Phylogenetic Relationships and Molecular Dating of the Genus *Meconopsis*

(by Alan Elliott in March 2012, written up by Alan and edited by Evelyn Stevens)
(a.elliott@rbge.ac.uk)

I realise that this was a complicated talk but at the same time it could in no way be a comprehensive outline of the molecular techniques and the concepts of phylogenetics. I understand that this is a new area for many people and I am happy to receive questions about any of the molecular techniques, phylogenetics, taxonomy and nomenclature that people may have (see email address above).

Meconopsis Literature in 2011

In 2011 there were a number of scientific, peer reviewed publications about aspects of *Meconopsis*. These ranged from the publications of new species, the extraction of novel compounds, genetic relationships, molecular dating and conservation implications for ethnomedicinal species. See the references at the end.

What is Phylogenetics and why use it?

Phylogenetics is the grouping of individuals, species, genera etc. based on their evolution, but as evolution cannot be directly observed we have to infer these relationships based on characters. These characters can be, as in the past, morphological (e.g. flower shape), or they can be the more recently used molecular-based characters. Molecular data give us an opportunity to study relatively independent sets of characters that can be dealt with avoiding the potential bias that is associated with morphological characters.

The standard method for visualising phylogenetic relationships is by producing cladograms, also referred to as phylogenetic trees. Generally this is done using molecular sequence data which comprise long lines of C,G,A & Ts that correspond to sequences of bases of an organism's DNA (see p. 2 for an explanation of C, G, etc and bases). These data can be analysed in a number of ways using different statistical tests to give weight to the groupings that are produced. The tests are done using specialist computer software packages but are all based on statistical methods of data analysis. One very good reason that computers are used is because an analysis of 10 species will result in over 2 million possible sets of relationships (possible phylogenetic trees). And for 50 species, the estimated number of species of *Meconopsis*, the analysis would result in approximately 2 .68 x 10^{74} possible sets of relationships. Reducing these vast numbers to much more meaningful ones is accomplished by means of a number of statistical methods. One of these is called Parsimony.

Parsimony.

This method assumes that evolution is "lazy" and that the changes in the structure of the DNA between species occurs with the least number of changes. The software compares all the possible trees and only keeps the ones that have the least number of changes. This can quite often be hundreds if not thousands of trees with different topologies, i.e. different groupings of species. The software then looks for the groupings that occur most often and gives these a statistical value. For example, species W, X, &Y occur as a related group 90% of the time in the different trees, whereas W, X and Z only occur 30% of the time as a closely related group.
Other statistical methods include "Maximum likelihood" and "Baysian inference, these generally result in more robust statistical outcomes than parsimony, but no more details will be given of these here.

DNA

DNA is the blueprint and building block of life. It carries all the hereditary traits, good and bad, that are passed down from parents to children.

DNA comprises a double helical structure. Each strand of the helix is made up of large number of four different nucleotide bases. Each of these nucleotide bases only interacts with one other type of nucleotide base on the other strand. This is called complementary base pairing.

- Adenine (A) bonds only to Thymine (T),
- Cytosine (C) bonds only to Guanine (G).

This arrangement of two nucleotides binding together across the double helix is called a base pair. In the laboratory, the two strands of DNA can be separated by high temperatures or chemicals. As a result, all the genetic information in the double-stranded sequence of a DNA helix is made available for the molecular laboratory techniques in which DNA is replicated. This replication is carried out many times and is essential in order to produce enough material for laboratory analysis.

Other useful properties of DNA are: i). it is negatively charged, because of its phosphate backbone, and ii). large fragments of DNA move more slowly than small fragments. These two properties are useful when it comes to visualising the DNA on agarose gel columns. Agarose gel is a cast, clear, porous jelly, made from algae polysaccharides, that allows DNA to move through it. Firstly, the DNA is stained with a dye and loaded on to the agarose gel column. An electric current is then passed through the gel and as a result of the DNA being negatively charged, it will migrate to the positive electrode. Secondly, large fragments move slowly through a gel while small fragments move more quickly. This process results in numerous discrete bands and the resulting stained bands fluoresce when the gel is placed under UV-light so that they can then be compared against each other and to DNA that is of a known fragment length

Genome

A genome is the entirety of an organism's hereditary information. Within plants there are three genomes that DNA can be sourced from, each with its own properties.

Nuclear Genome (nDNA)

- DNA stored in the cell's nucleus
- It has biparental inheritance, information is passed from the male and female
- Generally evolves more quickly
- Contains 35-45,000 genes
- Arranged in multiple chromosomes

Chloroplast Genome (cpDNA)

- DNA stored in the photosynthesising organelles, i.e. the chloroplasts, of a plant cell
- Usually Single parent inheritance
    - Angiosperms – mother
    - Gymnosperms - father
- 120(ish) genes
- Complete sequence has been elucidated for 144+ species

Mitochondrial Genome (mDNA)

- DNA in the organelles, i.e. mitochondria, that create chemical energy in almost all living things
- Usually single parent inheritance
- 50-60 genes
- Complete sequence has been elucidated for 50+ species

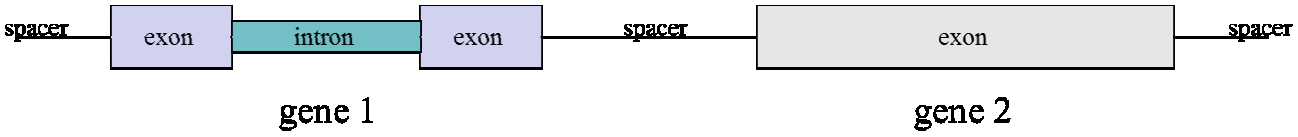
- Complicated restructuring- the gene order is different between different species and it is therefore difficult to extract DNA and compare species.

Within a genome, DNA is characterised by three distinct pieces (see the diagram below).

- The Exons are the coding genes. These are functional in that they code for proteins and RNA and therefore evolve very slowly.
- Introns are between exons. They have some function and evolve a bit faster.
- Spacers – the only function of these is to separate genes and so these evolve more quickly

Evolution of organisms depends on mutation (changes) in the genes. If *Exons* mutate there is the distinct possibility that this could cause a defect that would affect the fitness of the plant. An unfit plant may not reach maturity and would therefore not be able to pass the mutation on through sexual reproduction. The same is true for *Introns*.

Spacers are the most useful part of DNA to look for relationships between taxa as any changes here tend to have no effect on the health of the organism because this DNA has no real function. This is termed selective neutrality, the mutations reflect evolutionary change but do not apply any selection pressure that may improve or hinder the fitness of a plant, and through time, the genetic line.



Molecular techniques

There are many molecular techiques that could be applied to *Meconopsis*, but I will only go into detail about the ones that have been published in the literature.

Microsatellite Markers are most likely to be the best method for dealing with closely related groups that normal sequencing and morphology does not help with. This technique has shown potential in dealing with the *horridula* complex and I am sure will be useful in identifying the Big Blue cultivars.

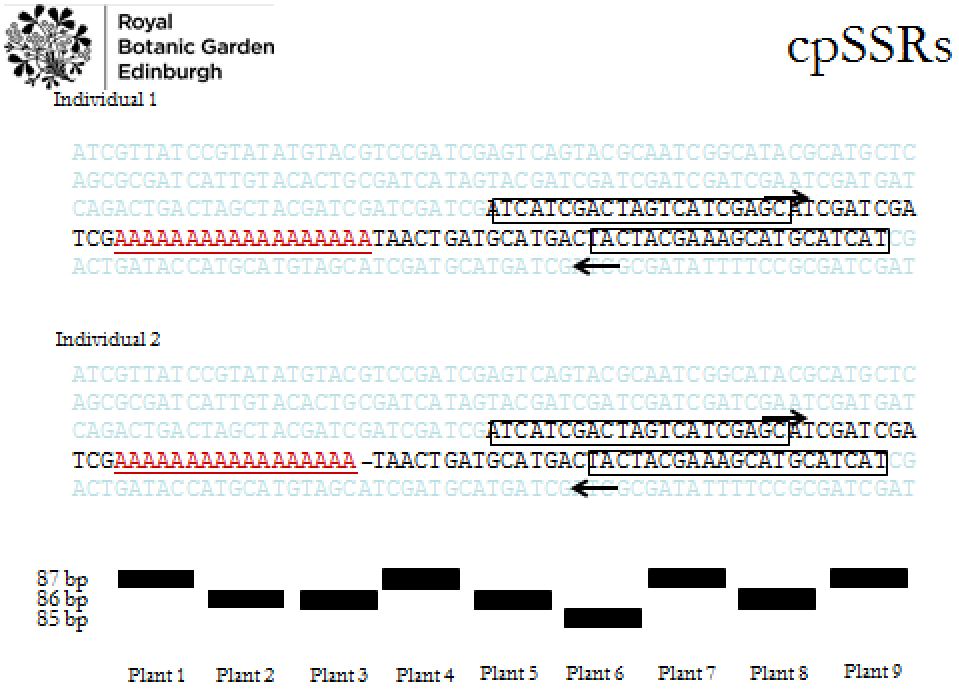
Sequence Data is what is most often used to create phylogenetic trees. This is because it yields the most useful data for elucidating genetic relationships and allow for closely and distantly related groups to be compared, depending on the specific bit of DNA are you looking at. However, sequence data falls down when there are groups of recently evolved species. This is because they have not yet acquired enough in the way of mutations to show relationships clearly.

Microsatellite Markers

A microsatellie marker is a short fragment of the molecular code that allows closely related species or cultivars to be differentiated and often identified. Typically these are short mononucleotide arrays e.g "AAAAAAAAA" (8-14 repeats) but you can also have dinucleotide arrays such as "CGCGCGCGCG". Different length variants represent distinct genetic markers.

As an example, let us look at Cholorplast DNA, only inherited down the maternal line, and you have samples from morphologically close-looking plants but you suspect they might be different. Say Sample 1 has a repeat of AAAAAAAAAA and Sample 2 has a repeat in the same place in the genome but only has AAAAA. This difference will pass down the maternal line for both samples. If you test enough plants and this distinction hold trues then you have a potential marker.

The next step is to find DNA at either end of the repeats that are the same in all the samples. This becomes the Microsatellie site. This can be used to amplify the small regions, from new and potential unknown samples, and it becomes a useful identification tool.



In the example above there are two different length repeats also known as markers. Individual 1 has a repeat of 18As, and Individual 2 has a repeat of 17As. This single base reduction is not enough of a difference to be seen on a standard gel. However using a short very specific primer set, i.e the code in the black box in the diagram above, you can extract a much smaller fragment and therefore get rid of the DNA that is before and after the smaller section. When this is then run on a gel then the difference in size would now be visible. So for the 9 tested plants, looking for the markers, they fall into three groups.

- Plants 1, 4, 7, & 9. The full length of the fragment including the primers is 87 base pairs (87bp in the diagram) so it must have the 18A marker
- Plants 2, 3, 5 & 8. The full length of the fragment including the primers is 86 base pairs so it must have a 17A marker
- Plant 6. The full length of the fragment including the primers is 86 base pairs so it must have a new 16A marker indicating a greater diversity than previously known.

There have been two recent publications that have used microsatellites in the context of *Meconopsis*.

The first paper Zhao et al. (2011) published 13 microsatellites designed to help the investigations with the Chinese species in the *M. horridula* complex. Hopefully these markers will aid in the identification of this group where the morphological characters are at best unreliable.

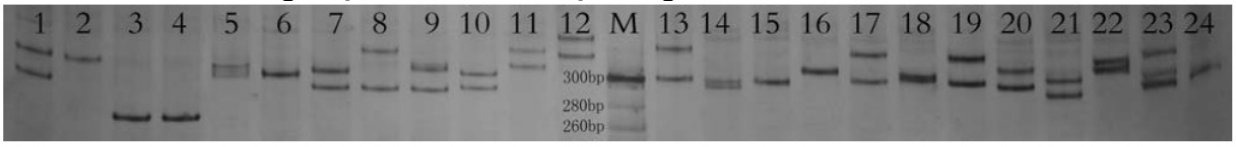


Figure 1 - SSR (Simple Sequence Repeats) profile of the MC26 locus in 24 specimens of *M. horridula*. Lane M: 20 bp DNA ladder, Lanes 1-24: samples.

The second paper, Rong & Zhiling (2011b) used a species specific marker, a short piece of code that was not a sequence repeat but a change in a few of base pairs. This basically facilitated a "presence or absence test" using as the marker, the particular fragment of DNA, to differentiate between *Meconopsis impedita* and *Meconopsis racemosa*. Tibetean medicine, according to the paper, relies heavily on *Meconopsis impedita* but often *Meconopsis racemosa* is sold in markets as *M. impedita*. The use of microsatellites in this case is as an identification tool. When the morphology is too similar to tell what the plant is, or it is lacking key characters, the unique microsatellite marker is enough to identify the plant. In the paper the researchers even managed to extract DNA from the crude drugs, obviously completely lacking any of the morphological characters to say what plant it came from. The DNA from the drug revealed the presence of *Meconopsis impedita*. It was a case of tackling a specific problem but it demonstrates the potential of microsatellite markers in the identification of difficult to identify species.

The use of microsatellites, once found, are what is most likely to be useful in working out molecular relationships between the Big Blue cultivars.

Sequence Data

This is the most expensive technique at approximately £3-6 per sample. This covers from extraction to sequencing. This method does give you the most scope for analysing and revealing evolutionary relationships. Sequence data is what is used to elucidate phylogenetic relationships and can give statistical weight to the grouping of genera, species etc.

The primers that extract DNA for sequencing and phylogenetic work are often termed "universal" as they work on many different groups and this allows for testing between closely and often distantly related groups of genera and species.

Quite a lot of work has been successfully done in recent years on *Meconopsis.* The current protocol for sequencing *Meconopsis* is to use one region from the nuclear genome, (the Internal Transcribed Spacer (ITS)) and one from the Chloroplast genome, (the TrnL-F spacer region). The use of these two regions between them offer relatively good resolution when it comes to demonstrating relationships between species of *Meconopsis* and also the relationships of sister groups (the next most closely related branch on the tree) within the Papaveraceae (see diagram below).

At the moment the published data covers about 50% of the total species in the Papaveraceae, but this does include representatives of all of George Taylor's series and subgenera.

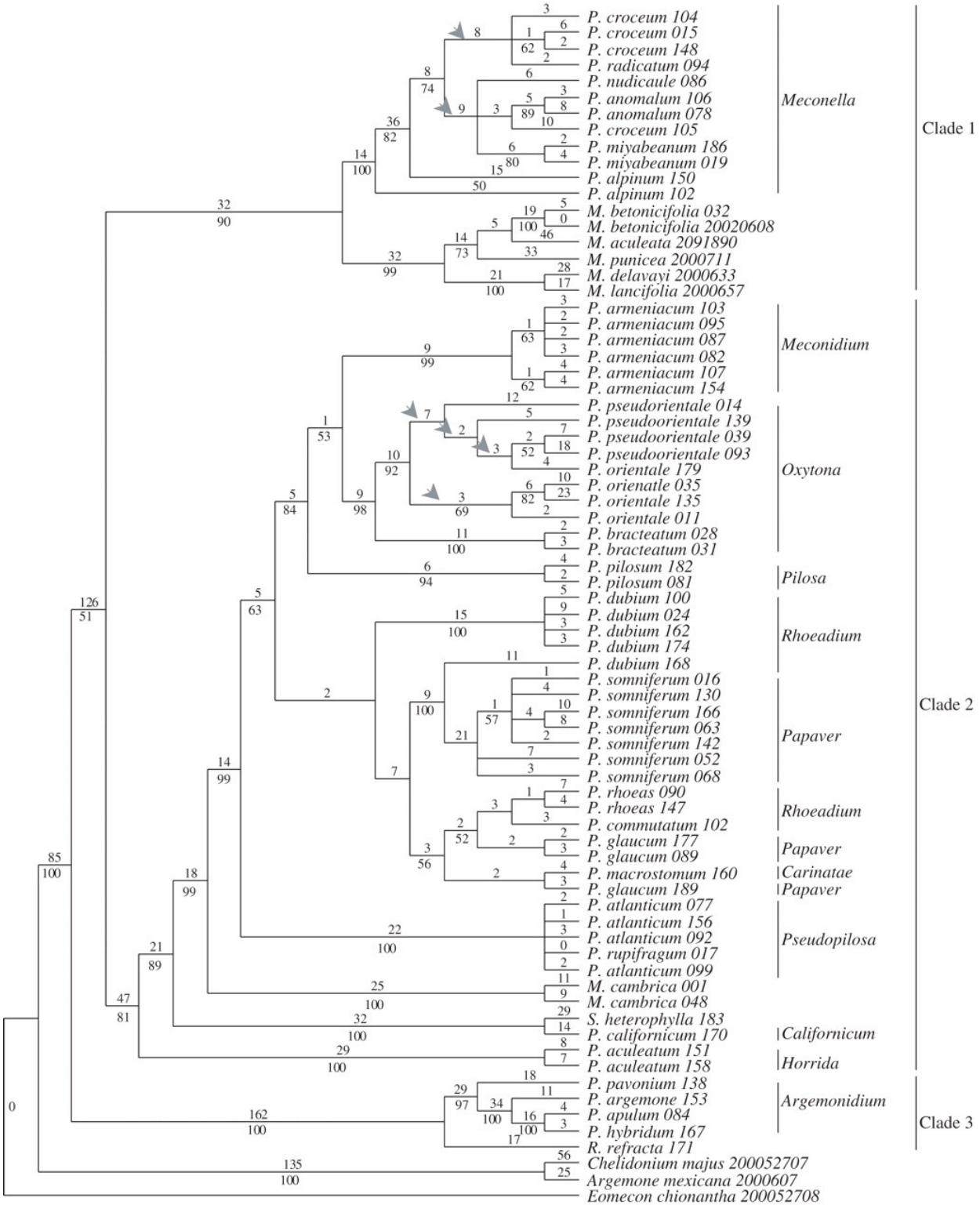
Relationships in Papaveraceae

Carolan et al. (2006) carried out a fairly comprehensive study on the relationships within Papaveraceae found that they resolve into three distinct groups (clades). (See phylogenetic tree below)

- Clade 1 (90% bootstrap support – the percentage of times a particular clade appears in all the possible different trees showing the relationships) comprises P. sect. *Meconella* (100% bootstrap support) and Asian *Meconopsis* (99% bootstrap support).
- Clade 2 (81% bootstrap support) comprises the remaining sections of *Papaver*, *Meconopsis cambrica* and *Stylomecon heterophylla*.
    - Within clade 2, the main group of sections (*Carinatae*, *Meconidium*, *Oxytona*, *Papaver*, *Pilosa*, *Pseudopilosa* and *Rhoeadium*) are evident and well supported (99% bootstrap support). Of these, sect. *Pseudopilosa* is most divergent and monophyletic within *Papaver* (100% bootstrap support). Support for the positioning of *Meconopsis cambrica* as sister, the next closest related clade, to the core sections of clade 2 and

its separation from the other representatives of *Meconopsis* was 99% bootstrap support.

- Clade three *Papaver* sect. *Argemondium*. Is sister to the genus *Roemeria*.



One of eight equally most-parsimonious trees generated from the combined ITS and *trnL–F* data sets* using maximum parsimony. Support for each node is represented by bootstrap percentages (BP) below the branch (shown only when >50% and consistent with the strict consensus tree). Numbers above each branch indicate the numbers of character changes along each lineage. (*see page 5 for an explanation of ITS and *trns.-F* data sets

Relationships within *Meconopsis* (see phylogenetic tree below)
The molecular results showed clearly that *Meconopsis chelidonifolia*, *M. smithiana*, *M. villosa* should be better treated as *Cathcartia* which is an older lineage than the main group of Asian *Meconopsis*

The phylogenetic tree also allowed the testing of Taylor's concept of how species group together in his monograph. The results showed that the groups indicated from the genetic (molecular) relationships are remarkably similar to Taylor's, which were based only on morphology.

The only differently placed species are *Meconopsis simplicifolia* and *Meconopsis sinuata*.

- *M. simplicifolia* came out in series Grandes instead of Simplicifoliae
- *M. sinuata* came out in series Primulinae instead of Aculeatae.

Both of these different placements do actually make a little bit of sense when the morphology is looked at.

- *M. simplicifolia* is a big blue poppy like *M. grandis* and *M. betonicifolia*.
- *M. sinuata* shares a remarkably similar black style with *Meconopsis primulina*

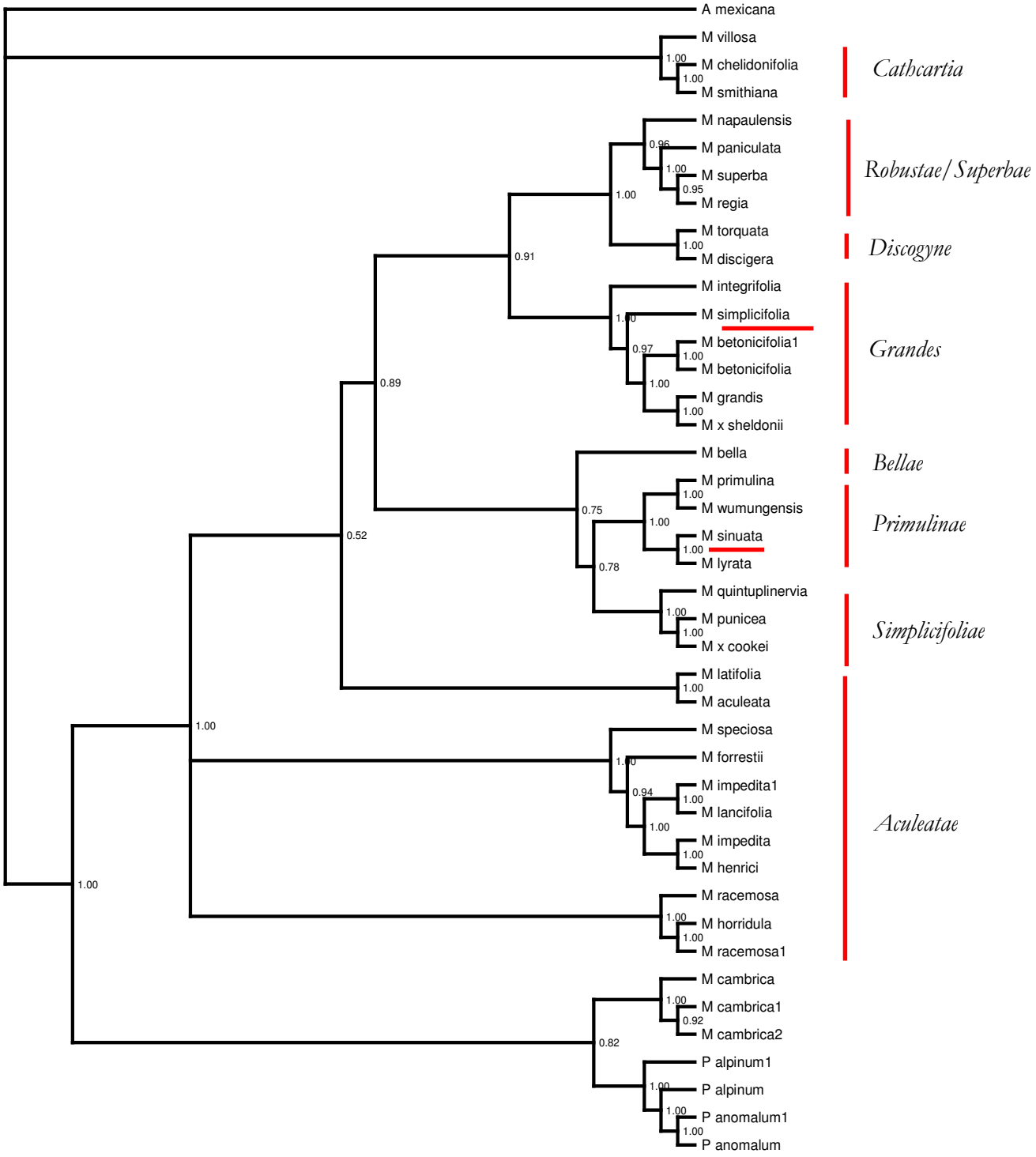


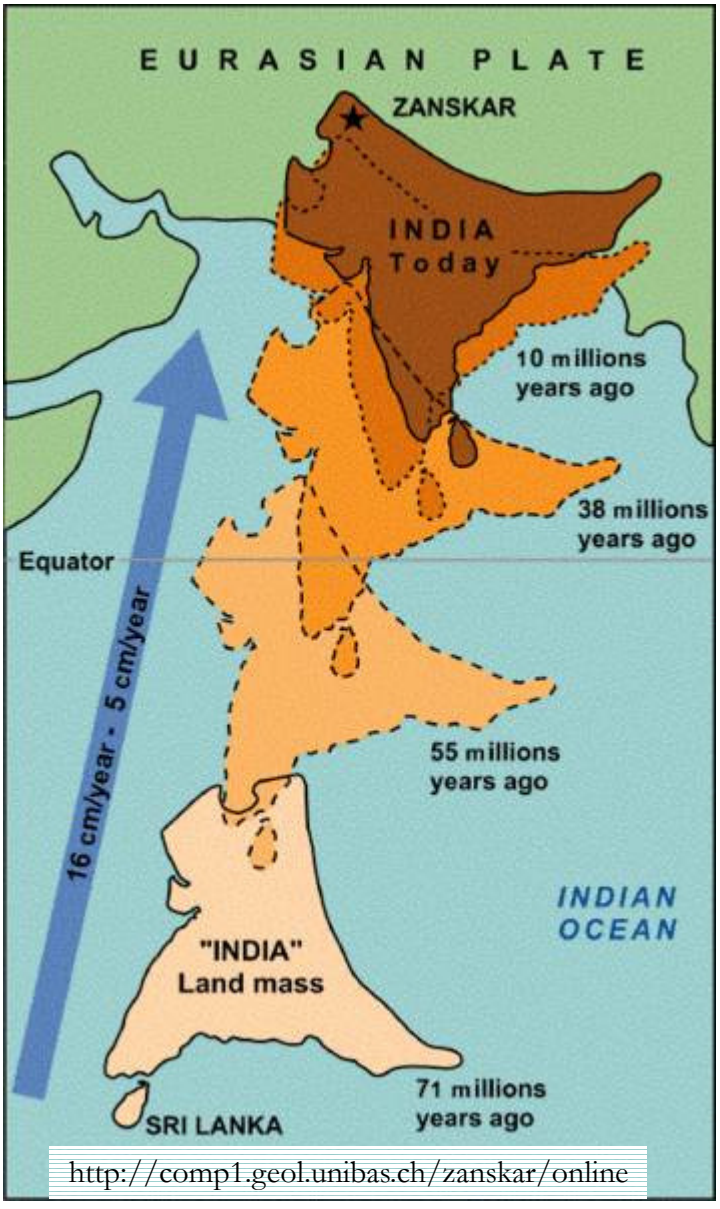
Figure 1 Cladogram of relationships based on ITS and trnL-F intergenic spacer regions. Testing Taylor's intra-generic ranks, the only two incongruent taxa *Meconopsis simplicifolia* and *Meconopsis sinuata* are underlined in red. Values are pp-values (posterior probabilities): this means < 0.90 poor support, >0.90 reasonable statistical supports, >0.95 good statistical support, 1.00 very good statistical support.

Molecular Dating

Is a technique where analysis software can estimate the divergence times, this being the point in a phylogenetic tree where the different branches split. The timings are based on what is known about the rate of change in genomes. So basically the more changes that are in a particular lineage the longer "something" has been around and the software can only tell you, for example, if something is twice the age of something else. But the real beauty of this software is that once you are able to incorporate fossil or geological dates into the data you can calibrate particular branch splits. The software can then put ages on to the other splits, rather than relative dates.

Kadereit *et al.* (2011) used a number of events e.g. fossils, plate tectonic dates etc., to calibrate their tree to make their dates as accurate as possible. The three main calibration dates they used to date the divergence dates within Papaveraceae were:

- Eudicots 130 millions of years ago (myr ago)– Papaveraceae is an early evolving lineage. This put a date on when the family first appeared.
- *Paleoaster* 74-64 myr ago – the earliest known fossil poppy that shared characters with existing *Papaver* taxa.
- Split of old world from new world *Papaver* occurred 52myr ago – a value based on plate tectonic dates



Using the estimates of when groups have diverged it can be tied into dates from other fields such paleoclimatology and dates in the geological record to try and make sense of the divergence dates. This map illustrates the position of the Indian plate at various points in time prior to its colliding with the Eurasian Plate, the cause of the uplift of the Himalaya and Tibetan Plateau.

For example the estimated divergence dates of *Papaver* from *Meconopsis* and *Papaver* sect. *Meconella* is given as 28mya +11my. These tie with the significant period of cooling and increased aridity c.28mya at the end of the Oligocene. And this is what Kadereit *et al.* (2011) suggest is the cause of the difference in an aspect of the ecology of the two groups. *Papaver* began to exploit the increasing availability of semi-arid ecological niches that came with the global climate trend of cooling and increased aridity; whereas *Meconopsis* and *Papaver* sect. *Meconella* retained the more mesic (wet/humid) ecological habitats of the ancestral lineage of *Cathcartia*. Kadereit's study focussed on the dates between the different genera and did not look at any of the internal dates of diversification within *Meconospis*. There is one that stands out in my mind to talk about.

To make sense of the results from molecular dating work, additional data from other scientific fields such as geology or climatology are used. For example a technique that used lasers to work out the ratio of Argon isotopes in crystals of metamorphic and igneous minerals dated the beginning of the post-mid-Miocene volcanism and therefore the time of uplift of the Tibetan plateau to 13 Myr ago (Turner et al. 1993). This date ties in with the estimates of the diversification of the Superbae/Robustae/Discogyne suite of species of *Meconopsis* at 14myr ago. This group has its centre of diversity firmly in the Himalaya and just over into the Tibetan plateau. This is a good example of work from different fields combining to add more weight to each others' results as they are independent of each other.

Something that is interesting is the suggestion from the results that *M. horridula* complex diverged c. 20myr ago from the next closest group, but from the only two samples of *M. horridula* and *M. racemosa* investigated, it was shown that they diverged as late as in the last million years. However the limited sampling of the aggregate would need to be much increased before a more accurate time could be given.

Taxonomic Implications of the Phylogenetic Work

This is not new information. As far back as 1997 a paper on the relationships of genera within Papaveraceae showed that *Meconopsis* formed three distinct and not closely related lineages.

The basal lineage of *Meconopsis* should be treated as the genus *Cathcartia* Hook.f.. This is already being done by taxonomists. David Long in the Flora of Bhutan (1984) treated *Meconopsis villosa* as *Cathcartia villosa* based purely on morphological grounds. More recently, in the Flora Nepal project account of *Meconopsis* written by Paul Egan, his taxonomy followed the molecular distinction and gave useful characters to distinguish the groups.

Major herbaria such as The U.S. National Herbarium in Washington are following the distinction and Edinburgh has already reordered the herbarium at family and genus level to reflect the molecular relationships. Genera will be reordered and species moved accordingly as new data become available so Papaveraceae, in particular *Meconopsis*, *Cathcartia* and *Papaver,* should be reordered in the near future.

Meconopsis villosa (Hook.f ex Hook.) G.Taylor and *Meconopsis smithiana* (Hand.-Mazz.) G.Taylor should be called *Cathcartia villosa* Hook. ex Hook. and *Cathcartia smithiana* Hand.-Mazz. respectively. However, there is a problem with the two other described species: *Meconopsis chelidonifolia* Bur. & Franch. & *Meconopsis oliverana* Franch. & Prain ex Prain. These two taxa have never been described as anything but *Meconopsis* and there is therefore no validly published *Cathcartia* name at the moment.

Nomenclatural Implications of Phylogenetic Work

The large Asian *Meconopsis* lineage needs the name conserved with a new Type specimen before the name conservation can take place. Under the rules of botanical nomenclature if the Type of a genus, in this case *Meconopsis cambrica*, is removed from the rest of the species in the genus then the generic name goes with the Type and an alternative name is needed. There are mechanisms in the Botanical Code of Nomenclature to conserve a name with a new Type. However this is a fairly long process. The new Type should be the earliest unambiguous name. Working through the possibilities:

De Candoelle's original publication of *Meconopsis napaulensis* DC. questioned if it was truly a *Meconopsis* and therefore this species name is excluded. *Meconopsis paniculata* (D.Don) Walp.

and *Meconopsis simplicifolia* (D.Don) Walp. were both originally described as *Papaver by* David Don in Prodromus Florae Nepalensis (1825) and this fact therefore excludes those.

The next available suitable candidate to become the Type for the genus is *Meconopsis wallichii* Hook.f.

(Since giving the talk, Grey-Wilson has published the Conservation proposal for the name *Meconopsis* using *Meconopsis regia* G. Taylor as the new Type for the genus in *Taxon*, Volume 61, Number 2, 13 April 2012 , pp. 473-474(2) which can be accessed online freely on the Taxon website.)

Taxonomic Implications of Phylogenetic Work

Wider implications of the molecular phylogenetic work being done are that *Papaver* is likely to become a much smaller genus: a significant amount of taxonomic work needs to be done.

Firstly *Papaver* Sect. *Argemonidium* should be merged into the genus *Roemeria* and secondly the Arctic-Montane species of *Papaver* sect. *Meconella* must either be combined with *Meconopsis* or given generic status. Despite close genetic affinities between *Meconopsis* and *Papaver* sect. *Meconella* there is no apparent overlap in species as they resolve to two distinct clades. Also with the morphological differences which pertain, it seems sensible that *Papaver* sect. *Meconella* should not be lumped with *Meconopsis* but be given generic status.

Future Work

Xiao Wei's current phylogenetic work will include many more taxa and another chloroplast spacer region to help in the understanding of the relationships between taxa. She is also revising the taxonomy of Papaveraceae and as a result there will be a major overhaul of names and potentially, as James Cobb predicted in 1994, it's "Good-Bye *Meconopsis*".

There have been three Botanical Congress meetings, 1999, 2005 & 2011, where the name *Meconopsis*, could have been conserved under the rules of Botanical Nomenclature and it has been well known about. Being pragmatic if the name changes and is done in accordance with the rules, I'll go with it. The plants we know and grow will remain the same, only they'll be called something different, but it doesn't mean we have to. A good example is from 1834 when *Azalea* was sunk into *Rhododendron* and caused an outcry from gardeners, but 180yrs later we still call them Azaleas despite their generic name being *Rhododendron*.

For my part I am looking to sequence as much Nepalese material as I can as part of my PhD project. The main focus is *Clematis* but I am looking at 10 other genera as well, including *Meconopsis.* I am sure *Meconopsis* will have a very different story to tell when compared with *Clematis* regarding their separate evolutionary paths in the Himalaya.

I am also sure that molecular data can and will be able to bring to light the parentage of the Big Blue cultivars and may even yield simple markers that can be used to distinguish them in a lab and with the cost coming down and the advent of DIY kits ultimately you might even be able to do much of the work in your kitchen.

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