommendation.

We also uncovered an interesting evolutionary trend between major clades within *Zygogynum s.l.,* where a single strongly supported clade is united by having syncarpous gynoecia as opposed to all other clades and genera within the family that have apocarpous gynoecia.

Our research has also contributed to on-going efforts to revise and update New Caledonia's vast floristic diversity. Having investigated only two groups during this thesis, it becomes apparent just what a momentous task it is to achieve. Many of New Caledonia's floral treatments are out of date and in need of revision (Morat *et al.*, 2012), including those used within this research (Vink, 1993; Mabberley & de Kok, 2002). Due to ever increasing technology and theory associated with molecular, biogeographic, and morphological research our thoughts on species taxonomy is constantly being challenged and revised. With advances in all of these fields it is becoming easier and indeed more common for

research to use such comprehensive and holistic approaches. This is the future goal of our own research in which we hope to thoroughly investigate both biogeographic and morphological aspects related to both *Vitex* and the Winteraceae.

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