



M.Sc: Biology + Chemistry

Poul Møllers Vej 7, DK - 2000 Frbg.

FAX + (45) 38 34 34 07

Email: info@conifers.dk

Morphology of the hypothetical ancestor of the recent conifers based on phylogenetic evidence

JENNY HELANDER

Address: Poul Mollers Vej 7, 2000 Frbg., Copenhagen, Denmark. Email: info@conifers.dk

Abstract.

Since 1999 it has been possible to state the conifer family tree by means of base sequences from GenBank using *rbcL*, 18S and 28S rDNA. Using independently the three algorithms NJ, MP, and ML plus three different outgroups Gnetales, Ginkgo/Cycadales, Angiosperm/Pteridophyte there is a total of $3 \times 3 \times 3 = 27$ phylogenetic trees each with different assumptions, but apart from a few obvious artifacts all yielding identical results in relation to the conifer family tree, which has later been confirmed by *matK* as well. Since 2000 it has been possible to do the Pinaceae genera tree using the same method, but partly different genes, although the result is not quite as safe.

Based on these phylogenetic trees and the knowledge of the morphology of the recent conifers it is possible to suggest some of the morphological features like morphology of leaves, shoots, and female cones, which may have been present in the common hypothetical ancestor of the recent conifers. In *Sciadopitys verticillata* the verticils are compared to the dwarf shoots in Pinaceae, and may be two long shoots infrequent grow partly together.

KEY WORDS: —phylogeny - multi factorial cladistic analysis - Coniferales - Pinaceae -
outgroup - hypothetical ancestor - *Sciadopitys*.

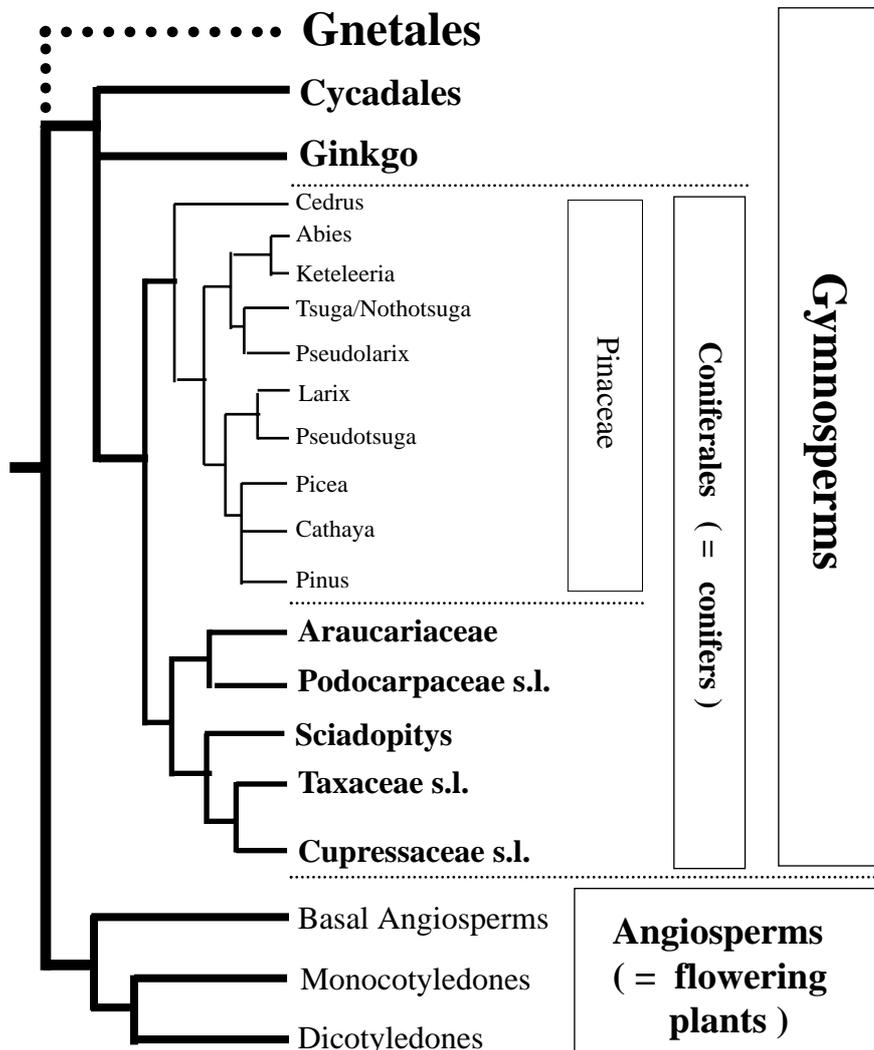
Introduction.

In order to understand the evolution of the conifers it is necessarily not only to state the family relations and phylogeny of the extant species, but also the fossils must be systematized. During the last decade molecular cladistics has shown to be superior to reveal the phylogeny of recent plants, because the number of available molecular characters are immense and the problems weighting them are small compared to working with morphological and chemical data, where the number of significant characters are small and the problems of weighting them are immense. Unfortunately many molecular cladists still prefer to perfect their results using very limited assumptions and thereby instead reducing the reliability considerably. New critical interpretation of morphological characters which yield conflicting phylogenetic results may solve specific disagreements like in Doyle (1998), but new interpretation is usually based on previous knowledge and it does not increase the very limited number of morphological characters.

The needed base sequences for calculating the molecular phylogeny of the conifers on family level have been available in GenBank for about 5 years, but most investigators have concentrated on solving the problem of the position of Gnetales, which has confused and distorted the statement of the conifer family tree. Therefore the position of Gnetales is not discussed here, but it is assumed as a fact that Coniferales is a monophyletic group, which means that Gnetales is not a part of the conifers.

Having stated the conifer family phylogeny by use of molecular cladistics it is exciting to compare the results with the morphological and chemical characters for the different families. Furthermore, a correct morphological phylogeny gives the possibility to estimate some characters of the common hypothetical ancestor of the recent conifers. The knowledge of the evolution of the recent conifers provides the paleobotanists with a better tool for analyzing and systematizing their fossils, most of which are thought to be extinct lineages. This procedure is opposite to the work of Miller (1988), where he tries to deduce the relationships for the living families from the fossils.

Phylogeny of Coniferales



Phylogeny of conifers

in relation to other seedplants

Traditionally the higher plants are divided into the class of Angiosperms (= flowering plants) and the class of Gymnosperms (= plants with naked seeds), but it is not known yet, whether Gnetales is a third class.

The family tree of Coniferales is quite safe.

The genera tree of Pinaceae is not quite as safe, but it is most likely that Cedrus is basal in close connection with the Abies/Keteleeria/Tsuga/Pseudolarix-clade, which is sister to the Larix/Pseudotsuga-clade plus the very weak Picea/Cathaya/Pinus-clade which are sisters.

Figure 1.

Molecular phylogeny of Coniferales on family level, own investigation.

The phylogenetic tree of Coniferales is exclusively based on my own unpublished multi factorial investigation from 1999-2001 using *rbcL*, 18S and 28S rDNA base sequences from GenBank by means of PAUP 4.0b2. In order to raise the reliability of the investigation and reveal possible doubt all factors were used independently, i.e. separate trees were made for each of the 3 genes (*rbcL*, 18S, 28S) using each of 3 different algorithms (NJ, MP, ML) and using 3 different outgroups (Gnetales, Cycadales/Ginkgo, "a close Pteridophyte + a close Angiosperm" yielding same results as "all" Pteridophytes and some Angiosperms) making a total of 27 trees (in reality more than 200 trees have been made). Details are given in addendum I.

All these 27 phylogenetic trees of Coniferales were identical on the family level apart from small artifacts due to a few trees placing Gnetales or Cycadales/Ginkgo inside the conifers, which is improbable as Coniferales is monophyletic not only according to the very strong evidence by Raubeson & Jansen (1992) but also on account of the very specialized morphology of the conifers!

NOTE: The fact that all 27 trees yielded the same result made the resulting tree more than 100 times safer than one tree done by adding all the different base sequences and using one single algorithm and one single outgroup.

Molecular phylogeny of Coniferales on family level, investigations done by others.

Contrary to the statement in Farjon (2003, fig. 2, 3 and 4) all molecular phylogenetic investigations, which include all major conifer families, agree with the in Figure 1 shown phylogenetic tree.

— All the three different cp-cladograms, which are stated in Farjon (2003) fig. 2 are rather obsolete in respect to both the low number of taxa and the lack of all major conifer families. The two cladograms in fig. 3 and the one in fig. 4 are mutually identical and identical with Figure 1 in this paper (apart from the small and artifactitious Taxaceae s.l. splitting in Chaw *et al.* (1997a)) —.

Chaw *et al.* (1997a): 18S; NJ, MP. — Stefanovic *et al.* (1998): 28S; MP. — Chaw *et al.* (2000)*: *rbcL*, 18S (= nuSSU), mtSSU, *rbcL*+18S+mtSSU; ML. — Bowe, Cota & dePamphilis (2000)*: *rbcL*+18S+*cox1*; NJ, MP, ML. — Gugerli *et al.* (2001)*: *nad1*; MP. — Quinn, Price & Gadek (2002): *rbcL*, *matK*, *rbcL*+*matK*; NJ, MP. — Rydin, Kjällersjö & Friis (2002): 28S (= 26S), *atpB*, *rbcL*+*atpB*, 18S+28S, *rbcL*+18S+28S+*atpB*; MP. — Quinn & Price (2003): *rbcL*+*matK*+18S+28S; MP — Price (2003): *rbcL*+*matK*; MP — And (without Araucariaceae) Kjällersjö *et al.* (1998): *rbcL*; MP.

XX*: Some of the trees from Chaw *et al.* (2000)*, the tree in Bowe *et al.* (2000)* using almost the same genes as Chaw *et al.* (2000), and the tree in Gugerli *et al.* (2001)* show Gnetales within the Coniferales.

Some of the cladograms, which are using a small datamatrix, seem to indicate that Taxaceae s.l. (incl. Cephalotaxaceae) is not monophyletic, which is an artifact. The transformation of the common conifer female cone into the unique "Female reproductive structures in Taxales" described by Stützel & Röwekamp (1998) is the one and only event, which creates and defines Taxaceae s.l. (including Cephalotaxaceae) and proves the monophyly. Furthermore the morphological similarities of Taxaceae s.l. are stated by Pant (2000) and Anderson & Owens (2003). The monophyly of Taxaceae s.l. is also inferred from *matK* + nrITS by Cheng *et al.* (2000).

Molecular phylogeny of Pinaceae on genera level.

Pinaceae (Coniferales I) is sister to all the other conifers usually mentioned as Coniferales II. Therefore the genera of Pinaceae can be compared with the families of Coniferales II in some respects, taking in account that Pinaceae is even more conservative than the Coniferales II.

Molecular phylogeny of Pinaceae on genera level has caused much trouble because the cladists have not been aware of the importance of choosing an appropriate outgroup. All the early investigations, which have used a "cousin" (a sister does not exist) from Coniferales II as outgroup, have obtained results, where a genus from the *Picea/Cathaya/Pinus*-clade usually is prior to the rest. Using an ancestor as a more appropriate outgroup the result is quite contrary showing *Cedrus* basal followed by the *Abies* (*Keteleeria*)/*Tsuga* (*Nothotsuga*)/*Pseudolarix*-clade then comes the *Larix/Pseudotsuga*-clade with the very weak *Picea/Cathaya/Pinus*-clade as sister.

NOTE: *Keteleeria* is rather close to *Abies*; *Nothotsuga* is rather close to *Tsuga*; and *Pseudo-tsuga* is rather close to *Larix* in all molecular investigations according to Liston *et al.* (2003).

As only few phylogenetic investigations within *Pinaceae* includes all the known genera it is necessary also to mention the results of the investigations, which include only an essential part of the genera.

Coniferales II as outgroup yielding *Picea/Cathaya/Pinus* basal:

Tsumura *et al.* (1995): RFLP of cpDNA; Wagner parsimony. — Wang, Han & Hong (1998b): RFLP of *trnK*; Wagner and Dolly parsimony; (no outgroup). — Chaw *et al.* (1997b): 18S (base sequences not released); NJ, MP, ML. — Conran *et al.* (2000): *rbcL*; Wagner parsimony. — Liston *et al.* (2003)*: *rbcL*+5,8S,+ITS-2; MP; *(*Cedrus* is prior to *Picea/Cathaya/Pinus*).

Cycadales/Ginkgo as outgroup yielding *Cedrus* basal followed by the *Abies (Keteleeria)/Tsuga (Nothotsuga)/Pseudolarix*-clade which is sister to the two resting clades as shown in Figure 1:

Chase *et al.* (1993): *rbcL* (agreement); MP; {outgr. Ginkgo}. — Wang, Han & Hong (1998a): *rbcL* (NJ agreement); NJ, (Fitch parsimony yields the *Picea/Cathaya/Pinus*-clade basal); {outgr. Cycadales/Araucariaceae/Podocarpaceae}. — Wang, Tan & Sang (2000): *matK+nad5*+4CL (agreement), *matK* (agreement), *nad5* (no disagreement in strict consensus), 4CL (small disagreement); MP, ML; {outgr. Cycadales for *matK+nad5*}. — Rydin *et al.* (2002): *rbcL*+ 18S+28S+ *atpB* (agreement); *atpB*, and *rbcL+atpB* (*Cedrus* and *Abies*-clade both basal); 18S+28S (no disagreement); 28S (disagreement); MP; {outgr. Cycadales/Ginkgo+others}.

Assuming the Cycadales/Ginkgo "ancestor"-outgroup to be the most appropriate for the Pinaceae genera tree, very few of the above mentioned results disagree with the in Figure 1 shown phylogenetic tree.

Morphological phylogeny of Coniferales on family level.

Analyzing the results of the 4 earlier papers mentioned by Farjon (2003):

1. Nixon *et al.* (1994) try to make a seed plant phylogeny using 102 characters in all, 9 of which are used to state the phylogeny of the conifers. One of the nine main characters is a simple distinction between woody cones or not, which in the paper causes a near connection between Podocarpaceae and Taxaceae s.l. against the rest of the conifers, and therefore it is difficult to regard the resulting phylogeny of the conifers as a serious work!

2. Hart (1987) tries to make a phylogeny for the conifers on family level (as well as phylogenies for the families on genera level) including 63 genera of extant conifers by using 123 characters in all and using Ginkgo, Lebachiaceae and Cordaitales as outgroup. The investigation is a tremendous work including almost 10,000 polarized characters with references! Even the introduction is amazingly good. Although he correctly works out that Pinaceae is basal, that Sciadopityaceae is a family of its own, that Taxaceae and Cephalotaxaceae are very closely connected, and that Taxodiaceae and Cupressaceae is one family, and he thus only have 6 families (Pinaceae, Araucariaceae, Podocarpaceae, Sciadopityaceae, Taxaceae s.l., and Cupressaceae s.l.) to account for, none of his five cladograms on family level have the faintest similarity with the molecular tree stated above in Figure 1. In addition there are serious mistakes in all his family cladograms on genera level (with Cupressaceae s.s. and Taxodiaceae as two families) except for Araucariaceae, which is only split up into *Araucaria* and *Agatis*.

This is not the place to analyze all 123 characters many of which are uninformative, but most of the characters which make the essential differences are rather insignificant. Taking the Pinaceae tree as an example, *Cedrus* is placed in the *Larix/Pseudotsuga*-clade on account of the similarities in shoot morphology between *Cedrus* and *Larix* (but not *Pseudotsuga*), although *Pseudolarix* is in the *Abies*-clade, and also *Cathaya* and *Pinus* have dwarf shoots. Furthermore, the characters (stated and not stated) for the female cones do not clearly reveal the close connection between the *Cedrus* and the *Abies* cone.

NOTE: It is quite impossible to construct a tree based on morphological data without weighting the characters, and the weighting of morphological characters is a rather impossible task per se!

3. Miller (1988) tries to construct an extant conifer family phylogeny on the basis of his knowledge of fossils. He is using 16 characters, and his resulting tree is disastrous. Again, looking at the characters, which make the differences, gives the whole explanation.

4. Doyle (1996) tries to find the phylogenetic relationships of Gnetales in relation to Gymno-sperms, Angiosperms and many fossils (36 taxa in all) using 91 different characters. Contrary to the work of Miller (1988) he gets the conifers as a monophyletic group with Pinaceae basal and, as a matter of fact, if Podocarpaceae was changed from a Pinaceae sister to an Araucariaceae sister his cladogram had been correct with respect to his 6 conifer families (Sciadopity is included in Cupressaceae s.l.; Taxaceae and Cephalotaxaceae are sisters).

— The different authors have tried to polarize their characters, but none of them know the morphology of the conifer ancestor, and therefore it is impossible for them to guess the evolutionary development, which has led to the extant families. This can only be done knowing the actual phylogeny. Taking a prominent character like the female cone as an example, while it is easy to see the development from *Sciadopitys* through the former *Taxodiaceae* to *Cupressaceae* s.s. it is very difficult to put it in relation to *Araucariaceae*/*Podocarpaceae*, and it is complete impossible to guess, where the change to *Taxaceae* s.l. has occurred.

Morphological phylogeny of Pinaceae on genera level.

Contrary to the totally unsuccessful attempts to find a morphological solution of the conifer family phylogeny some morphologists have been rather successful in relation to the Pinaceae genera phylogeny, facilitated by the fact that Pinaceae is a very conservative family. The main problem is that morphology of the shoots, which is the most prominent character, is misleading and completely useless in the Pinaceae phylogeny.

The dendrogram shown in Farjon (1990) is remarkable close to the molecular phylogeny. It is a development of Frankis (1988) and the immunology by Price, Olsen-Stojkovich & Lowenstein (1987), but the real basis is the root anatomy by Tieghem (1891) who divides the genera into two groups, and the mechanisms of pollination by Doyle (1945) who divides the genera into the same two groups and subdivides one of the groups. The groups of Tieghem (1891) and Doyle (1945) contradict a classifying according to the morphology of the shoots, but agree well with a classifying according to the morphology of the female cones, which was not sufficient evident by itself to determine the different groups. Besides the morphology of female cones is considered to be more conservative than the morphology of the shoots.

The most remarkable difference between the dendrogram of Farjon (1990) and the molecular tree is that *Cathaya* is situated together with the *Larix/Pseudotsuga*-clade in the dendrogram. But according to the anatomical studies by Hu & Wang (1984) *Cathaya* is not closely related with *Larix/Pseudotsuga*, although the vegetative anatomy seems to indicate that, because according to Gaussen (1971) both the cone scales and the pollen anatomy contradict the hypothesis and more weight must be laid on the reproductive organs than the vegetative organs. According to Wang & Chen (1974) the embryology of *Cathaya* is very similar to that of *Pinus*. Moreover Hu & Wang (1984) strongly agree with the view of Greguss (1955) so the wood anatomy of *Larix/Pseudotsuga*, *Picea*, *Cathaya*, and *Pinus* with ray tracheids and normal resin ducts is considered to be the highest stage in evolution and therefore the most advanced. Hereby they are in agreement with the molecular cladogram (Figure 1) using ancestors as outgroup and contradictory to the Pinaceae cladogram using *Coniferales II* as outgroup.

Hypothetical common ancestor of the recent conifers.

Knowing the correct phylogeny it is possible to suggest some morphological features of the hypothetical common ancestor to the recent conifers. I will try to propose a few of the most evident features accessible for everyone.

Leaves: The leaves of the ancestor are supposed to have been needles as found in the seedlings of all conifers. Besides needles are found in all the most basal conifer families.

Shoot morphology: Both *Cedrus* and *Larix* have obvious long shoots with the needles spirally arranged and a possibility of growing as well new long shoots as dwarf shoots from the long shoots. The needles on the dwarf shoots are arranged in pseudowhorls and even the dwarf shoots can create both new dwarf and long shoots. This structure of shoots is described in Dallimore & Jackson (1966) under *Larix*; new long shoots on long shoots are in *Cedrus* easily observed on *C. deodara* (Roxb.) G. Don in Loudon. (Possibly the end of the long shoots of *C. atlantica* (Endl.) Manetti ex Carrière have a tendency of getting the needles like pseudowhorls at the end of the season, but I am not sure.)

Supposing a similar morphology of shoots by the hypothetical common ancestor all known shoot morphology by the basal conifer families are easily explained. Not only the different shoot morphologies within Pinaceae but even the puzzle of *Sciadopitys verticillata* Sieb. & Zucc. are easily illustrated. The *Sciadopitys* long shoots (3 to about 200 mm in length) covered with leaves, which are reduced to scales, are interpreted equal to the long shoots of *Cedrus*, but have lost the ability to produce all sorts of shoots. The verticils ("umbrellas") with the famous double-needles are interpreted as short shoots (or derived from short shoots), All new shoots start as long shoots (some of which may be very short), and they grow exclusively from the verticils, generally there is only one, but up to four (may be even five) long shoots from the same verticil are possible. Then there is a possibility that two (infrequent; and even three which is extremely rare) of the new long shoots seem to be grown together in a part of their lengths as shown in Figure 2.

Normally new long shoots grow from a verticil from last year, but as a matter of fact a verticil up to an age of about teen years is able to create a new long shoot.

NOTE: Takaso & Tomlinson (1991) states that *Sciadopitys* besides new long shoots from the verticils also is able to create new long shoots from anywhere of any other long shoot. This statement is misleading. *Sciadopitys* has infrequent side shoots which seemingly do not originate from the verticils, but even a temporary glance will cause suspicion, partly is the proximal end of the shoot (from which the side-shoot seems to be created) not at all circular, and partly is the diameter of the forward shoot in question decreased considerably after the separation from the side-shoot. According to my observation, which are documented by consecutive photos during a season from buds to shoots, it seems that the long shoots, which apparently grow almost orthogonal out from a few of the long shoots are created from two buds, each of which causes its own shoot, but these two shoots are united in the first part of the length and then they separate abruptly giving the false impression that the one of the two shoots is a side shoot grown from the shoot which grow straight forward after the separation.



Figure 2.

**Young shoots from a
Sciadopitys verticil.**

Seemingly there are three shoots, the two of which are grown together in a part of their length. Next year the central shoot will look like a forward shoot and the shoot on the left will leave orthogonal and look like a side shoot.

NOTE: Proposed by different authors and investigated by Roth (1962) the famous double-needles of *Sciadopitys* are interpreted as reduced shoots and not two needles grown together. But in both cases the verticils must originate from dwarf shoots, because even the possible reduced shoots must have grown from a dwarf shoot.

NOTE: The new long shoots of Pinaceae and *Sciadopitys* get woody with brown bark much faster than the new shoots of all other families of Coniferales.

Shoot conclusion: Because dwarf shoots are found in as well in *Cedrus* and all tree *Pinaceae* clades (A: *Pseudolarix*, B: *Larix*, C: *Cathaya*, and *Pinus*) from Coniferales I as in *Sciadopitys* from Coniferales II it is very likely that the common ancestor has had dwarf shoots or at least has had very strong genetic predisposition for dwarf shoots.

Cone morphology: The cone of the hypothetical ancestor is thought to be very much like the cone of *Cedrus* and Araucariaceae with scales and bracts spiraled around a cone axis. The cone is supposed to have remained upright at maturity, because it is difficult to imagine a hanging cone raising. On account of the upright cone there are good reasons to suppose that the original cone has disintegrated at maturity, but it is not certain. Likewise the scale and bract not fused is thought to be the original.

Number of seeds per scale: It is an extremely important point because the palaeobotanists seem to define cones with one seed per scale/bract as Araucariaceae and cones with two seeds per scale/bract as Pinaceae. If the hypothetical ancestor have had one seed per scale/bract, which seems natural and is supposed by Hart (1987), the pre-Pinaceae must have changed from one to two seeds per scale/bract, when it turned into the lineage leading to extant Pinaceae. Which means that both pre-Pinaceae before the change and possible extinct descendants, which have not raised the seed number, wrongly are defined as Araucariaceae by the palaeobotanists. (If it is the other way around, and two seeds per scale/bract is the original, pre-Araucariaceae may wrongly be interpreted as Pinaceae).

Resin ducts in the wood: Because the wood anatomy is an important subject for the palaeo-botanists, it will be mentioned here although the details are not seen with the naked eye. According to Hu & Wang (1984), who refer several other authors, the wood of *Cedrus* and the *Abies*-clade is primitive without regular resin ducts (but traumatic resin ducts may occur), whereas the wood from the *Larix/Pseudotsuga*-clade and *Picea*, *Cathaya* and *Pinus* is more advanced. The wood of Araucariaceae does not have resin ducts either, which seem to indicate that the conifer ancestor did not have resin ducts in the wood, and therefore the palaeobotanists can't use the lack of resin ducts in the wood as proof for an Araucariaceae relationship.

Note that the wood anatomy of Pinaceae is in agreement with the polarity of the Pinaceae genera, which is shown in the tree based on the molecular phylogeny, Figure 1.

Chemistry: The well known "Christmas" aroma from the needles of *Picea* and *Abies* and a similar aroma in some other *Pinaceae* needles, which are described by Frankis (1989) is caused by some monoterpenes according to Price (1989). Yet not all *Tsuga* species are without the aroma as stated by Frankis (1989), it is present for instance in *T. canadensis* (L.) Carrière. More essential, the same aroma is also present in some Podocarpaceae (genus *Podocarpus* and *Saxegothaea*) plus a similar aroma in *Sciadopitys* and *Torreya* (but no other genera than the here mentioned according to my knowledge). The aroma is also present in *Cedrus* and may originate from the common ancestor.

CONCLUSION.

All modern molecular investigations of the conifers show the same family tree (Figure 1), which is therefore supposed to show the correct phylogeny.

The phylogeny shown by the molecular investigations of Pinaceae is not as safe, because the resulting tree seems to be dependant of the outgroup. Using ancestral outgroups seem to indicate that *Cedrus* is basal. The *Abies* (*Keteleeria*)/*Tsuga* (*Nothotsuga*)/*Pseudolarix*-clade is sister to the *Larix/Pseudotsuga*-clade plus the very weak *Picea/Cathaya/Pinus*-clade, which are sisters. This tree seems to be in agreement with the morphology. Using "cousin" outgroups from *Coniferales* II (no direct "sister" exists) seem to indicate that *Picea/Cathaya/Pinus* is rather basal in *Pinaceae*, which among other things is in conflict with the shared advanced wood anatomy of all three genera. The ML tree based on the molecular clock, which is shown as Fig. 3 in Wang et al. (2000) outlines a very good explanation for the evolution of all Pinaceae genera (except that *Cedrus* is not included in the tree), and it is shown in addendum II.

Knowing the phylogeny of the conifers it is possible to guess some features of the hypothetical ancestor to all extant conifers, which turn out to be a tree with many features close to the recent *Cedrus*.

P.S. The fact that almost any ancestral outgroup in this case seems to be more appropriate than all sister/"cousin" outgroups must be taken into account first and foremost in all molecular investigations including conifers but it must also be in mind in other molecular phylogenetic investigations.

For instance in the Podocarpaceae investigation by Conran et al. (2000) conifers are used as outgroup, but C.J. Quinn (personal communication) has kindly shown that adding Ginkgo/Cycadales to the outgroup do not change the resulting tree.

ACKNOWLEDGEMENTS

I want to thanks all the many persons, who have helped me during my long investigation.

A special tanks to Aljos Farjon, who helped and encouraged me, when I was still a student, and to C.J. Quinn as well for both help and rewarding discussions.

References

- Anderson ED, Owens JN. 2003.** Analyzing the Reproductive Biology of *Taxus*: Should it be Included in Coniferales? *Acta Horticulturae* **615**: 233-234.
- Bowe LM, Cota G, dePamphilis CW. 2000.** Phylogeny of Seed Plants Based on All Three Genomic Compartments: Extant Gymnosperms are Monophyletic and Gnetales' Closest Relatives are Conifers. *Proceedings of the National Academy of Sciences, U.S.A.* **97**: 4092-4097.
- Chase MW, Soltis DE, Olmstead RG, and 39 others. 1993.** Phylogenetics of Seed Plants: An Analysis of Nucleotide Sequences from the plastid gene *rbcL*. *Annals of the Missouri Botanical Garden* **80**: 528-580.
- Chaw S-M, Zharkikh A, Sung H-M, Lau T-C, Li W-H. 1997a.** Molecular Phylogeny of Extant Gymnosperms and Seed Plant Evolution: Analysis of Nuclear 18S rRNA Sequences. *Molecular Biology and Evolution* **14**: 56-68.
- Chaw S-M, Long H, Hsu W-L, Chiang GM-Y. 1997b.** Subdivision and Intergeneric Relationships of the Pinaceae Inferred from Nuclear 18S ribosomal RNA Sequences. *American Journal of Botany* **84**(6 suppl.): 182.
- Chaw S-M, Parkinson CL, Cheng Y, Vincent TM, Palmer JD. 2000.** Seed Plant Phylogeny Inferred from All Three Plant Genomes: Monophyly of Extant Gymnosperms and Origin of Gnetales from Conifers. *Proceedings of the National Academy of Sciences, U.S.A.* **97**: 4086-4091.
- Cheng Y, Nicolson RG, Tripp K, Chaw S-M. 2000.** Phylogeny of Taxaceae and Cephalotaxaceae Genera Inferred from Chloroplast *matK* Gene and Nuclear rDNA ITS Region. *Molecular Phylogenetics and Evolution* **14**: 353-365.
- Conran JG, Wood GM, Martin PG, Dowd JM, Quinn CJ, Gadek PA, Price RA. 2000.** Generic relationships within and between the gymnosperm families Podocarpaceae and Phyllocladaceae based on an analysis of the chloroplast gene *rbcL*. *Australian Journal of Botany* **48**: 715-724.
- Dallimore WD, Jackson AB. 1966.** *A Handbook of Coniferae and Ginkgoaceae*. Ed. 4, revised. London: Edward Arnold LTD.
- Doyle JA. 1996.** Seed Plant Phylogeny and the Relationships of Gnetales. *International Journal of Plant Science* **157**: S3 - S39.
- Doyle JA. 1998.** Molecules, Morphology, Fossils, and the Relationship of Angiosperms and Gnetales. *Molecular Phylogenetics and Evolution* **9**: 448-462.
- Doyle JC. 1945.** Developmental line in pollination mechanisms in the Coniferales. *The Scientific Proceedings of the Royal Dublin Society* **24**: 43-62.
- Farjon A. 1990.** *Pinaceae*. Germany: Koeltz Scientific Books.
- Farjon A. 2003.** The Remaining Diversity of Conifers. *Acta Horticulturae* **615**: 75-89.
- Frankis MP. 1988.** Generic Inter-Relationships in Pinaceae. *NOTES Royal Botanical Garden Edinburgh* **45**: 527-548.
- Gaussen H. 1971.** Les *Cathaya* ne sont pas de *Pseudotsuga*. *Comptes rendus de l'Académie des sciences* **273**: 1098-1099.
- Greguss P. 1955.** *Identification of Living Gymnosperms on the basis of Xylotomy*. Budapest: Akademiai Kiado.
- Gugerli F, Sperisen C, Büchler U, Brunner I, Brodbeck S, Palmer JD, Qui Y-L. 2001.** The evolutionary split of Pinaceae from other conifers: Evidence from an intron loss and a multigen phylogeny. *Molecular Phylogenetics and Evolution* **21**: 167-175.
- Hart JA. 1987.** A Cladistic Analysis of Conifers: Preliminary Results. *Journal of the Arnold Arboretum* **68**: 269-307.
- Hu YS, Wang FH. 1984.** Anatomical Studies of *Cathaya* (Pinaceae). *American Journal of Botany* **71**: 727-735.
- Isoda K, Shiraishi S, Kisanuki H. 2000.** Classifying *Abies* species (Pinaceae) based on the sequence variation of a tandemly repeated array found in the chloroplast DNA *trnL* and *trnF* intergenic spacer. *Silvae Genetica* **49**: 161-164.
- Källersjö M, Farris JS, Chase MW, Bremer B, Fay MF, Humphries CJ, Petersen G, Seberg O, Bremer K. 1998.** Simultaneous parsimony jackknife analysis of 2538 *rbcL* DNA sequences reveals support for major clades of green plants, land plants, seed plants and flowering plants. *Plant Systematics and Evolution* **213**: 259-287.

- Liston A, Gernandt DS, Vining TF, Campbell CS, Piñero D. 2003.** Molecular Phylogeny of Pinaceae and *Pinus*. *Acta Horticulturae* **615**: 107-115.
- Miller CN. 1988.** The origin of modern conifer families. In Beck CB (ed.): *Origin and Evolution of Gymnosperms*. New York: Columbia University Press, 448-486.
- Nixon KC, Crepet WL, Stevenson D, Friis EM. 1994.** A re-evaluation of seed plant phylogeny. *Annals of the Missouri Botanical Garden* **81**: 484-533.
- Pant DD. 2000.** Inclusion of Taxaceae in a separate order Taxales. *Current Science* **79**: 278-279.
- Price RA. 2003.** Generic and Familial Relationships of the Taxaceae from *rbcL* and *matK* Sequence Comparisons. *Acta Horticulturae* **615**: 235-237.
- Price RA. 1989.** The genera of Pinaceae in the southeastern United States. *Journal of the Arnold Arboretum*. **70**: 247-305.
- Price RA, Olsen-Stojkovich J, Lowenstein JM. 1987.** Relationships among the genera of Pinaceae: An immunological comparison. *Systematic Botany* **12**: 91-97.
- Quinn CJ, Price RA. 2003.** Phylogeny of the Southern Hemisphere Conifers. *Acta Horticulturae* **615**: 129-136.
- Quinn CJ, Price RA, Gadek PA. 2002.** Familial concepts and relationships in the conifers based on *rbcL* and *matK* sequence comparisons. *Kew Bulletin* **57**: 513-531.
- Raubeson LA, Jansen RK. 1992.** A Rare Chloroplast-DNA Structural Mutation is Shared by all Conifers. *Biochemical Systematics and Ecology* **20**: 17-24.
- Roth I. 1962.** Histogenese und morphologische Deutung der Doppeltnadeln von *Sciadopitys*. *Flora* **152**: 1-23.
- Rydin C, Kjällersjö M, Friis EM. 2002.** Seed plant relationships and the systematic position of Gnetales based on nuclear and chloroplast DNA: Conflicting data, rooting problems and the monophyly of conifers. *International Journal of Plant Science* **163**: 197-214.
- Stefanovic S, Jager M, Deutsch J, Broutin J, Masselot M. 1998.** Phylogenetic Relationships of Conifers Inferred from Partial 28S rRNA Gen Sequences. *American Journal of Botany* **85**: 688 -697.
- Stützel T, Röwekamp I. 1999.** Female reproductive structures in Taxales. *Flora* **194**: 145-157.
- Takaso T, Tomlinson PB. 1991.** Cone and Ovule Development in *Sciadopitys* (Taxodiaceae-Coniferales). *American Journal of Botany* **78**: 417-428.
- Tieghem P van. 1891.** Structure et affinités des *Abies* et des genres les plus voisins. *Bulletin de la Société Botanique de France* **38**: 406-415.
- Tsumura Y, Suyama Y. 1998.** Differentiation of Mitochondrial DNA Polymorphisms in Populations of Five Japanese *Abies* Species. *Evolution* **52**: 1031-1042.
- Tsumura Y, Yoshimura K, Tomaru N, Ohba K. 1995.** Molecular Phylogeny of Conifers Using RFLP Analysis of PCR- amplified Specific Chloroplast Genes. *Theoretical and Applied Genetics* **91**: 1222-1236.
- Wang FH, Chen TK. 1974.** The embryogeny of *Cathaya* (Pinaceae). *Acta Botanica Sinica* **16**: 64-69.
- Wang X-Q, Han Y, Hong D-Y. 1998a.** A molecular systematic study of *Cathaya*, a relict genus of the Pinaceae in China. *Plant Systematics and Evolution* **213**: 165-172.
- Wang X-Q, Han Y, Hong D-Y. 1998b.** PCR-RFLP Analysis of the Chloroplast Gene *trnK* in the Pinaceae with Special Reference to the Systematic Position of *Cathaya*. *Israel Journal of Plant Sciences* **46**: 265-271.
- Wang X-Q, Tang DC, Sang T. 2000.** Phylogeny and Divergence Times in Pinaceae: Evidence from Three Genomes. *Molecular Biology and Evolution* **17**: 773-781.

Addendum I

Methods. Own cladistic investigation.

During my study within biology I realized in 1999 that GenBank had listed a number of *rbcL* base sequences from different conifers, which had not been used. Out of curiosity I decided to make some cladograms using the beta version PAUP 4.0b2. My theoretical knowledge within the area was poor at that time and I was not able to achieve tutoring, therefore I started my work as a practical approach based on learning by doing and my own knowledge of dealing with numbers and judging scientific results. Contrary to the common praxis by the Scandinavian cladists I chose to do the thinking myself and only use the computer as a tool. Of course I made some mistakes during my work, for instance I started to use the inappropriate Coniferales II as outgroup for Pinaceae; and, when I for the first time got Ginkgo/Cycadales nested within Coniferales, I really was misled by the cladogram for a few days and thought that the conifers may be a paraphyletic order — but my mistakes of that kind is now long gone history!

All alignments of the base sequences were done manually, and I started to do *rbcL* for Pinaceae, which was very fascinating. Not only was the sequencing, which originally was carried out by many different persons, confirmed, but some relationships were evident from the very beginning. Although not many different taxa were sequenced in 1999 it was easy to separate *Pinus haploxylon* from *P. diploxylon*, and it was possible to construct the base sequences for the hypothetical ancestors of *P. haploxylon* and *P. diploxylon* as well as their common ancestor. With a little more uncertainty it was possible to estimate the hypothetical ancestor of all Pinaceae and even Coniferales (using all ancestors back to green algae and the other families within Coniferales as well). Out of a total of five 3 out of 4 species of Japanese *Abies* seemed closely related, whereas the fourth (*A. mariesii* Masters) seemed more related to the Californian *A. magnifica* A. Murray. Also Tsumura & Suyama (1998) using mtDNA polymorphism found *A. mariesii* very different from the other Japanese *Abies*, and Isoda, Shiraishi & Kisanuki (2000) using *trnL* and *trn-IS* found *A. mariesii* different from the 4 other Japanese *Abies*. — Besides *Keteleeria* seemed closely related to *Abies*.

— By the way, when I tried to run my "hypothetical Pinaceae ancestor" as a taxa in a tree using PAUP it came out very close to *Abies*, which I at that time thought was a misleading result, later in my investigation it made more sense, but I have not yet had the time to play more with my hypothetical ancestors. —

The above mentioned observations during the alignment were rather familiar with the NJ-principles and had no relation at all to MP, which algorithm is almost indispensable in relation to morphological investigations. Therefore I got confidence in the NJ-principle, although I dislike that the NJ-algorithm only yields one single tree not leaving the possibility for me to judge if another second best NJ tree may be more plausible.

Rejection of the usual cladistic method of perfecting a single gene by use of MP and a single outgroup.

Rather soon I realized that results from only one gene using only one single algorithm were extremely unsafe no matter how perfect the cladistic analysis was carried out, and still in contrast to my Scandinavian colleagues I decided to make a multi factorial analysis using separately all the 3 genes (*rbcL*, 18S, and 28S), which were at my disposal in 1999, each in 9 independent investigations using the following 3 different algorithms NJ, MP, and ML (based on very different assumptions), and using the following 3 different outgroups for each (1. Ginkgo/Cycadales; 2. Gnetales; 3. Lycopodium/Nymphaea, a "close" Pteridophyte and a "close" Angiosperm; (which yielded identical results as: "All" ancestors down to green algae.))

This method left me with an enormous amount of $3 \times 3 \times 3 = 27$ trees to be compared, but owing to the limited number of six conifer families only in 5 points of ramifications. Because of the different species for the different genes and in order to exclude the few artifacts caused by Ginkgo/Cycadales and Gnetales, which rarely turned up inside the conifer tree, this comparison was done manually in tabular form. (By the way, I have made more than 200 different trees, and later than 1999, when more taxa became available in GenBank, it showed that adding a couple of incidental taxa to the tree may cause one of the above mentioned artifacts).

Besides I tried to investigate how a different weighting of the first, second and third position bases turned out, but according to my opinion an equal weighting of all bases seemed to give the best and most reliable results in this case, and therefore I treated all single mutations equal.

It ought to be common knowledge from physics and chemistry that the safety of the result only doubles if the number of observations are increased by a factor of four, whereas 3 different and independent investigations each with an uncertainty of 10%, where the results are in agreement with each other, will yield a resulting uncertainty of $0,1 \times 0,1 \times 0,1 = 0,001 = 0,1\%$, which is extremely more safe!

Example: If the uncertainty of the conifer family tree is judged to 10% for *rbcL*, 20% for 18S, and 40% for 28S, when artifacts are excluded, and the results from using different algorithms and outgroups are in agreement, then the resulting uncertainty is $0,1 \times 0,2 \times 0,4 = 0,008 = 0,8\%$ if all the trees are in agreement. If the uncertainty of the single genes are only considered to be 5%, 10%, and 20% respectively the resulting uncertainty is 0,1%. The above mentioned percentages is my best judgement for the uncertainties on the different genes in relation to the conifer family tree when the 3 different algorithms NJ, MP, and ML generates the same result using different reasonable outgroups. Thus the uncertainty on the stated conifer family phylogeny based on my own investigation lies somewhere between 0,1% - 0,8%. Taking in account that *matK* used by Quinn *et al.* (2002) creates the same tree and that *matK* is about double as safe as *rbcL* the uncertainty lies between 0,005% - 0,04%, which is beyond any reasonable doubt!

My way of calculation of the uncertainty of the final conifer family tree is so elementary that it should be understood by every high-school student, who ought to be able both to check the premises and to carry out the calculation. Note that I have judged the uncertainties rather high; if the uncertainty of the 28S really is 40%, it is almost like tossing up a coin for the 28S result.

The 3 different algorithms (NJ, MP, and ML) based on very different assumptions yields the same conifer family tree, which means that for this special purpose they are equal reliable. On the other hand NJ is the only algorithm, who draws the *rbcL* Pinaceae genera tree just the way Wang *et al.* (2000) presume it to be.

Addendum II

"Molecular clock tree" from Wang *et al.* (2000):

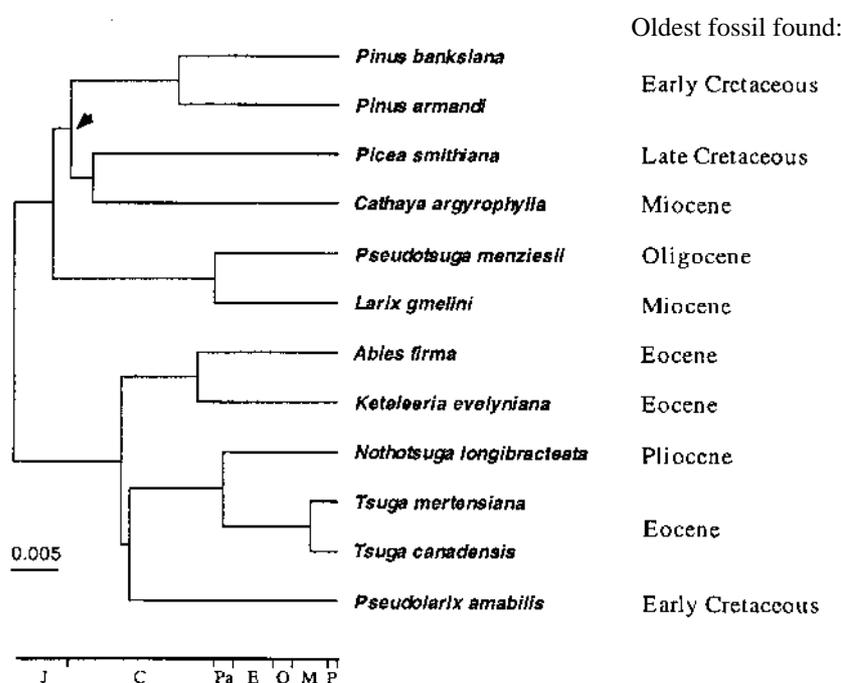


FIG. 3.—Maximum-likelihood tree of Pinaceae based on *matK* sequences with a molecular clock enforced. The earliest fossil record of each genus (not necessarily the species sampled in this study) is indicated. Branch lengths are proportional to sequence divergence estimated by maximum-likelihood and are measured by the scale bar. The geological timescale was calculated from the branch lengths according to the molecular clock: J, Jurassic; C, Cretaceous; Pa, Paleocene; E, Eocene; O, Oligocene; M, Miocene; P, Pliocene. The arrow indicates the point at which the molecular clock is calibrated (140 MYA).