BIODIVERSITY RESEARCH

Regional assessment of the impact of climatic change on the distribution of a tropical conifer in the lowlands of South America

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ABSTRACT

For decades, palynologists working in tropical South America are using the genus *Podocarpus* as a climate indicator although without referring to any modern data concerning its distribution and limiting factors. With the aim to characterize the modern and past distribution of the southern conifer *Podocarpus* in Brazil and to obtain new information on the distribution of the Atlantic rainforest during the Quaternary, we examined herbarium data to locate the populations of three Brazilian endemic *Podocarpus* species: *P. sellowii*, *P. lambertii*, and *P. brasiliensis*, and extracted DNA from fresh leaves from 26 populations. Our conclusions are drawn in the light of the combination of these three disciplines: botany, palynology, and genetics. We find that the modern distribution of endemic *Podocarpus* populations shows that they are widely dispersed in eastern Brazil, from north to south and reveals that the expansion of *Podocarpus* recorded in single Amazonian pollen records may have come from either western or eastern populations. Genetic analysis enabled us to delimit regional expansion: between 5° and 15° S grouping northern and central populations of *P. sellowii* expanded *c.* 16,000 years ago; between 15° and 23° S populations of either *P. lambertii* or *sellowii* expanded at different times since at least the last glaciation; and between 23° and 30° S, *P. lambertii* appeared during the recent expansion of the *Araucaria* forest. The combination of botany, pollen, and molecular analysis proved to be a rapid tool for inferring distribution borders for sparse populations and their regional evolution within tropical ecosystems. Today the refugia of rainforest communities we identified are crucial hotspots to allow the Atlantic forest to survive under unfavourable climatic conditions and, as such, offer the only possible opportunity for this type of forest to expand in the event of a future climate change.

Keywords

Atlantic rainforest, Brazil, climatic change, interglacial refugia, *Podocarpus*, Tropics

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INTRODUCTION

Podocarpus is an ancient conifer genus that today is mostly diversified in the Southern Hemisphere. *Podocarpus* remains have been widely used by palynologists and palaeoclimatologists to reconstruct Quaternary climatic changes in tropical South America even though the determinism of their modern distribution is not well understood (Enright & Hill, 1995). The bisaccate pollen grain is easy to identify, and the ecosystems where the trees grow are well defined climatically, i.e. permanent atmospheric or edaphic moisture. As pollen analysis using light microscopy has not yet been able to identify the grain at species level, palynological interpretations are the subject of several hypotheses. For instance, evolution of the Amazonian rainforest during the Quaternary is based on the presence/absence of this taxon in available pollen counts. Some authors interpreted the presence of this pollen grain as evidence of the expansion of Andean forest towards the Amazon basin (Colinvaux *et al.*, 2000; Bush *et al.*, 2001). What is more, neotropical palynologists usually infer the continuation of moist climatic conditions during the last glacial maximum from the presence of *Podocarpus*, and deduce a 5° C to 6 °C drop in temperature from a simultaneous

increase in the frequency of the taxa *Podocarpus*, *Hedyosmum*, *Symplocos*, and *Alnus* (Colinvaux *et al.*, 1996; Haberle & Maslin, 1999) due to the shift of the Andean forest tree line to a lower elevation. However, botanical data on the current distribution of *Podocarpus* show that the Amazonian rainforest is surrounded by different species of this genus from eastern gallery forests within the dry Brazilian Cerrados to western Andean rainforest at higher elevations, and in refuges in the northern lowlands of Venezuela (Andrade-Lima, 1982; Gentry, 1993; Oliveira Filho & Fontes, 2000). In western Amazonia, one species, *Podocarpus celatus*, even occurs in lowland rainforest as far east as Iquitos, Peru (Vasquez, 1997). In any case, the question whether *Podocarpus* originates in the lowland gallery forests, the Andean rainforest, or the Atlantic rainforest has never been discussed in the interpretation of palaeo records. *Podocarpus*, *Hedyosmum*, and *Symplocos* are sparse today in the Amazon lowlands, but common in savanna-like vegetation (Prado & Gibbs, 1993; Pennington *et al.*, 2000) as well as in Andean forest, and in all vegetation types that border the Amazonian rainforest. In addition, *Alnus* is a high pollen producer and a wind-transported taxon. Three endemic species of *Podocarpus* have been reported in Brazil: *P. sellowii* Klotzch ex Endl, *P. lambertii* Klotzch, and *P. brasiliensis* Laubenfels (de Laubenfels, 1985). Morphological characters used to distinguish the species include internode length, leaf, and cone size. These species occur either in the Atlantic rainforest (*P. lambertii* and *P. sellowii*) or in the Cerrado (*P. sellowii* and *P. brasiliensis*) domains. However, *P. lambertii* and *P. sellowii* sometimes occur in the same area and *P. sellowii* develop under a wide range of climates from north-eastern to southern Brazil, from lowlands to 1800 m a.s.l. In addition, few modern distribution maps of South American trees and forests are available and those that do exist are not very accurate, which makes it difficult to assess the contribution of each plant association.

To improve our knowledge of present and past distributions of tropical forests and more particularly of tropical conifers such as *Podocarpus*, we decided to interview Brazilian botanists and ecologists and also to examine several Brazilian herbaria. The first part of this paper presents the results of this study and reveals the complexity of the distribution of *Podocarpus* in Brazil, which does not appear to correspond to rigorous ecological or climatic patterns. Consequently, we decided to analyse the three Brazilian endemic species, which are part of the Atlantic forest floristic composition, using a molecular approach to detect a possible link between these sparse populations in the light of the available Quaternary pollen records.

THE ATLANTIC RAINFOREST

General presentation

The Atlantic rainforest has the second highest biodiversity in Brazil after the Amazonian rainforest and is considered one of the three most threatened ecosystems on Earth (Fig. 1) (Myers *et al.*, 2000). Its extension has been strongly reduced because of intensive deforestation and only approximately 7% of the
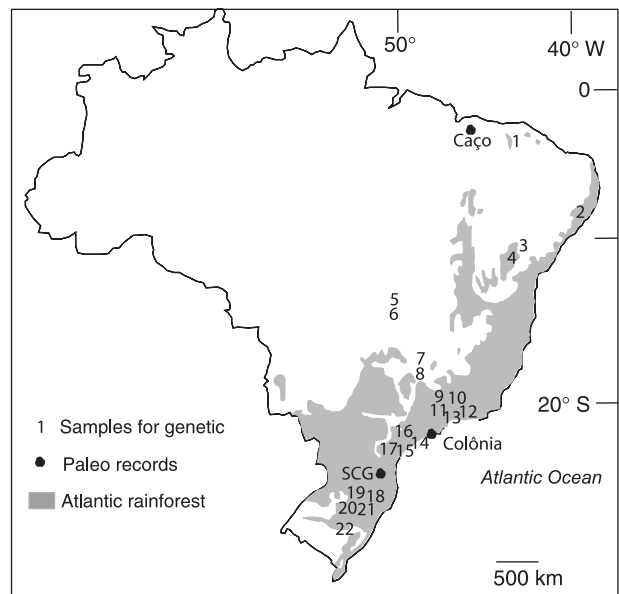

Figure 1 Map of Brazil showing the distribution of the Atlantic forest domain (in grey), location of the sample numbers (listed in Table 1) used for DNA extraction, and location of the pollen records discussed in the text (SCG for Serra Campos Gerais, see reference in text).

original cover remains, most of which is located on the slopes of the Serra do Mar in the south-eastern region or in ecological reserves. For many years the absence of a rigorous definition of this biome delayed its recognition by international conservation policy groups due to a long-standing academic controversy about whether it should be restricted to the ombrophilous dense forest which is under the direct influence of the moisture brought by the Atlantic Ocean, or should be expanded to include the semideciduous forest that extends into the interior of the states of Minas Gerais and São Paulo, or even include deciduous forests. It has also been proposed that the mixed forest dominated by *Araucaria angustifolia* should also be considered as part of the Atlantic rainforest. To help clarify this controversy, Oliveira Filho & Fontes (2000) investigated the tree flora in south-eastern Brazilian Atlantic forests with respect to variations in floristic composition of both rainforests and semideciduous forests. These authors concluded that the definition of the Atlantic forest biome should encompass all forest physiognomies occurring east of the dry corridor that passes through north-eastern Brazil, eastern Paraguay, north-eastern Argentina, and southern and south-eastern Brazil. All the following vegetation physiognomies: ombrophilous dense, ombrophilous open, ombrophilous mixed, semideciduous and deciduous forests, mangroves, restinga, sand dune vegetation, estuaries, lagoons, and high altitude rocky scrubs, found in the Atlantic rainforest domain are now considered Atlantic forest *sensu lato* (Joly *et al.*, 1999; Oliveira Filho & Fontes, 2000; Instituto Socio Ambiental, 2001). In this paper, we follow this widely accepted concept.

The Atlantic forest covers a large region in Brazil from the Equator to 30° S, and consequently includes a wide range of

climatic conditions but is generally characterized by nearly constant water availability through seasonal rainfall, frequent cloud cover, and drizzle and/or soil moisture content. Extensive deforestation in the interior of the two most cultivated states of Brazil, Minas Gerais and São Paulo, has made it difficult to define the geographical and climatic limits of the two species of conifer *P. sellowii* and *P. lambertii* we are studying, both of which are scarce in the forest understorey. *P. sellowii* is present in all the gallery forests and in high elevation forests in these regions, while *P. lambertii* appears to be restricted to the *A. angustifolia* forest on the mountain ridges between the cities of São Paulo and Rio de Janeiro and the southern middle-elevation plateaus.

Mountain forest

These forests are generally restricted to areas fed by spring waters on the top of the mountains located between 19° and 23° S and 44° to 45° W, at an elevation of 1100–1600 m a.s.l. Floristic and ecological descriptions are still rare in these areas. The climate at Carrancas (21°36′° S, 44°37′ W) in the State of Minas Gerais, at 1500 m a.s.l. is characterized by a mean annual rainfall of 1500 mm with a 3-month dry season and a mean winter temperature of 11.5 °C. Ten families make up 75.2% of the total number of plants: Lauraceae 17.7% (*Ocotea, Nectandra, Persea*), Myrtaceae 13.9% (*Myrcia, Eugenia*), Rubiaceae 10.3%, Melastomataceae 7.2% (*Miconia, Tibouchina*), Apocynaceae 6% (*Aspidosperma*), Euphorbiaceae 5.2% (*Croton*), Myrsinaceae 5.1% (*Myrsine*), Asteraceae 4.5%, Annonaceae 2.9%, Araliaceae 2.5%, and Cyatheaceae (*Cyathea*); and the seven most abundant species are *Ocotea odourifera, Psychotria sessilis, Aspidosperma parvifolium, Nectandra grandiflora, Miconia chartacea, Eremanthus erythropappus,* and *Myrsine umbellata*. Sparse individuals of *P. sellowii, Weinmannia paulliniifolia, Hedyosmum brasiliense,* and *Drymis brasiliensis* were also identified (Oliveira Filho *et al.*, 1994, 2004).

Refugia in north-eastern Brazil

On the northern coast, near the city of Fortaleza, *Podocarpus* formations are located on the top of the mountains where there is a permanent supply of moisture brought by Caribbean trade winds. Along the eastern coast, semideciduous tropical forests and rainforests occur in a narrow belt less than 100 km long which marks the transition between the ocean and the semi-arid Caatingas; but the same types of forest also occur in the hinterland as mountain forest enclaves (Andrade-Lima, 1982). For instance, the climate of the Serra dos Cavalos (14°14′ S, 38°56′ W) is driven by winds blowing inland from the Atlantic that bring permanent moisture that is deposited on the slopes of the mountains. This vegetation is associated with the formation of cloud forest and has been classified as 'refugia of the Atlantic rainforest' along the north-eastern coast. Botanical surveys identified the following species as being the most abundant: *Hymenea courbaril* (Fabaceae), *Byrsonima sericea* (Malpighiaceae), *Bowdichia virgilioides* (Caesalpiniaceae), *Pterocarpus violaceus* (Fabaceae), *Cedrela odourata* (Meliaceae), *Inga subnuda* (Mimosaceae), *Didymopanax morotoni* (Araliaceae), *Tapirira*

guianensis (Anacardiaceae), *Tabebuia serratifolia* (Melastomataceae), *Manilkara rufula* (Sapotaceae), *Ocotea glomerata* (Lauraceae), and *Podocarpus sellowii* (Podocarpaceae) (Andrade-Lima, 1982; Viana de Lima, 1991).

Araucaria forest

In southern and south-eastern Brazil, the climate is subtropical with frequent frosts, no dry season, and annual precipitation ranging between 1300 mm and 3000 mm. These conditions are propitious to the development and expansion of *Araucaria* forests. Today these forests are restricted to the southern mid-level plateaus at an elevation of around 500 m a.s.l., and to the mountain ridges along the coast between 18° S and 23° S, at an elevation of 1600 m a.s.l. (Hueck, 1953; Klein, 1960; Rambo, 1960; Aubréville, 1961). They are characterized by a combination of vegetation mosaics where species from a relic coniferous forest including several Andean genera, such as *Weinmannia* and *Hedyosmum*, are mixed with typical Atlantic forest species. In these forests *P. lambertii* grows with *A. angustifolia*. Floristic patterns provide evidence for an increase in Melasto-mataceae (*Miconia* and *Tibouchina*), Solanaceae (*Solanum*), Lauraceae (*Ocotea* and *Nectandra*), Aquifoliaceae (*Ilex*) and Asteraceae, and a decrease in Myrtaceae (*Eugenia*) and Moraceae (*Ficus*) with an increase in altitude (Oliveira Filho & Fontes, 2000).

Cerrado and gallery forests

The Cerrado biome, a savanna-like ecosystem, covers 22% of Brazil. Many different physiognomies can be observed as a function of the density of the tree layer, soil characteristics, and climate. The Cerrado has a unique flora that distinguishes it from other Brazilian ecosystems. The different physiognomies of the Cerrado show a gradient of savanna formations from pure grass-lands 'campo limpo' to forested formations 'cerradão' which differ from one another in the relative abundance of woody and herbaceous species (especially grasses). Gallery forests are part of the numerous mosaics of vegetation that make up the Cerrado biome and develop on hydromorphic soils on the banks of the many rivers that cross the Cerrado (Oliveira & Marquis, 2002; Oliveira-Filho & Ratter, 2002). The presence of some species suggests ancient connections with Atlantic and Amazonian rainforests (Ratter *et al.*, 2000). The five families that are always identified in the gallery forests are: Anacardiaceae (*Tapirira guianensis*), Annonaceae (*Xylopia emarginata*), Leguminosae (*Copaifera langsdorffii*), Myrtaceae, and Rubiaceae (many species). *P. brasiliensis* Laubenfels and *P. sellowii* Klotzch ex Endl, *H. bra-siliense* Mart., seven species of *Myrsine* and five species of *Ilex* also grow in these gallery forests (Silva Junior *et al.*, 1998). Mean annual rainfall is 1600 mm, 75% of which falls between November and January. Mean annual temperatures range between 18 °C and 20 °C. Relative atmospheric moisture varies between 70% and 85% in summer and decreases to 50–65% in winter, sometimes reaching less than 20%. Annual evapotranspiration varies between 1700 mm and 1800 mm, resulting in a hydric deficit.

General climatic features

In the Atlantic rainforest, the presence of *Podocarpus* is related to specific local or regional climatic conditions characterized by moisture availability throughout the year. Moisture is provided either by permanent drizzle, by cloud formation in the mountains, or by specific edaphic conditions, such as well-drained soils in the gallery forest, or abundant rainfall. In addition, throughout the Atlantic forest *sensu lato*, low winter temperatures seem to be the limiting factor for the development of *P. sellowii*, which preferentially grows in northern and central Brazil, whereas *P. lambertii* is abundant in southern Brazil where frost is often recorded.

PALYNOLOGICAL RESULTS

In pollen records, a change to *Podocarpus* is often associated with groups of taxa (called spectra) that vary as a function of their modern geographical location. Each type of pollen spectrum provides climatic information that is then used to reconstruct the past extension and evolution of the Atlantic forest. In the following sections, ages are all expressed in calendar years BP and written as year BP.

Podocarpus, Hedyosmum, Weinmannia, and the Atlantic rainforest

In southern Brazil, an increase in the pollen frequencies of *Podocarpus, Hedyosmum*, *Weinmannia*, and *Ilex* is associated with the development of the cloud forest and, when *A. angustifolia* is also recorded, with the expansion of the *Araucaria* forest. Results attest to an expansion of the cloud forest that started 3000 years ago. Between 25° and 30° S, a pollen record located inside the *Araucaria* forest enclave attests to an extension of the distribution of *Podocarpus* in the last 3000 years. Expansion of the *Araucaria* forest started from gallery forests *c.* 3000 years ago and was followed by an expansion towards the highlands in the last 1000 years. Species associated with *A. angustifolia* include *Podocarpus, Ilex, Mimosa scabrella*-type, and *Symplocos lanceolata*-type (Ledru *et al.*, 1994; Behling, 1997a,b). In São Paulo at 23° S, the Colônia record (Fig. 2) shows an increase in *Podocarpus, Weinmannia*, and *Hedyosmum* pollen frequencies between 29,000 and 21,000 year BP. Today, only pollen grains of *Weinmannia* are recorded at this site. An earlier increase in *Podocarpus* pollen frequencies is dated between *c.* 60,000 and 45,000 years ago. Associated taxa are Myrtaceae, Melastomataceae/Combretaceae, *Celtis*, and *Alchornea*, all considered as indicator taxa for semideciduous forest (Ledru, 2002). Finally, an increase in *Podocarpus* pollen frequencies is dated between 100,000 and 90,000 years BP and recorded in association with semideciduous taxa such as Myrtaceae, Melastomataceae/Combretaceae, *Myrsine*, *Ilex* and also but with low frequencies of *Araucaria* (Ledru & Mourguiart, in preparation). Although an increase in *Podocarpus* pollen frequencies is recorded throughout these three events, each is characterized by different spectra. When referring to modern analogues, the presence of *Araucaria* at the base of the
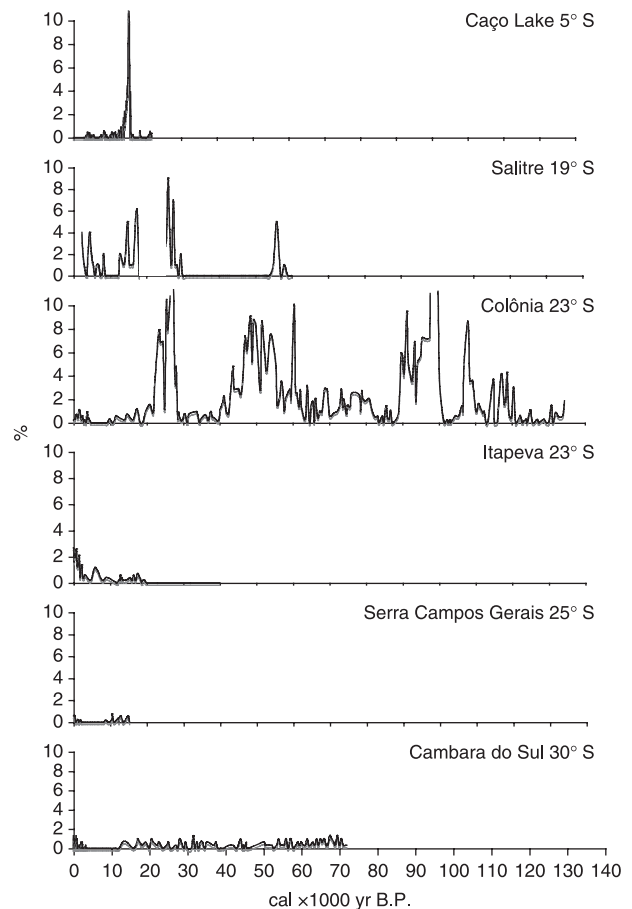

Figure 2 Changes in *Podocarpus* frequencies as a function of a time scale in the core MA 97–1 from Caço Lake Maranhão (Ledru *et al.*, 2001), in the core LC3 from Salitre (Ledru *et al.*, 1996), in the core CO3 from Colônia São Paulo 23° S (Ledru *et al.*, 2005), in the core from Itapeva (Behling, 1997a), in the core from Serra Campos Gerais (Behling, 1997b), and in the core from Cambara do Sul (Behling *et al.*, 2004).

record supports the hypothesis of an expansion of *P. lambertii*, whereas the two others are related to later expansions of *P. sellowii* characterized by the absence of *Araucaria* forest indicators and the presence of cloud forest spectra. Today, *P. sellowii* grows sparsely in the vicinity of the city of São Paulo, while *P. lambertii* is found 200 km further south in the State of Parana or 200 km further north in the Serra da Mantiqueira.

The springs of some of the main Brazilians rivers are located in high elevation mountain ridges in the State of Minas Gerais, in Central Brazil. In the pollen record of Salitre, 19° S 46°, 46′ W 1000 m a.s.l., low frequencies of *Podocarpus* associated with semideciduous forest taxa are observed from 60,000 to 3000 years BP onward with interruptions between 50,000 and 30,000 years BP and a hiatus in sediment deposition between 26,000 and 18,000 years BP. As observed in Colônia, two significant increases in *Podocarpus* frequencies are recorded, one between 60,000 and 50,000 and one between 29,000 and 26,000 years BP. *Hedyosmum* and *Weinmannia* are not recorded (Ledru *et al.*, 1996) (Fig. 2).

In the Serra da Mantiqueira, the pollen record of Morro de Itapeva, 22°47′ S, 45°35′ W, 1850 m a.s.l., is located within an enclave of *Araucaria* forest. Low frequencies of *Podocarpus* (Fig. 2) and *Weinmannia* and single pollen grains of *Araucaria* are recorded between 19,000 and 13,000 years BP and between 11,500 and 2500 years BP. *Hedyosmum* is not recorded. Pollen frequencies of these taxa increased in the last 2500 years, attesting to a recent expansion of the *Araucaria* forest to its modern limits (Behling, 1997a) in agreement with the data obtained in the southern *Araucaria* forests.

In southern Brazil, 24°40′ S, 50°13′ W, 1200 m a.s.l., low frequencies of *Podocarpus* (Fig. 2), *Hedyosmum*, and *Weinmannia* are recorded along with *Araucaria* since the end of the Pleistocene *c.* 12,000 years BP but these taxa only really expanded in the last 3000 years BP (Behling, 1997b). In the southernmost part of the Brazilian highlands, *Araucaria*, *Podocarpus* (Fig. 2), and single grains of *Weinmannia* are well documented in the pollen record of Cambara do Sul, 29°24′ S, 50°34′ W, 900 m a.s.l., while *Hedyosmum* is not recorded (Behling *et al.*, 2004).

Podocarpus, Hedyosmum, Myrsine, Ilex, and Amazonian rainforest

The pollen record of Lagoa Pata in Amazonia, 0°16′ N, 66°41′ W, 300 m a.s.l., attests to the association of *Podocarpus-Myrsine-Hedyosmum-Weinmania-Ilex* at the beginning of the deglaciation *c.* 18,000 years BP (Colinvaux *et al.*, 1996; Ledru *et al.*, 2001). Climatic cooling has been inferred from the increased frequencies of this pollen assemblage although the lack of a modern analogue during the late glacial prevents accurate climatic reconstruction. At Carajas, 6°20′ S, 50°25′ W, 700–800 m a.s.l., located on the eastern edge of the Amazonian basin, an increase in the pollen association between *Podocarpus* and *Myrsine-Ilex-Hedyosmum* is recorded twice during the late Pleistocene (Ledru, 2002). At the base of the record, when total tree pollen frequencies decrease abruptly from 90% to 20%, an increase in the frequencies of some other tree pollen grains is progressively recorded: *Podocarpus* followed by *Ilex, Hedyosmum* and finally *Myrsine*. The second decrease during deglaciation, low total tree pollen frequencies of between 20% and 30% are also recorded. This phase is characterized by a simultaneous increase in *Podocarpus*, *Myrsine*, and *Hedyosmum* but no *Ilex* (Absy *et al.*, 1991). In southern Amazonia, Laguna Chaplin (Bolivia), another record located at 14°28′ S and 61°04′ W, attests to an increase in the frequency of the *Podocarpus-Myrsine-Ilex* association but the absence of *Hedyosmum* at the end of the Pleistocene with a final peak in *Podocarpus* during the late glacial (Burbridge *et al.*, 2004). In Amazonia, due to the lack of species identification, it is not possible to conclude whether the pollen of *Podocarpus* identified originated from the mountain cloud forest which today is located further north on the slopes of the Pico da Neblina, or from the entry into the lowland rainforest of significant populations of the Andean forest, or if it was restricted to gallery forest formations within a grassland area, or if it originated from an expansion of eastern Atlantic forest populations.

Podocarpus, Myrsine, Ilex, Hedyosmum, and Cerrado

In the Cerrado, an increase in the frequency of *Podocarpus* is often recorded together with an increase in *Ilex*, *Myrsine*, and *Hedyosmum*, although sometimes out-of-phase with *Ilex*. Today, all these taxa grow in the Cerrado and in the Atlantic and the Andean forests. In the Andes, *Myrsine* grows at intermediate elevations, while *Ilex* is mostly found at higher elevations; in the Cerrado *M. umbellata*, *M. ferruginea*, *I. cerasifolia*, *I. martinsiana*, and *I. conocarpa* have been identified in lowland Amazonia; and *I. inundata*; and *M. umbellata*, *I. paraguariensis*, and *I. brasiliensis* have been identified in the *A. angustifolia* forest (Ledru *et al.*, 2001). Such widespread occurrence could correspond to a common area of distribution in the past. In central Brazil, in the 'true' Cerrado, the pollen records are often poorly dated with low analytical resolution. However, all the records attest to an abrupt increase in *Podocarpus*, *Ilex, Myrsine*, and *Hedyosmum* frequencies in the last 20,000 years (Salgado-Labouriau, 1997; Barberi *et al.*, 2000; Ledru, 2002).

In the northernmost part of the Cerrado, the Caço Lake pollen record dated the *Podocarpus-Ilex-Myrsine* expansion between 16,000 and 15,000 years BP (Fig. 2). The shortness of this period (not more than five centuries) and the abrupt increase in the forest taxa that characterize this event support the hypothesis of the location of *Podocarpus* populations close to where the forest originated and was able to expand within a very short period (Ledru *et al.*, 2001; Ledru *et al.*, 2006). The speed of this expansion also explains why these species were not detected in low-resolution records.

CONCLUSION

However, these results – single pollen record interpretations in a wide geographical area – are still far too scarce to allow any conclusions to be drawn with respect to the general evolutionary and historical pattern of the Atlantic rainforest. Two questions that remain to be answered are: was the expansion of the cloud forest expansion that is recorded in northern Brazil between 16,000 and 15,500 years BP just a local event or part of a regional change in vegetation? Is there a link between the recent increase in *P. lambertii* in southern Brazil and the northernmost populations which were well established during glacial times? More palaeoenvironmental records are needed to cover such a wide territory, especially in transition areas.

MOLECULAR ANALYSIS

Materials and methods

When the aim is to reconstruct past evolution and distribution on a wide spatial scale, molecular analysis has been shown to be more powerful than palynology (Petit *et al.*, 1997; Abbott *et al.*, 2000; Davis & Shaw, 2001; Cheddadi *et al.*, 2006), which is why we chose to use this approach.

Plant material was collected in the field, then dried and preserved in silica gel. In total, 26 populations of *Podocarpus* were selected

Table 1 Sites where samples were collected, with their geographical data. Codes refer to numbers in Figs 1 and 3.

Code	Herbarium	Species	Collector	Number of collect	Date	Elevation (m)	Latitude South	Longitude West
1	UFCE	*P. lambertii*	A. Silveira	472	20 May 2003	880	14°14′	38°56′
2	UFCE	*P. sellowii*	A. Ribeiro	613	Nov 2000	1100	8°18′	36°
3	SPF	*P. cf. lambertii*	G. Ceccantini	1991	12 Feb 2003	1400	13°32′	41°57′
4	SPF	*P. sellowii*	G. Ceccantini	1988	11 Feb 2003	1000	13°7′	41°7′
5	SPF	*P. sellowii*	G. Ceccantini	1964	7 Feb 2003	1000	14°13′	47°51′
6	SPF	*P. brasiliensis*	G. Ceccantini	1769	5 Jun 2003	750	15°86′	47°9′
7	SPF	*P. sellowii*	J.R. Pirani	5098	9 Jun 2003	1100	19°17′	43°35′
8	SPF	*P. lambertii*	J.R. Pirani	5090	9 Jun 2003	1100	19°17′	43°35′
9	ESAL	*P. sellowii*	A.T. Oliveira Filho	4554	15 Nov 2003	1050	21°33′	44°38′
10	SPF	*P. lambertii*	M.P. Ledru	4	21 Nov 2002	1200	22°75′	46°14′
11	SPF	*P. sellowii*	R. Mello Silva	1996	9 Nov 2002	950	20°11′	46°25′
12	SPF	*P. lambertii*	M.P. Ledru	3	22 May 2002	1700	22°73′	45°59′
13	PMSP	*P. sellowii*	R. Garcia	2163	29 Nov 2002	1100	23°59′	46°45′
14	ESAL	*P. sellowii*	Mazine	403	8 Dec 2002	0	25°01′	47°92′
15	SPF	*P. sellowii*	G. Ceccantini	1781	20 Oct 2002	26	25°8′	48°6′
16	SPF	*P. lambertii*	G. Ceccantini	1768	2 June 2002	875	25°43′	49°08′
17	SPF	*P. sellowii*	P. Labiak	118	7 Nov 2002	1200	25°47′	48°33′
18	PACA	*P. lambertii*	M.P. Ledru	2	10 Apr 2002	970	29°44′	50°58′
19	PACA	*P. lambertii*	M.P. Ledru	1	21 Mar 2000	466	30°34′	52°44′
20	SPF	*P. lambertii*	G. Ceccantini	1784	28 Dec 2002	267	30°21′	53°37′
21	SPF	*P. lambertii*	G. Ceccantini	1790	28 Dec 2002	351	30°51′	53°09′
22	SPF	*P. lambertii*	G. Ceccantini	1960	29 Dec 2002	466	30°34′	52°44′
23	SPF	*P. cf. montanus*	M.P. Ledru	5	29 Dec 2002	150	42°3′	72°15′
24	SPF	*P. parlatorei*	R. Mello Silva	2069	10 Dec 2002	2210	18°36′	64°01′
25	SPF	*P. parlatorei*	R. Mello Silva	2077	9 Dec 2002	2370	18°32′	64°02′
26	SPF	*P. parlatorei*	R. Mello Silva	1873	7 Dec 2001	1700	24°01′	65°23′

ESAL, Universidade Federal de Lavras; PACA, Porto Alegre Colégio Anchieta; PMSP, Prefeitura Municipal de São Paulo; SPF, Universidade de São Paulo; UFCE, Universidade Federal do Ceará.

(Fig. 1). They represent the biotopes where three endemic Brazilian species, *P. sellowii*, *P. lambertii*, and *P. brasiliensis*, grow today: gallery forest in Cerrado, Araucaria forest, and Atlantic rainforest *sensu lato*. We also collected specimens outside Brazil, *P. saligna* in Chile and *P. parlatorei* in northern Argentina and Bolivia (Table 1).

For DNA analysis, five individuals were collected at each site. Total genomic DNA was processed for AFLP analysis according to established procedures of restriction, adapters ligation, and pre- and selective PCR amplification (Vos *et al.*, 1995). EcoRI and MseI, respectively, and combination primers AA-CAT, AA-CAC, and AG-CTG were used in the analyses. Electrophoretic analyses were carried out in an ABI 310 automatic sequencer, using GenScan-500 Rox fluorescent markers; GENESCAN and GENOTYPER software (Applied Biosystems, Foster City, CA, USA) was used for fragment score and matrix making. A total of 126 informative AFLP binary-coded characters were obtained. UPGMA analysis using Jacquard distance was computed from these data using NT-SYS software (Rohlf, 1994). Bootstrap analysis was performed with 1000 replications using PAUP 4.0.b10 (Swofford, 2002). The level of genetic differentiation between the main clusters of the UPGMA tree was calculated using AMOVA (Machalakis & Excoffier, 1996) and significance level was tested with 1000 permutations as implemented in the ARLEQUIN software (Schneider *et al.*, 2000).

Results

Although we included five *Podocarpus* species in the analysis, both UPGMA and AMOVA only recovered two strongly differentiated groups, one including *P. sellowii*, *P. brasiliensis*, and *P. saligna*, and another grouping *P. lambertii* with *P. parlatorei* (Fig. 3). Our main results were the following:

1 *P. brasiliensis* is embedded within *P. sellowii*.

2 *P. lambertii* and *P. sellowii* are separated in two distinct clusters.

3 *P. sellowii* splits into two main geographical groups: the first one (yellow and red dots in the figure) between 5° and 17° S and the second one (green dots in the figure) between 17° and 24° S, southern Minas Gerais and the state of São Paulo.

4 *P. lambertii* shows a wider distribution than *P. sellowii* from north to south.

5 The main area of *P. lambertii* is located in southern Brazil between 24° and 30° S, and in the mountain ridges between Rio and São Paulo within the *A. angustifolia* region.
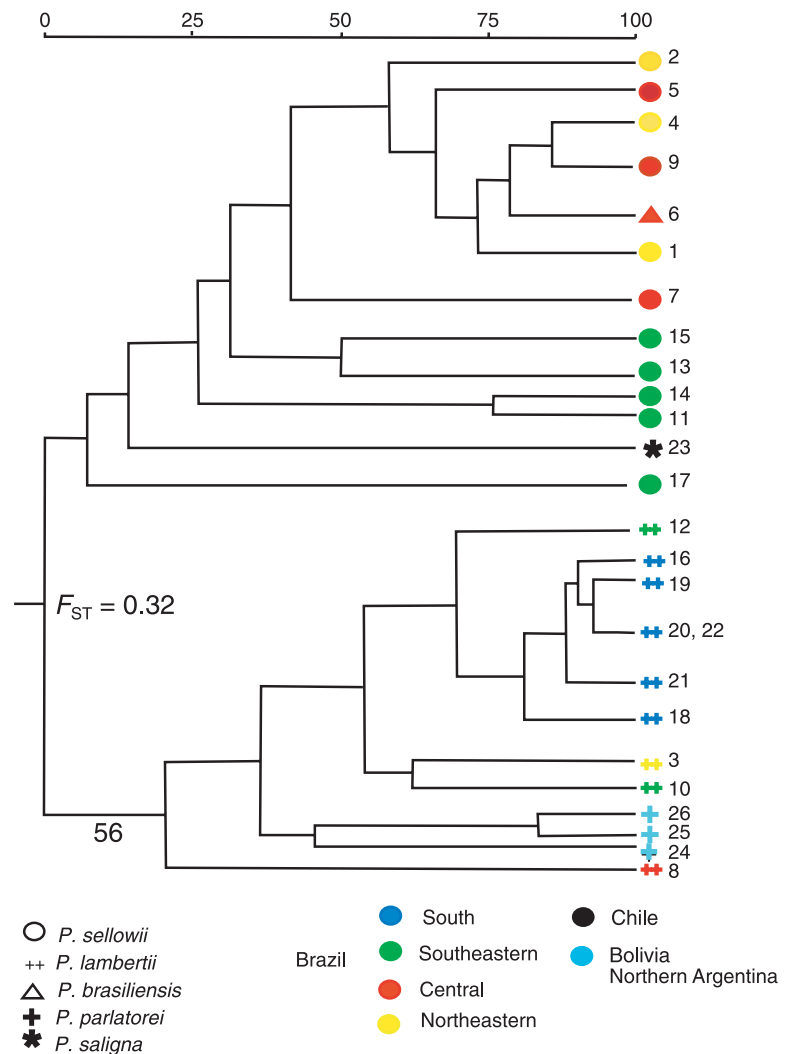
Figure 3 UPMGA dendrogram showing the relationship between all the samples of *Podocarpus* analysed based on Jaccard distance. Numbers refer to the samples listed in Table 1. Colours show the different regions where the samples were collected. Level of differentiation between the two main clusters (F_{ST}) is indicated as well as a bootstrap value (56) on the lower cluster. All other branches did not have significant bootstrap support except those grouping individuals from the same location which have been collapsed.

6 Rare groups of *P. lambertii* are found in north-eastern and central Brazil, close to *P. sellowii* populations; this could be explained by a pre-Quaternary distribution at the scale of the continent.

7 Chilean groups of *P. saligna* fall into the *P. sellowii* group while Bolivian groups of *P. parlatorei* fall into the *P. lambertii* group; this could be due to similar evolution.

AFLP analysis thus suggests the existence of two main *Podocarpus* groups in South America (*P. sellowii* + *P. brasiliensis* + *P. saligna* and *P. lambertii* + *P. parlatorei,* as far as we can tell from our taxonomic sampling). Differentiation between these two groups is supported by the very high F_{ST} value (0.32) from the AMOVA analysis. Within the *P. sellowii* group, two subgroups are distinguished that indicate specific ecological conditions: mountain vs. lowland gallery forest. The *P. lambertii* group is genetically more homogeneous (i.e. shorter distances between populations), and occurs mainly in the southernmost latitudes of Brazil in the *Araucaria* forest. Such high genetic homogeneity probably reflects the recent expansion of this genus in the area.

The other group of samples is composed of individuals attesting to drastically different ecological conditions, with longer distances between the populations, most from higher latitudes. This observation and the affinity between south-eastern *Podocarpus* populations and *P. parlatorei* from Argentina and Bolivia suggest an ancient connection with other South American regions. In both main clusters, longer genetic distances mainly characterize populations from south-east Brazil, whereas populations within the limits of *Podocarpus* distribution show considerably shorter genetic distances, again probably reflecting older expansion in south-eastern Brazil and more recent expansion in north-eastern and southern Brazil.

RESULTS AND DISCUSSION

Combining botany, palynology, and genetics enabled us to relatively rapidly (at least more rapidly than by analysing pollen records) distinguish and delimit three main populations of *Podocarpus* linked to three different patterns of Quaternary
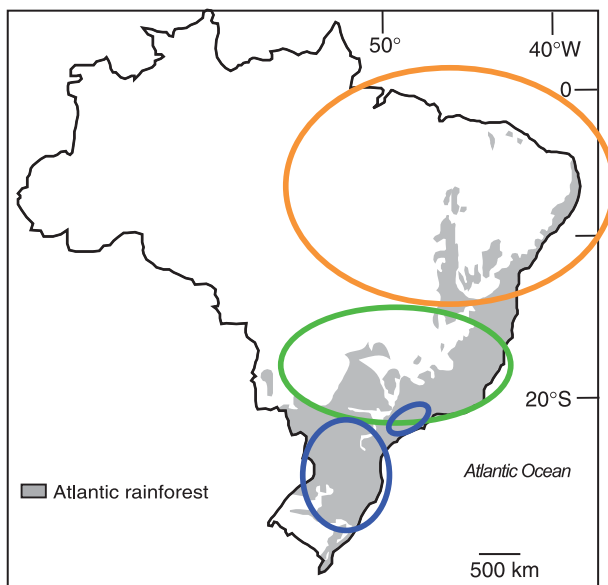
Figure 4 Map of Brazil showing the three groups of *Podocarpus* within the Atlantic rainforest, either interglacial patches refugia or main forest, based on results provided by both palynological and genetic analyses. Line colours correspond to sample distribution in Fig. 3 except the red and yellow areas which are here grouped as orange.

climatic evolution: between 5° and 15° S, between 15° and 23° S, and between 23° and 30° S (Fig. 4).

The modern distribution of *Podocarpus* shows that they are widely dispersed in eastern Brazil, from north to south. The patches of *Podocarpus* are formed by sparse populations restricted to refugia such as higher elevations of mountain ridges, gallery forests, or moist slopes in the Cerrado or the Caatinga, mainly *P. sellowii*, or under the canopy of *A. angustifolia* in the southern Atlantic forest for *P. lambertii*. They all form dense and moist forest patches that are included in the Atlantic rainforest domain (Oliveira Filho & Fontes, 2000). This first information enables us to propose a species name for palynological interpretations (rather than only limiting ourselves to the genus) when the record is located in a well-defined area. For instance, we can say that *P. lambertii* expanded in southern Brazil in the last 3000 years (dark blue samples 16, 18, 19, 20, 21, 22 in Fig. 4) because the *Araucaria* forest is involved, while *P. sellowii*, which grows in Nordeste patches and gallery forests within the Caatinga or Cerrado biotopes of central Brazil, expanded *c.* 16,000 years ago (in yellow and red in Fig. 4). The third group (in green in Fig. 4), composed of either *P. sellowii* or *lambertii*, and located in the mountain or coastal areas of eastern Brazil (samples 10, 11, 12, 13, 14, 15, and 17), shows a different pattern of evolution (Fig. 2). In this group, two pollen records attest to the fact that the last marked expansion of *Podocarpus* occurred *c.* 29,000 years ago, during the last glaciation and lasted *c.* 2000 years. A decline is then progressively recorded until 21,000 years BP, after which only single pollen grains from isolated trees are recorded until

today. Comparison with modern distribution indicates that the species grows in association with other cloud forest species such as *Hedyosmum*, is *P. sellowii*. Both before 30,000 years ago and after 3000 years BP, the peaks in *Podocarpus* are always associated with elements of *Araucaria* forests (e.g. *Araucaria*) and, based on modern analogues, are consequently linked with expansion of *P. lambertii*. In addition, isolated patches of *P. lambertii* in central Brazil (samples 3 and 8 in Fig. 1 and Table 1) and their genetic links with Bolivian and Northern Argentinean populations (samples 24, 25, and 26) support the hypothesis of a pre-Quaternary wider distribution of *P. lambertii* on the South American continent, probably before the formation of the Andes. As a consequence, three regions can be delimited as a function of genetic regrouping and in-phase 'palaeo' patterns. The first region includes all the populations between 5° S and 15° S, the second region is delimited by 15° and 23° S and the third region includes all the populations growing between 23° and 30° S (Fig. 4). The expansion of all these populations appears to have been rapid, lasting between 50 and 100 years depending on the resolution of the pollen record, which supports the hypothesis of the existence of several small patches of refugia such as the ones observed today, as well as different migration paths between each patch.

These regional patches are remnants of several phases of past tropical forest expansion at least since the last glaciation. The successive climatic phases detected in the pollen records caused fragmentation into refugia and/or expansion from these refugia and still have a detectable impact on the current distribution of these species. In regions 1 and 2, since the beginning of the Holocene, *c.* 11,500 years ago, these conifers continue to live or survive in restricted areas where they show different forms of physiological adaptation, for instance vegetative instead of sexual reproduction in north-eastern Brazil and in high elevation populations in Bolivia. In addition, the five populations of *P. lambertii* from northern to southern Brazil attest to adaptative differences in wall thickness or tracheid diameter as a function of their location, and to climatic features such as insolation and evaporation (G. Ceccantini, unpublished data).

Another conclusion is that, in contrast to what happened in temperate latitudes, these tropical conifers never expanded during the interglacials. This is evidenced by the fact that the southern populations of *P. lambertii*, which expanded in the past 3000 years, are more subtropical. They thus acted in a reverse way to European conifers which expanded during the interglacials (Cheddadi *et al.*, 2006). In the Tropics, forests of conifers appear to have expanded during the glaciations when the regional climate was cool and moist enough for their development. In Australia, southern conifers such as *Araucaria* have progressively disappeared since the mid Pleistocene and are almost extinct today (Kershaw, 1986; Moss & Kershaw, 2000). The same conclusion can be drawn about the behaviour of *A. angustifolia* in south-eastern Brazil. Today, many populations are isolated on the top of mountain ranges or on plateaus with highly specific climatic conditions due to the equatorward trajectory of the polar air masses (Ledru, 1993), but results of palaeobotanical and palynological studies attest to a much wider expansion of these conifers in the past (Enright & Hill, 1995; Wijninga, 1996a,b). Our

hypothesis is that these trees, which are relics of the Gondwana and subjected to the equatorward shift of the South American continent towards the Equator, did not survive the multiple successions of glacial–interglacials of the Quaternary. The interglacials, such as the one we are experiencing today, obliged the tropical conifers to adopt a refugia, and each interglacial characterizes a loss in southern conifers (Enright & Hill, 1995). In addition, during recent centuries, the superimposition of climatic changes, deforestation, and logging are accelerating factors that could lead to their extinction.

CONCLUSION

Podocarpus proved to be a very powerful bio-indicator both for palaeo- and for neo-ecologists, and the combination of botany, pollen, and molecular analysis proved to be a rapid way of inferring distribution limits of sparse populations and regional evolution within tropical ecosystems. First, we confirmed the high distinctiveness of two endemic species *P. sellowii* and *P. lambertii* and the similarities between *P. sellowii* and *P. brasiliensis*, which should lead to a taxonomic revision of these two species. Second, we identified their currently restricted areas of development throughout Brazil, indicating that the expansion of *Podocarpus* in Amazonian pollen records may have come from the eastern populations as well as from the western populations. Further studies are now needed to characterize Amazonian populations. Third, we confirmed that past *Podocarpus* expansion may have been associated with an increase in moisture rates, either edaphic moisture or the length of the dry season, but not necessarily with a decrease in temperature. This last point depends on the association of species represented in the palaeo record. Finally, we also demonstrated that historical events linked to climatic changes during the Quaternary such as range expansion or range fragmentation appear to have had a strong influence on patterns of conifer distribution in the Tropics, and we defined three main regions of evolution linking the genetic groups to similar past climatic forcings. The next step in our research will be to identify the different migration paths they used and the mode and timing of these migrations based on the hypothesis of a possible future expansion when climatic conditions become more favourable.

In addition, the definition of in-phase responses over large areas in Brazil will enable palaeoecologists and palaeoclimatologists to re-organize their sampling policy, e.g. the environment in north-eastern Brazil acts differently to the environment in south-eastern Brazil but is identical to that in central Brazil. Identification and recognition of the vegetation refugia, which are part of our natural environment today, are indispensable for a policy for the preservation of the Atlantic rainforest. Today, these refugia enable species conservation as well as the genetic flow that keeps populations viable. They are crucial to the survival of the Atlantic forest under unfavourable climatic conditions and represent the only possible way for this forest to expand and develop in the event of a future climate change. Preservation of these identified refugia is of equal importance in policies for the conservation of the Atlantic forest in Brazil for future generations.

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