Molecular Systematics of the *Lomandra* Labill. Complex (Asparagales: Laxmanniaceae)

by

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4. Combined Molecular and Anatomical Analysis of the *Lomandra* Complex (Asparagales: Laxmanniaceae)

4.1 Introduction

As inferred in the previous chapter there appears to be deviation between the phylograms obtained from each facet of this study. The phylograms have limited regions of homologous structure (as exemplified by small groups like section *Typhopsis*, the *L. micrantha* conglomeration, the bulk of the *L. longifolia*/*confertifolia* complex and the anticipated ingroup branches). Nevertheless, the distribution of three of the four sections recognised by Lee and Macfarlane (1986) over multiple branches of the molecular phylogeny (refer to Figure 2-13) is notably different to the more classically congruent anatomical phylogenies. In this final section of this study, the two apparently divergent data sets have been combined into one large master matrix which has then been analysed with the same maximum parsimony and bayesian inference methods as previously used in an attempt to reconcile the two conflicting sources of information. This process of utilisation of multiple domains of information for phylogenetic elucidation is correspondent to those used in other studies, prior examples of which include Betulaceae (Chen *et al*. 1999), Rubiaceae: Ixoroideae (Andreasen and Bremer, 2000) and notably in Rubiaceae: Vanguerieae (Lantz and Bremer, 2004) where they utilised DNA regions very similar to those in this study. The details of combining data and various methods for dealing with partitioned data models with bayesian inference is expounded in Nylander *et al*. (2004) with the analysis of the gall wasp family Cynipidae.

4.2 Materials and Methods

As with the previous examinations, the master matrix has been analysed with two complementary software methods. Once again PAUP* version 4.10beta has been utilised to perform maximum parsimony analysis; with MrBayes version 3.1.2 employed to perform bayesian inference. In contrast to the molecular aspect of this work, in order to expedite the computational aspects of this project the combined trees have been analysed with the less computationally intensive F81 model with equal rate variation across sites (*lset nst=1 rates=equal*). Given the high degree of similarity between the GTR and F81 results in the pure molecular phylogenies and the significant reduction in the required processing time, the selection of the dramatically faster model seems reasonable. Nevertheless, in order to quantify the differences, a GTR model (*lset nst=6 rates=invgamma*) analysis was undertaken and produced a result which was indistinguishable from the spectrum of F81 results.

The complete data matrix was 1,618 characters long, with 52 coming from the morphology/anatomy and 1,566 from the DNA sequence data. When the data are loaded into MrBayes, the software reports 52 unique site patterns for the anatomical data and 840 for the molecular data. PAUP* reports 850 constant characters, 273 parsimony-uninformative variable characters and 495 parsimony-informative characters. In both analyses all characters were treated with equal weightings.

4.3 Results and Discussion

At first observation, the trees presented in Figures 4-1, 4-2, 4-3, 4-5, and 4-6 appear to represent a median between the two disparate phylogenies. Unfortunately the results generated by processing the combined data set must be considered incomplete and thus unreliable at best. Compared to the analyses of the individual information domains, all three process methods applied to the master matrix required computer process time vastly in excess of what was anticipated. Furthermore, the results generated were either of reduced resolution with larger polytomies as in the case of maximum parsimony (Figure 4-1) or with reduced convergence diagnostic values with any of the bayesian inference models (Figures 4-2 and 4-3).

As expected, at the sections where the anatomical and molecular phylogenies agreed on structure, the confidence values for these branches were increased. This is demonstrated by the ingroups branch where almost all confidence values from both analysis methods were improved. The negative effect on the overall strength of the phylogenetic arrangement becomes obvious when the analyses are repeated under identical constraints and the results deliver subtle changes in the arrangement of the larger groups.

Figure 4-3 demonstrates the effect the reduced convergence values have on the results. Despite being an identical copy of the dataset and processed with identical parameters, there are rearrangements of the main branches and subtle changes in the structure and arrangement of the terminal clades. This variable result is in stark contrast to the highly confident and extremely replicable molecular results,

where repetition of the analysis produced virtually identical tree structures. The phylogenies obtained from the molecular analysis are sufficiently reliable that by changing the model or the process technique (bayesian inference to maximum parsimony) the results have absolutely minimal variation (refer Chapter 2). When performing these analyses, it was routine to repeat the processing in order to eliminate the potential of stochastic error and confirm the result; however, despite multiple iterations with the combined data matrix the results were never entirely replicated as utterly precisely as the molecular data alone was able to be. The lowered confidence and variability in the results is directly linked to the MrBayes convergence diagnostic, which in this aspect of the study was unable to reach a suitably low value. When replicating the maximum parsimony analyses, these also were not able to reproduce identical results, although the variation seen between the strict consensus trees was less than that observed with maximum parsimony.

The influence of each data source on the final generated trees is interesting. The maximum parsimony analyses consistently place the ingroups (*Acanthocarpus*, *Chamaexeros* and *Romnalda*) together in a structure directly inherited from the molecular work, but place the branch at the base of the tree, indicating that it may have been ancestral to *Lomandra*. Conversely, bayesian inference was more variable in its placement of this branch, while the internal structure still has origin in the molecular dataset. Bayesian inference consistently places the ingroups taxa as a sub-branch of *Lomandra*, but is variable in how deeply it is attached. In some analyses (Figure 4-2) this group echoes the maximum parsimony results with the group attached towards the base of *Lomandra*; however it differs in the co-attachment of the primary *Capitatae* branch to the same source location which has been influenced

directly by the molecular results. This motif is common through all of the bayesian trees, although its overall location and distance in steps from the root of the tree does vary. The repeated analyses (Figure 4-3), the increased chain temperature analyses (Figure 4-6), and the predefined maximum likelihood user tree (Figure 4-7) have all exhibited the attachment of this particular complex as sister to the secondary assembly of *Sparsiflora*e and *Lomandra* sections, rather than superior as in Figure 4-2 and the molecular trees (Figure 2-13).

The islands of classically defined sections and series have remained relatively consistent from tree to tree across the spectrum of results. The inclusion of anatomical data has had very little influence on this, although it must be remembered that the purely anatomical trees were also not forming well resolved groups. In many cases in the combined trees, the inclusion of anatomical data has induced minor rearrangement in the branching; and in some cases re-orientated the branches to group more anatomically similar specimens. The main example of this is the four taxon clade of '*L. multiflora* subsp. *multiflora* (Salvator Rosa) 70731.5', '*L. glauca* (Broadleaf) 61207.1', '*L. elongata* 70614.7' and '*L. longifolia* 61130.3' embedded within the largest *Sparsiflorae* grouping. In the molecular anatomy these species are more widely distributed amongst this group, but in three of the four bayesian analyses (Figures 4-3, 4-6 and 4-7), this group has been subtly rearranged to facilitate the closer affinities of these specimens.

The placement of the monotypic *X. divaricata* is broadly similar across the methodologies, where it is placed in the lowest branch and grouped together with the largest *Sparsiflorae* clades. Maximum parsimony unexpectedly places this specimen

in a small clade with '*L. glauca* (Broadleaf) 61207.1' and '*L. elongata* 70614.7' which is an arrangement not present in any of the individual phylogenies. Bayesian inference however, takes its primary influence from the molecular data and arranges *Xerolirion divaricata* towards the root of the largest *Sparsiflorae* clade (which encompasses all sampled members of the *L. filiformis* complex). As this branch is rearranged from method to method, the precise location and affinity of *Xerolirion divaricata* does change, but its relationship towards the root of this clade is fairly consistent.

FIGURE 4-1 (1 of 2): *Lomandra* complex cladogram generated with maximum parsimony methods on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Bootstrap values of all uncollapsed branches are indicated. (Image continues next page.)

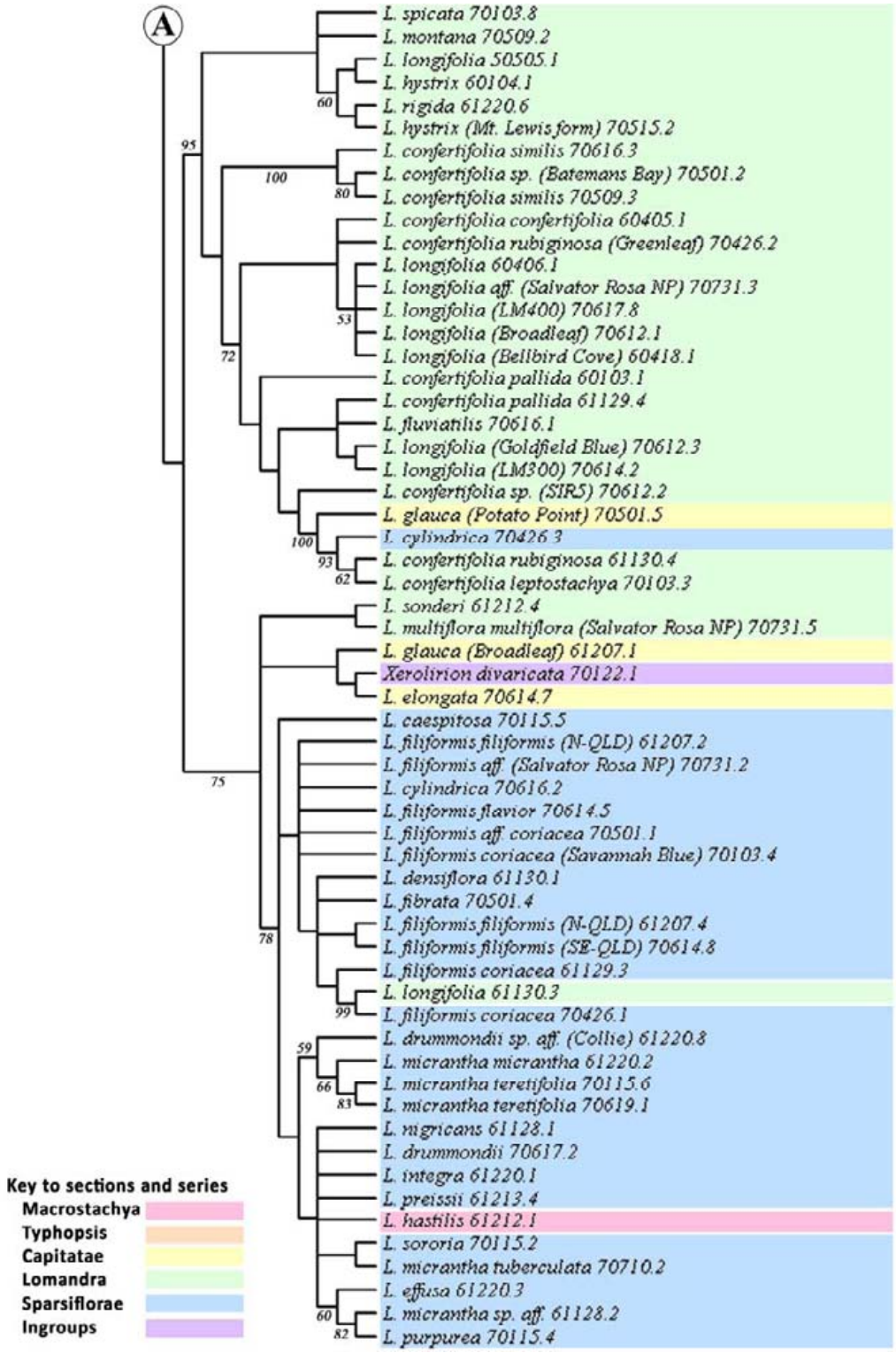

FIGURE 4-1 (2 of 2): *Lomandra* complex cladogram generated with maximum parsimony methods on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Bootstrap values of all uncollapsed branches are indicated.

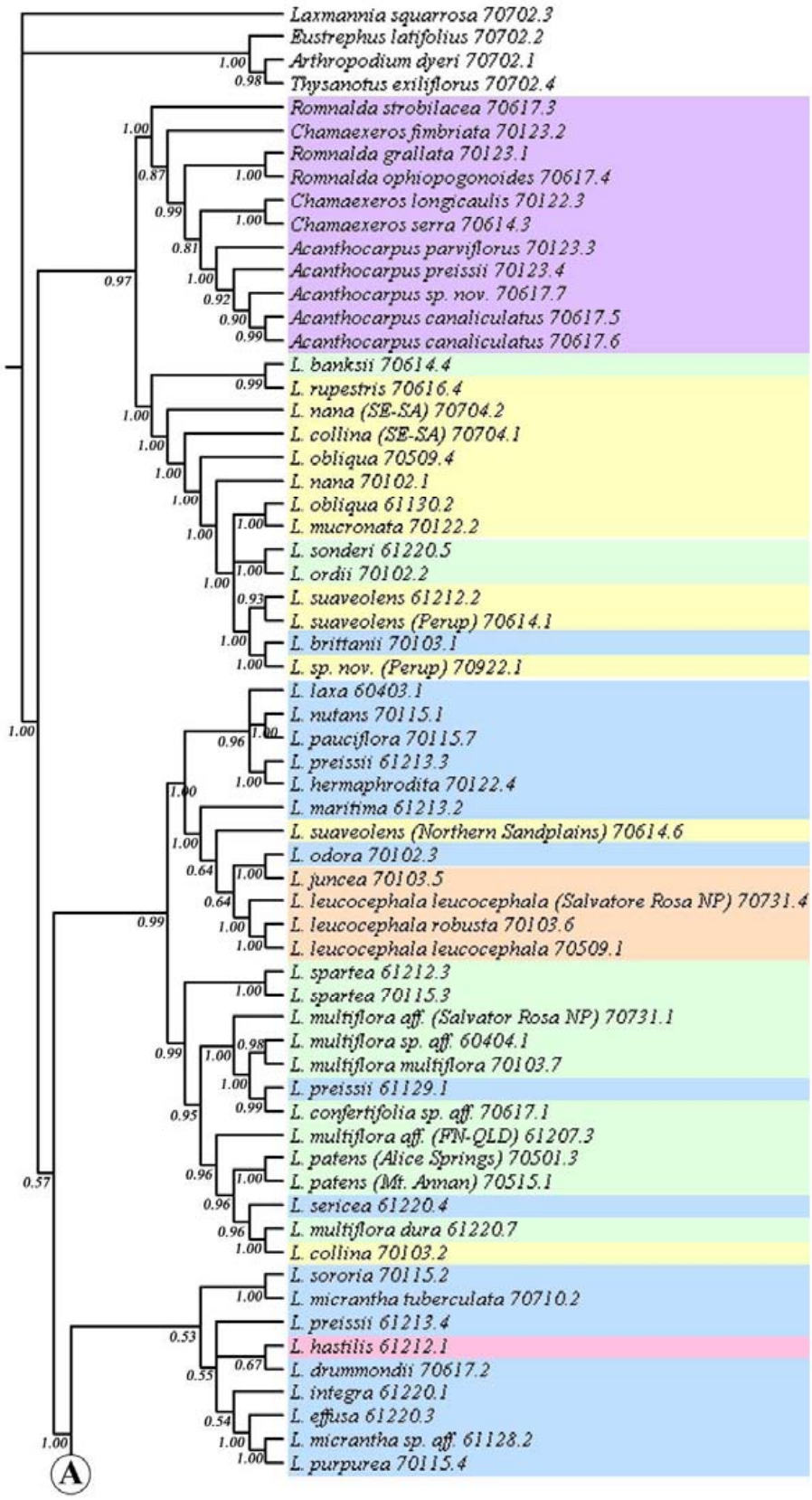

FIGURE 4-2 (1 of 2): *Lomandra* complex cladogram generated with bayesian methods (F81 model) on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated. (Image continues next page.)

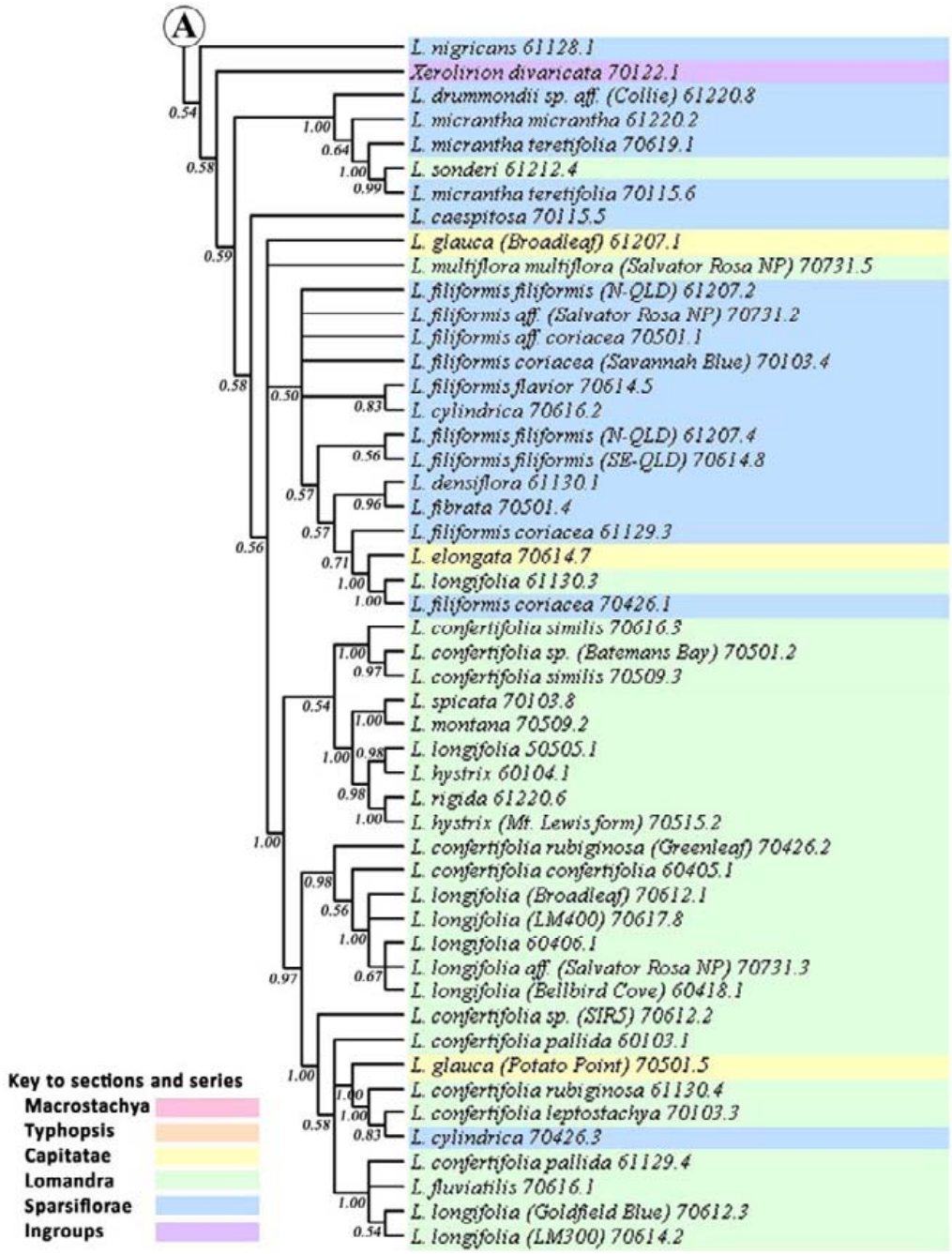

FIGURE 4-2 (2 of 2): *Lomandra* complex cladogram generated with bayesian methods (F81 model) on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated.

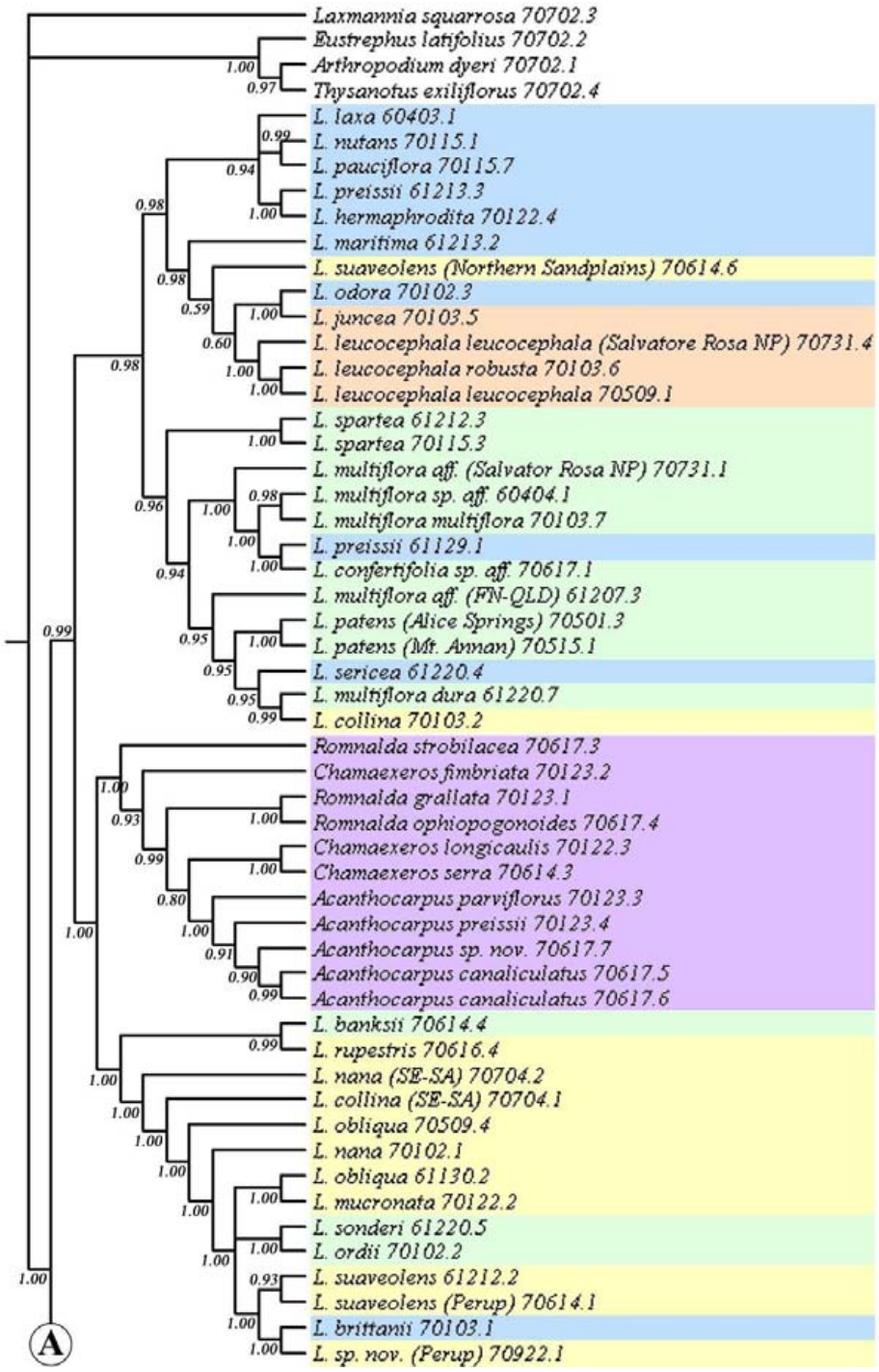

FIGURE 4-3 (1 of 2): *Lomandra* complex cladogram generated with bayesian methods (F81 model) on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated. Of note is the different result from Figure 4-2, despite starting with identical initial data and bayesian process parameters. (Image continues next page.)

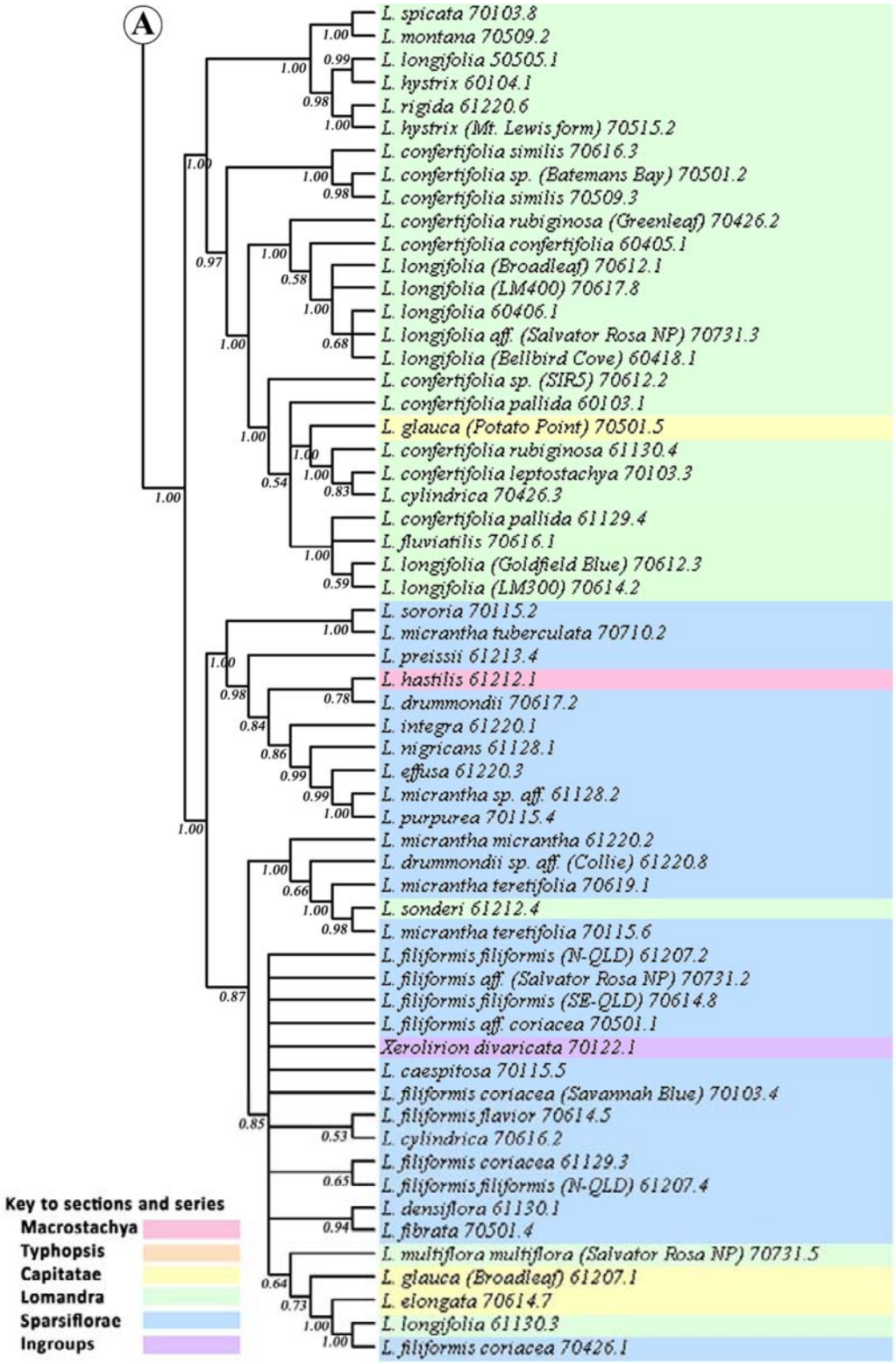

FIGURE 4-3 (2 of 2): *Lomandra* complex cladogram generated with bayesian methods (F81 model) on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated. Of note is the different result from Figure 4-2, despite starting with identical initial data and bayesian process parameters.

Careful monitoring of MrBayes while it processed the master matrix revealed a curious anomaly in the procession of the run and possible insight as to the poor performance of the convergence diagnostic and resultant lowering of confidence. Typically, the MrBayes process involves two simultaneous and completely independent analyses starting from different randomly generated trees and undergoes Markov-Chain-Monte-Carlo (MCMC) sampling (Ronquist, 2005). By default, MrBayes uses Metropolis coupling to improve the MCMC sampling of the target distribution and spreads this across four chains per process, three which have been "heated" and one "cold" chain. As the two independent analyses proceed, they should converge on the same (or extremely similar) resultant tree. MrBayes prints a diagnostic value as part of its display output called "average standard deviation of the split frequencies" which is an indication of how closely the two process threads have become converged on the same solution and thus how close the entire process is to acceptable completion.

Normally as MrBayes performs the MCMC calculations, the standard deviation starts as a high value (>0.25) and rapidly recedes towards zero. This value of the standard deviation is used as a direct measure of the completeness of the process run, with values under 0.05 being considered acceptable and values of less than 0.01 being indicative of high levels of convergence and thus strong confidence in the results (Hall, 2007).

When processing the *Lomandra* complex master matrix the convergence diagnostic initially behaves as expected, but then as the process continues, exhibits curious behaviour as the average standard deviation begins to pendulum between

high (>0.25) and low (≤0.055) values. Normally only a small amount of variation of this kind is exhibited in the very early iterations in a run (if at all) before the convergence diagnostic steadily progresses towards zero. The tendency of this dataset to induce oscillation of the convergence diagnostic has significant effect on the end result as it greatly extends the required numbers of iterations to reach confident convergence. In some analyses, it appears to increase the required number of iterations to beyond reasonable if convergence is to be reached. Examples of MrBayes diagnostic overlay plots can be found in Figures 4-4 and 4-5 which clearly show the normal end result progression giving a random distribution of plot points from the two process threads and the anomalous progression of the master matrix where the two threads oscillate and are unable to achieve convergence despite a large number of generations of analysis.

Given the time constraints of the project and the requirements for running multiple iterations of the data analysis for confirmation of results, MrBayes was limited to a maximum 100 million generations, an amount that was significantly greater than what is required for convergence in either of the individual information domains. This vastly increases the required processing time into rather inconvenient months with currently available computing power, even when utilising the parallel processing version of MrBayes (Altekar *et al*. 2004) on multiple CPU platforms. Further experiments where the MrBayes process runs were limited to 25 million, 50 million, 300 million and 600 million generations, the results demonstrated no variation outside of the spectrum of results obtained from the 100 million generation analyses. With the oscillation behaviour induced by this data, we estimate that it is likely to require many billions of generations to reach convergence, if it is even

achievable at all. Equally, manipulating the maxtrees variable of the PAUP* parameters with the same intentions and increasing it by factors of ten resulted in the same conclusion: that gargantuan increases in processing time were incapable of providing any additional resolution.

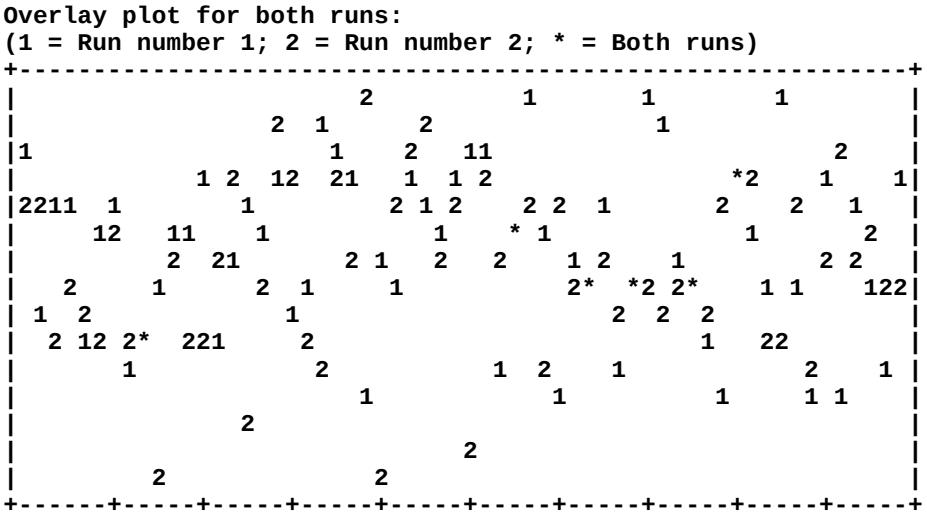

FIGURE 4-4: MrBayes successful diagnostic overlay plot.
The output of a successful run where the result is a random distribution of points indicating convergence between the two process threads as the plot points intermix.

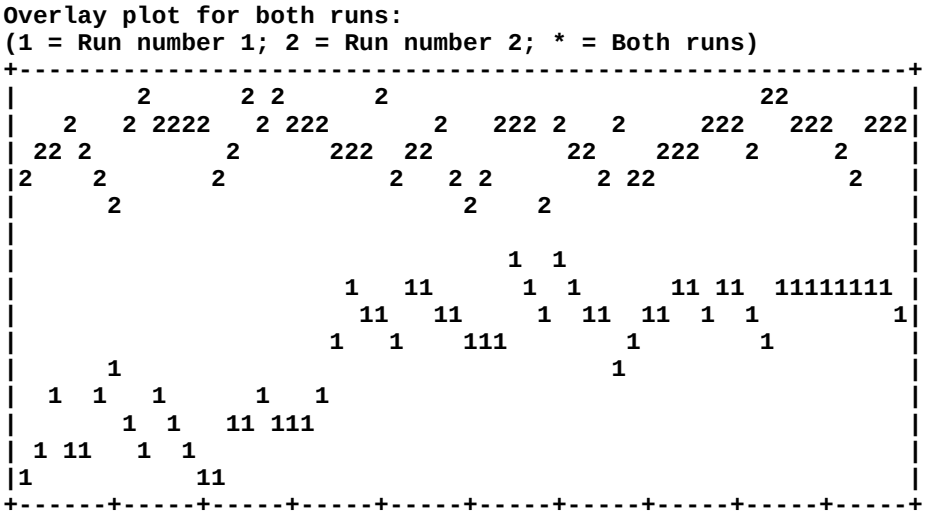

FIGURE 4-5: MrBayes oscillation diagnostic overlay plot.
A low confidence plot output demonstrating the pendulum oscillation of two runs that failed to reach convergence despite 100 million generations. The process threads appear to continue to waver without apparent resolution virtually indefinitely.

These issues with disparate data influences were not without a potential solutions (Hall, 2007). Possible solutions included changing the temperature of the heated chain from its default 0.20 to higher (0.25) or lower (0.15) values. Experiments with changing the temperature to the higher value on our dataset proved inconclusive with very little positive effect on the convergence diagnostic by the 100 millionth generation, or on its frustrating oscillation behaviour (Figure 4-5).

An alternative solution to trees which would not readily converge was to supply a topology only maximum likelihood (ML) tree generated by PhyML (Guindon, 2003) as a "usertree" variable in the MrBayes arguments. MrBayes will then use this maximum likelihood tree as the default starting tree instead of its normal random tree for each of the process threads. Given the maximum likelihood tree to guide the analysis of the combined dataset MrBayes displayed a much-improved initial behaviour with a strong trend for simple direct reduction of the convergence diagnostic towards zero. Unfortunately in this case, specifying a maximum likelihood tree reduces the oscillation effect to ranges typically between 0.15 and 0.055 but it does not entirely suppress the oscillation of the convergence diagnostic sufficiently well to supply high confidence results. This again results in a phylogeny with less resolution and lower confidence values (Figure 4-6). Interestingly this maximum likelihood tree bears the greatest similarity to the increased chain temperature tree.

I hypothesise that the conflicting dynamics of the two disparate information domains was directly responsible for the inability of the software to resolve the phylogeny with a high level of confidence. Comparison of the trees between

individual domain results shows a greater affinity for these results with the molecular phylogenies, which demonstrates the comparatively large effect the molecular data exerts over the anatomical subset. With the application of the anatomy to the DNA, a small amount of resolution is lost from the molecular results with the net gain consisting mostly of stronger grouping via branch rotations of the classical anatomical sections within DNA defined branches. This greater tendency to form more homogenous classically defined clusters within the clades comes at the price of overall detail; in particular the *longifolia* / *confertifolia* complex and the most distal *Sparsiflorae* group both have lower levels of differentiation between species as they blend into a broader polytomy. This reduction in intra-clade division does not however, come with a concomitant solution to the islands of classically defined sections being distributed amongst the terminal clades identified by the molecular data. Nor does it provide any measure of alleviation of the anomalous individuals (such as '*L. suaveolens* (Northern Sandplains) 70614.6') interrupting the otherwise contiguous groupings.

These variations in the results between analyses of the separated and combined data compartments indicated that in the example of the *Lomandra* complex, anatomy is not closely following the genetics and the internal familial relationships are more complex than those predicted by prior anatomical studies.

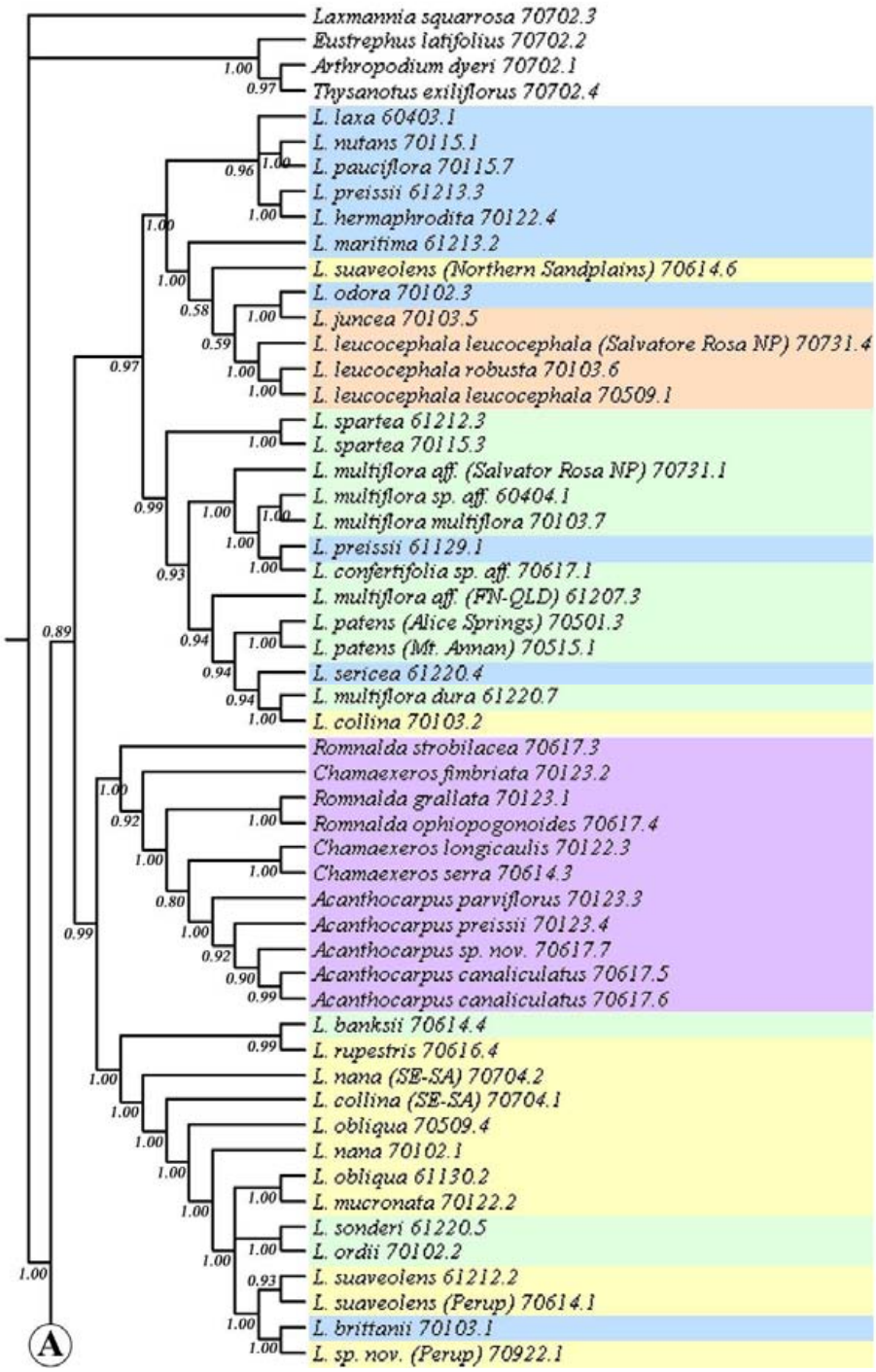

FIGURE 4-6 (1 of 2): *Lomandra* complex cladogram generated with bayesian methods modified with higher chain temperature on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated. (Image continues next page.)

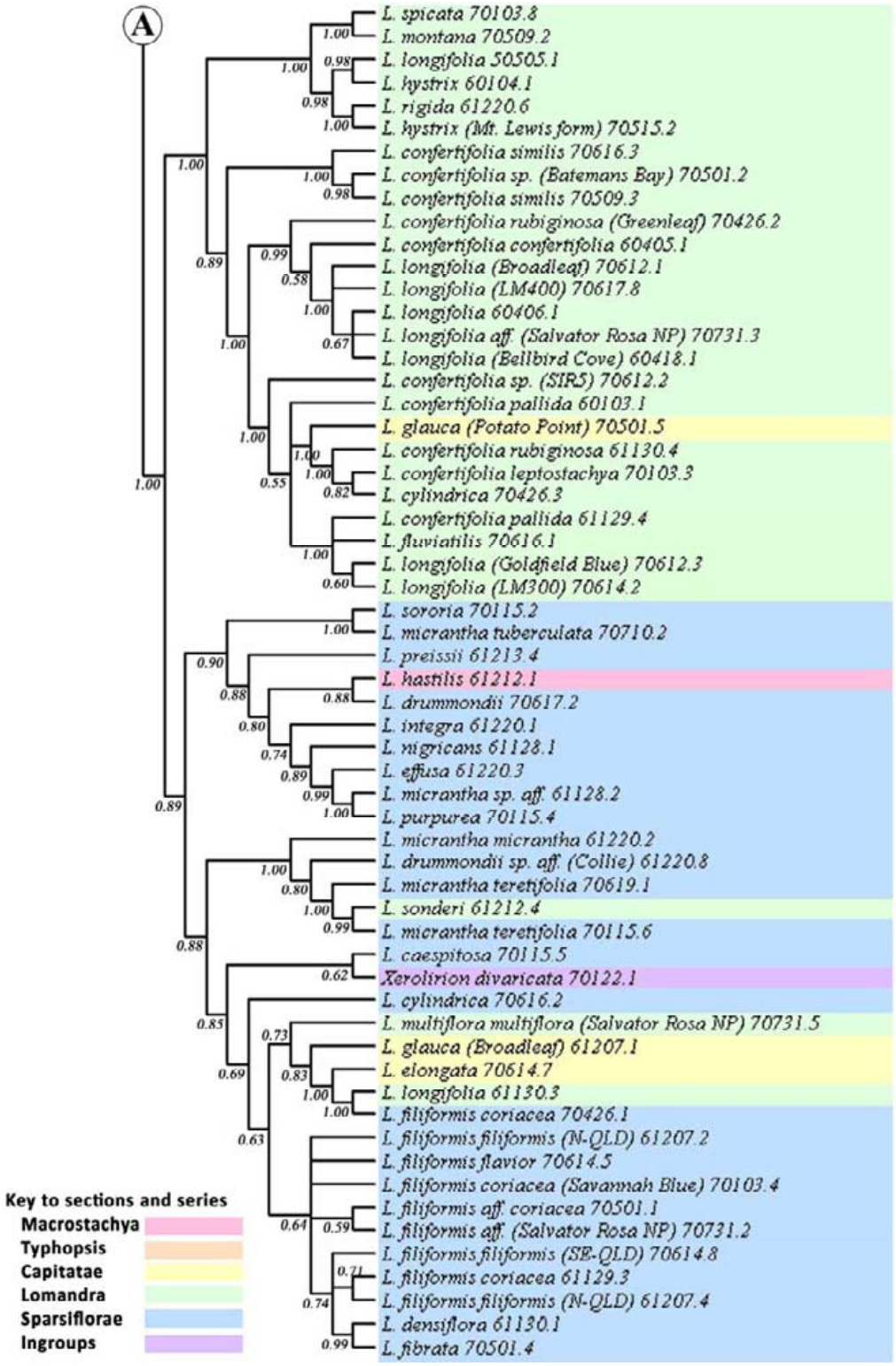

FIGURE 4-6 (2 of 2): *Lomandra* complex cladogram generated with bayesian methods modified with higher chain temperature on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated.

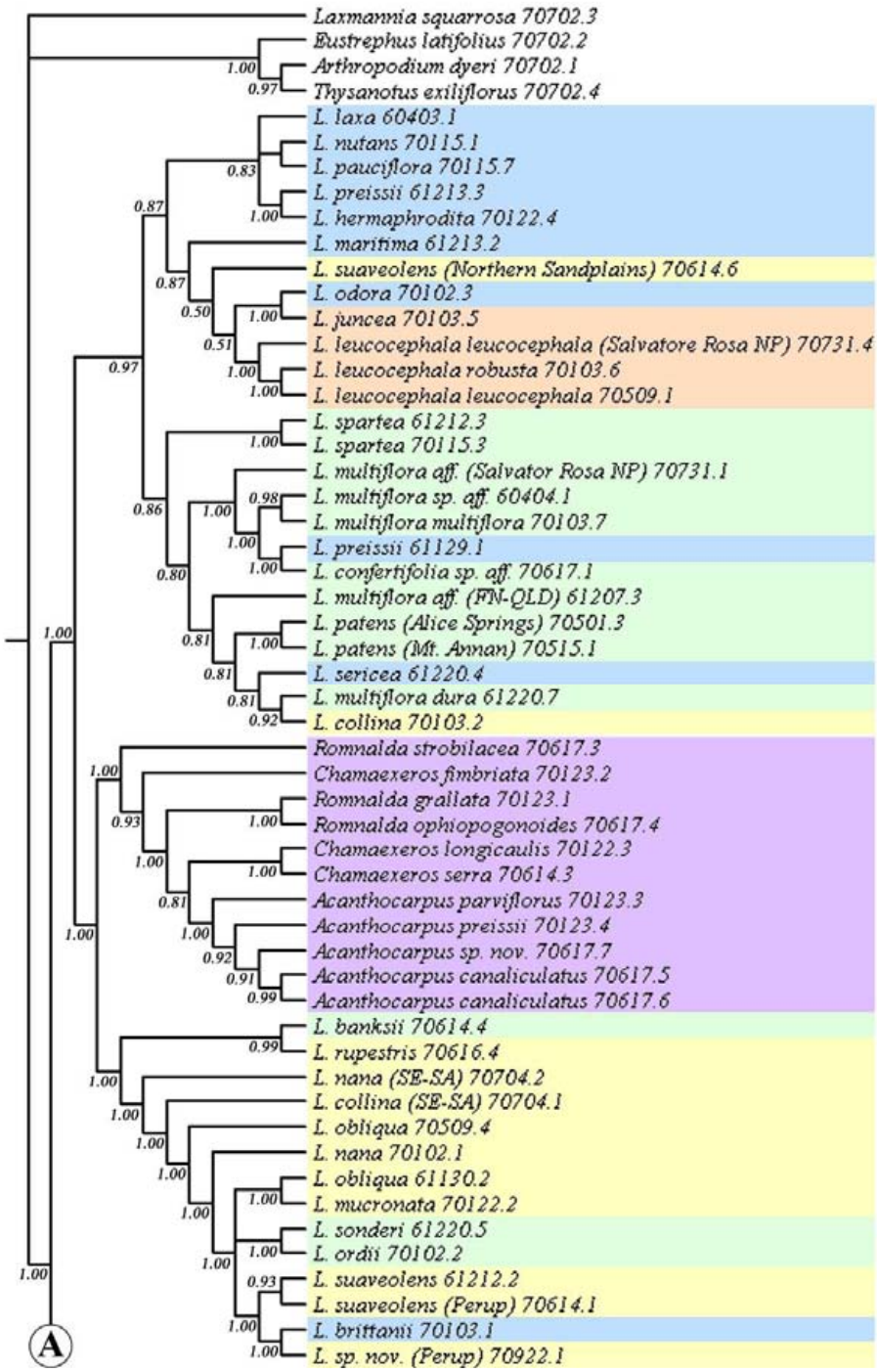

FIGURE 4-7 (1 of 2): *Lomandra* complex cladogram generated with bayesian methods modified with maximum likelihood usertree on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated. (Image continues next page.)

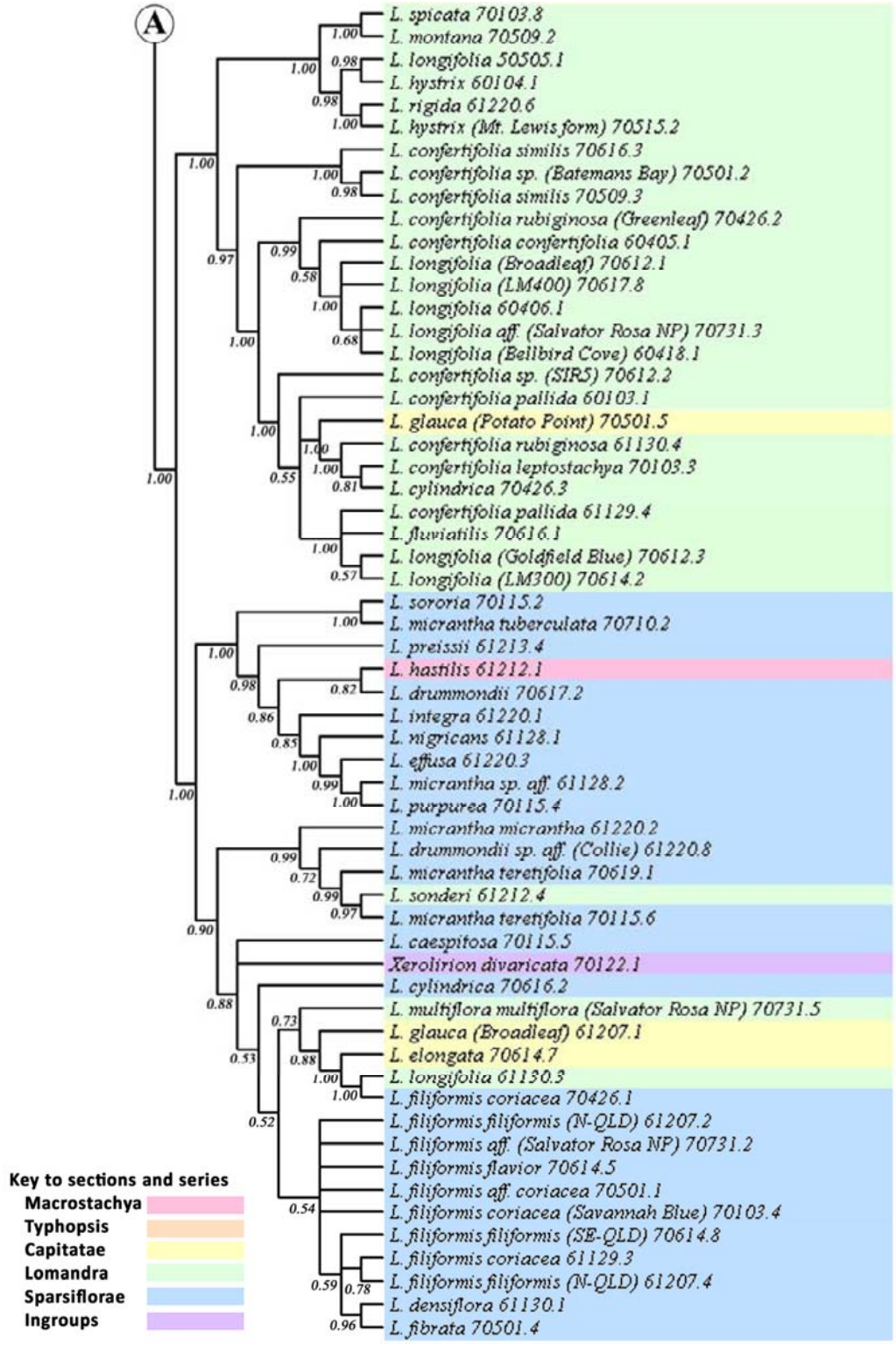

FIGURE 4-7 (2 of 2): *Lomandra* complex cladogram generated with bayesian methods modified with maximum likelihood usertree on a combined anatomical and molecular dataset. Sections and series per Lee and Macfarlane (1986) have been colour coded for clarity. Posterior probability values have been indicated.

5. General Conclusions, Summary and Recommendations

5.1 General Summary and Discussions

While there are limited regions of homology between the two opposing phylogenies with small groups such as the *Typhopsis* group, the *L. micrantha* group, the bulk of the *L. longifolia*/*confertifolia* complex and the ingroups branch; the subdivision of many of the four sections of *Lomandra* over multiple branches in the molecular phylogeny and the resultant creation of numerous discrete islands (refer to Figure 2-13) disagrees with the traditional section and series classification and suggests that there is a manifestly more complex relationship between the species represented in this study than was appreciated previously. The difficulty experienced in reconciling the two data sources and the comparative relationship between the combined phylogeny and each of the contributing phylogenies where the molecular data adds resolution and resolves polytomies for the anatomical data, but the addition of the anatomical data reduces the definition of the molecular data by reducing segregated groups into polytomies suggests that one of the data sources is misleading. The complexity experienced in rationalising a common phylogeny indicates the inconsistent branching exerts an influence that competes against the cumulative effects of the common branches. Considering an example such as the Elephant Shrew where anatomical similarity was proven with molecular methods to be highly misleading of affinity (Nishihara, 2005); I am inclined to assign greater emphasis on the molecular results being the more appropriate interpretation of the relationships and affinities within the *Lomandra* complex.

It is possible that some of the species utilised in this study were misidentified, however efforts to retain and reuse the precise same sample for both molecular and

morphological aspects of the work gives a high measure of certainty that the correlations between anatomy and DNA are consistent, even if the actual specimens themselves have been misidentified. This consistency between molecular and morphological sampling lends weight to the morphological variability conclusions drawn from the image maps (Figures 3-6 and 3-7) and clearly demonstrates the anatomical inconsistency that can be found between *Lomandra* complex species that are closely related at a genetic level. Reviewing the image maps of both transect (Figure 3-6) and cuticle (Figure 3-7) also gives insight to the wide disparity of microscopic features present in species aligned side-by-side by DNA methods. This too adds weight to the argument that morphology and anatomy in *Lomandra* are only a partial guide to the actual intra-species relationships.

Consideration of the DNA phylogram marked with respect to collection location (Figure 2-14) clearly demonstrates only partial regionalisation of the genus, with each of the four sub-clades defined in the tree containing representatives from the wide habitats of the species. There does exist a measure of concurrence where western species tend to group with central, and central species with eastern, however, this grouping is not strictly enforced and again suggests a more complex evolutionary distribution of the *Lomandra* complex species.

The molecular and combined phylogenies imply the existence of four ancestral *Lomandra* lineages; representing each of the main branches of the trees and existing or spreading into virtually all of the locations in which modern species are found. Over time, populations became isolated through geography or genetics and ecological change has forced the ancestral species to adapt and evolve. The

tendency of each of the classical sections (and hence inflorescence similarity) to dominate one of the four branches of the phylogenies may represent each of the ancestral forms, or it may demonstrate each ancestral species had an exaptation to more readily adopt certain morphological features in response to environmental influence. The anomalous presence of occasional other sections and series within these larger groups may be indicative of species where environmental factors have dictated a common successful form. With the common genetic heritage of all *Lomandra*, this repeated development of homoplasious features is assumed to be an example of parallel evolution, rather than convergent.

Alternatively, the anomalous specimens distributed throughout the phylogenies may be examples of natural interspecies hybridisations. In the cases where specimens were obtained from neighbouring regions this does not seem entirely impossible, especially when considering that some specimens in this study appear to be accidental hybrids (cf. '*L. longifolia* LM300 70614.2') facilitated by anthropomorphic intervention. However, in the example of '*L. banksii* 70614.4' the large geographic distance between the collection location of this specimen and the locations of those affiliated with it in both molecular and combined phylogenies indicates hybridisation is improbable in this case. If hybridisation is occurring at any significant frequency within the genus, this opens the possibility that the complexity of the phylogenetic tree is due, at least in part, to lateral gene transfer between species.

The phylogenies developed in this study lead me to suggest there may be examples of both situations within the *Lomandra* complex. Individual examples

are discussed in section 5.2. The multiple islands of inflorescence type are not the only morphological features to demonstrate this patterning in *Lomandra*. Leaf anatomical features also demonstrate this clustering effect, particularly with some stomatal features such as the overhanging or bridging papillae and the creasing or invagination of the leaf surface. The majority of invaginated-leaved specimens form an exclusive clade within both the molecular and combined phylogenies which spans vast geographic separation and encompasses three classically defined sections. This character also appears sporadically elsewhere in the phylogeny, well removed from this main clade. It even manages to cross genera, appearing in *Acanthocarpus*. In total, this character occurs in three widely separated branches on the phylogenies, which implies that it has either arisen independently three times through accumulation of unknown numbers of mutations to leaf structure genes, or that the underlying genetic potential already exists in all *Lomandra* and just requires a comparatively simple mutation to be activated in concert with favourable environmental conditions to persist. The resolution of the precise genetic mechanism of this unique character may give indications of genetic inheritance patterns across the genus.

Hypothesising parallel evolution within *Lomandra* raises the question of the mechanisms by which this may operate. With their common genetic heritage, it does not seem unreasonable to suggest that across the species the same genes and/or gene expression patterns are responsible for influencing the same anatomical character. However, the nature and complexity of the mutation(s) leading to particular anatomical features in the *Lomandra* complex currently remains unknown. This is however, not without possible resolution. Developmental genetics has a well

established history in plants, and there have been many candidate genes identified in various species that may make excellent subjects for further research in the nature of the adaptability of *Lomandra*. By elucidating the genes which effect development of the observed *Lomandra* characters, it may be possible to determine if the features distributed around the molecular phylogeny are examples of parallel evolution. There exists numerous prior examples on which this additional research could be based; the recent review by Theissen and Meltzer (2007) summarises the current knowledge of inflorescence developmental genetics. Correspondingly, Fleming (2005) reviewed formation and development of leaves; with practical examples including McHale (1993) with *Nicotiana* (Solanaceae). With a firmer grasp of the mechanism(s) controlling the observed anatomical features, it may be possible to improve comprehension of the relationships between the anatomical and molecular phylogenies.

The innate diversity of anatomical form exhibited by *Lomandra* and its allies is proposed to be a confounding factor in the construction of an anatomical phylogeny of the group. The presence of wide diversification of leaf and flower morphology within each of the four primary molecular based clades – features which are controlled both by environmental influences as well as genetics, obfuscate the true relationships.

Additional sequence data may assist to unlock the incongruence between the phylogenies and better understand the relationship. Supplementary molecular data from other organelles (such as the mitochondria) may be suitable. Preliminary review of cpDNA *rbc*L sequence data from selected species in our study and from

related species with data obtained from Genbank, suggest that despite the highly conserved nature of the gene which generally confines it to discerning higher level taxonomy, the *rbc*L in the *Lomandra* complex may be sufficiently varied to add further refinement to the phylogenies. Expanding the molecular data to include additional genes with different rates of conservation, such as the maturase K (*mat*K) gene with an estimated mutation rate some three times that of *rbc*L (Hilu *et al*., 2003), or the chloroplast nicotinamide dehydrogenase subunit gene *nhd*F at twice the rate (Sugiura, 1989; Olmstead and Sweere, 1994) may be more informative again than the addition of a single sequence.

5.2 Unexpected Species Placements

In the course of this study, a number of specimens appeared in the molecular and combined phylogenies with unexpected affinities outside of the classically defined sections and series. Intriguingly, some of these specimens grouped by molecular data have become associated together with other specimens in small groups possessing consistent leaf anatomical structures. Some of these specimens may represent hitherto unknown species. They will benefit from further attention to properly elucidate their taxonomic individuality and where required, appropriate descriptions and recognition as new species. Specific examples from the molecular phylogeny have been discussed below.

5.2.1 '*L. banksii* 70614.4'

The placement of *Lomandra* series specimen '*L. banksii* 70614.4' was unexpected. This specimen had very different leaf morphology from the other specimens which resolved to this branch, however molecular data from both nuclear and chloroplast organelles firmly locates it to this point. Given the anatomy, classical affiliations and ranges, this species was expected to be more closely allied to *L. multiflora* species. However, given its tropical distribution, specifically far northern Cape York, New Guinea and New Caledonia (where it is segregated as *L. insularis*; see Jaffré *et al*. 2001) and the inflorescence anatomy of section *Lomandra*, the broad thin leaves and the inflorescence structure in *L. banksii* may represent adaptations to tropical conditions and/or pollination strategies, although this would require further investigation.

5.2.2 '*L. sonderi* 61220.5' and '*L. ordii* 70102.2'

These two specimens from sect. Sparsiflorae were placed unexpectedly within the largest *Capitatae* assembly in the *trn*L C–D analysis. This is strongly corroborated by the *trn*L E–F and also broadly supported by the ITS2 phylogeny as well. Anatomically, these specimens have thicker, broader leaves than the specimens surrounding them which tend towards fine leaf profile. Unlike '*L. banksii* 70614.4' these species share geographic location with those Capitatae with which they have been associated.

5.2.3 '*L. brittanii* 70103.1'

This specimen resolves distantly from the primary or secondary *Sparsiflorae* clades and shows significant affinity with the *L. suaveolens* clade. The clade branching structure of this section is a duplicate of that found in the ITS2 results, which is loosely supported with the polytomy members of this branch in both of the *trn*L phylogenies. Anatomically, the three other specimens of this branch ('*L.* sp. nov. (Perup) 70922.1', '*L. suaveolens* 61212.2' and '*L. suaveolens* (Perup) 70614.1') all have very similar fine leaves, with five vascular bundles in each. '*L. brittanii* 70103.1' appears very similar, except with a thinner and finer leaf section again and only three vascular bundles.

5.2.4 '*L. multiflora dura* 61220.7' and '*L. collina* 70103.2'

These species initially seem to be a somewhat eclectic combination; however, closer anatomical examination reveals that in cross section *L. collina* has resemblance as a laterally abbreviated *L. multiflora* subsp. *dura*. These specimens also have very similar cuticle surfaces, with stomata appearing to be embedded below the leaf surface and protected by protrusions (possibly papillae) from the longitudinally adjacent epidermal cells. This grouping is directly derived from *trn*L C–D, is poorly supported in the *trn*L E–F and absent in ITS2 molecular phylogenies. While these species do have potentially overlapping geographic domains, the specimen of *L. collina* used in this study was sourced from western populations, well removed from the central location of the *L. multiflora* subsp. *dura* collection site. It is also possible that the (sterile) sampled plant represented a juvenile *dura* as these can look like plants of *collina.*

5.2.5 '*L. suaveolens* (Northern Sandplains) 70614.6'

Located in a separate node of the phylogeny and thus distantly removed from the *Capitatae* clade, this species has distinct differences in both molecular and leaf anatomy from the other *L. suaveolens* specimens. This location is primarily from the *trn*L E–F, but both *trn*L C–D and ITS2 place this group very distant to the other two *L. suaveolens* species, the affinities of which are well supported across all domains of information obtained for this study. This specimen is one of the unusual "comb section" leaves, with thickened epidermis and stomata only on one side located at the bottom of channels in the leaf surface. This species is also basal to this arm of the

phylogeny which is heavily characterised by leaf surface invagination. More than half of the invaginated-leaved specimens found in this study occur in this exclusive branch of the phylogeny. For these reasons, this accession from the northern, exposed sandy heath near Eneabba in Western Australia is suspected to be a new species of *Lomandra*, different from *L. suaveolens*, and further characterisation of this species including detailed flower morphology is highly recommended, particularly as the collections on which this study was based are sterile.

5.2.6 '*L. maritima* 61213.2' and '*L. odora* 70102.7'

As with '*L. suaveolens* (Northern Sandplains) 70614.6' these two examples of *Sparsiflorae* seem misplaced, however as with the previous example, these species form a clade characterised by surface invagination. Individual molecular results do not specifically define this branching, although as a point of interest the ITS2 result places *L. odora* immediately with *L. nutans*, a species which has a high degree of similarity in its leaf cross sections and cuticle to *L. odora*.

5.2.7 '*L. longifolia* (LM300)'

This species is a commercial cultivar, described in its patent (US Patent PP15420) as being discovered as an anomalous specimen in a large scale cultivar of *Lomandra longifolia* 'var katrinus' (an unpublished, horticulturally-derived name). It is noted as being unable to produce viable seed, and requiring asexual techniques (division or tissue culture) to be propagated. The high-confidence value affiliation with '*L. confertifolia* subsp. *pallida* 61129.4', has been primarily influenced from the

*trn*L E–F region results, partially by the common grouping present in *trn*L C–D and only at a broad series level in the ITS2. When the close affiliation with *L. confertifolia* is taken into context with the apparent sterility of this species, this may be a potent indicator of an interspecific hybrid, possibly facilitated by the dioecious nature of the genus and the artificially close proximity of other species in commercial production facilities. In a natural environment, a sterile species such as LM300 is highly unlikely to survive very long, whereas this unique event and anthropomorphic intervention has allowed it to persist which, given the uniqueness of the specimen, may give it extraordinary utility. Chromosomal studies, particularly karyology or further molecular work covering alternative organelle genomes may provide the answer to the habit of this unusual specimen, and in turn, this may give insight to the reproductive patterns of *Lomandra*. Assuming that LM300 is a hybrid of *L. longifolia* and *L. confertifolia*, it would confirm the ability of disjunct species to hybridise and additionally suggests that in *Lomandra*, the chloroplast genome is paternally inherited which is rare in angiosperms (Birky, 1995) but not unknown, as in the example described by Yang (2000) for creosote (*Larrea*: Zygophyllaceae).

5.2.8 '*L. glauca* (Potato Point) 70501.5'

This specimen has resolved to a branch of the tree distant from the main group of the *Capitatae*. This arrangement is moderately supported across all three DNA domains and not sufficiently refuted by anatomical observations to be repositioned elsewhere in the phylogeny. Its placement in the analyses suggests that it may be an aberrant *L. confertifolia* form, but the sample was sterile at the time of collection.

5.2.9 '*L. hastilis* 61212.1'

As the sole representative of section *Macrostachya*, the position of this species in the middle of an otherwise complete *Sparsiflorae* branch was unexpected. This placement of this specimen is consistent over all three individual molecular domains. When leaf anatomy is considered, this specimen with its distinct leaf margin consisting of large non-staining fibres is associated with a number of other species with similar features. This suggests that the molecular results are consistent and correct. The inclusion of the other *Macrostachya* species (*Lomandra teres*, which was unfortunately unobtainable for this study) may assist to further elucidate this relationship.

5.2.10 '*Xerolirion divaricata* 70122.1'

The placement of *Xerolirion divaricata* within the primary *Sparsiflorae* branch is supported by all three DNA regions. The unique anatomy of this species of reduction of leaves to virtual points on long stems and the terminal unisexual flowers suggests that *Xerolirion* represents a highly specialised and aridity-adapted species. The position of *X. divaricata* inside the lowest branch of *Lomandra* makes a compelling argument for the reclassification of this species and direct inclusion as a member of the *Lomandra* genus. This result reinforces commentary made by Rudall and Chase (1996), who noted that unpublished *rbc*L sequence data placed *Xerolirion* within *Lomandra*.

5.2.11 '*L. elongata* 70614.7'

This specimen resolves away from its section and was allied closely with another similarly displaced specimen, '*L. longifolia* 61130.3'. These species were all obtained from the same general geographic region, although since this spans very large areas, this is of low relevance. In their anatomy, '*L. elongata* 70614.7' and '*L. longifolia* 61130.3' share a number of leaf section features, including a distinctive diamond shape to the vascular bundles caused by gross enlargement of the parenchymatous outer bundle sheath cells. This affiliation also receives strong support from *trn*L E–F and ITS2 molecular trees with weaker generalised grouping from *trn*L C–D, which suggests this affiliation of disjunct species is correct and that the identification of these vouchers may be erroneous.

5.2.12 '*L. glauca* (Broadleaf) 61207.1'

Another commercial cultivar, this specimen has very different anatomy and genetics to the other *L. glauca* specimen used in this study. This specimen is thought to represent a probable error in identification or naming by the commercial entity, although as with previous commercial examples it may also represent an accidental hybridisation influenced by the artificial proximity of unrelated species during industrial propagation. As with '*L. longifolia* (LM300)' the association of this specimen with other taxa is primarily driven by *trn*L data, which when working under the assumption of functional hybridisation and assuming accidental pollination, again suggests that *Lomandra* species inherit chloroplasts from the staminate parent. Uniparental inheritance of non-nuclear DNA compartments is commonplace for

living systems. For reviews of this phenomenon, refer to Birky (1995, 2001), and Xu (2005).

5.2.13 '*L. sonderi* 61212.4'

With two accessions of *L. sonderi* in this study with highly congruent leaf anatomies, the vast distance between the locations of these specimens was entirely unexpected. The association of both species into their respective clades is supported on all three individual molecular phylogenies. Anatomically, '*L. sonderi* 61212.4' is very different from '*L. micrantha* subsp. *teretifolia* 70115.6', however they were obtained from the same western region. This association may represent a natural hybrid, and will require further work to define clearly.

5.2.14 Salvator Rosa/Carnarvon National Park Specimens

Five specimens from the Salvator Rosa section of Carnarvon National Park in the Queensland central tablelands were obtained as part of this study.

'*L. multiflora* sp. aff. (Salvator Rosa) 70731.1'

'*L. filiformis* aff. (Salvator Rosa) 70731.2'

'*L. longifolia* aff. (Salvator Rosa) 70731.3'

'*L. leucocephala* subsp. *leucocephala* (Salvator Rosa) 70731.4'

'*L. multiflora* subsp. *multiflora* (Salvator Rosa) 70731.5'

Of these specimens, '*L. longifolia* aff. 70731.3' and '*L. leucocephala* subsp. *leucocephala* 70731.4' appear the most appropriately organised, as both resolve into

clades with related taxa. In contrast, the affiliations of the other samples are not quite so apparent. In the molecular phylogeny '*L. multiflora* sp. aff. 70731.1' grouped strongly with '*L. multiflora* subsp. *multiflora* 70103.7', although the close relationship here within the *L. multiflora* and *L. patens* species complex at both genetic and anatomic level makes absolute identification difficult. Nevertheless, based on these results this specimen has been inferred to be *L. multiflora* subsp. *multiflora*, as has the '*L. multiflora* sp. aff. 60404.1' specimen in the same branch.

Similarly, '*L. filiformis* aff. 70731.2' appears nested deep within the lowest *L. filiformis* complex, but unlike the previous example, this specimen is strongly associated with two other examples of *L. filiformis* subsp. *coriacea*. Unexpectedly, '*L. multiflora* subsp. *multiflora* 70731.5' resolves to the same branch of the molecular phylogeny despite its tentative *L. multiflora* identification. The molecular results are all consistent in their placement of these two taxa within the larger *L. filiformis* species cluster. Conversely, when considering the leaf anatomy of this group, the broad flat '*L. multiflora* sp. aff. 70731.5' appears to be more similar to surrounding species whereas '*L. filiformis* aff. 707031.2' showed a highly-unusual semi-ovoid leaf transect. It is important to note the significant homology between the cuticles of either of these specimens, and also how closely they resemble the cuticle of the seemingly more appropriately placed '*L. longifolia* aff. (Salvator Rosa) (70731.3)'. The relative geographic isolation of this national park may be influencing the evolution of common characters from shared environmental pressures. Additionally, the intermix of genetics and anatomy with these species may represent long periods of isolation from external gene pools and repeated hybridisation events. These specimens may benefit from additional attention over

other genes and propagation of flowering specimens to determine their precise affiliations.

5.2.15 *Lomandra preissii* (various specimens)

Lomandra preissii was sampled independently three times for this study. Two of the putative *L. preissii* specimens (61313.3 and 61213.4) resolved with other *Sparsiflorae* taxa on the molecular phylogenies. Although separated into the two clades encompassing this Section they do bear some anatomical similarity to those specimens associated with them. Conversely '*L. preissii* 61129.1' occurs in a branch with closer affinity to '*L. preissii* 61213.3', but as a sole representative of *Sparsiflorae* amidst the smaller sect. *Lomandra* clade of the phylogeny. The distribution of these three specimens over the entire phylogeny is influenced by all three DNA domains and the notable differences in their leaf anatomy (refer to Appendix A for sections and cuticle preparation images). '*L. preissii* 61129.1' and '*L. preissii* 61213.4' have some similarities in their leaf anatomy, however '*L. preissii* 61213.3' is strikingly different from either; having a significant thickening of the abaxial surface which is lacking stomata, visible rhombohedral crystals in both surface cuticle preparations and short to medium length papillae on both sides of the leaf. The abaxial side of the leaf is mildly corrugated with stomata primarily restricted to the lower regions of the corrugations; however these are not as well developed as the deep invaginations present on a high proportion of specimens in this branch of the phylogeny. The genetic and anatomical diversity of these *L. preissii* specimens strongly suggest that a single species epithet is insufficient to describe this group of specimens and warrants further investigation.

5.3 Summary and Conclusions

The phylogeny of the *Lomandra* complex is vastly more complex than the *Flora of Australia* review of the group by Lee and Macfarlane (1986) would suggest. The distribution of species with widely varied leaf morphology which shows precious little comprehensive pattern forming; combined with the non-contiguous arrangements of the anatomically derived sections and series as defined by Lee and Macfarlane implies a complex ancestry and evolutionary patterning across the genus. The morphological similarity of species that appear to be only genetically distant is interpreted as the direct result of environmental selection pressures favouring a particular leaf design, or the presence of a particular reproductive strategy selecting for a generalised flower structure in a process analogous to "convergent evolution". Convergent evolution is often quoted as the "fly in the ointment" of morphology based studies (Chase 2004) and his review of the monocot relationships noted that not all molecular studies had morphological support. Returning to the example of the Elephant Shrew (*Macroscelididae*) examined by Nishihara (2005), environmental adaptations can be a powerful force in the shape and development of organisms and this must always be considered, especially when examples have relatively recent common ancestors.

The possibility of hybrids (natural or otherwise) in the genus as suggested by the data gathered for '*L. longifolia* LM300' and '*L. glauca* (Broadleaf) 61207.1' specimens may be a source of the complexity of the phylogeny. Given the basic assumptions in phylogenetic studies of rare natural hybrids and consistent biparental inheritance of genomic domains, if hybrids have been occurring and persisting

naturally, these will have significant effect on the interpretation of the data, as discussed in Hansen *et al*. (2007) and exemplified by Chat *et al*. (2004) in their assessment of Kiwifruit (*Actinidia*: Actinidiaceae). Determining inheritance patterns of organelles with discrete genomes and expanding the sampled gene regions to encompass mitochondrial (Laroche *et al*. 1997) and additional nuclear regions (Small *et al*. 2004, and Syring *et al*. 2005) may resolve this difficulty.

The combination of molecular phylogeny with leaf anatomy identified a number of specimens with unexpected placements and features. These specimens may represent putative new species of *Lomandra*. Some, as in the examples of '*L. suaveolens* (Northern Sandplains) 70614.6' and '*L. preissii* 61213.3' encompass both leaf features and positions in the molecular phylogeny that reinforces the unique nature of these specimens. Others, such as the Salvator Rosa section of Carnarvon National Park specimens '*L. multiflora* subsp. *multiflora* 70731.5' and '*L. filiformis* aff. 70731.2' have unique leaf anatomy that contrasts their position within the molecular phylogeny.

This study has shown that the assumptions of evolutionary relationships within *Lomandra* on the basis of staminate inflorescence and the consequential division of the *Lomandra* into four sections and two series is erroneous (Choo 1969). While the anatomy of the *Lomandra* is extremely useful for the identification of species and not diminished in the slightest by the results of this study, the fragmented island distribution of the classically defined sections and series across the molecular phylogeny advocates that these anatomical divisions within *Lomandra* are unreliable indicators of phylogeny. The parallel or

convergent evolution of multiple concurrent *Lomandra* lineages under similar environmental conditions towards homologous anatomy suggests an intrinsic genetic variability and extensive adaptability embedded in the *Lomandra* genome; and that the anatomical variability used in the prior segregation of the genus may be obscured by the adaptive response to dynamic environmental conditions rather than a specific inherited character.

Expansion of the anatomical analysis to encompass leaf anatomy also does not provide a useful phylogeny of *Lomandra*. As noted in Chapter 3, broad macroscopic-scale leaf anatomy is too plastic to be useful for phylogenetic reconstruction. Investigations of microscopic scale features showed similar results to inflorescence characters, where small islands of characters occasionally occur, but there is no underlying relationship between leaf anatomy and molecular phylogeny. However, as with inflorescence, the use of microscopic features of both the mid-leaf section and the leaf cuticle, in particular the stomatal structures, has proved useful for the identification of individual species. A key has been generated on the basis of this data and has been presented in Appendix B (p. 341).

The other genera grouped with *Lomandra* (*Acanthocarpus*, *Chamaexeros*, *Romnalda* and *Xerolirion*) by Conran (1998) as the informal "*Lomandra* complex" all fall within *Lomandra* on the molecular phylogeny, which is highly supportive for the formal recognition of the "*Lomandra* complex". Three of the four genera (*Acanthocarpus*, *Chamaexeros* and *Romnalda*) group together on a single branch of the phylogeny. Within this branch, *Acanthocarpus* forms a close-knit, well-supported clade; however the relationships between the other ingroup genera are less

well defined. The representative specimens from *Chamaexeros* and *Romnalda* intergrade in this study which is suggestive of a closer relationship between these genera which may benefit from additional study with additional gene sequences. The affiliation of these bisexual, hermaphroditic genera within the unisexual, dioecious *Lomandra* clade supports the recognition of the '*Lomandra* complex' but as an expanded *Lomandra* and likely represents an example of sexual evolution and then reversion. This association of hermaphroditic taxa within bisexual taxa, and the apparent reversal of sexual development represent an opportunity for further exploration of the underlying mechanisms in the evolution of plant sexuality.

The results of this study are supportive of previous affiliations of these taxa as per Kunth (1903), where *Acanthocarpus* and *Chamaexeros* are placed as sections of *Lomandra* and Lauterbach (1913), where the type species of what is now *Romnalda* was described as a species within *Lomandra*. The location of *Xerolirion* within *Lomandra* on the molecular phylogeny, along with strong measures of confidence indicates that a monotypic generic rank for this species is unwarranted. I propose that *Xerolirion* be reduced to synonymy and reassigned as a new combination: *Lomandra divaricata*.

Molecular systematics has proved invaluable for the determination of the phylogeny of *Lomandra*. It has revealed putative new species as well as some surprising affiliations with other genera and has been an important step forward in the understanding of the true structure of the Laxmanniaceae. This study has highlighted the difficulty that can be encountered when attempting to combine morphological features with gene sequence data and the potential error that may

occur when relying on a single domain of information. Determination of the manner in which the genes controlling anatomical features have evolved across the *Lomandra* may answer how the separate branches of *Lomandra* have arrived at morphologically similar adaptations to ecological opportunities that have resulted in the cladistically confounding classical sections and series. The circumscription of *Lomandra* should be revised to include *Xerolirion*, with further investigation encompassing additional molecular markers ought to be devoted to the taxonomic status of *Acanthocarpus*, *Chamaexeros* and *Romnalda* as these taxa potentially should also be redefined under synonymy as part of an expanded *Lomandra*.

This study has successfully resolved the relationships within the *Lomandra* and revealed unexpectedly close associations within the *Lomandra* complex subsection of Laxmanniaceae. The molecular results presented advocate a complex evolutionary history where prior groups derived from anatomical features were mostly polyphyletic. The results also suggest that intra-species hybridisation may have influenced the modern relationships of taxa; however, the precise method by which the four main branches of the *Lomandra* appear to have evolved so many common characters was indeterminate. Additionally, this study has also successfully determined leaf features useful for species identification and generated an identification key for *Lomandra* based on microscopic leaf features.

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