Plant Syst Evol (2010) 284:1–15
DOI 10.1007/s00606-009-0228-y

ORIGINAL ARTICLE

Taxonomy and systematics of the genus *Pinus* based on morphological, biogeographical and biochemical characters

Shiv Shankhar Kaundun · Philippe Lebreton

Received: 30 October 2008 / Accepted: 14 September 2009 / Published online: 6 October 2009

Abstract For a long time, systematists have subdivided the genus *Pinus* into Diploxylon and Haploxylon according to morpho-anatomy and the number of needles. Nevertheless, divergent views remain regarding the structure of these two subgenera, mainly at the section and subsection levels. We propose to clarify some of these uncertainties by studying 45 *Pinus* taxa of different origins. Our results, based on morphometric and biochemical (flavonoids) parameters, complement those obtained from classical anatomical and morphological studies, and also modern macro-molecular markers (proteins, DNA). We confirm the subdivision of the genus into *Pinus* = Diploxylon versus *Strobus* = Haploxylon and the further sectioning of the first subgenus into sections *Pinus* and *Trifoliae*. Moreover, we specify the different subsections, whereby the contents of the methylated flavonol isorhamnetin coupled with needle morphometry play a significant role (subsections *Pinus* vs. *Pinaster* in section *Pinus*, *Australes* + *Ponderosae* vs. *Contortae* in section *Trifoliae*). Given that isorhamnetin proceeds from quercetin by the irreversible action of an *O*-methyl-transferase, this methylated flavonol becomes a dynamic marker in such way that the taxa rich in isorhamnetin can be considered as more "derived = evolved". In addition, there exists a highly significant negative correlation between methylation index and number of needles. Consequently, the pines from the Holarctic *Strobus* group (with five needles and low isorhamnetin contents) can be considered as "ancestral", in reference to a Laurasian origin of the genus. In the subgenus *Pinus*, the Nearctic group (=section *Trifoliae*) remains near the ancestral base. On the other hand, the Holarctic subset "densiflorae" is connected to the other members (mainly European) of the polyphyletic subsection *Pinus*, in particular with series "sylvestres". Because of their very high contents of isorhamnetin, the Mediterranean pines result from an accentuation of this evolutionary trend (=subsection *Pinaster*). In fact, the pines growing under hot and dry climates (Mediterranean region) are highly evolved compared to those from cold and/or wet regions (Eurasia and North America but also, to a lesser extent, the south-eastern USA and East Asia). Our dynamic propositions based on plant phenolics data complete those from more modern macromolecular (DNA, proteins) studies.

Keywords *Pinus* · Needles · Morphometry · Biogeography · Flavonoid biochemistry · Taxonomy · Systematics

S. S. Kaundun · P. Lebreton
Laboratoire de Biochimie végétale, Université Claude Bernard Lyon I, 69622 Villeurbanne, France

Present Address:
S. S. Kaundun (✉)
Syngenta Jealott's Hill International Research Center, RG42 6EY Bracknell, UK
e-mail: deepak.kaundun@syngenta.com

Present Address:
P. Lebreton
56 Chemin du Lavoir, 01700 Beynost, France

Introduction

> "With the wealth of data available for *Pinus*, it is perhaps surprising that key phylogenetic relationships remain unresolved" (Syring et al. 2005).

The genus *Pinus* (Pinaceae) consists of around 110 species. It is the most abundant among the conifers, a

group of 630 species (Farjon 1984, 2005). A century ago, following Koehne's observations, Shaw (1914) proposed to split the genus into two subsets, Haploxylon (with only one fibrovascular bundle in the needles) and Diploxylon (with two fibrovascular bundles). The first subgenus (*Strobus*, "soft pines") includes the Asiatic and North American pines with five needles per fascicle, while the second subgenus (*Pinus*, "hard pines") possesses three or two needles with more or less persistent fascicle sheaths and populates the whole Paleartic region, including Middle Europe and the Mediterranean area.

This subgeneric differentiation was confirmed by Swedish chemists as early as the middle of the 20th century following structural analysis of phenolic substances isolated from the wood: "the consistency of the (phenolic) pattern appears typical of Diploxylon…. Subgenus *Strobus* has a more complex heartwood chemistry. The main feature is the occurrence of the stilbenes together with their hydrogenation products, the dibenzyls, and of the flavanones and their dehydrogenation products, the flavones.…It is possible that these chemical differences are due to a mutation in which case the Haploxylon pines could be regarded as being more ancestral than Diploxylon pines" (Norin 1972). Two decades later the advent of gas chromatography and high performance liquid chromatography allowed for the precise analyses of resins and oleoresins (terpenoids) and phenolics and other "micromolecules" respectively (Lebreton 1995). More recently relayed and even somewhat submerged by "macromolecular taxonomy" (proteins, DNA), chromatographic analyses have provided accurate and reproducible data which are very convenient for chemotaxonomical discussions at different levels. In addition phenolic compounds can act as dynamic markers depending on their positions in the biochemical pathway involved. Thus they can be ordered from "ancestral" (primitive) to "derived" (evolved).

Following four doctoral theses (Laracine (1984): *Pinus sylvestris*; Idrissi-Hassani (1985): *Pinus pinaster*; Lauranson-Broyer (1989): *Pinus uncinata*, *mugo* and *nigra*; Kaundun (1995): *Pinus halepensis*, *brutia* and *eldarica*) and several subsequent publications, the objective of this paper is to analyse flavonic data at the mesosystematic level (from species to genus). These are subsequently compared with macromolecular parameters (proteins, DNA) studied in the past decade. At the macrosystematic level (from genus to order), the abundance of proanthocyanidins (including prodelphinidin) and the absence of C-glycoflavones allows distinction of pines from the other Pinales; they are themselves characterised by the absence of biflavones, present in Cupressaceae and Araucariaceae (Lebreton 1990).

Materials and methods

Sampling

The present work is based on 45 taxa originating from 56 populations and a total of 620 individual plants. These include several species complex—for example *Pinus* grex *nigra*. Of the remaining 37 species 10 are from *Strobus* and 27 from *Pinus* subgenus. Morphometric and biochemical analyses were carried out on needles collected from forest trees in nature or in controlled plantations (French National Agricultural Research Institute; INRA) and botanical gardens (Les Barres Arboretum and Lyon Parc de la Tête d'Or, France).

Systematics

We have used the classification recently proposed by Gernandt et al. (2005), supported by the seminal works of Little and Critchfield (1969) and Price et al. (1998). Some considerations were given to classical works carried out by Gaussen (1960), Mirov (1967) and Krüssmann (1972). Number and size of needles were taken into consideration, as well as biogeographical localisation (New vs. Old World, Middle Europe vs. Mediterranean) and corresponding climates (hot vs. cold, dry vs. wet). In this way seven different biogeographical subsets were defined.

Biochemical analyses

Biochemical analyses were carried out on mature pine needles collected during winter and dried at room temperature in the absence of light. Following hot hydrochloric acid (2 N) hydrolysis in the presence of air, the anthocyanins generated from proanthocyanidins and the flavonol aglycones from native *O*-glycosides were extracted with relevant solvents and subsequently identified and measured with HPLC. Details of the biochemical analyses are included in similar works conducted in our laboratory (Idrissi-Hassani and Lebreton 1992; Lauranson-Broyer and Lebreton 1995; Kaundun et al. 1997).

Morphometric analyses

Morphometric analyses were also carried out on mature pine needles dried at room temperature. The length and weight of the needles were measured to the nearest 0.5 mm and 0.1 mg respectively and averaged on a minimum of ten fascicles for each individual tree (20–50 depending on the species).

Results and taxonomic findings

The raw data for each of the 45 taxa are given in Tables 1 (geography and morphometry) and 2 (biochemistry). The

Table 1 Geographical and morphometric characteristics of 45 *Pinus taxa* studied here

		Sampling[a]	Origin	Geoset	Needles (*n*)	*L* (mm)	*W* (mg)	GSI
Subgenus *Pinus*								
Section *Trifoliae*								
Subsection *Contortae*	*banksiana*	1 (1)	USA (N)	3	2	23	5.2	2.21
	contorta	1 (1)	USA (W)	3	2	39	8.6	2.27
	muricata	1 (1)	Mexico (W)	2	2	105	28.4	1.85
	virginiana	1 (1)	USA (E)	1	2	33	23.3	2.62
Subsection *Australes*	*palustris*	1 (1)	USA (SE)	1	3	230	90.6	0.85
	rigida	1 (1)	USA (E)	1	3	112	22.5	1.66
	taeda	1 (1)	USA (E)	1	3	137	33.9	1.35
Subsection *Ponderosae*	*coulteri*	1 (1)	USA (SW)	3	3	146	44.6	1.09
	engelmanii	1 (1)	Mexico	2	3	201	55.8	1.20
	jeffreyi	1 (1)	Mexico	2	3	147	30.7	1.60
	ponderosa	1 (1)	America (N)	3	3	153	39.0	1.31
Incertae sedis	*patula*	1 (1)	Mexico	2	3	163	23.3	2.33
Section *Pinus*								
Subsection *Pinus*								
Series densiflora	*densiflora*	1 (1)	Japan	4	2	88	23.0	1.91
	massoniana T+	1 (1)	China	5	2	140	29.7	2.36
	resinosa	1 (1)	America (N)	3	2	123	29.8	2.06
	tabuliformis T+	1 (1)	Asia (E)	5	2	85	24.2	1.76
	thunbergi	1 (1)	Japan	4	2	60	17.4	1.72
Series silvestres	*silvestris T+*	1 (20)	Vosges (F)	6	2	74	27.1	1.36
	silvestris T-	1 (25)	Valais (CH)	6	2	26	6.5	1.98
	uncinata	1 (25)	Pyrenees (F)	6	2	44	17.3	1.28
	mugo	1 (20)	Poland	6	2	36	13.8	1.31
	x *uliginosa*	1 (20)	Poland	6	2	41	15.1	1.42
	leucodermis	1 (1)	Balkans	6	2	87	37.0	1.18
	pinea	1 (1)	Mediterr.	7	2	128	44.4	1.44
Pinus grex *nigra*	*calabrica*	1 (25)	Italia (S)	7	2	120	36.1	1.58
	laricio	1 (25)	Corsica	7	2	126	47.0	1.34
	salzmanii	2 (25)	Mediterr. (W)	7	2	148	46.8	1.58
	nigricans	3 (25)	Europe (Ctr)	6	2	90	34.9	1.29
	pallasiana	3 (25)	Europe (SE)	6	2	123	44.7	1.38
Subsection *Pinaster*	*canariensis*	1 (1)	Atlantic	7	3	183	29.7	2.05
	brutia	3 (25)	Turkey	7	2	79	21.0	1.88
	eldarica	1 (25)	Iran	7	2	68	18.4	1.85
	halepensis	3 (20)	Mediterr. (W)	7	2	66	12.0	2.74
Pinus pinaster	"*maritima*"	2 (20)	Atlantic	7	2	197	182	0.54
	"*mesogeensis*"	2 (20)	Mediterr.	7	2	162	116	0.70
Subgenus *Strobus*								
Section *Strobus*								
Subsection *Gerardianae*	*bungeana*	1 (1)	China	5	3	63	23.3	0.90
Subsection *Strobus*	*armandii*	1 (1)	China	5	5	112	19.1	1.17
	ayacahuite	1 (1)	Mexico	2	5	79	9.1	1.74
	cembra	1 (1)	Europe (E)	6	5	71	13.0	1.09
	koraiensis	1 (1)	Asia (E)	4	5	73	13.1	1.11
	parviflora	1 (1)	Japan	4	5	36	3.9	1.85

Table 1 continued

		Sampling[a]	Origin	Geoset	Needles (*n*)	*L* (mm)	*W* (mg)	GSI
	peuce	1 (1)	Balkans	6	5	114	9.3	2.45
	pumila	1 (1)	Asia (NE)	5	5	49	5.7	1.72
	strobus	1 (1)	America (NE)	1	5	84	6.5	2.58
	wallichiana	1 (1)	Asia (Ctr.)	5	5	101	9.9	2.04
Means for the genus *Pinus*					2.8	101	31.0	1.64
Standard deviation					1.2	51	32.0	0.52

GSI Global slimness index = $L/n \times W$

[a] In the sampling column, the first figure corresponds to the number of populations studied, and the corresponding number of trees analysed is given in parentheses

chemical values are expressed as the percentage of the sum of absolute contents (mg/g dry weight) of homologous substances, proanthocyanidins (two molecules) or flavonols (six molecules). For a better readability, the values are sometimes expressed to the nearest whole digit even if calculations were performed with an additional decimal. Two indices were defined based on flavonic data: (1) an index of phenyl-*O*-methylation (Me-O), which is the sum of the relative amounts of the methylated flavonols isorhamnetin, larycitrin and syringetin; syringetin, i.e. di-*O*-methyl-3′,5′ myricetin, was attributed a coefficient of 2; and (2) an index of phenyl-tri-hydroxylation (Tri-OH), which is the sum of the relative amounts of prodelphinidin and myricetin; the former was attributed a coefficient of 1/3 due to the presence of two proanthocyanidins and six flavonols. The results are further summarized based on taxonomical (subgenera, sections, subsections) (Tables 3, 4), morphological (Table 5) or geographical (Table 6) subsets.

Morphometric aspects

Pines with two, three or five needles show very distinct needle lengths and weights. Likewise, if an index of slimness is defined by length (*L*) divided by weight (*W*) of a needle, clearly distinct values are obtained for each type of fascicle. *L*/*W* values are 3.36, 4.29 and 8.75 for pines with two, three and five needles, respectively (Table 5). A significant difference is even obtained between the groups of pines with two and three needles ($t = 1.88$; $P = 0.03$). These results tend to show that the greater the number of needles per fascicle, the finer the needles. There is an obvious and positive correlation between individual needle length and weight ($r = +0.694$; $P \ll 0.001$). There is also a highly negative correlation between weight and fineness ($r = -0.612$; $P \ll 0.001$): the longer the needles, the heavier they are and the heavier they are, the stockier the needles.

If the calculation is based on the whole fascicle (global slimness index GSI = $L/n \times W$, where n is the number of needles), no significant differences are observed between the two subgenera *Pinus* and *Strobus* or between the two sections *Pinus* and *Trifoliae*. The same is true for subsections *Pinus* and *Pinaster* ($1.6 < \text{GSI} < 1.7$). Taking into consideration the ratio *L*/*W* does in fact mean implicitly using the number of needles as a taxonomical character. At this level, it is thus better to use the number of needles directly rather than having recourse to the *L*/*W* index. Nevertheless, the GSI can be useful at lower taxonomical levels, for example when series "densiflorae" is compared to "sylvestres". The equivalence of the GSI at the intrageneric level as well as its relatively low dispersion around the mean for the 45 taxa [$L/n \times W = 1.64$ (0.52)] leads to the conclusion that needle ontogenesis proceeds from an initial structure common to the genus *Pinus* as a whole, which was then subjected to subsequent dichotomies by ontogenesis.

Geographical aspects

If the samples are grouped based on the three relevant continents (considered at the present geological time), clear differences are observed for some characters (Table 6 means and standard deviations). Significant differences are observed between Asia and Europe, and Asia and Europe + America for the number of needles (respectively, $t = 2.40$, $P = 0.01$ and $t = 1.98$, $P = 0.03$) but neither for length nor fineness. For *O*-methylation (but not for phenyl-tri-hydroxylation), significant differences exist between Asia and America ($t = 2.64$; $P = 0.007$), Asia and Europe ($t = 5.74$; $P < 0.001$) and even between Europe and America ($t = 3.10$; $P = 0.002$). There is thus a relationship between geographical location, number of needles and intensity of *O*-methylation. If climatic criteria (cold vs. hot, wet vs. dry) are taken into consideration, seven principal biogeographical regions can be identified: south-eastern USA, southern USA with Mexico, western and northern America, eastern and south-eastern Asia, continental Asia, continental Europe and Mediterranean region. The differences are clear as far as the proportion of the number of species of the two subgenus *Strobus* and

Table 2 Biochemical characteristics (flavonoids) of 45 *Pinus* taxa studied here

		LD	Myr	Lar	Syr	IRh	Tri-OH	MeO
Subgenus *Pinus*								
Section *Trifoliae*								
Subsection *Contortae*	*banksiana*	94	18	6	0	11	49	17
	contorta	94	10	7	4	7	41	22
	muricata	95	17	8	3	12	49	26
	virginiana	95	8	3	1	12	40	17
Subsection *Australes*	*palustris*	83	1	0	0	3	29	3
	rigida	89	9	0	1	2	39	4
	taeda	93	8	0	0	1	39	1
Subsection *Ponderosae*	*coulteri*	95	9	2	0	6	41	8
	engelmanii	91	6	0	0	8	36	8
	jeffreyi	96	15	10	4	4	47	22
	ponderosa	94	6	2	3	6	37	14
Incertae sedis	*patula*	95	0	0	0	31	32	31
Section *Pinus*								
Subsection *Pinus*								
Series densiflora	*densiflora*	95	2	0	1	3	34	5
	massoniana T+	76	0	0	0	3	25	3
	resinosa	88	0	0	0	13	29	13
	tabuliformis T+	43	2	0	0	4	16	4
	thunbergi	89	6	2	0	17	36	19
Series silvestres	*silvestris T+*	79	4	1	0	16	30	17
	silvestris T-	91	3	0	0	17	33	17
	uncinata	91	0	0	0	37	30	37
	mugo	92	0	0	0	37	27	37
	x *uliginosa*	85	1	0	0	34	29	34
	leucodermis	94	1	0	0	15	32	15
	pinea	96	1	0	0	32	33	32
Pinus grex *nigra*	*calabrica*	84	0	0	0	12	28	12
	laricio	89	0	0	0	19	30	19
	salzmanii	82	0	0	0	23	27	23
	nigricans	75	0	0	0	10	25	10
	pallasiana	77	0	0	0	11	26	11
Subsection *Pinaster*	*canariensis*	93	1	0	0	34	32	34
	brutia	94	4	2	1	23	35	27
	eldarica	94	3	3	3	40	34	49
	halepensis	93	12	9	2	23	43	37
Pinus pinaster	"*maritima*"	93	5	9	3	21	36	36
	"*mesogeensis*"	93	4	9	4	21	35	38
Subgenus *Strobus*								
Section *Strobus*								
Subsection *Gerardianae*	*bungeana*	95	4	0	0	3	36	3
Subsection *Strobus*	*armandii*	96	2	0	0	9	34	9
	ayacahuite	91	5	1	0	7	35	8
	cembra	87	3	0	0	8	32	8
	koraiensis	94	2	0	0	2	33	2
	parviflora	96	11	0	0	1	43	1

Table 2 continued

		LD	Myr	Lar	Syr	IRh	Tri-OH	MeO
	peuce	90	3	0	0	3	33	3
	pumila	92	4	1	2	2	35	7
	strobus	90	2	0	0	5	32	5
	wallichiana	93	2	0	0	4	33	4
Means for the genus *Pinus*		89	4	2	1	14	34	17
Standard deviation		9	4	3	1	11	6	13

LD Prodelphinidin (expressed as % of total proanthocyanidins), *Myr* myricetin, *Lar* larycitrin, *Syr* syringetin, *IRh* isorhamnetin (expressed as % of total flavonols), *Tri-OH* phenyl-tri-hydroxylation index, *MeO* phenyl-O-methylation index (for index see "Materials and methods"). *T+* with taxifolin, *T−* without taxifolin

Table 3 Morphometric characteristics of *Pinus* subsets recognised in this study

	Needles (*n*)	Length (mm)	Weight (mg)	GSI
Subgenus *Pinus* (35)	2.3 (0.4)	108 (54)	36 (34)	1.63 (0.51)
Section *Pinus* (23)	2.1 (0.2)	100 (47)	38 (38)	1.60 (0.49)
Subsection *Pinus* (16)	2.0 (0.0)	88 (39)	28 (12)	1.59 (0.34)
Series "densiflorae" (5)	2.0 (0.0)	99 (32)	25 (5)	1.96 (0.26)
Series "sylvestres" (11)	2.0 (0.0)	83 (42)	30 (15)	1.43 (0.22)
Subsection *Pinaster* (7)	2.1 (0.4)	126 (56)	61 (64)	1.60 (0.78)
Section *Trifoliae* (12)	2.7 (0.5)	124 (65)	32 (24)	1.70 (0.56)
Subsection *Contortae* (4)	2.0 (0.0)	50 (37)	12 (11)	2.24 (0.32)
Subsection *Australes* (3)	3.0 (0.0)	160 (62)	49 (37)	1.29 (0.41)
Subsection *Ponderosae* (4)	3.0 (0.0)	162 (26)	43 (11)	1.30 (0.22)
Incertae sedis (*P. patula*)	3.0	163	23	2.33
Subgenus *Strobus* (10)	4.8 (0.6)	78 (26)	11 (6)	1.67 (0.59)
Section *Strobus* (10)	4.8 (0.6)	78 (26)	11 (6)	1.67 (0.59)
Subsection *Gerardianae* (1)	3	63	23	0.90
Subsection *Strobus* (9)	5.0 (0.0)	80 (27)	10 (5)	1.75 (0.55)
Genus *Pinus* (45)	2.8 (1.2)	101 (51)	31 (32)	1.64 (0.52)

GSI Global slimness index = $L/n \times W$

Pinus and the methoxylation index MeO are concerned (Fig. 3).

Correlation between variables (45 taxa × 12 traits)

The number of needles per fascicle (two, three or five) is significantly correlated (negatively) to the level of isorhamnetin ($r = -0.482$; $P = 0.001$) (Table 7). This relationship is accentuated with the *O*-methylation (=methoxylation) index MeO ($r = -0.527$; $P < 0.001$): the greater the number of needles, the lower the level of methylated flavonols. In fact, the methoxylation index differs more significantly when the difference in the number of needles is higher: between the pines with three and five needles, $t = 1.92$, $P = 0.035$; between two and three needles, $t = 2.12$, $P = 0.020$; between two and five needles, $t = 6.59$, $P \ll 0.001$. Quercetin and isorhamnetin are negatively correlated ($r = -0.418$; $P = 0.004$); isorhamnetin derives from quercetin by *O*-methylation of the lateral phenyl group of this flavonoid.

The level of prodelphinidin, a phenyl-trihydroxylated pro-anthocyanidin, is correlated to the relative amount of myricetin, a phenyl-trihydroxylated flavonol ($r = +0.345$), larycitrin = methyl-3′ myricetin ($r = +0.274$) and syringetin = dimethyl-3′,5′ myricetin ($r = +0.289$; $P = 0.02$–0.06), thus reflecting the existence of the phenyl-trihydroxylation pathway. There is indeed a highly positive correlation between myricetin and its two mono- and di-methylated derivatives (larycitrin, $r = +0.684$, $P \ll 0.001$; syringetin, $r = +0.475$, $P = 0.001$) and between the latter two ($r = +0.824$, $P \ll 0.001$). However, the number of needles is neither correlated to the tri-*O*-substitution index ($r = +0.120$, NS), nor is the latter to the methoxylation index MeO ($r = -0.085$, NS).

Table 4 Biochemical characteristics of *Pinus* subsets recognised in this study

	LD (%)	Myr (%)	Lar (%)	IRh (%)	Tri-OH	MeO
Subgenus *Pinus* (35)	88 (10)	5 (5)	2 (3)	16 (12)	34 (7)	20 (13)
Section *Pinus* (23)	86 (11)	2 (3)	2 (3)	20 (11)	31 (5)	23 (13)
Subsection *Pinus* (16)	83 (12)	1 (2)	Tr.	17 (11)	29 (5)	17 (11)
Series "densiflorae" (5)	78 (21)	2 (2)	Tr.	8 (7)	28 (8)	9 (7)
Series "sylvestres" (11)	85 (6)	1 (1)	0	21 (10)	29 (3)	21 (10)
Subsection *Pinaster* (7)	94 (1)	4 (4)	5 (4)	27 (8)	35 (4)	36 (7)
Section *Trifoliae* (12)	93 (4)	9 (6)	4 (4)	9 (8)	40 (6)	14 (10)
Subsection *Contortae* (4)	95 (1)	13 (5)	6 (2)	11 (2)	45 (5)	21 (4)
Subsection *Australes* (3)	88 (5)	6 (4)	0	2 (1)	36 (6)	3 (2)
Subsection *Ponderosae* (4)	94 (2)	9 (4)	4 (4)	6 (2)	40 (5)	13 (7)
Incertae sedis (*P. patula*)	95		0	31	32	31
Subgenus *Strobus* (10)	93 (3)	4 (3)	Tr.	4 (3)	35 (3)	5 (3)
Section *Strobus* (10)	93 (3)	4 (3)	Tr.	4 (3)	35 (3)	5 (3)
Subsection *Gerardianae* (1)	95	4	0	3	36	3
Subsection *Strobus* (9)	92 (3)	4 (3)	Tr.	5 (3)	34 (3)	5 (3)
Genus *Pinus* (45)	89 (9)	4 (4)	2 (3)	14 (11)	34 (6)	17 (13)

LD Prodelphinidin (expressed as % of total proanthocyanidins), *Myr* myricetin, *Lar* larycitrin, *Syr* syringetin, *IRh* isorhamnetin (expressed as % of total flavonols), *Tri-OH* phenyl-tri-hydroxylation index, *MeO* phenyl-O-methylation index (for index see "Materials and methods"). Mean; standard deviation in parentheses

Table 5 Morphometric and biochemical data according to the number of needles

	Length (mm)	*L/W* (mm/mg)	$L/n \times W$	IRh	Me-O	Tri-OH
Two needles (26)	89 (46)	3.36 (1.06)	1.68 (0.53)	18 (11)	22 (12)	33 (7)
Three needles (10)	154 (46)	4.29 (1.44)	1.43 (0.48)	10 (12)	13 (12)	37 (5)
Five needles (9)	80 (27)	8.75 (2.75)	1.75 (0.55)	5 (3)	5 (3)	34 (3)

IRh Isorhamnetin (%), *Tri-OH* phenyl-tri-hydroxylation index, *Me-O* phenyl-O-methylation index

Table 6 Morphometric and biochemical data according to geographical origins

	Number of needles	Length (mm)	$L/n \times W$	IRh	Me-O	Tri-OH
Asia ($n = 10$)	3.6 (1.5)	81 (31)	1.65 (0.46)	5 (5)	6 (5)	33 (7)
Europe ($n = 19$)	2.4 (1.0)	99 (49)	1.52 (0.53)	22 (11)	24 (13)	32 (4)
America ($n = 15$)	2.9 (1.0)	118 (60)	1.78 (0.55)	9 (7)	13 (9)	38 (7)

IRh Isorhamnetin (%), *Tri-OH* phenyl-tri-hydroxylation index, *Me-O* phenyl-O-methylation index

Differentiation between subgenera *Pinus* and *Strobus*

The two subgenera *Strobus* (=Haploxylon) and *Pinus* (=Diploxylon) taken globally differ from each other by their average number ($t = 11.90$, $P \ll 0.001$), length ($t = 2.44$, $P = 0.009$) and individual weight of the needles ($t = 4.12$, $P < 0.001$). The needles of subgenus *Strobus* are shorter and lighter than those of the subgenus *Pinus*. Also, based on flavonoid data, subgenus *Pinus* is significantly richer in isorhamnetin than subgenus *Strobus*: 16 versus 4% ($t = 5.55$, $P < 0.001$). This complete differentiation between subgenus *Strobus* and *Pinus* has been recognized since long ago by many authors and disciplines (Bergmann and Gillet 1997; Kutil and Williams 2001) and thus validates our methodology based on flavonoid and needle characteristics.

Based on the species analysed here (which neglects section *Parrya*, including subsection *Cembroides* of Mexican pines), we observe the homogeneity of subsection *Strobus* Loud., which groups species formerly distinguished as *Strobi* and *Cembrae* by some authors (Klaus 1989; Liston et al. 1999). With the exception of *Pinus bungeana*, all other species analysed belong to the section *Strobus* (= *Quinquefoliae*). Gernandt et al. (2005), Wang et al. (1999) or Liston et al. (1999) did not differentiate among them either based on chloroplastic or ribosomal DNA analyses though *P. strobus*, *P. peuce* or *P. parviflora*

Table 7 Correlations between morphometric and/or biochemical variables

Morphometric or biochemical variable	Morphometric or biochemical variable	Correlation coefficient (r)	P
Needle number	Weight	−0.288	(*)
	MeO index	0.527	***
	Tri-OH index	0.120	NS
Needle length	Weight	0.694	***
Global slimness index (GSI)	Length	−0.347	*
	Weight	−0.612	***
Prodelphinidin	Myricetin	0.345	*
	Larycitin	0.274	(*)
	Syringetin	0.289	(*)
	Tri-OH index	0.728	***
Myricetin	Larycitin	0.684	***
	Syringetin	0.475	***
	Tri-OH index	0.894	***
Larycitrin	Syringetin	0.824	***
	Tri-OH index	0.628	***
	MeO index	0.465	**
Syringetin	Tri-OH index	0.481	***
	MeO index	0.425	**
Tri-OH index	Cf. prodelphinidin, myricetin, larycitin, syringetin		***
Isorhamnetin	Needle number	−0.482	***
	Quercetin	−0.418	**
	MeO index	0.907	***
	Tri-OH index	−0.184	NS
MeO index	Needle number	−0.527	***
	Isorhamnetin	+0.907	***
	Larycitrin	+0.465	**
	Syringetin	+0.425	**
	Tri-OH index	0.085	NS

$df = 43$; $*P = 0.05$ for $r = 0.294$, $**P = 0.01$ for $r = 0.380$, $***P = 0.001$ for $r = 0.474$, (*) $= 0.05 < P < 0.10$, $NS = P > 0.10$

were detached from the lot as subsection S*trobi*. Moreover, *Pinus bungeana* (subsection *Gerardianae*) is not biochemically distinguishable from the other species of the same subgenus, although it has fewer ($n = 3$) and heavier needles (average 23 vs. 11 mg). Thus, our discussion will be focused mainly on the morphometric and flavonic taxonomy of species of subgenus *Pinus* and its diverse sections and subsections, better represented in this study.

Structure of subgenus *Pinus*

The two sections *Pinus* ($n = 23$) and *Trifoliae* ($n = 12$) are completely distinguished by their average number of needles ($t = 4.19$; $P < 0.001$) but not by morphometry; the first is significantly richer in phenyl-trihydroxylated flavonoids (LD: $t = 2.65$, $P = 0.006$; Myr: $t = 3.93$, $P < 0.001$; Tri-OH index: $t = 4.39$, $P < 0.001$) and poorer in methylated flavonols (IRh: $t = 3.56$, $P < 0.001$; MeO index $t = 2.19$, $P = 0.017$). Thus, the number of needles and flavonic biochemistry validate the two sections recently explained by Gernandt et al. (2005).

The Nearctic section *Trifoliae* Duhamel is heterogeneous, with complete individualisation of subsection *Contortae* (enriched with *P. muricata*) according to various criteria: number of needles (n) (2.0 vs. 3.0, $P \ll 0.001$), length (L) (161 vs. 50 mm, $t = 4.60$, $P < 0.001$), weight (W) (45 vs. 12 mg, $t = 3.27$, $P = 0.005$) and GSI (1.29 vs. 2.24, $t = 4.95$, $P < 0.001$). Myricetin content (8 vs. 13%, $t = 1.87$, $P = 0.046$) and Tri-OH index (38 vs. 45, $t = 2.02$, $P = 0.036$) are weaker, as are isorhamnetin (4 vs. 11%, $t = 4.09$, $P = 0.001$) and MeO index (9 vs. 21, $t = 3.39$, $P = 0.004$). Separated and amended, the two subsections with three needles, *Australes* (hereby reduced to three species of south-eastern America: *P. palustris*, *rigida* and *taeda*) and *Ponderosae*, share a common foliar biometry ($L = 160$ and 162 mg, GSI = 1.29 and 1.30) and a close combination of trihydroxylated flavonoids (Tri-OH index = 36 and 40). This conclusion can justify a posteriori the recognition by Gaussen (1960) of the subclass *Taedoponderosoides* which today is obsolete. Nevertheless, the methylated flavonol content allows individualisation of the two subsections: IRh = 2 versus 6% ($t = 4.00$, $P = 0.005$) and MeO = 3 versus 13 ($t = 3.01$, $P = 0.015$).

There remains the case of *Pinus patula*, a California pine with three needles per fascicle. The needles are of identical length to those of the two precedent similar subsections; this species is in fact positioned (but with *P. muricata*) by Gernandt et al. (2005) in the Southern subsection discussed earlier. However, the heavy and slim needles (GSI = 2.3 vs. 1.3; cf. subsection *Contortae*) do not favour this insertion, nor does a very low trihydroxylated flavonoid content (Tri-OH = 32) and a very high isorhamnetin content (IRh = 31%), the only methyl-flavonol present. For many authors (Little and Critchfield 1969; Price et al. 1998; Liston et al. 1999), *Pinus patula* should be placed in an *Oocarpeae* subsection, which was unfortunately not analysed here. In this context, this species is considered as *incertae sedis* within the *Trifoliae* section.

Contrary to the *Trifoliae*, section *Pinus* is heterogeneous from a geographical standpoint. It comprises some Nearctic representatives (such as *P. resinosa*) together with a few Palearctic members ranging from the Canary Islands (*P. canariensis*) to Japan (*P. thunbergi*). The first dichotomy proposed by our biometric and biochemical data

properly separates the two subsections, *Pinus* and *Pinaster*, distinguished by Gernandt et al. (2005). The needles of the first subsection are relatively shorter on average (88 vs. 126 mm, $t = 1.63$, $P = 0.057$) and have lower levels of phenyl-trihydroxylated flavonoids (LD: 83 versus 94%, $t = 3.62$, $P < 0.001$; Myr: 1 versus 4%, $t = 2.09$, $P = 0.023$; Tri-OH: 29 vs. 35, $t = 3.85$, $P < 0.001$), and also low methylated flavonol content (IRh: 17 vs 28%, $t = 2.71$, $P = 0.006$; MeO: 17 vs. 36, $t = 5.06$, $P < 0.001$). This dichotomy is equally accepted by Wang et al. (1999) based on chloroplastic DNA data. Their study recognizes two clades in subgenus *Pinus* including Mediterranean pines on the one side and species from subsection *Sylvestres* on the other side.

The subsection *Pinus* in turn reveals a dichotomy based on geography. The holartic subgroup have very slim needles (GSI: 1.96 vs. 1.43, $t = 4.00$, $P < 0.001$), and the European subgroup are significantly richer in isorhamnetin (IRh: 21 vs. 8%, $t = 3.04$, $P = 0.004$; MeO index: 21 vs. 9, $t = 2.80$, $P = 0.007$). Dividing subsection *Pinus* into segments would make things clearer but would further complicate the phylogenetic scheme. We prefer not to question this subsection, but at the same time distinguish within it two series designated as "densiflorae" and "sylvestres". Yet, an argument of coherence resides in the fact that subsection *Pinus* is the only one with species containing taxifolin (=dihydro-2,3 quercetin) (one European *P. sylvestris*, one North American *P. tabuliformis*, one Asian *P. massoniana*). The synthesis of taxifolin is genetically controlled in mountainous and meadow populations of *Pinus sylvestris* (Laracine-Pittet and Lebreton 1988; Lebreton et al. 1990; Yazdani and Lebreton 1991). Actually, on the basis of chloroplastic DNA, Krupkin et al. (1996) recognized that "the North American species *P. resinosa* is closely allied to *P. sylvestris*" (following the same analysis, *P. canariensis* is close to the Mediterranean pines *P. pinea* and *brutia*).

In fact, attention must now be focused on the distinction between the two European subgroups, "sylvestres" (series) and *Pinaster* (subsection), which questions the autonomy of the Middle European and Mediterranean pines, recognised by numerous authors (Schirone et al. 1991; Piovesan et al. 1993) following seed enzyme analysis. The morphometric arguments are inefficient due to the presence, in both cases, of long and/or heavy needles (*salzmanii* vs. *pinaster*) and short and/or light needles (*uncinata* vs. *halepensis*). Flavonic characteristics are however different; methylation of flavonols is certainly high in "sylvestres", but lower than that of *Pinaster*: MeO = 21 versus 36 ($t = 3.74$, $P < 0.001$). This is mainly due to the almost complete absence of larycitrin (0.1 vs. 5%) in series "sylvestres". The path of phenyl-trihydroxylation is equally less active in the latter series as compared to subsection *Pinaster*. The Tri-OH index is respectively 29 versus 35 ($t = 4.26$, $P < 0.001$), as a result of lower levels of prodelphinidin (LD: 85 vs. 94%, $t = 4.83$, $P < 0.001$) and myricetin (Myr: 1 vs. 4%, $P < 0.001$) in series "sylvestres".

Our biochemical analyses therefore favour the recognition of a group of Mediterranean pines sensu lato, previously suggested by various authors including Klaus (1989) who clearly differentiates the Mediterranean pines from the "diploxyl mountain pines from the area surrounding the Mediterranean". This is typically the case in the species complex *Pinus nigra* (*laricio* + *salzmanii* vs. *nigricans* + *pallasiana*), as mentioned below. On the other hand, we refuse to agree with same author on the fact that the Mediterranean pines could be in "close relationships with Central American and Caribbean pines", which is rejected mainly based on MeO index or the abundance of two-needled pines in the Atlanto-Mediterranean area. Referring to these pines, Krupkin et al. (1996) made a close connection between *P. canariensis* and the Mediterranean pines *P. pinea* and *brutia*. Also Liston and co-workers (1999) group the *canariensis* and *pinaster* species with *pinea* and *halepensis*. The same year, Wang and co-workers connected *P. halepensis*, *brutia* and *pinaster* to *pinea* and *canariensis*. Moreover, the close relationship among the three species *P. halepensis*, *P. brutia* and *P. eldarica* has been documented by means of flavonols (Kaundun et al. 1997). The correlation between drought (summer) and *O*-methylation will be discussed below.

As a conclusion to this section devoted to the comparison of our biometric and biochemical results from a taxonomic (=static) point of view, we can acknowledge, on one hand, the boldness of the new data, and on the other hand, their general adequacy as compared to more recent propositions made by different authors (Lopez et al. 2002), even if certain modifications have been suggested and uncertainties exist here and there, which are discussed later on from a phylogenetic perspective.

Discussion and systematic aspects

Flavonic chemotaxonomy

The concept of "flavonic evolution" is based on two principles (Lebreton 1990). The first and of higher importance here is that the methylation of the hydroxyl of the lateral phenyl group is a derived character. This is founded upon the irreversible nature of phenyl-*O*-methyl-transferase activity and on its macrosystematic expression in the phanerogams (increasing from ligneous to herbaceous plants). In this respect, isorhamnetin is more "evolved" than quercetin from which it is derived; similarly larycitrin

(monomethylated) and syringetin (dimethylated) are more advanced than myricetin.

The second principle consists of tri-hydroxylation of the lateral phenyl group of flavonoids being a primitive (ancestral) character, founded upon the primordial nature of the shikimate biosynthesis pathway and its macrosystematic expression in the phanerogams (decreasing from the gymnosperms to the monocotyledons). In this respect, prodelphinidin and myricetin are more "ancestral = primitive" than procyanidin and quercetin respectively.

Actually, the situation can be more complex: not only the second character can be masked by methylation of the phenolic group but the first is consecutive to the second one (syringetin, for instance, cannot appear if the myricetin pathway is closed), and both can be uncoupled. This can be illustrated with the species complex *Pinus nigra* Arn. of which the Calabrian taxa (LD = 84%, IRh = 12%) of the Mediterranean region can be considered as the strain of two (postglacial) phyla, respectively occidental/meridional (*laricio* and *salzmanii*: LD = 85 ± 4%, IRh = 21 ± 2%) and oriental/continental (*nigricans* and *pallasiana*: LD = 76 ± 1%, IRh = 11 ± 1%). On the first phylum, prodelphinidin remained constant whilst isorhamnetin took an upward trend; on the second, isorhamnetin remained stable and prodelphinidin took a downward trend (Lauranson-Broyer and Lebreton 1995). Overall based on the relative contents of myricetin, isorhamnetin, larycitrin, syringetin and prodelphinidin, two global indices were defined: an index of methoxylation, MeO, and an index of phenyl-trihydroxylation, Tri-OH (cf. section "Material and methods"). It is noteworthy that the methoxylation index is of greater amplitude than that of hydroxylation. The means and standard deviations are respectively 16 (12) and 34 (6) with variation coefficients 74 and 19%. Thus methylation generates more information than hydroxylation, the latter being of minor importance for the present discussion.

Phylogenetic relationships

Statistical analysis of the whole genus *Pinus*, coupled with that of its taxonomic subgroups (statistical comparison of means), shows a net negative correlation between the number of needles and amounts of *O*-methylated flavonols that are "derived". It can therefore be safely concluded that a large number of needles, that is five needles as compared with three or two needles per fascicle, is an ancestral character. The same is true for subgenus *Strobus* when compared to subgenus *Pinus*. This proposition is in contradiction with Gaussen (1960), but concurs with Norin (1972). This being said, it would be unwise to conclude without any other considerations that there is a direct derivation of subgenus *Pinus* from *Strobus*. On the one side the phenyl-tri-*O*-substitution level does not differentiate between the two subgenera (Table 1b), and on the other side subgenus *Pinus* is manifestly heterogeneous in its two flavonic characteristics. In particular, section *Trifoliae* shows a significantly higher Tri-OH index than subgenus *Strobus*: 40 versus 35 ($t = 2.57$, $P = 0.009$). In this section, myricetin and its two methylated derivatives together represent not less than 12% of total flavonols, thus testifying to the persistence of the archaic shikimate pathway in contrast with the subgenus *Strobus* (only 5% for the total of the tri-*O*-substituted flavonols). The same holds for the subsection *Pinaster* (15%), making it most unlikely for subgenus *Pinus* to be directly derived from *Strobus*.

Under these circumstances a common original strain for the different species of *Pinus* and its subsets can be considered to have the following archaic characteristics: a large number of needles per fascicle (originally five), low level of *O*-methylation (<2) and high level of phenyl-trihydroxylation (around 45). As indicated in Table 7 the first two characters are highly correlated. From a phylogenetic point of view, a first dichotomy could have generated two groups, the first leading to subgenus *Strobus*, the second to subgenus *Pinus*, with subsequent subdivision into Neartic *Trifoliae* and Holartic *Pinus* sections (Fig. 1a, b). In the first case, the MeO index progresses less than in the second (14 vs. 23) and the Tri-OH index remains high (40 vs. 31); thus, Eurasiatic pines are globally more "evolved = derived" than the American pines. However, the situation is not homogeneous at other levels. In North America, two subsets appear, the first composed of subsections *Australes* + *Ponderosae*, with similar number and morphometry of needles, the second, *Contortae*, with two short and slim needles per fascicle (Fig. 1a). Taking into consideration the number of needles and MeO index, subsection *Contortae* is the more "evolved" of the group of American pines (Fig. 1b). This scheme agrees with the Dollo phylogenetic tree proposed by Govindaraju et al. (1992) on the basis of ribosomal DNA but differs from the Wagner tree, where the *Ponderosae* group is separated from *Australes* and *Contortae*. In the classification presented by Syring et al. (2005), *P. contortae* is detached from *P. ponderosa* + *P. taeda*. According to Krupkin et al. (1996), the "subsection *Contortae* emerged as a sister group to all the other North American pines". Contrary to Syring et al. (2005), we do not believe that "the position of (sub)sect. *Contortae* remains equivocal", and its evolution in the New World can be compared to that of subsection *Pinaster* in the Mediterranean with maximal methoxylation within these two subsections (Fig. 2).

In the Holarctic section *Pinus*, MeO index and taxifolin distinguish clearly the two subsections *Pinus* and *Pinaster*, as seen in the former taxonomical discussion (cf. Fig. 1b). Thus, *Pinus* is more ancestral than *Pinaster*, in spite of a higher level of prodelphinidin and trihydroxylation index

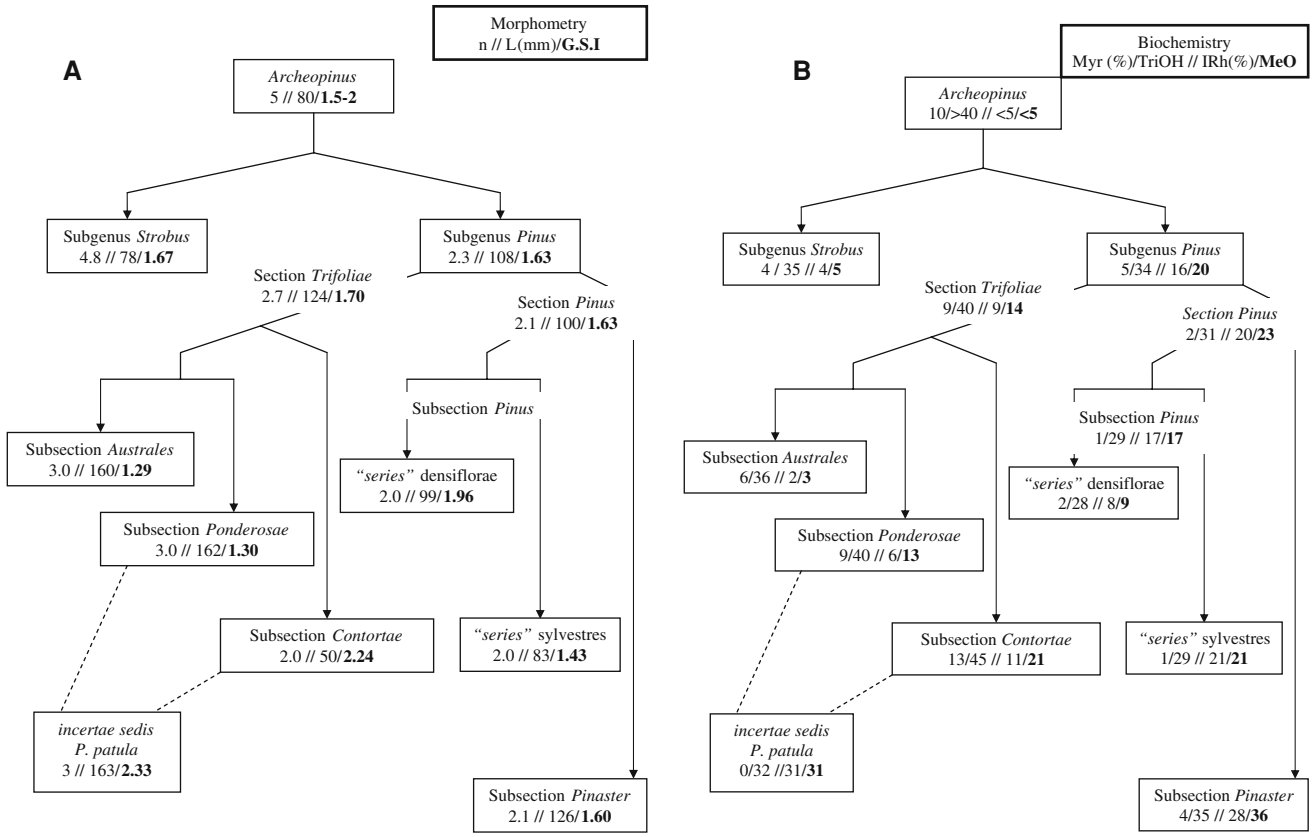

Fig. 1 **a** Phylogeny of genus *Pinus* and morphometrical characteristics. *n* Number of needles, *L* length of needle, *GSI* global slimness index. **b** Phylogeny of genus *Pinus* and biochemical characteristics. *Myr* Myricetin %, *Tri-OH* phenyl-tri-hydroxylation index, *IRh* isorhamnetin %, *MeO* phenyl-O-methylation index

in subsection *Pinaster*. This is confirmed by the near absence of larycitrin and syringetin in subsection *Silvestres* on the one hand, and their substantial presence (together around 9%) in *Pinaster* on the other. In fact, despite the common weakness in substituted phenyl-tri-*O* flavonols and the presence of taxifolin, the subsection *Pinus* as proposed by Gernandt et al. (2005) is heterogeneous for two reasons: slimness of the needles (GSI = 1.96 vs. 1.43) and low levels of methylated flavonol (MeO index = 9 vs. 21), hence the acknowledgment of the two "series", densiflorae and sylvestres, the latter being more evolved (=derived) than the former (Fig. 2). However, instead of concluding on a direct relationship (prohibited by geography), it seems more appropriate to propose two separate groups with distinct levels of evolution.

Biogeographical and ecophysiological phenomena

Over and above the few distinctions based on the strictly geographically oriented continental classification, we can also compare the influence of climate to the genetic/environmental interface, whereby biochemistry can be the ecophysiological mediator. In this respect, North America is not homogeneous with a north-western set which is cold, and two warm subsets, south-central and south-eastern, which are dry and humid respectively. Taking biogeography and the methoxylation index together, it can be emphasised that methylation is a chemophysiological character associated with lipophily and water balances for plants living in hot and dry regions. At equal masses, pines with five needles show a greater surface (thus more evapotranspiration) than those with two or three needles. This can explain the adaptive correlation between number of needles and MeO index. Yet, if both temperature and dryness are taken into consideration, seven "bioclimatic regions" A to G can be distinguished according to Critchfield and Little (1966). These include three regions in North America and four in Eurasia (Table 8 and map, Fig. 3). As for subsection *Pinaster* and the Mediterranean region, there is often a close correspondence between taxonomy and ecogeography: subsection *Australes* with south-eastern USA, subsection *Ponderosae* with southern USA and Mexico, and series "sylvestres" of the *Pinus* subsection with Middle Europe. Nevertheless, the collective species *Pinus nigra* is split between Middle and southern Europe.

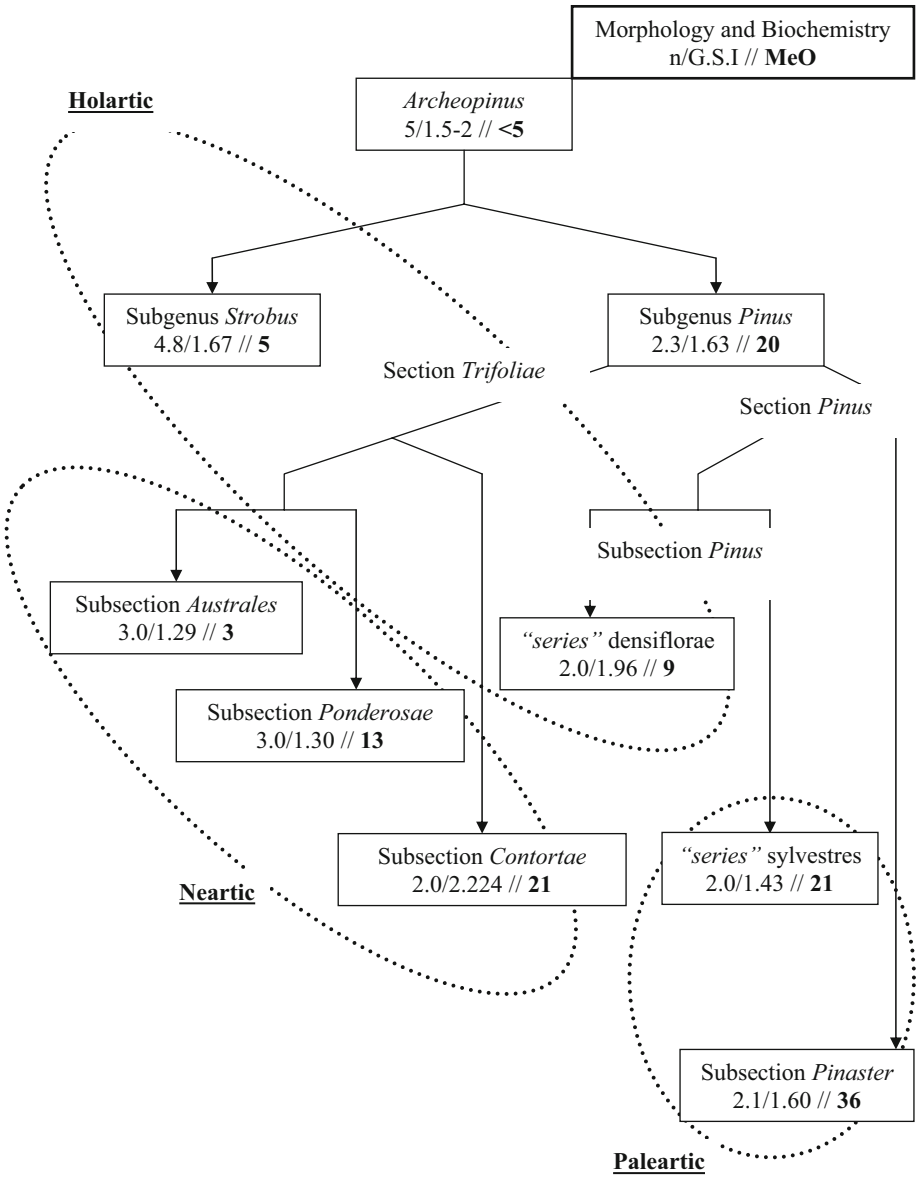

Fig. 2 Phylogeny of genus *Pinus* and biological characteristics: number of needles/global slimness index// MeO index

Table 8 Bioclimatic dependence of biological characteristics (number of needles, MeO index)

Geoclimatic subset	Climatic traits	*n*1	*n*2	Needles (*n*)	GSI	MeO	Strobus (%)
(A) North America, E/SE	Hot, wet	5	12	2.6	1.81	7 (6)	0
(B) North America, S. USA/Mexico	Hot, dry	5	30	3.4	1.74	19 (11)	27
(C) North America, W/SW	Cold, wet	5	18	3.6	1.79	14 (5)	44
(D) Eastern Asia	Wet	4	7	2.7	1.65	7 (8)	43
(E) Continental Asia	Cold	6	13	3.7	1.66	5 (2)	54
(F) Middle Europe (mountains)	Cold	10	10	2.6	1.37	20 (13)	20
(G) Mediterranean area	Hot, dry	10	10	2.1	1.53	31 (10)	0
Total (*n* taxa studied)		45	100	45	45	45	100

*n*1 number of taxa analysed here, *n*2 number of taxa recognised in each bioclimatic region

In continental Asia and northern America, with simultaneously cold and wet climates, the number of needles remains high: 3.7 and 3.6, and the MeO index is low or medium: 5 and 14; for the Mexican region compared to northern USA, the number of needles and MeO index are similar. In Middle Europe, the specific characteristics are

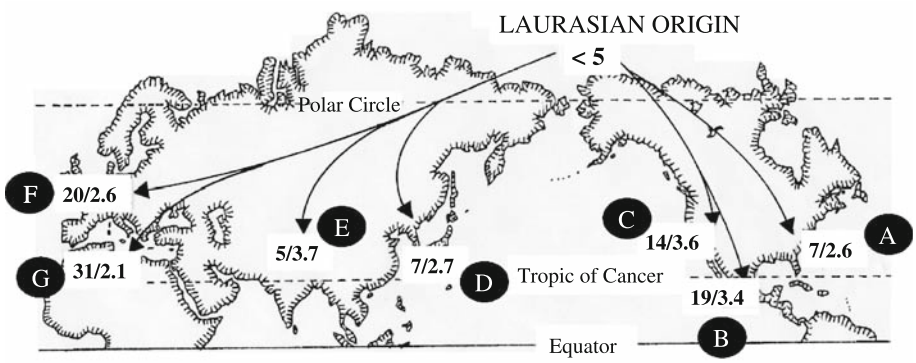

Fig. 3 Bioclimatic regions and biological characteristics (MeO index/number of needles) within the genus *Pinus*

very different compared to the Mediterranean ones: 2.6 versus 2.1 and 20 versus 31, respectively. Actually, bringing together the different species of the *halepensis* and *pinaster* groups, as well as *Pinus canariensis* and *Pinus pinea*, the subsection *Pinaster* constitutes an atlanto-mediterranean subset particularly evolved (=derived) within the Eurasian category. At the opposite, when the climate is hot but wet, as in south-eastern USA and Asia, the number of needles decreases (to 2.6 and 2.7 respectively) whereas the MeO index remains low (7 and 7 respectively), contrasting with the situation in other latitudes.

On other hand, other latitudinal clines can be observed concerning biological segregation in the genus *Pinus*. First, the number of species increases, for example from one to seven from northern to southern Europe (calculated from Atlas Florae Europaeae, Jalas and Suominen 1973) and from 18 to 30 in North America (based on 100 species including the 37 taxa studied here). Second, the importance of the subgenus *Strobus* and, correlatively, of the pines with five needles, decreases in the same way: if "cold" (continental Eurasia and America) and "hot" (Atlantic and Central America, south-eastern Asia, Mediterranean area) pines are compared, there are 17 and 11 species of the *Strobus* subgenus versus 22 and 50 of the *Pinus* subgenus, respectively. The difference between the two is highly significant ($\chi^2 = 7.71$; $P = 0.006$), the first subgenus being twice as rich for "cold pines" as the second (61 and 31% of species in each subgenus).

Conclusion

At the present time, the systematics of the genus *Pinus* cannot be understood without simultaneous recourse to classical morphological characters and geography, and those factors proposed more recently by biochemistry and molecular biology at two levels: macromolecular (proteins, DNA) and micromolecular ("chemotaxonomy", here polyphenolic markers). Beyond their length and weight (thus their fineness), the number of needles per bundle appears negatively correlated to the content of *O*-methylated foliar flavonols. Given that this biochemical character is directed biogenetically speaking because methylation is generally irreversible, it can be concluded that a low level of methylation (almost nil) and a large number of needles (up to five) are both ancestral characters, as observed for subgenus *Strobus*. Inversely, pines with two needles and a methoxylation index exceeding 30 are to be considered as evolved (=derived), as observed in subgenus *Pinus*. This is particularly the case for the two subsections *Contortae* (section *Trifoliae*) and *Pinaster* (section *Pinus*). On other hand, several characters show coherence with the two former subsections relative to taxonomy (balance between the two subgenus *Strobus* and *Pinus*), biogeography (northern vs. southern area) or ecophysiology (cold vs. hot, wet or dry regions).

From a dynamic point of view, there is a strong correspondence between the taxonomical subsets that have been recognised and the evolutionary stages of the genus defined by morphometric and biochemical characters given that the first generally shows a good biogeographical and climatic homogeneity; the Mediterranean case (subsection *Pinaster*) is a symptomatic one. In this respect, we postulate the existence of a common ancestor (or "Archeopinus") of present *Pinus* species of continental/septentrional location in the present geographical sense, allowing consideration of the geological phenomena which gave birth, about 180 millions years ago (during the Mesozoic era between the Cretaceous/Jurassic), to the two major blocks, Gondwana and Laurasia from Pangea. Our proposal of an Archeopinus taxon with a Laurasian origin nullifies the present distinction between Palearctic and Neartic domains, which would have previously populated the same continuous territory.

Starting from this hypothetical primary Laurasian origin, the migration and then the segregation following separation of present Eurasia and America could have thus allowed the divergence of two phyla: the first, corresponding to the subgenus *Strobus*, remains near the "Archeopinus" stem group; the second, corresponding to the subgenus *Pinus*, has generated two sub-phyla, the first mainly Palearctic (section

Pinus, with some species of the Nearctic "series" densiflorae) and North American (section *Trifoliae*). Considering the low number of needles and the high MeO index, subsection *Contortae* is the more derived subset of the North American pines. In Eurasia, the two Asian subsets, continental and eastern, share a low methylation index. There is a clear difference compared to the European bloc which in turn, also comprises two sub-classes, Middle European and Mediterranean. The latter with a low number of needles and high methylation index could thus be derived from the first. In the Old World, continental Asia (northern, cold and humid) and the Mediterranean area (southern, hot and dry) show extreme characteristics which are highly significant: 54 versus 0% for the presence of the subgenus *Strobus*, 3.7 versus 2.1 for the average number of needles per bundle, 5 versus 31 for the *O*-methylation index. Thus *Pinus* is a genus "that came from the cold"!

To conclude, it is hoped that our multi-disciplinary contribution, which adds a morphometric, biogeographical and micromolecular dimension to the many recent works of macromolecular nature, may partly be considered as an answer to the pertinent comment recently made by Syring et al. (2005): "Despite ca. 30 published studies over the past two decades, a well-resolved phylogeny of *Pinus* remains a work in progress."

Acknowledgments We would like to express our gratitude to Mr Paul Berthet and Mr Frédéric Pautz, former and present directors of the Parc de la Tête d'Or botanical garden (Lyon, France), who have given us free access to the plant samples and their institute. Our thanks also go to Mrs Christine Bayet who analysed the flavonoids from Asian and American pines and anonymous reviewers for their critical advice on the manuscript.

References

Bergmann F, Gillet EM (1997) Phylogenetic relationships among *Pinus* species (Pinaceae) inferred from different numbers of 6PGDH loci. Pl Syst Evol 208:25–34

Critchfield WE, Little EL (1966) Geographic distribution of the *Pines* of the world. USDA Forest Service 991:1–97

Farjon A (1984) *Pines*: drawings and descriptions of the genus, 2nd edn. Brill and Backhuys, Leiden (ibid. 2005, 235 p)

Farjon A (2005) A bibliography of conifers: selected literature on taxonomy and related disciplines of the coniferales, 2nd edn. Royal Botanical Gardens, Kew

Gaussen H (1960) Les Gymnospermes actuelles et fossiles. Genre *Pinus*. Trav Lab Forest Fac Sci Toulouse VI(11):1–272

Gernandt DS, Lopez GG, Garcia SO, Liston A (2005) Phylogeny and classification of *Pinus*. Taxon 54:29–42

Govindaraju D, Lewis P, Cullis C (1992) Phylogenetic analysis of pines using ribosomal DNA restriction fragment length polymorphisms. Pl Syst Evol 179:141–153

Idrissi-Hassani M (1985) Etude de la variabilité flavonique chez deux conifères mediterranéenne: le pin maritime *Pinus pinaster* Ait. et le genévrier thurifère *Juniperus thunifera* L. PhD Thesis, Université Claude Bernard Lyon 1, Lyon

Idrissi-Hassani M, Lebreton P (1992) Les flavonoïdes du Pin maritime: une approche chimiotaxonomique. Forêt méditerr 13:3–8

Jalas J, Suominen J (1973) Atlas Florae Europaeae. Finnish Museum of Natural History, Helsinki

Kaundun SS (1995) Contribution biochimique à la connaissance systématique et génétique du Pin d'alep *Pinus halepensis* Mill. et du Douglas *Pseudotsuga menziesii* Mirb. Université Claude Bernard Lyon 1, Lyon

Kaundun SS, Fady B, Lebreton P (1997) Genetic differences between *Pinus halepensis*, *Pinus brutia* and *Pinus eldarica* based on needle flavonoids. Biochem Syst Ecol 25:553–562

Klaus W (1989) Mediterranean pines and their history. Pl Syst Evol 162:133–163

Krupkin AB, Liston A, Strauss SH (1996) Phylogenetic analysis of the hard pines (*Pinus* subgenus *Pinus*, *Pinaceae*) from chloroplast DNA restriction site analysis. Am J Bot 83:489–498

Krüssmann G (1972) Handbuch der Nadelgehölze. Parey, Berlin

Kutil BL, Williams CG (2001) Triplet-repeat microsatellites shared among hard and soft pines. J Heredity 92:327–332

Laracine C (1984) Etude de la variabilité flavonique infraspécifique chez deux Conifères: le Pin sylvestre *Pinus sylvestris* L. et le genévrier commun *Juniperus communis* L. PhD Thesis, Université Claude Bernard Lyon 1, Lyon

Laracine-Pittet C, Lebreton P (1988) Flavonoid variability within *Pinus sylvestris*. Phytochemistry 27:2663–2666

Lauranson-Broyer J (1989) Exploration de la diversité biochimique chez les Conifères: contributions à l'étude de l'hybridation *Pinus uncinata* Ram. x *Pinus sylvestris* L., et à la connaissance du complex spécifique *Pinus nigra* Arn. Université Claude Bernard Lyon 1, Lyon

Lauranson-Broyer J, Lebreton P (1995) Flavonic chemosystematics of the specific complex *Pinus nigra* Arn. In: Baradat P, Adams WT, Müller-Starck G (eds) Population geenetics and genetic conservation of forest trees. Academic Publishing, Amsterdam, pp 181–188

Lebreton P (1990) La chimiotaxonomie des Gymnospermes. Bull Soc Bot Fr 137:35–46

Lebreton P (1995) Genetics and biodiversity of flavonoids in conifers. In: Baradat P, Adams WT, Müller-Starck G (eds) Population geenetics and genetic conservation of forest trees. Academic Publishing, Amsterdam, pp 41–54

Lebreton P, Laracine-Pittet C, Bayet C, Lauranson J (1990) Variabilité polyphénolique et systématique du pin sylvestre *Pinus sylvestris* L. Ann Sci For Fr 47:117–130

Liston A, Robinson WA, Pinero D, Alvarez-Buylla ER (1999) Phylogenetics of *Pinus* (Pinaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. Mol Phylogenet Evol 11:95–109

Little EL, Critchfield WB (1969) Subdivisions of the genus *Pinus*. Misc. Publ. 1144. USDA, Washington DC

Lopez GG, Kamiya K, Harada K (2002) Phylogenetic relationships of *Diploxylon* pines (subgenus *Pinus*) based on plastid sequence data. Int J Pl Sci 163:737–747

Mirov NT (1967) The genus *Pinus*. Ronald Press, New York

Norin T (1972) Some aspects of the chemistry of the order Pinales. Phytochemistry 11:1231–1242

Piovesan G, Pelosi C, Schirone A, Schirone B (1993) Taxonomic evaluations of the genus *Pinus* (Pinaceae) based on electrophoretic date of salt soluble and insoluble seed storage proteins. Pl Syst Evol 186:57–68

Price RA, Liston A, Strauss SH (1998) Phylogeny and systematics of *Pinus*. In: Richardson DM (ed) Ecology and biogeography of *Pinus*. Cambridge University Press, Cambridge. Cited by Gernandt et al. 2005, pp 49–68

Schirone B, Piovesan G, Bellarosa R, Pelosi C (1991) A taxonomic analysis of seed proteins in *Pinus* spp. (Pinaceae). Pl Syst Evol 178:43–53

Shaw GR (1914) The genus *Pinus*, vol 5. Arnold Arbor, Boston

Syring J, Willyard A, Cronn R, Liston A (2005) Evolutionary relationships among *Pinus* (Pinaceae) subsections inferred from multiple low-copy nuclear loci. Am J Bot 92:2086–2100

Wang X-R, Tsumura Y, Yoshimaru H, Nagasaka K, Szmidt AE (1999) Phylogenetic relationships of Eurasian pines (*Pinus*, Pinaceae) based on chloroplast *rbc*L, *mat*K, *rpl*20-*rps*18 spacer, and *trn*V intron sequences. Am J Bot 86:1742–1753

Yazdani R, Lebreton P (1991) Inheritance pattern of the flavonic compounds in Scots pine (*Pinus sylvestris* L.). Silvae Genet 40:57–59