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Variation in the *psb*C gene region of gymnosperms and angiosperms as detected by a single restriction site polymorphism

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Abstract This paper reports on a PCR-RFLP analysis in a chloroplast DNA region consisting of coding and intergenic spacer sequences of *trn*S and the adjacent *psb*C gene. This region was PCR-amplified in 62 woody plant species, predominantly tree species, that represent a broad systematic range in both gymnosperms and dicotyledonous angiosperms. The amplification products were digested by the restriction endonuclease *Hae*III (GG↓CC). Fourteen different restriction patterns occurred, 5 of which characterised representatives of the gymnosperms, and 9 angiosperm representatives. A single restriction site polymorphism revealed most of the species to share restriction patterns. Groups formed which showed relationships to plant systematic units. This phenomenon is discussed with regard to the *psb*C gene and the GGCC motif for tracing species' relationships on a high taxonomic level of gymnosperms and angiosperms.

Key words Trees · Chloroplast DNA · PCR-RFLP · Genetic variation · Evolution

Introduction

Chloroplast genes are assumed to have slower rates of evolution than plant nuclear genes (Clegg et al. 1991). This makes chloroplast DNA (cpDNA) variation a suitable tool for studying molecular evolutionary relationships among plant species. The existing molecular studies in this field range from the intrageneric up to the interfamilial level. Intrageneric analyses on closely related species are often performed in the frame work of hybridisation and introgression studies (e.g. Govarindaju et al. 1989; Hong et al. 1993; Kormuták et al. 1993; Wang and Szmidt 1993; Fregene et al. 1994; Rajora and Dancik 1995). At the familial level, phylogenetic relationships have been deduced between genera of the same family or between families as such (Frascaria et al. 1993; Bousquet et al. 1992; Sriboonma et al. 1993; Ehrendorfer et al. 1994; Manen et al. 1994; Tsumura et al. 1995). Most of the investigations have dealt with the analysis of chloroplast genes, although some also included investigations on non-coding intergenic cpDNA sequences (Ehrendorfer et al. 1994).

In a previous study we carried out a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis on a cpDNA region consisting of both the gene and intergenic sequences of the *trn*S and *psb*C genes in the conifer species silver fir (*Abies alba*, *Pinaceae*). Individuals from wide-ranging provenances of the silver fir natural range were sampled, the *trn*S-*psb*C region was PCR-amplified and the latter was subsequently digested by numerous restriction endonucleases (Ziegenhagen et al. 1995). No variation was detected, with one exception: digestion by the enzyme *Hae*III (GG↓CC) resulted in the detection of two distinct variants of restriction patterns. The detection of the two variants was not limited to the species *Abies alba*. Exactly the same variants also occurred in other species of the genus *Abies*. These findings suggest that the *trn*S-*psb*C region is possibly involved in species evolution of at least the genus *Abies*. This encouraged us to do this particular PCR-RFLP analysis for a systematically broad range of species. A total of 62 woody plant species were analysed, 34 of them representing 7 families of the gymnosperms, and 28 representing 13 families of dicotyledonous angiosperms.

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Materials and methods

Plant material

The selected species represent 60 tree and 2 shrub species (*Rosa spec.* and *Sambucus nigra*). Table 1 gives a survey of the investigated species and individuals, and their positions in plant systematics according to Strasburger et al. (1983). Most of the samples were taken from trees of the "Arboretum Tannenhöft", Institute for Forest Genetics, Grosshansdorf. Some species were sampled in the Botanical Gardens of the Universities of Hamburg and Bonn or collected in the surroundings of the Institute for Forest Genetics. Also, samples of conifer species were provided from the Royal Botanical Garden of Edinburgh. A minimum of two to three individuals per species or genus was analysed. If possible, the samples of conspecific individuals were taken from different localities. One gymnosperm (*Abies alba*) and 2 angiosperm species (*Fagus sylvatica* and *Quercus robur*) were selected for additional intraspecific analyses. For this study the sample size of the relevant species was enlarged, and individuals were chosen from different provenances or populations: 220 individuals from 12 provenances of *Abies alba*, 23 individuals from 10 provenances of *Fagus sylvatica*, 47 individuals from five populations of *Quercus robur*.

Isolation of genomic DNA

Genomic DNA was isolated from needle, leaf and bud material according to the minipreparation procedure of Ziegenhagen et al. (1993).

PCR amplification, restriction, and visualisation of DNA fragments

The cpDNA region comprising coding and non-coding sequences between flanking regions of the *trn*S gene [tRNA - Ser (UGA)] and the adjacent *psb*C gene (PS II 44kDa) was amplified by a pair of

Table 1 The species investigated

Class	Order	Family	Species
a) *Gymnospermae*			
1) *I. Ginkgoatae*	*Gingkoales*	*Gingkoaceae*	*Ginkgo biloba* (7)[a]
2) *II.Pinatae* Sub-class: *Pinidae*	*Pinales*	*Araucariaceae*	*Araucaria araucana* (3)
		Pinaceae	*Abies alba* (220)
			Abies cephalonica (1)
			Abies grandis (1)
			Abies homolepis (1)
			Abies nordmanniana (1)
			Abies numidica (1)
			Abies pinsapo (1)
			Picea abies (5)
			Tsuga canadensis (3)
			Pseudotsuga menziesii (1)
			Larix kaempferi (3)
			Cedrus atlantica (2)
			Cedrus deodara (1)
			Cedrus libani (1)
			Calocedrus decurrens (2)
			Pinus aristata (1)
			Pinus densiflora (1)
			Pinus sylvestris (3)
			Pinus thunbergii (1)
		Taxodiaceae	*Metasequoia glyptostroboides* (2)
			Sequoia sempervirens (1)
			Sequoiadendron giganteum (1)
			Cryptomeria japonica (5)
			Taxodium distichum (3)
		Cupressaceae	*Thuja occidentalis* (1)
			Thuja plicata (2)
			Chamaecyparis nootkatensis (1)
			Chamaecyparis obtusa (1)
			Chamaecyparis pisifera (1)
			Juniperus chinensis (2)
		Cephalotaxaceae	*Cephalotaxus harringtonia* (2)
Sub-class: *Taxidae*	*Taxales*	*Taxaceae*	*Taxus baccata* (3)

Table 1 Continued

Class	Order	Family	Species
b) *Angiospermae*			
Dicotyledoneae			
Sub-class:			
Magnoliidae	*Magnoliales*	*Magnoliaceae*	*Magnolia grandiflora* (2)
			Liriodendron tulipifera (2)
Sub-class:			
Hamamelididae	*Hamamelidales*	*Hamamelidaceae*	*Hamamelis intermedia* (1)
			Hamamelis japonica (1)
			Hamamelis vernalis (1)
			Hamamelis "Wildling" (1)
	Fagales	*Fagaceae*	*Fagus sylvatica* (23)
			Castanea sativa (2)
			Quercus robur (47)
			Quercus petraea (1)
		Betulaceae	*Corylus avellana* (3)
			Carpinus betulus (5)
			Betula pubescens (3)
			Alnus glutinosa (3)
	Urticales	*Ulmaceae*	*Ulmus glabra* (2)
Sub-class:			
Rosidae	*Rosales*	*Rosaceae*	*Rosa spec.* (3)
			Sorbus aucuparia (3)
	Fabales	*Fabaceae*	*Robinia pseudoacacia* (3)
	Sapindales	*Hippocastanaceae*	*Aesculus hippocastanum* (3)
		Aceraceae	*Acer carpinifolium* (1)
			Acer saccharum (3)
Sub-class:			
Dilleniidae	*Salicales*	*Salicaceae*	*Salix caprea* (3)
			Salix viminalis (3)
			Populus tremula (1)
			Populus tremuloides (2)
	Malvales	*Tiliaceae*	*Tilia cordata* (3)
Sub-class:			
Asteridae	*Dipsacales*	*Caprifoliaceae*	*Sambucus nigra* (5)
	Oleales	*Oleaceae*	*Fraxinus excelsior* (3)

[a] Number in brackets is the number of individual plants investigated

universal primers (Demesure et al. 1995); primer 1: 5-GGT TCG AAT CCC TCT CTC TC-3′, primer 2: 5′-GGT CGT GAC CAA GAA ACC AC-3′. The primers were synthesised by Pharmacia Biotech Europe GmbH (Freiburg, Germany).

The PCR was run in a pre-heated DNA Thermal Cycler TC1 (Perkin-Elmer & Co GmbH, Überlingen, Germany) with the following profile (Demesure et al. 1995): 94°C for 4 min, followed by 35 cycles of 93°C for 1 min, 57°C for 1 min and 72°C for 2 min. Last strand elongation (72°C) was allowed an additional 10 min. Amplification was performed in a 25-µl volume of total reaction mixture. The reaction mixture (Demesure et al. 1995) consisted of 20 ng genomic DNA, 16.6 m*M* $(NH_4)_2SO_4$, 67 m*M* *Tris*-HCL pH 8, 2 m*M* $MgCl_2$, 0.001% W_1 (Gibco BRL, Life Technologies GmbH, Eggenstein, Germany), 10 m*M* *β*-mercaptoethanol, 4.4 µg/ml bovine serum albumin, 100 µ*M* of each dNTP, 1 unit *Taq* polymerase (Gibco BRL, Life Technologies GmbH, Eggenstein, Germany) and 0.54 µ*M* of each of the two primers. Of the total PCR amplification volume 16 µl was digested by 10 units of the restriction endonuclease *Hae*III (GG↓CC) following the recommendations of the manufacturer (Boehringer Mannheim, Germany).

Undigested PCR products as well as the restriction fragments were separated by electrophoresis in a 1.5% (w/v) agarose gel run in 0.5 × TRIS-borate buffer at 13 V/cm for 3 h. DNA fragments were visualised by UV fluorescence after staining with ethidium bromide (0.25 µg/ml staining solution).

PCR amplification and restriction of the PCR products were repeated at least twice for all individuals. Amplification products as well as restriction patterns were shown to be reproducible.

Results

The PCR-RFLP analysis based on the amplification of a specific cpDNA region followed by *Hae*III restriction

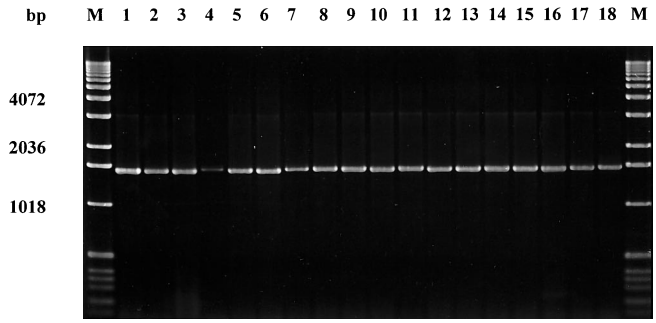

Fig. 1 Undigested amplification products for 18 conifer species, among which are *Gingko biloba* and representative species of gymnosperm families *Araucariaceae*, *Pinaceae*, *Taxodiaceae*, *Cupressaceae*, *Cephalotaxaceae and Taxaceae. Lane 1 Gingko biloba*, *2 Taxus baccata*, *3 Cephalotaxus harringtonia*, *4 Sequoia sempervirens*, *5 Cryptomeria japonica*, *6 Taxodium distichum*, *7 Thuja occidentalis*, *8 Chamaecyparis pisifera*, *9 Juniperus chinensis*, *10 Picea abies*, *11 Abies alba*, *12 Tsuga canadensis*, *13 Pseudotsuga menziesii*, *14 Larix kaempferi*, *15 Cedrus libani*, *16 Pinus sylvestris*, *17 Calocedrus decurrens*, *18 Araucaria araucana. M* Molecular weight marker (1-kb ladder, Gibco BRL)

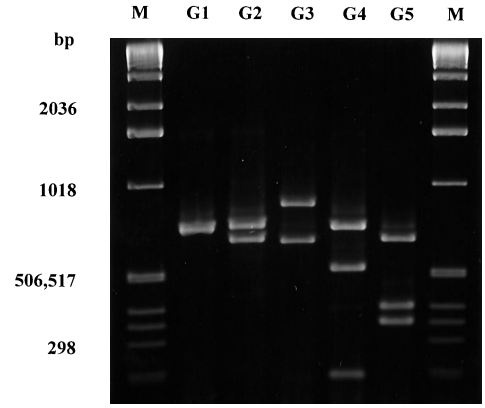

Fig. 2 *Hae*III restriction patterns of gymnosperm species G1–G5, each with one representative species. *G1 Abies nordmanniana*, *G2 Araucaria araucana*, *G3 Thuja occidentalis*, *G4 Larix kaempferi*, *G5 Gingko biloba. M* Molecular weight marker (1-kb ladder, Gibco BRL)

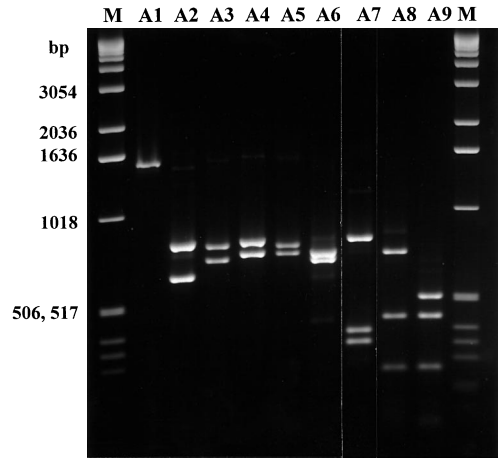

Fig. 3 *Hae*III restriction patterns of angiosperm species A1–A9, each with one representative species. *A1 Robinia pseudoacacia*, *A2 Corylus avellana*, *A3 Acer carpinifolium*, *A4 Alnus glutinosa*, *A5 Aesculus hippocastanum*, *A6 Fagus sylvatica*, *A7 Castanea sativa*, *A8 Liriodendron tulipifera*, *A9 Magnolia grandiflora. M* Molecular weight marker (1-kb ladder, Gibco BRL)

was performed on a total of 418 individuals, representing 62 woody species. Within the *Gymnospermae*, a total of 34 species were studied. *Gingko biloba* was analysed as the only species of the most ancient class *Gingkoatae*, followed by 33 species of the class *Pinatae*. These represent two sub-classes with 6 families in two orders. Within the *Angiospermae* (*Dicotyledonae*) 28 species were analysed. They represent five sub-classes with 13 families in 11 orders.

PCR analysis resulted in an amplification product of about 1600 bp for all individuals. Figure 1 demonstrates the amplification products for 18 representatives of the gymnosperms. Except for *Metasequoia* and *Sequoiadendron*, all of the genera under study are represented in this figure. As can be judged from the agarose gel depicted here, the amplification products did not significantly deviate in length. The same was found for the representatives of the angiosperms (data not shown).

Each of the 418 amplification products was digested by *Hae*III. As a result, 14 different restriction fragment patterns were visualised in the agarose gel. Gymnosperms and angiosperms did not have any of these patterns in common. The 34 representative species of the gymnosperms (3 orders, 7 families) were characterised by 5 patterns. In comparison, 9 patterns characterised the 28 representative species of the angiosperms (11 orders, 13 families).

Figure 2 shows the 5 different restriction patterns within the *Gymnospermae*, referred to here as patterns G1–G5. As far as could be judged from the agarose gels, G1 was characterised by one visible band, G2 and G3 by two, and G4 and G5 by three visible bands. In Fig. 3, the 9 different restriction patterns within the *Angiospermae* are shown, referred to here as patterns A1–A9. On the basis of the agarose gels, A1 is characterised by one visible band, A2 to A6 by two, and A7–A9 by three visible bands.

Of the 14 different patterns, 5 occurred only once, each in a single species: G2 (*Araucaria araucana*) and G5 (*Gingko biloba*) within the gymnosperms; A5 (*Aesculus hippocasanum*), A8 (*Liriodendron tulipifera*) and A9 (*Magnolia grandiflora*) within the angiosperms. The remaining 9 patterns were shared by at least 2 species.

The identity of fragment lengths for all species sharing the same restriction fragment pattern, was confirmed by grouping those species on the same agarose gels. Figure 4 is an example of such a grouping for selected gymnosperm species. Similar gels were run for the angiosperms (data not shown).

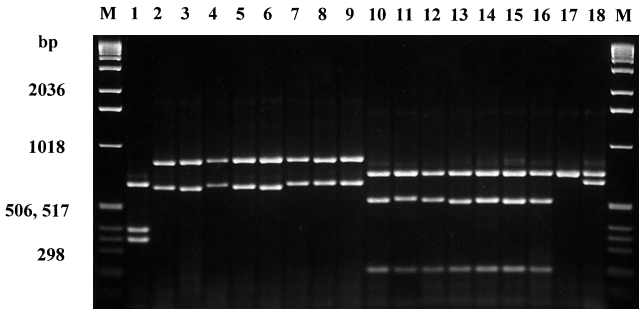

Fig. 4 Grouping of gymnosperm species having the same *Hae*III restriction fragment patterns. *Lane 1 Gingko biloba*, *2 Taxus baccata*, *3 Cephalotaxus harringtonia*, *4 Metasequoia glyptostroboides*, *5 Cryptomeria japonica*, *6 Taxodium distichum*, *7 Thuja occidentalis*, *8 Chamaecyparis pisifera*, *9 Juniperus chinensis*, *10 Picea abies*, *11 Abies alba*, *12 Tsuga canadensis*, *13 Pseudotsuga menziesii*, *14 Larix kaempferi*, *15 Cedrus libani*, *16 Pinus sylvestris*, *17 Abies alba*, *18 Araucaria araucana*. *M* Molecular weight marker (1-kb ladder, Gibco BRL)

Many of the species which were grouped in either G3 or G4 were confirmed to share identical patterns. There were, however, slight deviations in fragment lengths within the groups, specially within the G3 group. As a first approach, these deviations are acceptable given the distinct differences observed with respect to the other gymnosperm patterns.

The pattern G1 was found only in the genus *Abies* and was the only intraspecific variation detected. Whereas only 1 restriction fragment was visible in the agarose gel, the G1 pattern has been shown to consist of two fragments of identical length (Ziegenhagen et al. 1995). Figure 4 demonstrates the intraspecific variation of *Hae*III restriction patterns in *Abies alba* (lanes 11 and 17). These 2 distinct variants correspond to patterns G4 and G1. The ratio of the 2 patterns in 220 *Abies alba* individuals was 56 (G1) to 164 (G4). Most of the provenances under study were characterised by the occurrence of both variants, some by either G1 or G4.

On the basis of results from the agarose gels, no significant deviations in restriction fragment lengths could be observed within the pattern groups of the angiosperms. For 2 of the angiosperm tree species, beech (*Fagus sylvatica*) and oak (*Quercus robur*), the number of individuals studied was increased to 23 and 47, respectively, for possible detection of intraspecific variation. Although the individuals were taken from different provenances or populations no intraspecific variation was detected, in either species.

After confirmation of the pattern identity within the species, the question arose as to which species share which restriction patterns. Table 2 presents the species investigated according to the systematic arrangement presented in Table 1 (Strasburger et al. 1983). In addition, the restriction patterns G1–G5 and A1–A9 are grouped with respect to the species. In this example of systematic arrangement, two phenomena can be observed. First, many closely related species bear the same patterns and second, some distantly related species also share the same restriction patterns.

Table 2 Investigated species of gymnosperms (left) and angiosperms (right) according to the systematics in Table 1. The *Hae*III restriction patterns G1–G5 and A1–A9 are arranged with respect to species

Species		Species	
Gingko biloba	G5	*Magnolia grandiflora*	A9
		Liriodendron tulipifera	A8
Araucaria araucana	G2		
		Hamamelis intermedia	A6
Abies alba	G1/G4	*Hamamelis japonica*	A6
Abies cephalonica	G1	*Hamamelis vernalis*	A6
Abies grandis	G1	*Hamamelis "Wildling"*	A6
Abies homolepis	G4		
Abies nordmanniana	G1	*Fagus sylvatica*	A6
Abies numidica	G1	*Castanea sativa*	A7
Abies pinsapo	G4	*Quercus robur*	A7
Picea abies	G4	*Quercus petraea*	A7
Tsuga canadensis	G4		
Pseudotsuga menziesii	G4	*Corylus avellana*	A2
Larix kaempferi	G4	*Carpinus betulus*	A2
Cedrus atlantica	G4	*Betula pubescens*	A4
Cedrus deodara	G4	*Alnus glutinosa*	A4
Cedrus libani	G4		
Calocedrus decurrens	G4	*Ulmus glabra*	A4
Pinus aristata	G4		
Pinus densiflora	G4	*Rosa spec.*	A4
Pinus sylvestris	G4	*Sorbus aucuparia*	A1
Pinus thunbergii	G4		
		Robinia pseudoacacia	A1
Metasequoia glyptostroboides	G3		
Sequoia sempervirens	G3	*Aesculus hippocastanum*	A5
Sequoiadendron giganteum	G3		
Cryptomeria japonica	G3	*Acer carpinifolium*	A3
Taxodium distichum	G3	*Acer saccharum*	A3
Thuja occidentalis	G3	*Salix caprea*	A3
Thuja plicata	G3	*Salix viminalis*	A3
Chamaecyparis nootkatensis	G3	*Populus tremula*	A3
		Populus tremuloides	A3
Chamaecyparis obtusa	G3		
Chamaecyparis pisifera	G3		
Juniperus chinensis	G3	*Tilia cordata*	A6
Cephalotaxus harringtonia	G3	*Sambucus nigra*	A1
Taxus baccata	G3	*Fraxinus excelsior*	A1

Discussion

Novel PCR-based approaches to cpDNA polymorphisms are using conserved cpDNA regions for universal primer design (Taberlet et al. 1991, Demesure et al. 1995), with the amplification products undergoing subsequent investigations by direct sequencing or restriction analyses (Ehrendorfer et al. 1994, Tsumura

et al. 1995). In particular, PCR-RFLP analyses open up the field by providing a simple and fast screening of cpDNA regions in a large number of samples; for example, Tsumura et al. (1995) studied several chloroplast genes in 5 conifer families. To our knowledge, our investigation was one of the first to span a broad systematic range in both gymnosperms and angiosperms, it was based on 1–7 species per genus and on 1–220 individuals per species.

PCR-amplification of the *trn*S-*psb*C region was successful in all of the species under study, thereby supporting the universal usability of the primer pair designed by Demesure et al. (1995). Amplification products did not significantly differ in length, not even between the two sub-divisions of *Gymnospermae* and *Angiospermae*. As the two primers refer to flanking regions of two different genes we conclude that there is a highly conserved arrangement of the two chloroplast genes. For most of the vascular land-plants the genes are supposed to be arranged in the same order on the circular cpDNA molecule (Clegg et al. 1991). We did a search for homology between primer sequences and the complete cpDNA sequence of the gymnosperm species *Pinus thunbergii* [DDBJ Database, accession No. D17510 (Wakasugi et al. 1994)]. Primer sequence 1 was found to be 100% homologous to the relevant *trn*S gene sequence, primer sequence 2 shared 95% homology with the relevant complementary *psb*C gene sequence near to the 3′ end of the gene. Between the primer sequences, a *P. thunbergii* sequence 1521 bp in length was deduced: it comprises 23 bp of *trn*S gene, 168 bp of intergene region and 1330 bp of *psb*C gene, making 1521 bp in total. We therefore assume a high proportion of *psb*C gene sequences in all amplification products, at least for the gymnosperm species.

Because chloroplast genes show a relatively slow sequence evolution, genes like *rbc*L, *psb*A, *psb*D and numerous genes encoding for different tRNAs have frequently been used in phylogenetic studies (e.g., Frascaria et al. 1993; Bousquet et al. 1992, Kormuták et al. 1993, Tsumura et al. 1995). Our study revealed that the *psb*C gene seems to be of evolutionary relevance for both groups gymnosperms and angiosperms. The number of different restriction patterns reflects the different degrees of systematic sub-division in gymnosperms and angiosperms, as only 5 patterns characterised the wide-ranging gymnosperm species while 9 patterns were distinctive for the angiosperm species. Furthermore, a single restriction site polymorphism makes numerous species share patterns and form groups that show relationships to systematic units. Among the different fragment patterns, fragments of identical length appeared, e.g. G1, G2 and G4 or A8 and A9. As fragments of identical length are not necessarily fragments of identical sequence, at present it is not advisable to deduce any pattern hierarchy on the basis of band sharing. Sequencing alone will, for example, validate parsimony computations and establish phylogenetic relationships based on a single cpDNA region; e.g. as in the *atp*B-*rbc*L region for deducing the phylogeny of *Rubiaceae* (Manen et al. 1994). Interpretations of our results are determined by the observable phenomenon, the restriction fragment patterns as such.

In the gymnosperms and angiosperms studied here, our restriction site polymorphism was characterised by either different fragment lengths or different numbers of restriction fragments. The occurrence of both types is due to insertions/deletions, or reflects gains/losses of restriction sites by nucleotide substitutions. Both types of polymorphisms are frequently observed in the chloroplast genome, with site mutations moreover occurring in chloroplast genes and predominantly representing substitutions at the third codon position (Zurawski et al. 1984; Palmer 1987; Clegg et al. 1991). With regard to our fragment patterns, there was strong evidence for the gains or losses of restriction sites to be located in the *psb*C gene. As far as detected in the agarose gels, fragment lengths of all patterns clearly exceeded 200 bp. Site mutations in non-*psb*C sequences analysed by restriction should result in fragments smaller than 23 bp + 168 bp, at least for the gymnosperms.

The length polymorphisms observed between patterns cannot unequivocally be attributed to either the gene or the non-coding region. Different rates of sequence evolution have been discussed for genes on the one hand and intergenic non-coding sequences on the other (Clegg et al. 1991). Heterogeneity of sequence evolution should also be considered in the region under study. Sequence data on the *trn*S - *psb*C region in *Abies alba* provides preliminary evidence of high intraspecific variation in the non-coding intergenic region compared to the highly conserved *psb*C gene sequence (Ziegenhagen, unpublished data).

As already stated above, previous studies in the species *Abies alba* revealed that the restriction endonuclease *Hae*III is the only 1 out of 14 enzymes to detect cpDNA variation in the *psb*C region. The two detected intraspecific variants are supposed to be involved in sequence evolution throughout the genus *Abies*. This and the findings of our present study bring the nucleotide motif 'GGCC' into the focus of interest. Obviously, for both gymnosperms and angiosperms, evolution from 'older' to 'younger' species seems to correlate with a reduction in the number of bands as detectable in agarose gels resulting from the loss of at least one GGCC restriction site. The application of our particular PCR-RFLP analysis to other chloroplast genes of systematically wide-ranging species would be worthwhile. By this, the question of homogeneity/heterogeneity of sequence evolution among genes in major plant lineages could be inferred (Clegg et al. 1991). Finally, completed by sequence data the results might contribute to a deeper insight into the relationships of nucleotide substitutions and cpDNA sequence divergence (Zurawski et al. 1984). In this respect,

representatives of evolutionary old divisions in plant systematics but also representatives of angiosperm dicotyledonous annuals and monocotyledons should be analysed. Preliminary analyses of horse-tail (*Equisetum*), liverwort (*Marchantia*), tomato (*Lycopersicon*), and grasses (*Panicum*, *Zea*, *Miscanthus*) encourage to add further data in this field.

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