Wieringam J.J. & Gühl, K. (2005). Species and generic delimitation in Bikinia and Tetraberlinia (Leguminosae, Caesalpinioideae) using ITS an AFLP. In: S.A. Ghazanfar & H. Beentje (eds.), African Plants: Biodiversity, Ecology, Phytogeography and Taxonomy, pp. 00–00. Royal Botanic Gardens, Kew.

SPECIES AND GENERIC DELIMITATION IN *BIKINIA* AND *TETRABERLINIA* (LEGUMINOSAE, CAESALPINIOIDEAE) USING ITS AND AFLP

JAN J. WIERINGA[1,2] & KERSTIN GÜHL[1]

[1]Wageningen University, Biosystematics group, National Herbarium of The Netherlands – Wageningen University Branch, Gen. Foulkesweg 37, 6703 BL Wageningen, The Netherlands.
[2]corresponding author: Jan.Wieringa@wur.nl.

Abstract

We have assessed the monophyly and internal topology of *Bikinia* and *Tetraberlinia* along with some other systematic and methodological questions using AFLP analyses. In addition to AFLP analyses we tried to determine the sister group of these two genera by adding additional sequences to an existing ITS data set. Our analyses suggest *Julbernardia* is the closest related genus, but also *Icuria* is a candidate since the position of this genus remains unclear. Although ITS provides us with some good resolution at the generic level, we have to conclude ITS is not a good marker in this group due to the presence of several non-homologous copies. Evidence for a monophyletic *Bikinia* is quite strong. However, more evidence is needed to test the monophyly of *Tetraberlinia*. Within *Bikinia*, only a clade consisting of *B. aciculifera* and *B. durandii* was supported by a jackknife analysis. We show that *B. le-testui* and *B. pellegrinii* are separate species. Aberrant *Bikinia* material from the Crystal Mountains in Gabon proved to be a new species. A sapling collected under a tree of *B. le-testui* could be identified as a hybrid between this species and *B. media*. Another new species, *T. apiphila*, is clearly related to *Tetraberlinia*, although it also shares some morphological characters with *Bikinia*. We demonstrated that AFLP results can be reproduced and that the errors of such replications fall within the variation present at the population level. AFLP can discriminate between different populations of a single species, even with high jackknife support. In the sample of genera studied here the AFLP technique provides high resolution at generic level and we even expect it to work between several closely related genera. Finally, we describe how the AFLP technique can be used to identify hybrids.

Résumé

Délimitation spécifique et générique chez *Bikinia* et *Tetraberlinia* (Leguminosae, Caesalpinioideae) grâce à l'utilisation d'ITS et d'AFLP. Nous avons évalué la monophylie et la topologie interne de *Bikinia* et *Tetraberlinia* ainsi que d'autres aspects systématiques et méthodologiques, en utilisant des analyses AFLP. Par ailleurs, nous avons essayé de déterminer le groupe-sœur de ces deux genres en complétant les données ITS existantes par des séquences additionnelles. Nos analyses suggèrent que *Julbernardia* est le genre le plus proche mais également que le genre *Icuria* est un candidat car sa position reste incertaine. Bien que l'ITS permette une bonne résolution

au niveau générique, nous devons conclure que l'ITS n'est pas un bon marqueur dans ce groupe du fait de la présence de nombreuses copies non-homologues. Les preuves de la monophylie de *Bikinia* sont assez évidentes. Par contre, d'autres preuves sont nécessaires pour tester la monophylie de *Tetraberlinia*. Au sein de *Bikinia*, un seul clade constitué de *B. aciculifera* et *B. durandii* a été supporté par une analyse jackknife. Nous montrons que *B. le-testui* et *B. pellegrinii* sont des espèces distinctes. Le matériel aberrant de *Bikinia* provenant des Monts de Cristal au Gabon s'est révélé être une nouvelle espèce. Une plantule collectée sous un arbre de *B. le-testui* pourrait être un hybride entre cette espèce et *B. media*. Une autre nouvelle espèce, *T. apiphila*, est clairement apparentée à *Tetraberlinia*, quoiqu'elle partage aussi certains caractères morphologiques avec *Bikinia*. Nous avons démontré que les résultats AFLP pouvaient être reproduits et que les erreurs de telles réplications étaient expliquées par la variation inter-population. L'AFLP peut différencier des populations d'une même espèce, même avec l'aide d'une analyse jackknife. Au sein de l'échantillon de genres étudié, la technique de l'AFLP fournit une haute résolution au niveau générique et nous pouvons même nous attendre à ce qu'elle fonctionne entre différents genres très proches. Finalement, nous décrivons comment la technique AFLP peut être utilisée pour identifier des hybrides.

Key words: AFLP, *Bikinia*, hybrid, phylogeny, *Tetraberlinia*

1 Introduction

The genera *Bikinia* Wieringa and *Tetraberlinia* (Harms) Haumann comprise at present 17 species, which occur in rain forests in West and Central Africa. They are medium-sized to large trees, which may constitute co-dominant or even monodominant forest (Wieringa, 1999). *Bikinia* and *Tetraberlinia* are related to the genera *Aphanocalyx* Oliv., *Brachystegia* Benth. *Icuria* Wieringa, *Julbernardia* Pellegr., and possibly *Michelsonia* Haumann (Gervais & Bruneau, 2002; Wieringa & Gervais, 2003: Bambijt-clade). Although this group of genera has high support in phylogenetic analyses, the relationships within this clade remain unclear. In most analyses these genera come out as monophyletic, but relationships between the genera and between species within each genus are poorly resolved or without support.

Recent collections from Gabon and Cameroon provided material of a new species of *Tetraberlinia*. This new species, *Tetraberlinia apiphila* Wieringa ined., recognised on floral characters, clearly fits within *Tetraberlinia*, but because it also shares a few morphological characters with *Bikinia*, cladistic analyses based on morphological data that include this species result in a paraphyletic *Tetraberlinia*, though without support (Wieringa & Gervais, 2003).

Other new (sterile) collections of a *Bikinia* from the Crystal Mountains in Gabon fall in between *Bikinia le-testui* (Pellegr.) Wieringa and *B. pellegrinii* (A. Chev.) Wieringa. In the notes of both species Wieringa (1999: 227 & 239) mentions such intermediate material, which may either constitute a separate species, be of hybrid origin or challenges the delimitation of both species.

A pilot AFLP study that included only eight samples of *Aphanocalyx*, *Bikinia*, *Julbernardia* and *Tetraberlinia* provided some proof that AFLPs may resolve some nodes where other phylogenetic methods using morphology or sequencing fail, and that in this clade the technique may be applied around and above the generic level (Wieringa & Zevenbergen, 1999). However, AFLP studies focussing on more than one genus or on tropical groups like Caesalpinioideae are rare, and hence little data exists on the applicability of this method for different systematic questions.

This study aims to test whether AFLP results can be reproduced, at which level AFLPs can find differences among samples, whether AFLPs can be used to assess relationships between related genera and whether AFLPs can help identify hybrids. Next to these methodological questions, we would like to solve a range of systematic questions: What is the sister group to the *Bikinia–Tetraberlinia* clade? What is the phylogenetic position of *Icuria*? What is the internal topology of *Bikinia* and *Tetraberlinia* and are these two genera monophyletic? Are *Bikinia pellegrinii* and *B. letestui* separate species and does the aberrant material from the Crystal Mountains belong to a separate species or is it a hybrid between these two? Where does the new species *Tetraberlinia apiphila* fit and can AFLPs prove whether or not a seedling belongs to this (in sterile state) cryptic species? To address the questions above the generic level we will use sequences of the rDNA internal transcribed spacer (ITS), while we expect the questions on and below the generic level to be most powerfully resolved with AFLP.

2 Material and methods

2.1 ITS-sequences

To identify which genus should be considered sister to the *Bikinia–Tetraberlinia* clade and to gain a better understanding of the position of *Icuria*, we have sequenced ITS of 9 additional samples of *Aphanocalyx*, *Bikinia*, *Julbernardia* and *Tetraberlinia* (Table 1). DNA isolation and ITS sequencing follows the protocol of Bakker et al. (1998) using only fluorescent labelling. These sequences have been included in the data set as used by Gervais & Bruneau (2002) and Wieringa & Gervais (2003). Alignment was performed manually using Megalign. The data set was analysed using both parsimony and Bayesian inference. A second parsimony analysis was conducted that excluded *Icuria*.

2.2 AFLPs

AFLPs were performed on 26 samples of *Bikinia*, *Julbernardia* and *Tetraberlinia* (Table 1). To test reproducibility of the results, three samples were used twice. AFLPs were performed according to the ABI PRISM 377 protocol of Perkin-Elmer. As restriction enzymes we used EcoRI and MseI. For amplification we used Goldstar Taq polymerase. The first pre-amplification PCR was run using pre-selective primers E01 (Eco+A) and M02 (Mse+C). The selective amplification PCR was run using three different primer combinations: M48E35 (Mse+CAC, Eco+ACA), M59E35 (Mse+CTA, Eco+ACA) and M61E36 (Mse+CTG, Eco+ACC). We used fluorescent labelling; E35 is a 6-FAM (blue) fluorescent primer, E36 a JOE (green) fluorescent primer. The PCR products and an added internal size standard (ROX 500) were analysed on a 5% polyacrylamide Long Ranger (BMA) gel using an ABI PRISM 377 automated sequencer. Resulting trace-files were analysed using Genographer 1.6.0 (Benham, 2001). Bands were scored as either present or absent, in a few cases as unknown (?). The resulting data matrix was checked manually and subjected to a parsimony analysis.

2.3 Data analyses

The parsimony analyses were performed using PAUP* 4.0b8a (Swofford 2002) on a PowerMac G4. Heuristic searches were performed with 100 random addition sequence replicates and tree bisection-reconnection branch swapping. Branches of zero length were collapsed. Robustness of the results was tested by jackknife analyses (36% deletion, fast stepwise addition) with 10000 replicates. The outgroup in all analyses was

TABLE 1. Species and voucher specimens (first collector + number) of the samples used for the AFLP analysis and/or for additional ITS sequencing.

A	S	Species	Voucher	Provenance
	AY615899	*Aphanocalyx djumaensis*	Wieringa 4149	Gabon, Lopé
	AY615893	*Bikinia aciculifera*	Wieringa 4503 (tree)	Gabon, Chaillu Massif, Ikobey
*		*Bikinia aciculifera*	Wieringa 4503 (sapl.1)	Gabon, Chaillu Massif, Ikobey
*		*Bikinia aciculifera*	Wieringa 4503 (sapl.2)	Gabon, Chaillu Massif, Ikobey
*		*Bikinia aciculifera*	Wieringa 4533	Gabon, Chaillu Massif, Ikobey
*		*Bikinia durandii*	Wieringa 4515	Gabon, Chaillu Massif, Ikobey
*		*Bikinia durandii*	Wieringa 4479	Gabon, Chaillu Massif, Ikobey
*	AY615894	*Bikinia le-testui* ssp. *le-testui*	Wieringa 3795	Gabon, Mékambo
*		*Bikinia le-testui* ssp. *le-testui*	Wieringa 3927	Gabon, Mt. Sassamongo
**		*Bikinia le-testui* ssp. *le-testui*	Wieringa 4606	Gabon, Chaillu Massif, Mbigou
*		*Bikinia le-testui* ssp. *le-testui*	Wieringa 4681 (sapl.2)	Gabon, Chaillu Massif, Bongolo
*	+	*Bikinia le-testui* ssp. *mayumbensis*	J.J.F.E. de Wilde 11088	Congo (Brazzaville)
*		*Bikinia le-testui* × *B. media*	Wieringa 4681a (sapl.1)	Gabon, Chaillu Massif, Bongolo
	AY615891	*Bikinia media*	Wieringa 4543 (tree)	Gabon, Chaillu Massif, Mouila
**		*Bikinia media*	Wieringa 4543 (sapl.1)	Gabon, Chaillu Massif, Mouila
*		*Bikinia media*	Wieringa 4543 (sapl.2)	Gabon, Chaillu Massif, Mouila
*	AY615892	*Bikinia media*	Wieringa 4633	Gabon, Chaillu Massif, Mbigou
*	AY615896	*Bikinia pellegrinii*	Wieringa 3757	Gabon, Mékambo
*	AY615895	*Bikinia pellegrinii*	Wieringa 3944	Gabon, Mt. Sassamongo

TABLE 1. continued

A	S	Species	Voucher	Provenance
*		*Bikinia* spec. nov.	Wieringa 4695 (sapl.2)	Gabon, Crystal Mountains
*		*Bikinia* spec. nov.	Wieringa 4695 (sapl.4)	Gabon, Crystal Mountains
*		*Bikinia* spec. nov.	Wieringa 4695 (sapl.5)	Gabon, Crystal Mountains
*		*Bikinia* spec. nov.	Wieringa 4711 (tree)	Gabon, Crystal Mountains
*		*Julbernardia brieyi*	Wieringa 4678	Gabon, Lebamba
	AY615898	*Julbernardia pellegriniana*	Wieringa 4078	Gabon, Forêt des Abeilles
*		*Julbernardia* spec.[1)]	Wieringa 4037	Gabon, Mt. Sassamongo
**		*Tetraberlinia apiphila*	Wieringa 4129	Gabon, Forêt des Abeilles
*		*Tetraberlinia apiphila*	Wieringa 4148	Gabon, Forêt des Abeilles
*		*Tetraberlinia" bifoliolata"*	Wieringa 4518	Gabon, Ikobey
	AY615897	*Tetraberlinia korupensis*	Burgt 548	Cameroon, Korup
*	+	*Tetraberlinia polyphylla*	Wieringa 3123	Gabon, Chaillu Massif, Lebamba

Column A = used for the AFLP analysis; ** = used twice (replicate); S = used for ITS sequencing, the GenBank accession number is listed; + = already sequenced by Gervais & Bruneau (2002); sapl. = sapling; [1)] *J. seretii* or *J. hochreutineri*.

Microberlinia brazzavillensis A.Chev. A Bayesian Inference analysis was performed using MrBayes 2.01 (Huelsenbeck & Ronquist, 2001). The maximum likelihood model employed 6 substitution types, with base frequencies set to the empirically observed values. Rate variation across sites was modelled using a gamma distribution. The Markov chain Monte Carlo search was run with 4 chains for 1,000,000 generations, with trees sampled every 100 generations, while the first 1000 sampled trees were discarded to allow for the 'burn-in' process.

3 Results

3.1 ITS

The parsimony analysis of the ITS sequences resulted in a small set of trees (Fig. 1) which all show a monophyletic *Julbernardia* and a monophyletic *Tetraberlinia* which is sister to a clade containing both *Bikinia* and *Icuria*. Jackknife (jac) support for *Julbernardia* is fairly high (79%). Four of the included *Tetraberlinia* species receive high support (82% jac) as a clade, but the inclusion in this genus of the single sequence of the fifth species *T. bifoliolata* (Harms) Hauman (which has a long terminal branch) is not supported. The combined clade of *Bikinia* and *Icuria* hardly receives any support (55% jac). The clade containing all *Bikinia*, *Icuria*, *Julbernardia* and *Tetraberlinia* (BIJT-clade) is highly supported with 91% jac, which is a result not obtained previously, and may well be the result of the inclusion of a second sequence of *Julbernardia pellegriniana* Troupin which broke up the previously found long branch leading to this species (see Wieringa & Gervais, 2003). The Bayesian analysis results are similar to the parsimony analysis, except that *Bikinia* is now highly (100% posterior probability: pp) supported as monophyletic with *Icuria* as its sister, while *T. bifoliolata* has moved from within *Tetraberlinia* to being sister to all other *Tetraberlinia*, *Icuria* and *Bikinia*, with high support (98% pp for the clade containing *Icuria*, *Bikinia* and all other *Tetraberlinia*). *Julbernardia* receives high support (100% pp) for being monophyletic, so does the BIJT-clade (also 100% pp). The shift in position of *Icuria* may well be attributed to long branch attraction as discussed by Wieringa & Gervais (2003). In the parsimony analysis where *Icuria* was excluded the *Bikinia* clade is supported by 83% jac, suggesting that the previous lack of support was caused by *Icuria*. Also the *Julbernardia* clade receives a higher support (now 94% jac), while some support emerges for a clade containing both *Tetraberlinia* and *Bikinia* (58% jac). In all ITS analyses some internal structure is present in *Tetraberlinia*. However, in *Bikinia* different sequences of the same species end up in quite different parts of the genus. Gervais & Bruneau (2002) already noted similar discrepancies, but thought they might be due to misidentifications. The inclusion of more *Bikinia* sequences now reveals this is more a structural character of ITS sequences within *Bikinia*, probably due to the presence of different ITS copies as has already been observed in *Aphanocalyx* (Gervais & Bruneau, 2002; Wieringa & Gervais, 2003). Several additional ITS sequences we tried to include were uninterpretable because we did not clone the ITS DNA previous to sequencing. This happened both in *Bikinia* and *Tetraberlinia*.

3.2 AFLPs

The AFLP analysis resulted in 220 bands of which 218 were polymorphic. The parsimony analysis of these AFLP data reveals a highly resolved cladogram (Fig. 2), where the branches to all three incorporated genera (*Bikinia*, *Julbernardia* and *Tetraberlinia*) receive 100% jackknife support. The internal branches of *Tetraberlinia* receive support as well, but several of those within *Bikinia* are not or only moderately supported.

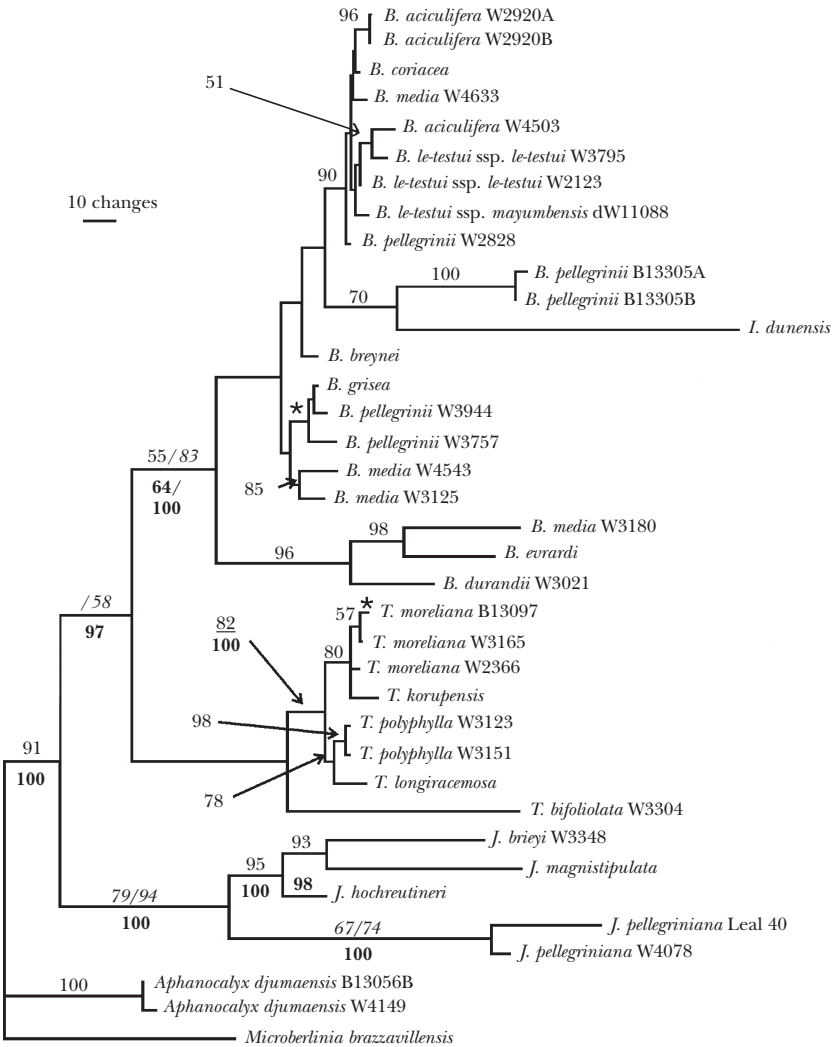

FIG. 1. One of the shortest trees resulting from the parsimony analysis of the ITS data set. For species with more than one sample present the voucher specimens are indicated, where B = Breteler, dW = J.J.F.E. de Wilde, W = Wieringa. When a single collection supplied more than one ITS clone these are referred to as A and B after the collection number. Abbreviated genera are B = *Bikinia*, I = *Icuria*, J = *Julbernardia* and T = *Tetraberlinia*. Jackknife values higher than 50% are given above the branches, branches that collapse in the consensus tree are marked with *. In case jackknife values for a clade (with *Icuria* pruned) differ substantially when *Icuria* is left out of the analysis these are given in italics after a slash. Bayesian posterior probabilities are given in bold below a branch, since the Bayesian analysis puts *Icuria* as sister to *Bikinia*, the Bayesian support for a monophyletic *Bikinia* is given in italics again.

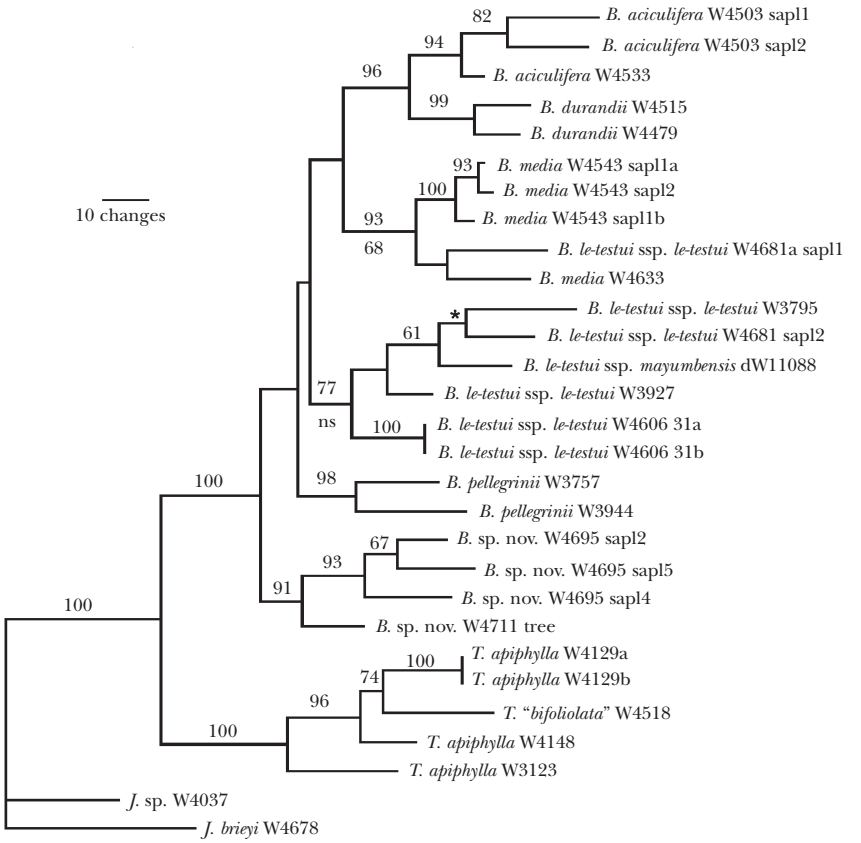

FIG. 2. One of the shortest trees resulting from the parsimony analysis of the AFLP data set. For abbreviations of genera and collectors of voucher specimens see Fig. 1. Samples that were used twice are marked as a and b. Jackknife values higher than 50% resulting from the analysis excluding the hybrid sample W4681 sapl1 are given above the branches, branches that collapse in the consensus tree are marked with *. Jackknife values that differ substantially (>2%) when the hybrid is included are given below the branch.

Of the three replicates that were incorporated in this analysis, one (W4606) came out as completely identical, one (W4129) was slightly different, but still similar enough to be included as sisters in the cladogram, while the third (W4543 sapling 1) was so different that the other included sapling of the same population (W4543 sapling 2, probably a sibling plant) was placed closer to one of the two replicates. Re-examination of the band scoring shows that one of the two samples produced weaker bands, resulting in some bands being scored negative, while the other replicate was scored positive. The sample with the stronger bands was linked to the sibling plant in the analysis, probably based on several shared bands that were missed in the second replicate. The three samples together still formed a 100% jackknife supported clade.

Samples originating from the same population that can probably be considered as siblings (saplings from under the same group of trees) were well supported (82–100% jac) clades in the cladogram (except one, see below), while for all these cases another specimen of the same species was included as well. This shows AFLPs can distinguish populations or groups of siblings of the same species from one another.

There was one striking exception to the grouping of siblings: *Wieringa et al. 4681* sapling 1 was grouped within *Bikinia media* Wieringa while its sibling, sapling 2, ended up between samples of *B. le-testui* ssp. *le-testui*, only the latter being as expected from the initial identification. Re-examination of the scored bands for sapling 1 showed that it shared some, otherwise species specific bands, with both *B. media* and *B. le-testui* (Fig. 3). If sapling 1 is a hybrid between these two species, we would expect it to: 1) have more bands than other samples, 2) share specific bands with both parent species, 3) cause a strong decrease in jackknife support for both parent species (because of the ambiguous signal that will cause placement in both parent species in different jackknife replicates) and 4) the actual plant to be morphologically intermediate between both parents. We have tested these 4 indications of a hybrid origin of sapling 1. Sapling 1 proved to show the highest number of bands of all samples included: 88, being 22% more than the average number of bands. As already reported it did share several specific bands with both parent species. To test the decrease in jackknife support caused by this sample we re-analysed the AFLP data but excluded sapling 1. This resulted in nearly the same cladogram with the same jackknife support values, except those of the two putative parents: the support for *Bikinia media* increased from 68 to 93%, while that for *B. le-testui* went from no jackknife support to 77% (see Fig. 2). Finally we examined the herbarium specimen of sapling 1. We discovered that this single sapling had unusual broad leaflets for *B. le-testui* and that it was not identifiable using the key of Wieringa (1999: 189B191). At the point in this key where *B. media* and *B. le-testui* split (point 3), all values of this sapling fall in the overlap range, while in other specimens always some leaves or leaflets definitely fall in one of the two ranges. A difference between these two species not used

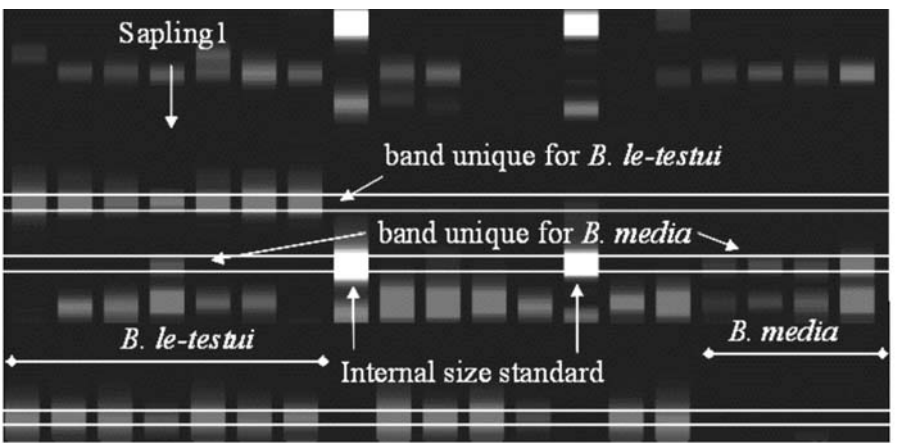

FIG. 3. Part of a composed gel image showing two bands that are unique for either *Bikinia le-testui* or *B. media*, but are both present in the hybrid sapling 1 of *Wieringa et al. 4681a*. The seven lanes on the left represent samples of *B. le-testui*, where the one in the middle (lane 4) is the hybrid sapling. The four lanes on the right represent *B. media*. Of the eight lanes in the middle, six belong to two other *Bikinia* specimens and two represent the internal size standard.

at this point in the key is that the leaf rachis of *B. le-testui* is glabrous above, while it is hairy in *B. media*. In sapling 1 it is sparsely hairy. The other saplings collected under *Wieringa et al. 4681* all key out nicely as *B. le-testui*, as did the two trees under which the saplings were collected. All four tests of sapling 1 being a hybrid proved positive, which leads us to the conclusion that this sapling is of hybrid origin; the herbarium specimen is now labelled *Wieringa et al. 4681a*. For assessing the phylogeny of *Bikinia* this sample should be excluded from further analyses.

The aberrant *Bikinia* material from the Crystal Mountains, morphologically resembling *B. le-testui* and *B. pellegrinii*, clearly concerns a third, yet undescribed, species (Fig. 2, *B.* sp. nov.). This new species is not of hybrid origin, since it has a number of unique bands, while some bands occurring in both *B. pellegrinii* and *B. le-testui* are absent. Although without jackknife support, the position of this new species within *Bikinia* seems to be basal.

Even if we exclude the hybrid sapling from the analyses, the internal topology of *Bikinia* is still not very well resolved. All species now are recognised as clades with between 77 and 99% jackknife support, but only the grouping of *B. durandii* (F.Hallé & Normand) Wieringa and *B. aciculifera* Wieringa in a clade has jackknife support (96%). Branch length between the species-bearing nodes is usually short. These results seem to indicate that the diversification of the genus *Bikinia* happened during a relatively short period.

Most species that have more than one sample in the analysis come out as monophyletic. The only exception appears to be *Tetraberlinia apiphila*. Sterile specimens of this species are indistinguishable from *T. bifoliolata*. The saplings of *Wieringa et al. 4129* were collected under a flowering tree (*Wieringa et al. 4125*) and were assumed to belong to the same species. By including them in this AFLP analysis we hoped to provide proof for this identity. However, these saplings link up with the single specimen of *T. bifoliolata* (*Wieringa et al. 4518*) that was included in this study. Our first interpretation of this result was that the seedlings, although collected under a definite specimen of *T. apiphila*, were actually seedlings of *T. bifoliolata*. However, doubt was cast on this conclusion when we realised that also the voucher for *Wieringa et al. 4518* is also a sterile sapling. It may well be that this specimen, although from an area where so far no unambiguous material of *T. apiphila* has been found, also belongs to this new species. Preliminary results of a larger AFLP analysis that includes several definite samples of *T. bifoliolata* indeed indicate that all four samples included in the present study belong to *T. apiphila* (Wieringa unpublished data).

4 Discussion

With this study we aimed to test several methodological questions. The first was whether AFLP results could be reproduced. The answer is that replicates may differ slightly, but that the differences as found in our study are only large enough to bring confusion within sibling or population level. For higher taxonomic level questions these differences are irrelevant and results can be reproduced.

The second question concerned the level where AFLPs are able to provide resolution. Given the origin of the method – fingerprinting - it is not a surprise it detects differences between individuals. However, it is good to demonstrate that in all cases (excepting the hybrid) all samples of a single species from a single locality not only are grouped together, but also receive a jackknife support of between 81 and 100%, indicating that AFLPs can discriminate between such populations.

A third methodological question concerned the applicability of AFLPs at the level of related genera. Our results at least show that up to the generic level AFLPs remain

very powerful (all three genera receive 100% jackknife support). To be able to test applicability for phylogenetic relationships between genera and to see if it can still generate jackknife or bootstrap support we need to include at least a fourth genus. In the present data set already several bands are present that are shared by all three genera, indicating that at least some signal will be present for a clade containing these three genera as soon as other genera are included.

Our last methodological question concerned the use of AFLPs to trace hybrids. As was demonstrated by the unexpected tracing of the hybrid sapling, AFLPs can indeed be used to trace such F1 hybrids. If this sapling had not been placed among samples of its other, less obvious, parent, it would have been more difficult to identify hybridisation. However, now we have identified hybridisation, we can define some criteria how such hybrids can be located, and these criteria could be used on any AFLP data set to look for such hybrids. The easiest criterion to check is whether any sample has a relatively high number of bands. However, this difference is not very high (20% in our case). A far more powerful tool could be developed from the decrease in jackknife support that is caused by these hybrids. One should develop an automated tool in phylogenetic reconstruction programs that after an initial analysis leaves out single accessions and sets of such accessions. Jackknife or bootstrap supports for resulting clades should be compared to the initial support values for these clades (with the left-out samples pruned). As soon as any positive support shift reaches a threshold value (say 10%) the concerning left-out accessions that caused the shift should be reported as suspect, where the clades that received a higher support can be regarded as a potential parent. A confirmation of a suspected hybrid would be that not one but two species receive significantly higher support values. However, a single increase of support could be caused by the absence of one of the parents in the analysis, in which case a smaller increase may be present only in a clade of species related to the other parent. Our third criterion to identify hybrids, looking for the sharing of species specific bands with more than one species, is very time consuming and can only be performed on samples that are already suspected of being a hybrid or after a run of the above mentioned taxon resampling procedure. We demonstrated AFLPs can easily trace these F1 hybrids. However, it is not sure AFLPs can as easily trace other types of hybrids. Backcrosses to one of the parent species will become less and less obvious. Allotetraploid species on the other hand should be recognisable, as is the case with F1 hybrids, although slowly the number of additional bands may shrink, as will the distortion in support values of both parent species. Still, such hybrid species should remain detectable for quite some time.

An additional methodological result from this analysis is that AFLPs prove to be a good method to delimit species, as has been shown previously by i.e. Zhang *et al.* (2001) and Richardson *et al.* (2003). All conspecific samples (except for the two cases already discussed above) are being grouped together with 77 to 99% jackknife support. Especially in the case of sympatrically occurring species AFLPs may help clarify specific circumscription. For tropical taxa this may be a very welcome tool, since populations of such species can usually not be followed over time or subjected to crossing experiments. However, it is easy enough to take silica gel dried leaf samples of two co-occurring specimens, or even seedlings as in our case, to be analysed later.

Several of the systematic questions we have addressed with this project could be (partly) answered: based on ITS *Icuria* is either sister to *Bikinia,* or to both *Bikinia* and *Tetraberlinia* when we consider the relatively low Bayesian support for the branch leading to *Icuria* & *Bikinia.* However, analyses (partly) based on other genes (Gervais & Bruneau, 2002; Wieringa & Gervais, 2003; Herendeen *et al.,* 2003) place *Icuria* somewhat further away from *Tetraberlinia* and *Bikinia.* To investigate this incongruence, other methods should be sought such as sequencing another region or inclusion of *Icuria* in the AFLP

data set. A definite result from our ITS analysis is the support for the BIJT-clade, indicating *Julbernardia* as the closest relative to *Bikinia* and *Tetraberlinia* (apart from *Icuria*). Since *Brachystegia* and *Michelsonia,* two other close relatives of the BAMBIJT-clade were missing from this analysis, the results should be treated with caution. What the support does tell us though is that the BAMBIJT-clade contains a subclade containing at least *Bikinia*, *Julbernardia*, *Tetraberlinia* and possibly *Icuria* but which does **not** contain *Aphanocalyx*, and that is something previous analyses were not able to establish. This result is corroborated by an analysis of the chemical content of these genera (Kite & Wieringa, 2003), where *Julbernardia* species contained hydroxypipecolic acids and/or hydroxyprolines which were also present in most species of *Bikinia* and *Tetraberlinia*, while these compounds were missing in *Aphanocalyx*, *Brachystegia*, *Icuria* and *Michelsonia*. *Michelsonia* did contain one hydroxypipecolic acid (ovalin), but this compound was not shared with any other species of this group, so no phylogenetic relationships could be established from it. The absence of any of these compounds in *Icuria* could be another indication that the ITS data put it too close to *Bikinia*, *Julbernardia* and *Tetraberlinia*. In three species of *Bikinia* these compounds are absent as well, but this is interpreted by Kite & Wieringa (2003) as a secondary loss, since these species already formed a clade within *Bikinia* in the morphological analysis (Wieringa, 1999) as the two included here are doing in the present AFLP analysis (see below).

Our questions on the *Bikinia le-testui* & *B. pellegrinii* complex have been answered quite well. The two samples of *Bikinia pellegrinii* and those of the five included populations of *B. le-testui* are grouped according to initial identification, and not according to provenance, even while for both sampled populations of *B. pellegrinii*, a sample of *B. le-testui* growing next to it was included. This means that they do represent two different species, although sometimes hard to tell apart. Further material of *B. pellegrinii*, especially from the Chaillu Massif area and from Estuaire province in Gabon, should be added to test if this species is a single species or is composed of an aggregate of sibling species as outlined by Wieringa (1999).

Another definite result from this analysis was that we were able to establish the identity of the aberrant *Bikinia* material from the Crystal Mountains. Both the presence (and absence) of unique specific bands and the phylogenetic analysis of the entire AFLP dataset clearly indicate it constitutes a separate species. So far no flowering material of this species has been collected, the only fertile collection that probably belongs to it is one with young pods (*Breteler, Wieringa & Nzabi 12857*). More material and further morphological research is needed to properly delimit this species. AFLPs may be used to test whether certain samples belong to it as well.

The internal topology of *Bikinia* and *Tetraberlinia* remains poorly resolved. Although we thought we started with three different species of *Tetraberlinia*, we now have to conclude we were probably dealing with only two species. In *Bikinia* we are dealing with a different situation. Since the aberrant material from the Crystal Mountains proved to be a new species, we have 6 different species of *Bikinia* in our analysis. Although the internal topology is entirely resolved, only a single clade has jackknife support within *Bikinia*. It is that of *Bikinia aciculifera* and *B. durandii* with 96% jackknife support. *B. aciculifera* is very closely related to *B. coriacea* (J.Morel ex Aubrév.) Wieringa, which was not included in this AFLP analysis. These three species already formed a clade in the morphological phylogenetic analysis of Wieringa (1999), but without support. These species also do not have hydroxypipecolic acids nor hydroxyprolines. Kite & Wieringa (2003) interpreted this as a secondary loss, which is now corroborated by the support we found for them forming a clade within *Bikinia*. The phylogenetic analysis based only on chloroplast sequences of Gervais & Bruneau (2002) also recognised a weakly

supported clade of *B. aciculifera* and *B. durandii*, while *B. coriacea* was considered as one of the possible sisters to it. Their analysis including also ITS sequences, as well as our present one based solely on ITS sequences, shows *B. durandii* and *B. aciculifera* fairly far apart. The majority of the characters of this Gervais & Bruneau (2002) analysis consists of ITS sequences, rendering it feasible that the different position in these two analyses is caused by the sequencing of non-homologous ITS copies.

Tetraberlinia apiphila (still to be described) shows yellow petals and free stipules, which are typical characters of *Tetraberlinia*. However, it has a few odd characters for this genus like the hairy pods (at least when young), the pink-purple anthers and more or less free adaxial sepals that would better fit in *Bikinia*. It is reassuring that the AFLP analysis indeed puts this species solidly next to *Tetraberlinia polyphylla* (Harms) J.Léonard ex Voorh., with 100% jackknife support. Whether or not the tested seedling belongs to this new species as well, will only become clear when the analysis of the larger data set that is being prepared at present becomes available, but the result will probably be that both the seedling and the alleged sapling of *T. bifoliolata* collected 120 km away from it, belong to the new species.

Concerning the monophyly of *Bikinia* and *Tetraberlinia* we have found good evidence that *Bikinia* is monophyletic. Probably only two species of *Tetraberlinia* were included in our AFLP study and the ITS analysis is not able to support the inclusion of the type of the genus, *T. bifoliolata*, in the clade including the other *Tetraberlinia* species, hence we cannot yet conclude *Tetraberlinia* is monophyletic. However, we were able to establish that the new species *T. apiphila* belongs to the main *Tetraberlinia* clade, even though it shares some morphological characters with *Bikinia*, thus eliminating at least one of the question marks around the monophyly of *Tetraberlinia*.

Wieringa & Gervais (2003) discussed the occurrence of several long terminal branches in the ITS data, and proposed that adding more samples that could break up these long branches might solve part of this problem. Although they did not presume this method to work for the case of *Julbernardia pellegriniana*, apparently it did, because not only support for the position of *J. pellegriniana* as being part of *Julbernardia* increased, but even the support for the BIJT clade was now established for the first time. The new sequence of *J. pellegriniana* differs considerably from the first one, pointing to either a high substitution rate in this taxon, or to the presence of different ITS copies, as are occurring in *Aphanocalyx*, *Bikinia* and *Tetraberlinia*.

4.1 ITS sequencing

As is quite evident from the results of the ITS sequencing, these data do give a signal appropriate for the level we are interested in. However, as also shown and discussed above, the sequencing itself is already problematic and only feasible after cloning. Although more elaborate, this would not be a definite set back for future sequencing. However, the presence of different ITS copies in the same sample gives us far larger problems for the phylogenetic analysis. Even analyses based on cloned ITS will result in gene trees not species trees, as demonstrated by the three different positions at which both *B. media* and *B. pellegrinii* can be found in the present ITS analysis, and the above discussed erroneous placement of a sequence of *B. durandii* relative to *B. aciculifera*. These problems for the analysis might be solved by using the coding method proposed by Wieringa & Gervais (2003), but only if each sequence can be assigned to a set of truly homologous copies. From the present ITS tree one gets the impression there are at least 4 different non-homologous ITS copies present within *Bikinia*, which would mean an enormous amount of cloning and sequencing to get each copy out of each species. In this case it seems better to start sequencing another marker. Regrettably, chloroplast markers so far proved to have

too little variation between these closely related genera, but a combination of several such markers may work. Next to that, AFLPs may still have enough resolution at and above the generic level to bridge the gap between the higher and the lower phylogenetic levels.

Acknowledgements

The authors wish to thank Xander van der Burgt and Jos van der Maesen for providing silica-gel dried DNA samples. James Richardson and two anonymous reviewers made useful comments on a draft of this manuscript.

References

Bakker, F.T., Hellbrügge, D., Culham, A. & Gibby, M. (1998). Phylogenetic relationships within *Pelargonium* sect. *Peristera* (Geraniaceae) inferred from nrDNA and cpDNA sequence comparisons. Pl. Syst. Evol. 211: 273–287.

Benham, J.J. (2001). Genographer 1.6.0. http://hordeum.oscs.montana.edu/genographer

Gervais, G.Y.F. & Bruneau, A. (2002). Phylogenetic analysis of a polyphyletic African genus of Caesalpinioideae (Leguminosae): *Monopetalanthus* Harms. Pl. Syst. Evol. 235: 19–34.

Herendeen, P.S., Bruneau, A. & Lewis, G.P. (2003). Phylogenetic relationships in caesalpinioid legumes: a preliminary analysis based on morphological and molecular data. In: B.B. Klitgaard & A. Bruneau (eds). Advances in Legume Systematics, part 10, higher level systematics. Royal Botanic Gardens, Kew.

Huelsenbeck, J.P. & Ronquist, F. (2001). MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755. see also http://morphbank.ebc.uu.se/mrbayes/

Kite, G.C. & Wieringa, J.J. (2003). Hydroxypipecolic acids and hydroxyprolines as chemical characters in *Aphanocalyx*, *Bikinia* and *Tetraberlinia* (Leguminosae: Caesalpinioideae): support for the segregation of *Monopetalanthus*. Biochem. Syst. Ecol. 31: 279–292.

Richardson, J.E., Fay, M.F., Cronk, Q.C.B. & Chase, M.W. (2003). Species delimitation and the origin of populations in island representatives of *Phylica* (Rhamnaceae). Evolution 57: 816–827.

Swofford, D.L. (2002). PAUP*: Phylogenetic analysis using parsimony, version 4.0b8a. Sinauer Associates, Sunderland, Massachusetts, USA.

Wieringa, J.J. 1999. *Monopetalanthus* exit. A systematic study of *Aphanocalyx*, *Bikinia*, *Icuria*, *Michelsonia* and *Tetraberlinia* (Leguminosae, Caesalpinioideae). Wageningen Agr. Univ. Pap. 99-4: I–XVI, 1–320.

Wieringa, J.J. & Gervais, G.Y.F. 2003. Phylogenetic analyses of combined morphological and molecular data sets on the *Aphanocalyx–Bikinia–Tetraberlinia* group (Leguminosae, Caesalpinioideae, Detarieae *s.l.*). In: B.B. Klitgaard & A. Bruneau (eds), Advances in Legume Systematics, part 10, higher level systematics, pp. 181–196. Royal Botanic Gardens, Kew.

Wieringa, J.J. & Zevenbergen, M.J. (1999). 7 AFLP analyses. Wageningen Agr. Univ. Pap. 99–4: 95–98.

Zhang, L.-B., Comes, H.P. & Kadereit, J.W. (2001). Phylogeny and quaternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. Amer. J. Bot. 88: 2331–2345.