



## Abstract

Several extant species of the Araucariaceae family (one of the families of conifers) were invested for the experimental artificial maturation by confined pyrolysis, in order to realize the transformation of biomolecules to geomolecules in laboratory conditions. The experimental study of diagenetized molecular signatures of the Araucariaceae species (common, inter- and infra-generic characteristics) allow to complete our knowledge in botanical palaeochemotaxonomy. Such knowledge is relevant to the reconstitution of palaeoflora and palaeoclimatic reconstruction, archaeology and environmental studies. In this work, major carbon skeleton types of Araucariaceae are detected in the organic solvent extracts of fresh and pyrolyzed plants using gas chromatography-mass spectrometry. The results show that all species of Araucariaceae are firstly characterized by a predominance of saturated tetracyclic diterpenoids. Moreover, the *Araucaria* genus shows a high relative abundance of bicyclic sesquiterpenoids, particularly compounds of the cadalane-type compounds accompanied by those of eudesmane-type, bisabolane-type as well as chamazulene, pentamethyl-dihydroindenes. Diterpenoids are of the labdane-type, isopimarane, abietane-type (essentially derived from abietanoic acids) as well as isohexyl alkylaromatic hydrocarbons. Compared to the tetracyclic diterpenoids, these compounds show a relatively lower abundance, reaching trace levels in the case of saturated abietanes. Distribution of sesqui- and diterpenoids of *Agathis* shows some similarities to that of *Araucaria*, with the exception of one species, in which the tetracyclic compounds are absent and the abietane-type (essentially derived from abietanoic acids) predominant. High similarities between the *Wollemia* and *Araucaria* genera are observed. Both are characterized by some high relative abundance of tetracyclic compounds with no predominance of other specific diterpenoids.

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## 1 Introduction

Numerous studies of the molecular composition of extant terrestrial plants have pointed out the chemotaxonomic values of many biological compounds bioterpenoids in particular (e.g. Aplin et al., 1963; Smith, 1976; Castro et al., 1996; Mongrand et al., 2001).

This means that these biomolecules are synthesized by a restricted number of plant taxa and can be used as specific markers. While most bioterpenoids are degraded and their atomic constituents recycled in the surface processes of the earth, a minor part is incorporated into sediments, thus joining the geological cycle. During this process, the bioterpenoids are transformed by diagenesis, leading to the formation of reaction products called geoterpenoids. Their initial chemotaxonomic value can be partially or totally retained (e.g. Simoneit, 1986; Otto and Simoneit, 2001). Geoterpenoids of ancient sediments may thus yield palaeochemotaxonomic information inherited from their biological precursors.

Botanical palaeochemotaxonomy has some specific attributes compared to palaeobotany and palynology in the reconstruction of palaeoflora and palaeoclimatic evolutions through geological times (e.g. Vliex et al., 1994; van Aarssen et al., 2000; Hautevelle et al., 2006a). Indeed, (1) plant biomarkers are more widespread in the stratigraphic record than well preserved plant macrofossils, (2) on the contrary to Palaeozoic and Mesozoic palynomorphs, they can be directly linked to specific taxa of plants and (3) biomarkers could be readily analyzed by usual organic geochemistry procedures on total rock samples.

In addition to be useful to palaeofloristic and palaeoclimatic studies, botanical palaeochemotaxonomy has also been recently applied in other instances. In environmental science, it proved to be helpful in the appreciation of past land-use and recent anthropic impacts to soil (e.g. Farella et al., 2001; Heim et al., 2010; Huang et al., 2011; Lavrieux et al., 2011) as well as to trace river pollution caused by paper mills (e.g. Leeming and Nichols, 1998; Wang et al., 2007). In archaeology,

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palaeochemotaxonomy is used to trace diet habits, to understand the use of natural products in craft and funeral rites (e.g. Colombini et al., 2005; Romanus et al., 2008).

Most of palaeochemotaxonomic investigations are derived from published chemotaxonomic data (Otto and Wilde, 2001) and to some part from the studies of fossil plants found in sedimentary rocks (Otto and Simoneit, 2001; Otto et al., 2005; Dutta et al., 2011). Unfortunately, our knowledge on botanical palaeochemotaxonomy is still very scarce. As pointed by Hautevelle et al. (2006b), difficulties are related to (1) available chemotaxonomic data (generally focus on specific biomolecules or on particular substances, like resin or essential oil); (2) degradation and diagenesis reactions, which may significantly modify the initial molecular fingerprint, making it difficult to perform a direct chemotaxonomic relationship between an extant plant and its fossil counterpart; (3) the scarcity of reference collections of well preserved and identifiable fossils plants containing organic molecules.

In order to fill these gaps, within the 7 extant conifer families (Fig. 1), we investigated the palaeochemotaxonomy of several extant species of the Araucariaceae family (Table 1) using an experimental method based on artificial maturation by confined pyrolysis (Hautevelle et al., 2006b). This procedure allows to simulate the conversion of biomolecules into their corresponding diagenetic geomolecules (Stankiewicz et al., 2000; Gupta et al., 2006, 2007). Aims of this study are (1) to determine the common palaeochemotaxonomic (diagenetized) signature of all extant Araucariaceae species, (2) to evaluate the inter- and infra-generic differences within the family, (3) to highlight the molecular characteristics which should allow their distinction from other conifer families in ancient sediment samples. This contribution could serve as database to future investigations on palaeoflora, palaeoclimatic reconstruction, archaeology, environmental research.

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## 2 Some generalities concerning the Araucariaceae family

Araucariaceae is considered as a Southern Hemisphere conifer family, and was among the first conifer families to be individualised from their “Voltziales” ancestors during Triassic (Stockey, 1994). Diversity and abundance of Araucariaceae are well preserved up to nowadays despite of some reduction of geographic distribution and the extinction of several species during their evolution (Axsmith et al., 2004; Kunzmann, 2007a).

### 2.1 Taxonomy, geographical distribution and living environment

#### 2.1.1 Extant Araucariaceae

Today, Araucariaceae species represent a dominant component of the South Hemisphere forests. The major native Araucariaceae species are restricted to South America, Southwest Asia and the Western Pacific region and cover a large rainfall region extending from subtropics to tropics (Enright and Hill, 1995). Nowadays, more than 40 species are described and consist of three well-defined genera which are (1) *Agathis* (Salisbury, 1807), represented by 21 species, (2) *Araucaria* (Jussieu, 1978) represented by 19 species and (3) *Wollemia* (Jones et al., 1995) represented by the solo species *Wollemia nobilis*. The latter, previously thought to be extinct, was rediscovered in 1994 in Australia, cloistered deep in the New South Wales (Jones et al., 1995).

These phylogenetic relationships of Araucariaceae species were also confirmed by Setoguchi et al. (1998) based on *rbcL* gene sequences:

- the three genera are all monophyletic (i.e. all species descend from an unique ancestor);
- *Araucaria* could be infragenerically classified into four sections: *Araucaria*, *Bunya*, *Eutacta*, and *Intermedia*. This sub-classification is in agreement with their morphological characteristics (Stockey, 1982);
- phylogenetic relationships within *Agathis* remain somewhat unclear;

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- *Wollemia* is the most basal extant taxon in the family, sharing morphologies with *Araucaria* and *Agathis*.

The geographic distribution of major Araucariaceae species is given in Table 1. Extant *Araucaria* species develop in the subtropical regions and extend into the marginal tropical regions where there is a lower climatic variability, while most of *Agathis* species occupy the tropical islands (Dettmann and Clifford, 2005). These two genera, extending within the equatorial region of New Guinea and Southeast Asia at lower latitude, grow under a mesothermal climate limited to the lower montane zone (Kershaw and Wagstaff, 2001). The monotypic genus *Wollemia* is by far the most endemic genus and its few wild species (about 40 adult plants) live deep in a wilderness rainforest of the Wollemi National Park in New South Wales (Australia).

### 2.1.2 Fossil Araucariaceae record

Appearance and evolution of Araucariaceae are recorded by both macrofossils and pollen in sedimentary series all around the world. The first pollen record is from the Early Triassic of Australia (as ancestor) just after the Permo-Triassic extinction and the unequivocal Araucariaceae representatives were found in North Yorkshire from the Middle Jurassic (Stockey, 1982; Kershaw and Wagstaff, 2001; Kunzmann, 2007b). Its maximum worldwide distribution was achieved in both hemispheres during the Cretaceous. Their distribution was then reduced during the Middle Palaeocene to the Southern Hemisphere, with an extension to the Northern Hemisphere (restricted to the Southeast Asia).

During the Jurassic, *Araucaria*, especially the *Eutacta* and the *Bunya* sections, was the first genus to appear and diversify. Their macrofossils and pollens broadened a wide range of habitats in the two hemispheres (Gondwana and Laurasia), where Jurassic climate condition was a subtropical to warm-temperate (Kershaw and Wagstaff, 2001). During the Cretaceous, the first effective *Agathis* and *Wollemia* fossils appeared, but remained restricted to Australia and New Zealand (Hill and Bigwood, 1987; Stockey,

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1994). This palaeogeographic limitation could have been due to the broken-up of palaeofloristic exchanges between Gondwana and Laurasia. Moreover, appearance of angiosperms during the Cretaceous also forced *Agathis* to be more competitive (Kershaw and Wagstaff, 2001), and it seems also be that for *Wollemia* genus. At the end of the Cretaceous, the *Araucaria* genus began to leave the Northern Hemisphere. As pointed out by Kunzmann (2007b), their retreat from Northern Hemisphere coincides with two major events in Earth history: (1) major changes in forest composition in Europe and North America; (2) rapid environmental changes across the K-T boundary.

Later, during the Cenozoic, *Wollemia* and especially *Agathis* became more prominent from the Early Eocene to the Early Oligocene. This development was synchronous with the diversification of angiosperms. Owing to their adaptation ability to many unfavourable conditions, such as the ultramafic soils on peridotites and the unstable climate in islands, *Agathis* species began to colonize and diversify in New Caledonia and other Asia-Pacific islands (Jaffré, 1995). In parallel, there was a reduction of *Agathis* from South-Eastern Australia due to the cooler conditions at high latitudes. Regarding *Araucaria* species, they regenerated from Early Miocene under subtropical to warm-temperate conditions (Kershaw and Wagstaff, 2001).

### 2.1.3 Araucariaceae interests

The worldwide patchy and sparse distribution of Araucariaceae in sedimentary deposits, as inferred by palaeobotanic and palynological data, can thus provide valuable palaeoclimatic information. Indeed, they preferentially grow under warm and wet conditions, mainly in equatorial, tropical or subtropical rainforests as well as peat swamps (Kershaw and Wagstaff, 2001).

Furthermore, many Araucariaceae produce resins having archaeological and economical values. *Agathis* is highly considered as a source of attractive, straight-grained, easily worked timber. The wood of *Agathis australis* has been intensively used in the past for the construction of canoes, houses and cult objects by native Maori people of New Zealand. Some of these objects were well preserved and show archaeological

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interest nowadays. The resin of *Agathis* (kauri copal, kauri gum or kauri resin) was also widely used for the manufacture of paints, varnishes and linoleum during the XIX and XX centuries. Other *Agathis* living in Indonesia and Philippines, as *Agathis dammara*, produce resin having an important economic value (Manilla resin or Manilla copal).

5 Copal has also been used for a long time in jewellery and related arts. Furthermore, *Araucaria* may not have an equivalent economical value as *Agathis* but provided wood for shipbuilding during several centuries.

## 2.2 Chemotaxonomy

Available data on the composition of monoterpenoids ( $C_{10}$ ), sesquiterpenoids ( $C_{15}$ ) and diterpenoids ( $C_{20}$ ) are here summarised.

10 Monoterpenoids ( $C_{10}$ ) are abundantly synthesized by Araucariaceae. Compounds, like pinene, thujene, limonene and cubebene are widespread in most of Araucariaceae species (Brophy et al., 2000). They represent the major components of araucarian essential oils (Brophy et al., 2000; Staniek et al., 2009). Furthermore, owing to their  
15 lower molecular weight, these chemicals are generally highly volatile and not readily preserved in sediments.

Sesquiterpenoids are composed by monocyclic (e.g. elemene-, germacrane- and humulane-types), bicyclic (e.g. cadinane-, copaane-, muurolane- and caryophyllane-types) and tricyclic (e.g. aromadendrane-, cubebane- and gurjunane-types) compounds. These chemicals are mainly represented by unsaturated hydrocarbons, alcohols and acids, constituents of essential oil and resins (e.g. Pietsch and König, 2000; Brophy et al., 2000). However, they are common in conifers as well as in other plants like angiosperms and bryophytes (Otto and Wilde, 2001).

25 Regarding diterpenoids, they are represented by bicyclic, tricyclic and tetracyclic compounds. Bicyclic diterpenoids mainly composed by labdane-type compounds are common in all conifers. They are synthesized as unsaturated hydrocarbons (like sclarenes and biformenes), alcohols (like sclareols) as well as acids (like agathic and communic acids) and isomers may occur (Caputo and Mangoni, 1974; Caputo et al.,

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1974a,b, 1976; Thomas, 1969, Pietsh and König, 2000; Otto and Wilde, 2001). Other bicyclic compounds, belonging to the clerodane-type, like clerodadienic acids seem to be restricted to two *Araucaria* species: *A. bidwillii* (Caputo and Mangoni, 1974) and *A. hunsteinii* (Otto and Wilde, 2001).

5 Tricyclic diterpenoids reported in Araucariaceae are composed essentially by the abietane- and pimarane-types. Tetracyclic diterpenoids belong to the beyerane-, kaurane-, phyllocladane-, trachylobane- and atisane-types (Brophy et al., 2000; Otto and Wilde, 2001). According to Thomas (1969), Brophy et al. (2000) and Pietsh and König (2000), both tri- and tetracyclic diterpenoids are synthesized as unsaturated hydrocarbons (like pimaradienes, abietadienes, kaurenes, etc.), acids (like abietic and isopimaric acids) and more rarely as alcohols (like phyllocladanol, phenolic abietanes which are only reported in few *Agathis* species).

15 Distribution of bioterpenoids varies from species to another in fresh plants (Thomas, 1969; Brophy et al., 2000). For instance, each species seems to be dominated by one or eventually two compounds. Their molecular composition could thus be theoretically specific to each araucarian species and/or genus. These data originate from a compilation of several studies and some inconsistencies may occur. For instance, Thomas (1969) insists on the presence of compounds like agathic, communic, isopimaric and abietic acids in *Agathis* species while these compounds are not reported by  
20 Brophy et al. (2000). Such kind of contradictions is certainly due to the fact that the former studied the resins while the last studied essential oil. Nevertheless, chemotaxonomic data provides valuable knowledge to palaeochemotaxonomy.

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### 3 Samples and experimental procedures

#### 3.1 Samples

12 Araucariaceae species were selected for this study and are represented by 3 *Agathis*, 8 *Araucaria* and 1 *Wollemia* (Table 1). This sampling is believed to be well representative of the intrinsic variability of the Araucariaceae family.

#### 3.2 Experimental and analytical procedures

This study focuses on the diagenetized molecular signature of Araucariaceae. Transformation of fresh material to its diagenetized counterpart was carried out by experimental artificial maturation by confined pyrolysis developed by Hautevelle et al. (2006b). Twigs and leaves of the selected plants were finely cut and dried under vacuum for 24 h in a desiccator at 45 °C before being crushed. The plants to be pyrolyzed were introduced into gold tubes with and without LiAlH<sub>4</sub> (Fluka No 62420, purity ≥ 97%, powder form). The use of LiAlH<sub>4</sub> favors the generation of saturated over aromatic terpanes during pyrolysis. The filed gold tubes were then sealed under an argon atmosphere and introduced into autoclaves and heated at 280 °C and 700 bar during 24 h. These parameters of pyrolysis were determined to obtain the broadest distribution of biomarkers (aliphatic, aromatic and polar). Biomolecules of the starting fresh plant samples of each representative were also studied.

The soluble organic compounds of fresh plants and their pyrolysates were extracted under 80 °C and 100 bar using CH<sub>2</sub>Cl<sub>2</sub> with an Accelerated Solvent Extractor (ASE 350, Dionex). The total extract was then fractionated into aliphatic, aromatic and polar fractions using liquid chromatography on alumina (separation of hydrocarbon and polar fraction) and silica (separation of aliphatic and aromatic fractions from the hydrocarbon fraction) columns. The fractions were successively eluted with CH<sub>2</sub>Cl<sub>2</sub> and CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub> (50/50 v/v) on the alumina column, then with pentane, followed by pentane/CH<sub>2</sub>Cl<sub>2</sub> (65/35 v/v) and CH<sub>3</sub>OH/CH<sub>2</sub>Cl<sub>2</sub> (50/50 v/v) on the silica column. The

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obtained fractions were diluted in hexane (4 mg ml<sup>-1</sup> for aliphatic fraction and 8 mg ml<sup>-1</sup> for the others) before analysis by GC-MS.

### 3.3 Gas Chromatography-Mass Spectrometry (GC-MS) and identification of compounds

Aliphatic and aromatic fractions were analysed using an HP 5890 Series II gas chromatograph coupled with an HP 5971 mass spectrometer (GC-MS). Polar fractions were silylated using N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) before analysis. The capillary column was a DB-5 J&W (60 m × 0.25 mm i.d., with 0.1 μm film thickness). The temperature programme was 70–315 °C at 15 °C min<sup>-1</sup> to 130 °C, and then 3 °C min<sup>-1</sup> followed by an isothermal stage at 315 °C for 15 min. Helium was the carrier gas (1 ml min<sup>-1</sup> flow rate). The MS operated in the electron impact mode (EI) at 70 eV ionization energy and mass spectra were scanned from 50 to 500 Da using a quadrupole detector. Data were acquired and processed using the Agilent ChemStation software. Compounds were identified by comparison of mass spectra with literature and library database (Wiley275) or by interpretation of mass spectrometric fragmentation patterns.

## 4 Results and discussion

Aliphatic, aromatic and polar fractions were studied for each Araucariaceae representative, including the extracts of fresh plants and of their pyrolysates. Since our study mainly focuses on palaeochemotaxonomy, the extracts of pyrolyzed plants were preferentially studied.

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## 4.1 Molecular characteristics of artificially diagenetized *Araucaria* genus

### 4.1.1 Sesquiterpenoids

Distribution of sesquiterpenoids of *Araucaria* is showed in Fig. 2. Di- and tri- methyl-naphthalenes are ubiquitous in each representative. *Araucaria* species are also commonly characterized by:

- Farnesane (C<sub>15</sub>H<sub>32</sub>) and bisabolane-type compounds. Acyclic farnesane, probably derived from isomers of farnesol (*trans*- and *cis*-) detected in the fresh plants, is identified in all *Araucaria* species. Bisabolane-type compounds, like isomers of saturated bisabolane (C<sub>15</sub>H<sub>30</sub>), *ar*-curcumene (C<sub>15</sub>H<sub>22</sub>) and dihydro-*ar*-curcumene (C<sub>15</sub>H<sub>24</sub>), are observed in the aliphatic and the aromatic fraction respectively. *Ar*-curcumene is often found in trace amounts for all species. Bisabolane-type compounds are generally derived from bisabolenes found in the fresh plants. They represent one of the major class of *Araucaria* sesquiterpenoids (especially in the aliphatic fractions), except for *A. bernieri* and *A. bidwillii*;
- cadalane-type compounds. They are mainly derived from cadinol and cadinenes which are ubiquitous in vascular plants. In the aliphatic fractions, a variety of saturated cadalane-type compounds (C<sub>15</sub>H<sub>28</sub>) are identified like cadinanes, muurolanes and amorphanes. Their spectra are commonly characterized by a molecular ion at *m/z* 208, a low abundance (even absent) of the M<sup>+</sup>-15 ion at *m/z* 193, a relatively high abundance of ion at *m/z* 165 (corresponding to the loss of a isopropyl unit, M<sup>+</sup>-43) and a base peak at *m/z* 109 (corresponding to a fragmentation of A/B-ring moiety after the loss of precedent units) (Fig. 3a). In the aromatic fraction, the cadalane-type compounds are represented by calamenene and calamene (C<sub>15</sub>H<sub>22</sub>, both partially aromatized), cadalene (C<sub>15</sub>H<sub>18</sub>, completely aromatized). One norcadalene (probably the 1-isopropyl-7-methylnaphthalene according to the spectrum from Singh et al., 1994) is detected in all species (Fig. 3b). Its formation is the result of the demethylation of cadalane-type compounds, and

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no more precision to its structure was proposed. In addition, cadalane-type compounds show a high abundance in most of species samples and represent also one of major *Araucaria* sesquiterpenoids, except for *A. araucana* and *A. cunninghamii*;

- eudesmane-type compounds.  $4\alpha$ (H)-eudesmane and  $4\beta$ (H)-eudesmane are identified according to Wiley275 database and the spectrum published by Alexander et al. (1983) (Fig. 3c, d). Their relative abundances of these two compounds vary from one specie to another. It is higher in *A. bernieri* and at trace level in *A. heterophylla* as well as in *A. cunninghamii*. Moreover, eudesmanes-type compounds are widely distributed in fossil vascular plants (Alexander et al., 1983). However, they were not reported in the previous studies of Brophy et al., (2000) and Otto and Wilde, (2001), which focus on fresh material;
- ionene ( $C_{13}H_{18}$ ), with a molecular ion at  $m/z$  174 and a base peak at  $m/z$  159 according to Achari et al. (1973). It was detected for most of *Araucaria* species. It could be derived from  $\beta$ -carotene like in sporopollenin (which derives from some pollen exines) (Achari et al., 1973; Simoneit, 1986). In our case, the ionene is probably directly derived from ionone that is abundant in the fresh plants;
- chamazulene ( $C_{14}H_{16}$ ) and other compounds identified as pentamethyl-2,3-dihydroindenes according to their spectra are also remarkable in the aromatic fraction. Chamazulene elutes after cadalene and its spectrum, according to the Wiley275 database, is quite similar to that of norcadalene but with a molecular ion ( $m/z$  184) more intense than its base peak at  $m/z$  169 (Fig. 3e). Due to its carbon skeleton composed of a ring composed of seven carbon atoms and a pentenic ring, it is probably derived from precursor of similar carbon skeleton, like aromadendrenes, alloaromadendrene and spathulenol, which are widespread in the fresh species (Brophy et al., 2000; Olawore, 2005). Pentamethyl-2,3-dihydroindene, identified according to Wiley275 database, is characterized by a spectrum with a molecular ion at  $m/z$  188 ( $C_{14}H_{20}$ ) and a base peak at  $m/z$  173

(M<sup>+</sup>-15) (Fig. 3f). Pentamethyl-2,3-dihydroindene and chamazulene show a higher abundance than other aromatic bicyclic sesquiterpenoids with the exception of cadalene;

- furthermore, 1,3,4-trimethyl-2-(4-methylpentyl)benzene (C<sub>15</sub>H<sub>24</sub>) with a molecular ion at *m/z* 204 and a base peak at *m/z* 133 was detected in all *Araucaria* species but with a low abundance. According to Ellis et al. (1996), it belongs to the family of isohexyl alkylaromatic hydrocarbons (see Sect. 4.1.2) with opened A-ring (Fig. 4a). Its occurrence could be due to the alteration of some bicyclic compounds (like drimane) during the pyrolysis.

#### 4.1.2 Diterpenoids

Diterpenoids in the extracts of pyrolysates of *Araucaria* species are characterized by a diversity of bicyclic, tricyclic and tetracyclic compounds (Fig. 5).

#### Bicyclic diterpenoids

Bicyclic diterpenoids are essentially composed by labdane-type compounds (C<sub>20</sub>H<sub>38</sub>). Several isomers of labdane are identified in *Araucaria* species, although they do not show a high abundance in each representative. As shown in the literature, they are generally derived from labdadiene, phenolic labdanes and communic acids present in the fresh plants (Caputo and Mangoni, 1974; Caputo et al., 1974a,b, 1976; Otto and Wilde, 2001). Moreover, according to de Paiva Campello and Fonseca (1975), the agathic acid could also be the origin of labdanes in the case of *A. angustifolia*.

A peak having a molecular ion at *m/z* 244 (C<sub>18</sub>H<sub>28</sub>) is detected in the aromatic fraction in all *Araucaria* species. It shows a significant abundance in most species, but in traces for *A. bidwillii*, *A. laubenfelsii* and *A. nemorosa*. According to its mass spectrum, its base peak at *m/z* 159 matches very well to fragment composed of two rings (including an aromatic ring) and two methylene units. This fragment is probably formed via the loss of a branched-chain with 6 carbon atoms (Fig. 6a). This kind of fragmentation

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seems to be consistent with a monoaromatic labdane. No aromatic labdane has been described in the literature until now.

Another peak heaving a molecular ion at  $m/z$  278 ( $C_{20}H_{38}$ ) and a base peak at  $m/z$  109 is detected with a low intensity in the aliphatic fraction of *A. bidwillii*. Its mass spectrum indicates that it could also be a bicyclic compound and has a similar carbon skeleton to labdane. Indeed, its fragment at  $m/z$  193 matches to a configuration including two rings (A and B) and three methylene units, while its A/B-ring fragment ( $m/z$  109) contains only two methylene units. The “third” methylene unit could thus only be bound at the carbon-9 position (Fig. 6b). This obtained configuration is consistent with a structure similar to that of clerodane. However, its report in extant conifers is limited to a few derivatives of *ent*-clerodadienes (Otto and Wilde, 2001) and is restricted to two *Araucaria* species including *A. bidwillii* (Caputo and Mangoni, 1974; Cox et al., 2007).

2,6-dimethyl-1-(4-methylpentyl)naphthalene and 6-ethyl-2-dimethyl-1-(4-methylpentyl)naphthalene are abundant for most species and in traces in *A. launbenfelsii*. These two compounds are identified from spectra published by Ellis et al. (1996) and given in Fig. 4b, c. They belong to the family of isohexyl alkylaromatic hydrocarbons just like the 1,3,4-trimethyl-2-(4-methylpentyl)benzene previously described. Indeed, their specific carbon skeleton with an opened A-ring could be due to some specific chemical reactions, like aromatisation, ring-opening processes and rearrangement, during the maturation (Ellis et al., 1996). Furthermore, the co-occurrence of 2,6-dimethyl-1-(4-methylpentyl)naphthalene and 6-ethyl-2-dimethyl-1-(4-methylpentyl)naphthalene suggests precursors like tricyclic diterpenoids (Ellis et al., 1996), for which the diagenetic products are structurally quite dissimilar to their precursors.

## Tricyclic diterpenoids

Few aliphatic but a high diversity of aromatic tricyclic diterpenoids are observed. Pimarane-type compound, like isopimarane ( $C_{20}H_{36}$ ), is detected in the aliphatic fraction with a significant abundance for numerous species, like *A. bernieri*, *A. heterophylla*,

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*A. laubentfelsii* and *A. nemorosa*. This kind of tricyclic aliphatic diterpenoids is probably derived from isopimara-7,15-dienes, 13-isopimardiene, isopimara-8(9),15-diene as well as 8 $\beta$ -hydroxyisopimarene and 3 $\beta$ -isopimadinol, that are detected in some of the fresh plants.

Other tricyclic diterpenoids, like abietane-type compounds, show a higher diversity, especially in the aromatic fraction. Related aromatic compounds are retene, dehydroabietane, 18- and 19-norabieta-8,11,13-trienes, tetrahydroretene, simonellite, bisnor-simonellite, 2- and 9-methylretenes. While the saturated abietane-type compounds like abietanes and fichtellite are detected at trace level.

These abietane-type compounds are the major diterpenoids of *Araucaria*. They are mostly derived from dehydroabietic acid that is observed in the polar fraction of pyrolysates and more generally from abietanoic acid in the fresh plants. As regards the phenolic abietanes including sugiol, hinokiol and ferruginol (Otto and Wilde, 2001; Otto and Simoneit, 2001), which are known as abietane-type compounds precursors for Araucariaceae as well, show a low relative abundance in the fresh plants and were mostly degraded during pyrolysis.

A peak having a similar spectrum to those of methylretenes is observed in the aromatic fractions of all *Araucaria* species (Fig. 6c). Compared to Alexander et al. (1995), the spectra of these compounds have a same molecular ion and mass fragments, but are different by the fragment intensities. They may rather be consistent with a trimethyl phenanthrene.

### Tetracyclic diterpenoids

Tetracyclic diterpenoids, mainly *ent*-beyerane, 16 $\alpha$ (H)- and 16 $\beta$ (H)-phyllocladanes and *ent*-16 $\alpha$ (H)- and *ent*-16 $\beta$ (H)-kauranes, show relative high abundance in each representative. They are identified according to the spectra published by Noble et al. (1985). Their spectra are all characterized by a molecular ion at  $m/z$  274 and a base peak at  $m/z$  123 (C<sub>20</sub>H<sub>34</sub>). Distribution and abundance of these compounds for each representative are given in Fig. 5a and Table 2. They are probably derived mainly from beyerene,

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ent-kaurene, isokaurene and phyllocladene, as well as kauran-16-ol, phyllocladanol and kaur-16-en-19-ol, observed in the fresh plants. Furthermore, beyerane-type compounds are detected in *A. araucana*, *A. bidwillii* and *A. cunninghamii* in both their fresh plant (beyerene) and the pyrolysates (*ent*-beyerane). Co-occurrence of phyllocladane-type and kaurane-type compounds is observed in most of *Araucaria* species, except of *A. angustifolia* and *A. nemorosa*. Only phyllocladane-type or kaurane-type compounds are observed in *A. angustifolia* and in *A. nemorosa* respectively.

A peak with a spectrum characterized by a molecular ion at  $m/z$  260 ( $C_{19}H_{32}$ ) with a base peak at  $m/z$  123 is detected in *A. angustifolia*. This spectrum matches very well to that of 17-nortetracyclic diterpane described by Noble et al. (1986) (Fig. 6d). Formation of this C-19 diterpane is likely due to the demethylation of a C-20 tetracyclic diterpane, while its precise molecular structure remains unclear. Furthermore, in the case of *A. angustifolia*, as only phyllocladanes are observed, the C-19 tetracyclic diterpane could rather be a 17-norphyllocladane.

Another peak, characterized by a molecular ion at  $m/z$  288 ( $C_{21}H_{32}$ ) and a base peak at  $m/z$  123 is detected in *A. angustifolia*, *A. bernieri* and *A. laubenfelsii* (Fig. 6e). It is likely a methylated tetracyclic diterpane, because of the positive difference of molecular weight of 14 Da to the well known tetracyclic diterpenoids corresponds to an additional  $-CH_2-$  group. This configuration is in correspondence to a C-21 tetracyclic diterpane. Moreover, (1) according to the spectrum, the ion at  $m/z$  231 is relatively more abundant than the ions at  $m/z$  259; (2) *A. angustifolia*, *A. bernieri* and *A. laubenfelsii* all show the presence of phyllocladanes. This C-21 tetracyclic diterpane is probably a 16(H)-homo-phyllocladane.

It is also interesting to point out that, according to Otto and Wilde (2001), tetracyclic diterpenoids like trachylobane-type and atiserane-type compounds are those exclusive for *Araucaria* genus among all conifers. Unfortunately, the diagenetic products of these two compounds are still unknown to our knowledge. In our study, trachylobane and atiserene/isoatiserene are identified in the fresh *A. araucana* and *A. nemorosa*. Yet, no possible corresponding pyrolysis products could be identified.

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## Unidentified compounds

Numerous compounds remain unidentified in the extract of *Araucaria* pyrolysates and some of them, their mass spectra are given in the Figs. 4d, e and 7a–g. Tentative identifications as proposed in Fig. 7a suggests, respectively saturated tricyclic diterpenoid.

5 They have, to our knowledge, never been reported in previous studies on vascular plant biomarkers. Nevertheless, many of them are quite abundant in several species. For instance, it was observed that two peaks having spectra with a molecular ion at  $m/z$  238 ( $C_{18}H_{22}$ ; Fig. 4d) and 254 ( $C_{19}H_{26}$ ; Fig. 4e), a base peak at  $m/z$  168 and 184 respectively co-appear with 2,6-dimethyl-1-(4-methylpentyl)naphthalene and 6-ethyl-2-dimethyl-1-(4-methylpentyl)-naphthalene. These two unidentified peaks show also respectively  
10 similar abundance to 2,6-dimethyl-1-(4-methylpentyl)naphthalene and 6-ethyl-2-dimethyl-1-(4-methylpentyl)-naphthalene. The relationship of the compounds is not clear; while their co-occurrence and similar fragmentation tend each couple to a similar structure. However, these unidentified compounds may have some relevant palaeochemotaxonomic values and special attention should be paid to these biomarkers in future studies.  
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## 4.2 Molecular characteristics of artificially diagenetized *Agathis* genus

### 4.2.1 Sesquiterpenoids

Composition of sesquiterpenoids in *Agathis* (*Ag.*) species is given Fig. 8a. Distribution and relative abundance of major sesquiterpenoids are given in Table 2. Some similarities to the molecular composition of *Araucaria* species are observed. Farnesane, cadalane-type compounds (saturated cadalanes, calamene, calamenene, cadalene and norcadalene), pentamethyl-dihydroindenes and chamazulene show some high relative abundances in each *Agathis* representative. 1,3,4-trimethyl-2-(4-methylpentyl)benzene is detected as well, but at trace level.  
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Furthermore, bisabolane-type compounds (bisabolanes and dihydro-*ar*-curcumene), which are widespread in the *Araucaria* genus, are observed only in *Ag. australis* and show a low abundance in the other *Agathis* species. Concerning the eudesmanes, they were only detected only in *Ag. australis*.

#### 4.2.2 Diterpenoids

As sesquiterpenoids, compositions of diterpenoids of *Agathis* species (Fig. 8b) show also several similarities to those of *Araucaria*. Distribution and abundance of major diterpenoids of the *Agathis* genus are given in Table 2.

Bicyclic diterpenoids are characterized by the presence of labdanes in the aliphatic fraction and monoaromatic labdane in the aromatic fractions (cf. Sect. 4.1.2), which essentially originate from agathic acid (Thomas, 1969). The tricyclic diterpenoids like isopimarane is observed in *Ag. australis* and *Ag. robusta*. The isohexyl alkylaromatic hydrocarbons (2,6-dimethyl-1-(4-methylpentyl)naphthalene and 6-ethyl-2-dimethyl-1-(4-methylpentyl)-naphthalene) show high relative abundance in most of *Agathis* species and are found as traces for *Ag. robusta* (Fig. 4b, c). Abietane-type compounds show a high abundance in all species. Aromatic abietanes, like retene, dehydroabietane, 18- and 19-norabieta-8,11,13-trienes, tetrahydroretene, 2- and 9- methylretenes, are much more abundant in *Ag. moorei* and *Ag. robusta* than in *Ag. australis*. The methylretenes and norabieta-8,11,13-trienes are detected only at trace level in *Ag. australis*. Saturated abietanes are generally in trace in *Ag. australis* and *Ag. moorei*, but in higher abundance in *Ag. robusta*. As for *Araucaria*, the detected abietane-type compounds are in majority derived from dehydroabietic acid (detected in the pyrolysates) as well as from other abietanoic acids precursors and (cetono)phenoilic abietanes (detected in the fresh plants). Only dehydroabietic acid is observed in the pyrolysats. Tetracyclic diterpenoids, the *ent*-beyerane is identified only in *Ag. australis*; 16 $\alpha$ (H)-phyllocladane, *ent*-16 $\alpha$ (H)- and *ent*-16 $\beta$ (H)- kauranes, are detected in high abundance except for *Ag. robusta*. Despite of the presence and absence, these tetracyclic compounds represent as the major components within the *Agathis* species. Regarding to the unidentified

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compounds, like those of Fig. 7a–e, are also detected with a significant abundance in *Agathis* species.

Furthermore, some peaks show relatively high abundance and their spectra are respectively characterized by a molecular ion at  $m/z$  264 and 274. A base peak at  $m/z$  109 and 259 are observed in the aliphatic fraction of *Ag. australis* and *Ag. moorei*. Their chemical formulas are respectively  $C_{20}H_{24}$  and  $C_{20}H_{34}$ , and their spectra are given in Fig. 7h, i. Since these spectra are neither referenced in literature nor in our spectral database, their molecular structures are not yet clear. However their frequent, significant but also unique presence in *Agathis* species could be interesting for future biomarker research.

### 4.3 Molecular characteristics of artificially diagenetized of *Wollemia nobilis*

*W. nobilis* shows several similarities to other Araucariaceae genera (Figs. 2, 6, 9). Distribution and abundance of major sesqui- and di- terpenoids of this monotypic genus are given in Table 2.

For the sesquiterpenoids, *W. nobilis* is characterized by the presence of eudesmane-type ( $4\alpha(H)$ - and  $4\beta(H)$ - eudesmanes), bisabolane-type (bisabolane, *ar*-curcumene and dyhydro-*ar*-curcumene), cadalane-type (saturated cadalanes, norcadalene, calamene, calamenene and cadalene) compounds. Pentamethyl-dihydroindene in *W. nobilis* is not as abundant as those observed in *Araucaria* and *Agathis* genera among the sesquiterpenoids. Chamazulene, which presents a relatively high abundance in both *Araucaria* and *Agathis* genera, is here absent. And do likewise 1,3,4-trimethyl-2-(4-methylpentyl)benzene.

Furthermore, *W. nobilis* shows a wide diversity of diterpenoids. On one hand, as the other Araucariaceae species, it is characterized by the presence of labdane-type compounds (like labdanes), isohexyl alkylaromatic hydrocarbons (like 2,6-dimethyl-1-(4-methylpentyl)naphthalene and 6-ethyl-2-dimethyl-1-(4-methylpentyl)-naphthalene), abietane-type compounds (like retene, dehydroabietane, 18- and 19- norabieta-8,11,13-trienes, tetrahydroretene, 2- and 9- methylretenes as well as dehydroabietic

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acid), tetracyclic diterpenoids (like *ent*-beyerane, *ent*-16 $\alpha$ (H)- and *ent*-16 $\beta$ (H)- kaurenes). Phyllocladane-type compounds, being largely widespread in most of Araucariaceae species, are neither detected in fresh nor in pyrolysates of *W. nobilis*. Moreover, due to the high abundance of tetracyclic diterpenoids, the saturated abietanes are present at trace level in the aliphatic fraction. The unidentified compounds like those cited in Sect. 4.1.2 (Fig. 7) show a significant abundance. The diversity of diterpenoids of *W. nobilis* is, on the other hand, demonstrated by a great variety of other unknown aromatic diterpenoids. The particularities of these compounds are that they are of similar abundance with no predominance of any specific compounds (Fig. 9d).

## 5 Conclusion

Palaeochemotaxonomy of the Araucariaceae family is evaluated using artificial maturation by confined pyrolysis of extant species. Major structures of sesqui- and diterpenoids allow to trace the transformation of biomolecules into geomolecules (biomarkers). The Araucariaceae family is characterized by a remarkable predominance of saturated tetracyclic diterpenoids. However, a high abundance of sesquiterpenoids and abietanes derived could also be pointed out. The palaeochemotaxonomy of the Araucariaceae could be summarized as follows:

- the *Araucaria* genus is mainly characterized by the sesquiterpenoids, like eudesmanes, bisabolanes, chamazulene, penatamethyl-dihydroindenes and more particularly by cadalane-type compounds. For the diterpenoids, saturated tetracyclic compounds are largely predominant. Compared to the tetracyclic diterpenoids, other diterpenoids, like labdane, isopimarane, abietanes as well as iso-hexyl alkylaromatic hydrocarbons, show a relatively lower abundance. Moreover, isopimarane is only detected in the *Eutecta* section species.
- the *Agathis* genus is characterized by sesquiterpenoids, like cadalane-type compounds, chamazulene and penatamethyl-dihydroindenes. Compared to the

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*Araucaria* genus, the bisabolanes and eudesmanes are occasionally present in only certain species. Concerning the diterpenoids, most of species are highly similar to those of *Araucaria* species. Two types of molecular signature could be otherwise distinguished. One is characterized, as most of *Araucaria* species, by a high abundance of tetracyclic diterpanes and isohexyl alkylaromatic hydrocarbons, but a low abundance of saturated abietanes. This is the case for *Ag. australis* and *Ag. moorei*. For the second type, the isohexyl alkylaromatic hydrocarbons show a relatively lower abundance and the tetracyclic diterpane are absent. The saturated abietanes represent thus a significant fraction of the aliphatics like it is the case for *Ag. robusta*. Moreover, within the two types of diterpenoid signatures, the aromatic abietanes show always a high relative abundance.

- *Wollemia nobilis*, presents high similarities to the *Araucaria* genus. Presence of cadalane-types, bisabolanes, eudesmanes, penatamethyl-dihydroindenes and also the absence of chamazulene characterize the sesquiterpenoids signature of *Wollemia nobilis*. The tetracyclic compounds, like *ent*-beyerane and *ent*-kauranes, are predominant among the diterpenoids. Other recurrent diterpenoids in the *Araucaria* and *Agathis* genera, like labdane and isopimarane, are at trace amount or absent. The recurrent abietane-type compounds are identified. The aromatic abietanes do not seem as abundant as those observed in the two other genera. Moreover, there is a great variety of unidentified aromatic diterpenoids and are of same relative abundance as much as the other well known biomarkers, like the aromatic abietane-type compounds and the isohexyl alkylaromatic hydrocarbons.

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Table 2. Major detected sesqui- and di- terpenoids types in Araucariaceae species.

Class of structure	Agathis			angustifolia	araucana	bidwillii	bernieri	Araucaria			nemorosa	Wollemia nobilis
	australis	moorei	robusta					cunninghamia	heterophylla	laubenfelsii		
Sesquiterpenoids												
Farnesane-type	+++	++	+	+++	+++	+	++	+	+++	+++	++	++
Bisabolane-type	+	tr	tr	+++	++++	+	+	tr	++	++	+	+
Eudesmane-type	+++	tr	tr	++	+	++	+	tr	tr	+	+	++
Cadalane-type	++++	++++	++	++++	++	++++	++++	++	+++	+++	+++	++++
Ionene	+	tr	tr	+	+	tr	+	tr	++	tr	tr	tr
Chamazulene	++	++	++	+++	++	++	+++	++	++	++	+++	-
Pentamethyl-dihydroindene	+	+	+	++	+	+	+	tr	++	++	++	+
Diterpenoids												
Labdane-type	+	+	++	++	++	+++	+++	++	++	+	+++	+
Isohexyl alkylaromatic hydrocarbons	+++	+++	+	++++	++++	++	+++	+++	++++	-	++	++
Abietane-type												
normal abietane (dehydroabietic acid)	+	+	+	+	+	+	+	+	+	+	+	+
phenolic abietane (feruginol, sugiol and hinokiol)	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
diagenetic products	++	+++	+++	+++	tr	++++	++++	+++	+++	+++	+++	++++
Pimarane-type	+	-	-	-	-	-	+	-	+	++	+	-
<i>Erit</i> -beyerane	+++	-	-	-	+++	+++	+	-	-	-	-	-
Phyllocladane-type	+	+	-	++++	++	tr	++++	++++	+++	+++	-	-
<i>Erit</i> -kaurane	++++	+++	-	-	++	-	++++	++	++++	++++	+	++++
?C <sub>19</sub> H <sub>24</sub> (252 Da)	+	++	+	++	++	+	++	++	++	+	++	++
?C <sub>18</sub> H <sub>22</sub> (252 Da)	tr	+	+	+	+	++	++	++	+	+	++	++

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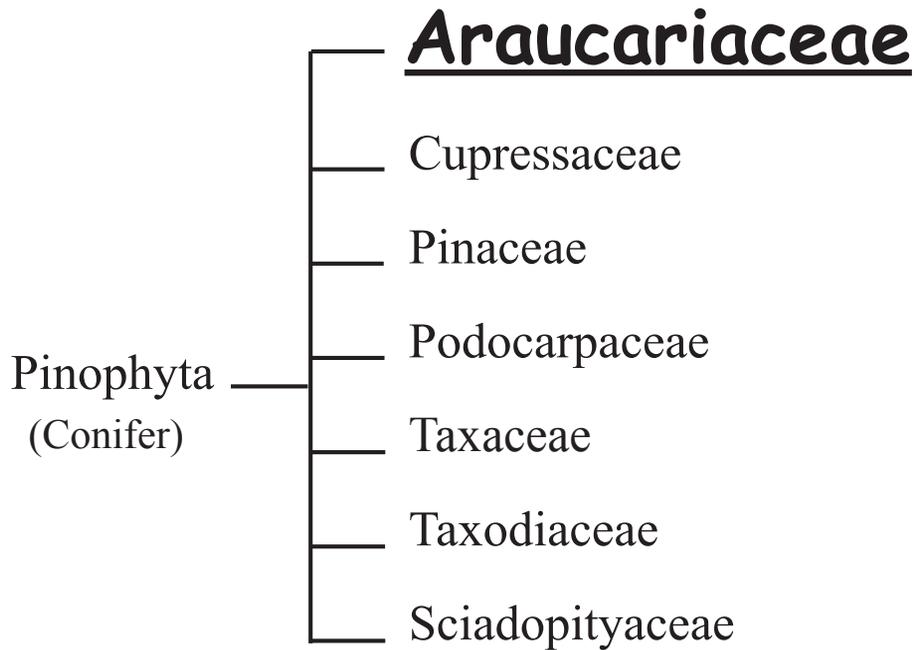
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**Fig. 1.** Phylogenetic classification of conifers.

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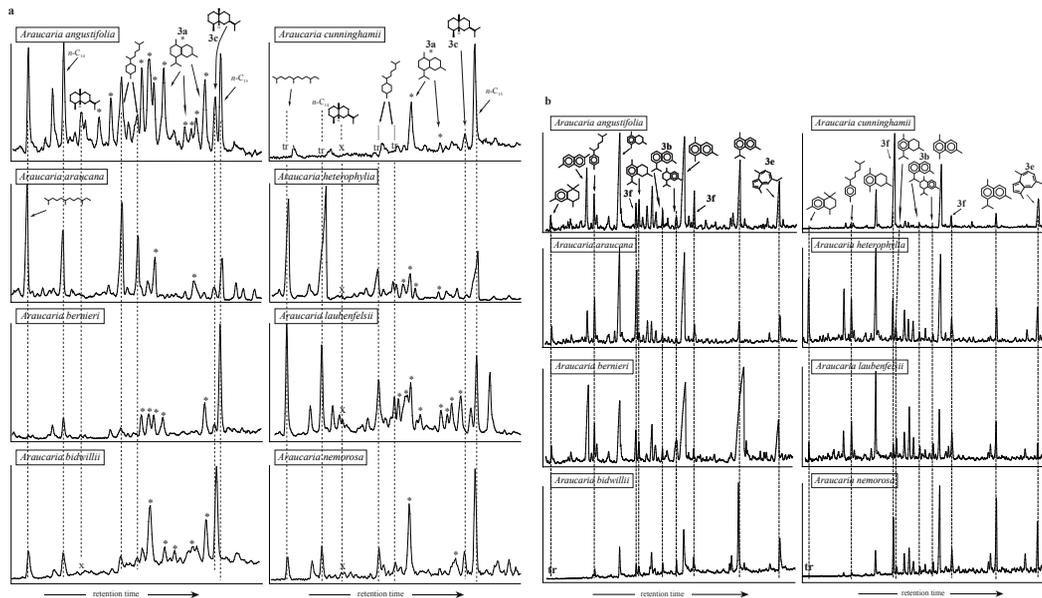
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**Fig. 2.** Partial chromatograms (sesquiterpenoids retention time window) of aliphatic **(a)** and aromatic **(b)** fractions of pyrolysis products of *Araucaria* species. The mass spectra of Fig. 3a–f are given in Fig. 3: x: absence of compound; tr: at trace level ; \*: cadalane-type compounds.

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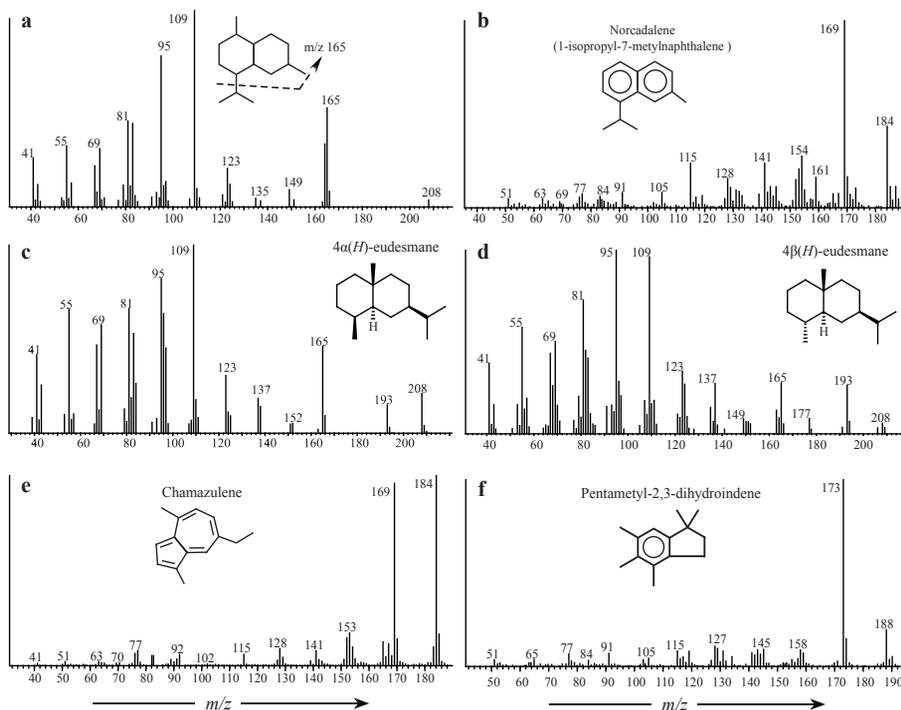
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**Fig. 3.** Mass spectra of some identified Araucariaceae sesquiterpenoids. **(a)** Saturated cadalane-type compounds; **(b)** norcadalene (identified from spectrum in Singh et al., 1994); **(c)** 4 $\alpha$ (H)-eudesmane (identified using Wiley275 database); **(d)** 4 $\beta$ (H)-eudesmane (identified from spectrum in Alexander et al., 1983); **(e)** chamazulene (identified using Wiley275 database); **(f)** pentametyl-2,3-dihydroindene (identified using Wiley275 database).

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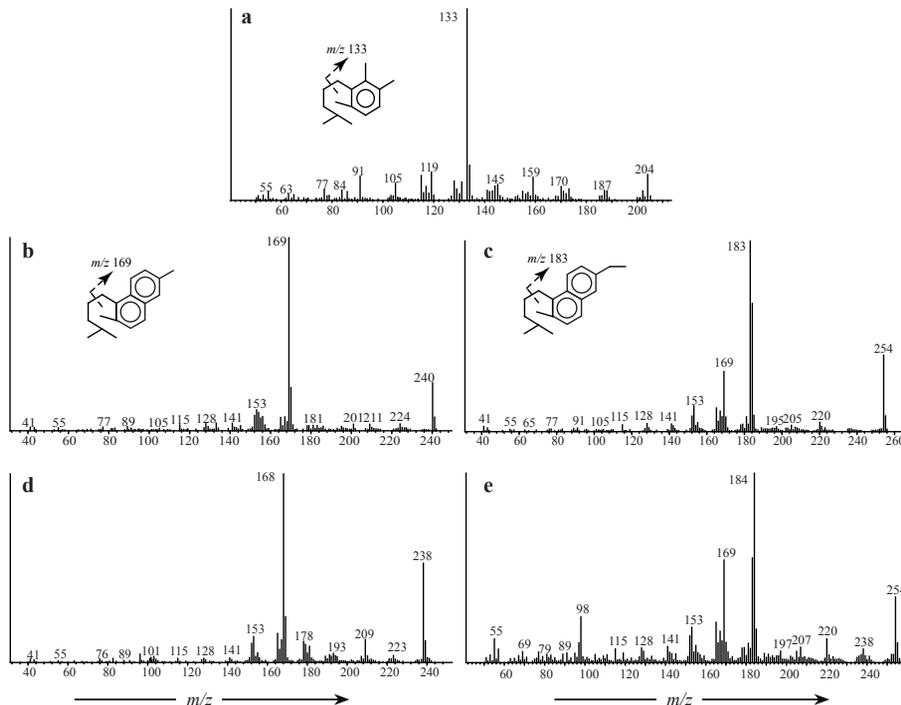
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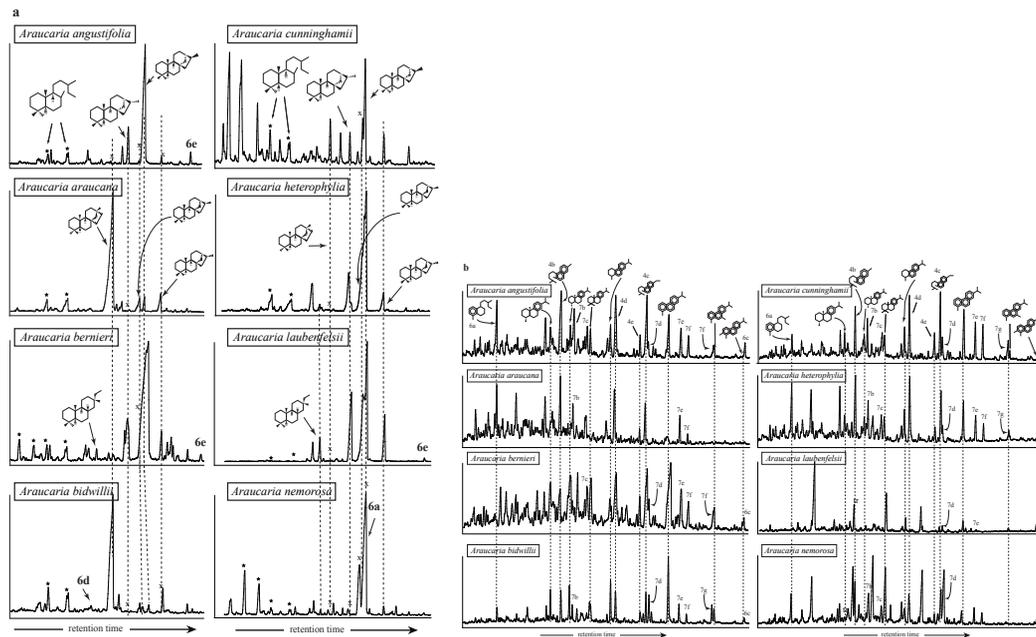
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**Fig. 4.** Mass spectra of identified isohexyl alkylaromatic hydrocarbons in Araucariaceae species and mass spectral cleavage patterns (identified after Ellis et al., 1996). **(a)** 1,3,4-trimethyl-2-(4-methylpentyl)benzene; **(b)** 2,6-dimethyl-1-(4-methylpentyl)-naphthalene; **(c)** 6-ethyl-2-dimethyl-1-(4-methylpentyl)-naphthalenemethylretene. Mass spectra of some unidentified Araucariaceae diterpenoids but similar to some of the identified isohexyl alkylaromatic hydrocarbons: **(d)** to **(b)** and **(e)** to **(c)**.

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**Fig. 5.** Partial chromatograms (diterpenoids retention time window) of aliphatic **(a)** and aromatic **(b)** fractions of pyrolysis products of *Araucaria* species. The mass spectra of Fig. 4b–e are given in Fig. 4. The mass spectra of Fig. 6a–e are given in Fig. 6. The mass spectra of Fig. 7a–g are given in Fig. 7. \*: labdanes; tr: in trace; x: absence of compound.

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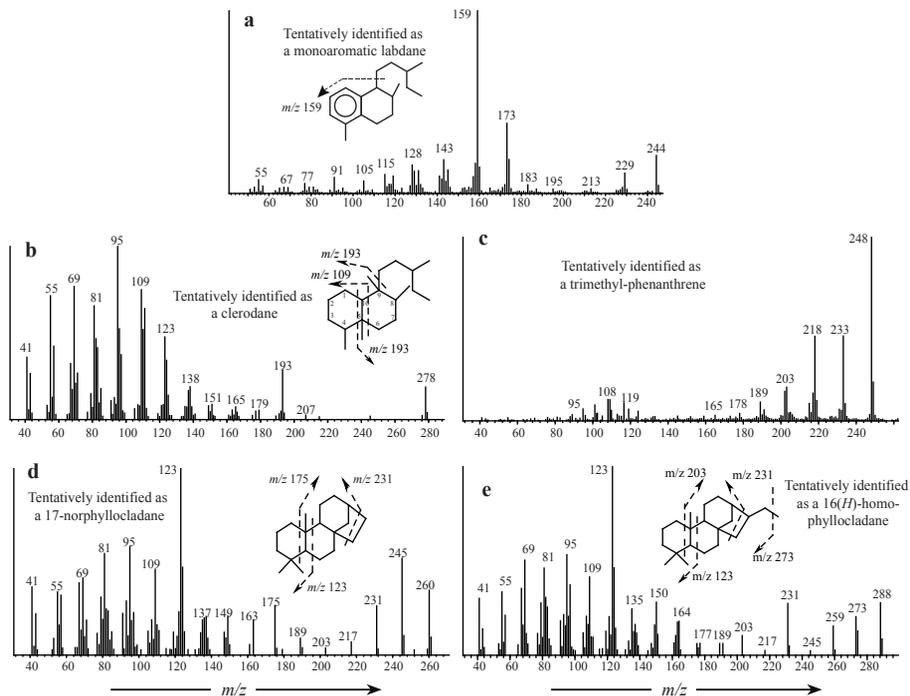
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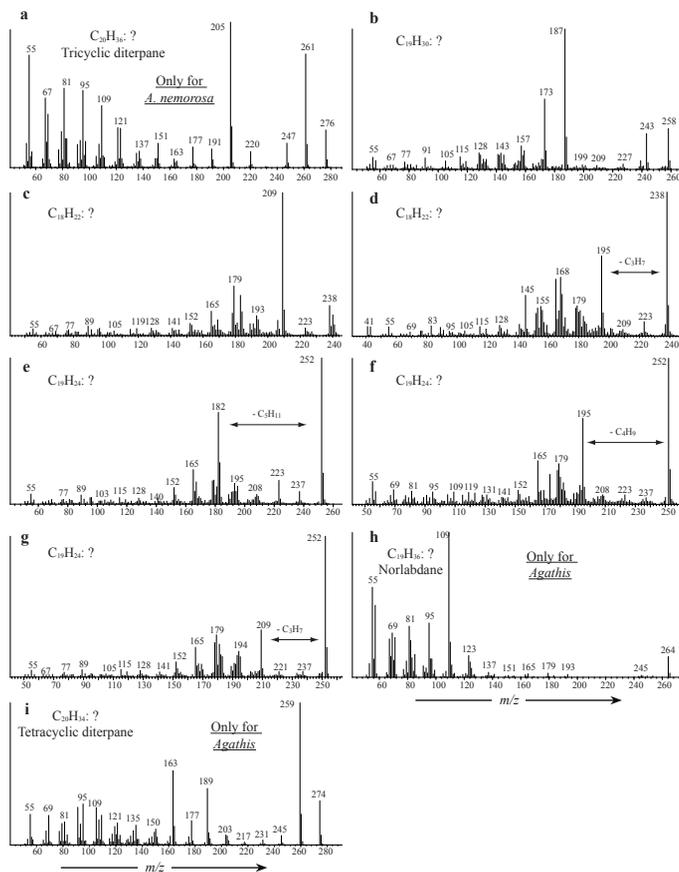
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**Fig. 6.** Mass spectra of some tentatively identified Araucariaceae diterpenoids and suggested mass spectral cleavage patterns for **(a)** monoaromatic labdane; **(b)** clerodane; **(c)** methylretene (tentatively identified according to Alexander et al., 1995); **(d)** 17-norphyllodane (identified according to Noble et al., 1986); **(e)** 16(H)-homo-phyllodane.

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**Fig. 7.** Mass spectra of some unidentified but recurrent Araucariaceae diterpenoids. **(a)** (Only for *A. nemorosa*, in Fig. 5a), **(h)** and **(i)** (only for *Agathis*, in Fig. 8a): in the aliphatic fraction; **(b, c, d, e, f, g)** in the aromatic fraction (Figs. 5b, 8b).

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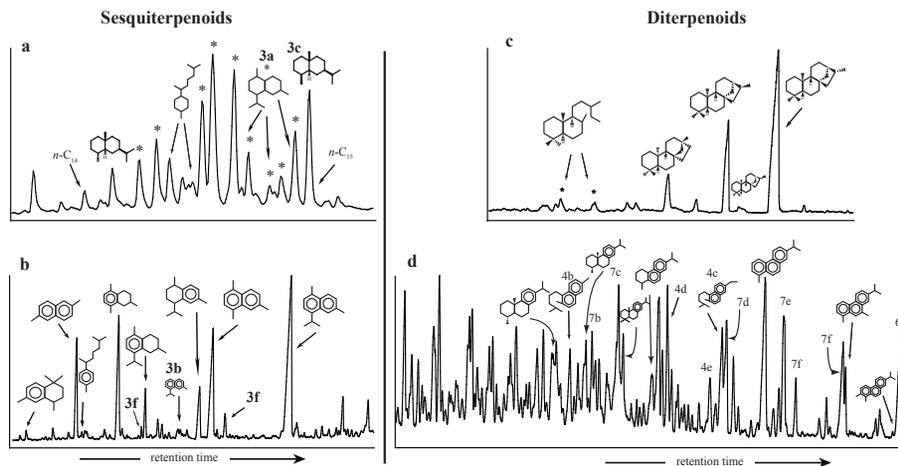
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**Fig. 9.** Partial chromatograms (sesquiterpenoids (**a**, **b**) and diterpenoids (**c**, **d**) retention time windows) of aliphatic (**a**, **c**) and aromatic (**b**, **d**) fractions of pyrolysis products of *Wollemia nobilis*. The mass spectra of Fig. 3a–c, f are given in Fig. 3. The mass spectra of Fig. 4b–e are given in Fig. 4. The mass spectra of Fig. 6c are given in Fig. 6. The mass spectra of Fig. 7b–f are given in Fig. 7. \*: cadalane-type compounds.

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