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Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (Asteraceae)

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Abstract

Pyrrolizidine alkaloids (PAs) of the macrocyclic senecionine type are secondary metabolites characteristic for most species of the genus *Senecio* (Asteraceae). These PAs are deterrent and toxic to most vertebrates and insects and provide plants with a chemical defense against herbivores. We studied the PA composition of 24 out of 26 species of *Senecio* section *Jacobaea* using GC-MS. The PA profiles of eight of these species have not been studied before and additional PAs were identified for most other species that were included in previous studies. With one exception (senecivernine) all 26 PAs identified in sect. *Jacobaea* can be regarded as derivatives of the biosynthetic backbone structure senecionine. Based on the PA profiles of the species of sect. *Jacobaea* and the results of previous tracer studies, we constructed two hypothetical biosynthetic scenarios of senecionine diversification. Both scenarios contain two major reactions: the conversion of the necine base moiety retronecine into the otonecine moiety and site-specific epoxidations within the necic acid moiety. Further reactions are site-specific hydroxylations, sometimes followed by *O*-acetylations, site-specific dehydrogenations, *E, Z*-isomerizations, and epoxide hydrolysis and chlorolysis. The GC-MS data and both biosynthetic scenarios were subsequently used to study the evolution of PA formation in sect. *Jacobaea* by reconstructing the evolutionary history of qualitative PA variation in this section. This was achieved by optimizing additive presence/absence data of PAs and types of enzymatic conversions on a maximum parsimony cladogram of section *Jacobaea* inferred from DNA sequence and morphological data. Besides showing large intra- and interspecific variation, PA distribution appears to be largely incidental within the whole clade. These results together with the finding that all but one of the PAs identified in sect. *Jacobaea* are also present in species of other sections of *Senecio* indicate that differences in PA profiles in *Senecio* can not be explained by the gain and loss of PA specific genes, but rather by a transient switch-off and switch-on of the expression of genes encoding PA pathway-specific enzymes.

Keywords: Section *Jacobaea*; *Senecio*; Character optimization; Pyrrolizidine alkaloid; Biosynthesis; Chemical diversity; Evolution; Chemical defense

1. Introduction

Pyrrolizidine alkaloids (PAs) are secondary metabolites which are only found in the angiosperms. They occur scattered and often sporadically in distantly related families. PAs are frequently found in some genera of the tribes Senecioneae and Eupatorieae of the family

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Asteraceae, many genera of the Boraginaceae, the genus *Crotalaria* (Fabaceae), several orchid genera (e.g., *Phalaenopsis*), and few genera of the Apocynaceae (e.g., *Parsonsia*. In addition, isolated PA occurrences are known from single species of the Celastraceae, Convolvulaceae, Ranunculaceae, Santalaceae, and Sapotaceae. Five structural PA-types representing monoesters and open-chain or macrocyclic diesters with more than 400 compounds are known (Hartmann and Witte, 1995; Rizk, 1991). PAs of the macrocyclic senecionine type comprise a diverse class of more than 100 structures, which are characteristic for PA containing species of the tribe Senecioneae, which are primarily species that are usually assigned to the genus *Senecio*.

PA biosynthesis has been extensively studied in *Senecio* (Hartmann and Ober, 2000). In all species studied so far, senecionine *N*-oxide was identified as the primary product of biosynthesis (Hartmann and Toppel, 1987; Toppel et al., 1987) and can be regarded as the backbone structure of most PAs. It is synthesized in the roots (Sander and Hartmann, 1989) and translocated into the shoots via the phloem-path (Hartmann et al., 1989) where it is transformed into the species-specific PA profiles (Hartmann and Dierich, 1998). Although PAs are spatially mobile within the plant, they are stored in vacuoles (Ehmke et al., 1988) and typically accumulate in the inflorescences and the peripheral stem tissues, i.e. epidermal and sub-epidermal cell layers (Hartmann and Zimmer, 1986; Hartmann et al., 1989). Tracer studies with ^{14}C-labeled senecionine *N*-oxide applied to a number of *Senecio* species (i. e., *S. erucifolius*, *S. inaequidens*, *S. jacobaea*, *S. vernalis*, and *S. vulgaris*) revealed that the structural diversification of senecionine *N*-oxide requires just one or very few enzymatic modifications (Hartmann and Dierich, 1998). Besides structural diversification, PAs do not underlie any turnover or degradation (Sander and Hartmann, 1989; Hartmann and Dierich, 1998).

PAs are known to be deterrent and toxic to most vertebrates and insects (Boppré, 1986; Schneider, 1987; Macel, 2003). Their toxicity bases primarily on the bioactivation of the molecules by means of cytochrome P450 enzymes creating pyrrolic intermediates that are highly reactive with biological nucleophiles, causing cytotoxicity, mutagenicity, and genotoxicity (Frei et al., 1992; Fu et al., 2004). PAs therefore probably evolved as plant defensive chemicals under the selection pressure of herbivory. The efficiency of PAs as defense compounds is highlighted by the fact that various adapted insects sequester PAs from their food-plants and utilize them for their own protection against predators and parasitoids. Among those adapted insects are leaf beetles, moths, butterflies, grasshoppers, and aphids (for a review see Hartmann, 1999, 2004; Hartmann and Ober, 2000).

In order to learn more about the evolutionary diversification of PAs in *Senecio*, we studied qualitative PA variation in *Senecio* section *Jacobaea* (Mill.) Dumort. This section has a mainly Eurasian distribution and contains 26 species, of which 24 were included in our studies. The biosynthetic relationships of all PAs identified in the species of sect. *Jacobaea* were deduced and related to the phylogeny of the section, which has recently been elucidated with phylogenetic analyses of nuclear and plastid DNA sequences, Amplified Fragment Length Polymorphism markers (AFLPs), and morphological characters (Pelser et al., 2002, 2003, 2004). This approach allowed us to quantify variation in PA composition within a group of closely related species and to infer the evolutionary origin of the variation found.

2. Results and discussion

2.1. PA diversity in Senecio sect. Jacobaea

The results of GC-MS analyses of samples of flowerheads of 24 *Senecio* species of sect. *Jacobaea* are summarized in Table 1. Eight of these species have not been studied before. A total of 24 PAs were detected and identified by their RIs, molecular masses, and mass fragmentation patterns in comparison to defined reference compounds or reference data from our PA database (Witte et al., 1993). The identified PAs cover exclusively macrocyclic alkaloids of the senecionine type, containing as necine base either retronecine or otonecine (Fig. 1(a)). The respective macrocyclic esters of the two necine bases give senecionine (**2**) and its analog senkirkine (**8**) and their various derivatives. According to their structures, six groups of macrocyclic ester PAs can be distinguished: (i) senecivernine (Fig. 1(b)), (ii) simple senecionine derivatives (Fig. 1(c)), (iii) simple senkirkine derivatives (Fig. 1(d)), (iv) jacobine-related epoxides and their derivatives (Fig. 1(e1)), (v) erucifoline-related epoxides (Fig. 1(e2)), and (vi) otosenine related epoxides and derivatives (Fig. 1(f)).

With one exception all identified PAs are also known to occur in species of other sections of *Senecio* (Hartmann and Witte, 1995). Only senecicannabine (**20**) appears to be unique for two species of sect. *Jacobaea* (i.e., *S. ambiguus* and *S. cannabifolius*). An evaluation of reference data from previous reports on the occurrence of PAs in species of sect. *Jacobaea* revealed the presence of additional PAs in some species that were not identified in the present study. In Table 1 these structures are indicated by letters and related to the respective references. Two PAs reported in the literature for species of sect. *Jacobaea* could not be identified in our study. These are retrorsine (Fig. 1(c), **A1**) (the 20-*Z*-isomer of usaramine (**16**), reported from *S. jacobaea*), and onetine (Fig. 1(f), **A2**) (the otonecine analog of jacoline (**9**), reported from *S. othonnae*) (Hartmann and Witte, 1995). Both PAs fit well into the PA profiles of

Table 1
Profiles (relative abundance)[a] and concentrations of PAs in the flower heads of 24 species of sect. *Jacobaea*

No	Pyrrolizidine alkaloids	*Incani* s.l.-group											*S. paludosus*-group				*S. jacobaea* s.l.-group										
		RI	M^+	abr	ado	boi[b,c]	car	hal[c]	inc	leu[c]	min[c]	per	can	del[c]	oth	pal	alp	amb[c]	aqu	chr	cin	eru	gig[c]	gna[c]	jac	pan	sub
1	Senecivernine	2286	335	1													1				0/1			0/1	1/0		1/0
2	Senecionine	2296	335	0/1		1	2	2	2/3	3	2/1	1/2	0/1	0/2	2/0	0/2	2	1/0	0/3	2/3	0/1		2/0	0/1	1/2	3/2	2/3
3	Seneciphylline	2312	333	0/1	1/0	3	4/5	5	5/4	4/3	3/2	5	0/4	2	5/0	6/5	5	0/1	0/4	2/4	1/2	1/0	3/6	1/4	1/0	4	4
4	Spartioidine	2347	333					1/0	1	2		1/0			1/0	0/1	1/0			0/1			1/0		1/0		
5	Integerrimine	2355	335					1/0	0/1	2				0/1	1/0	0/1	2	1/0	0/2		0/1	1/0	1/0	2/1	1/0	2	1/0
6	Jacobine	2438	351						0/1				3/0			a	a, b	0/2	b		0/2			2/1	2/0		a
7	Jacozine	2464	349										2				a, b	0/1			1/0			1/2	1/0		
8	Senkirkine	2477	365				1/0	1/0		1	0/1			4/3	1/0		a, b			0/2				0/2			
9	Jacoline	2489	369						0/2		0/1							0/1			1/2			1/0	1/0		
10	Dehydrosenkirkine	2516	363				2	1/0		1		1/0		2/3													
11	Erucifoline	2523	349			4					0/2	2/0						0/1	5/2	4/0		6			3/5		
12	Jaconine	2531	387						0/2				3/2				a, b	0/2			2/3			2	3/0		
13	Adonifoline	2549	365	2/0	3/6																						
14	Neosenkirkine	2549	365											2/1													
15	Dehydrojaconine	2568	385										0/2											1/0			
16	Usaramine	2588	351																		1/2			2/1	2/0		
17	Otosenine	2613	381	3/2	2/0										0/2			4/3	b		2			2/1	c		
18	Eruciflorine	2620	351																			d		2/0	1/0		
19	Acetylerucifoline	2621	391																2/0								
20	Sennecicannabine	2635	365										3/2					2/0									
21	Deacetyldoronine	2686	417	1/3	2/0										0/4			0/3			2			2/0			
22	Florosenine	2758	423	3/2	3/0													3/0	b		3/2						
23	Floridanine	2771	441	1/2	1/0										0/2			1			1/0						
24	Doronine	2795	459	1/2	1/0										0/2			1	b		3/2						
A1	Retrorsine																								e		
A2	Onetine														e												
Total PAs (mg/g)																											
Sample I				0.9	2.4	0.1	0.5	3.0	1.4	4.3	0.1	1.7	<0.1	0.3	0.7	<0.1	2.8	0.6	0.6	0.6	4.9	0.1	2.5	5.5	1.3	2.4	3.9
Sample II				0.8	0.1		0.7	2.3	0.4	7.6	0.2	1.2	0.3	0.5	1,3	0.3	7.9	0.9	3.9	1.6	0.8	<0.1	0.1	5.0	0.4	4.1	9.5

Abbreviation of the *Senecio* species: abr = *S. abrotanifolius*, ado = *S. adonidifolius*, boi = *S. boissieri*, car = *S. carniolicus*, hal = *S. halleri*, inc = *S. incanus*, leu = *S. leucophyllus*, min = *S. minutus*, per = *S. persoonii*, can = *S. cannabifolius*, del = *S. delphinifolius*, oth = *S. othonnae*, pal = *S. paludosus*, alp = *S. alpinus*, amb = *S. ambiguus*, aqu = *S. aquaticus*, chr = *S. chrysanthemoides*, cin = *S. cineraria*, eru = *S. erucifolius*, gig = *giganteus*, gna = *S. gnaphalodes*, jac = *S. jacobaea*, pan = *S. pancicii*, sub = *S. subalpinus.*

Occurrence of PAs reported previously but not confirmed in our study: a = see review Hartmann and Witte (1995); b = Hartmann and Lüscher (unpublished); c = Hagen and Hartmann (unpublished); d = Witte et al. (1992a); e = see Hartmann and Witte (1995), in our analysis not detected in any of the species of section *Jacobaea*.

The PA profiles were established by GC-MS.

[a] Figures are defined as follows: 0 = not detectable; 1 = trace amounts to 5%; 2 = > 5%; 3 = > 25%; 4 = > 50%; 5 = > 75%; 6 = 100%. Two samples (I and II), often combining material from more than one specimen, were independently analyzed; data separated by a backslash if different.

[b] Only one sample available.

[c] Species analyzed for the first time.

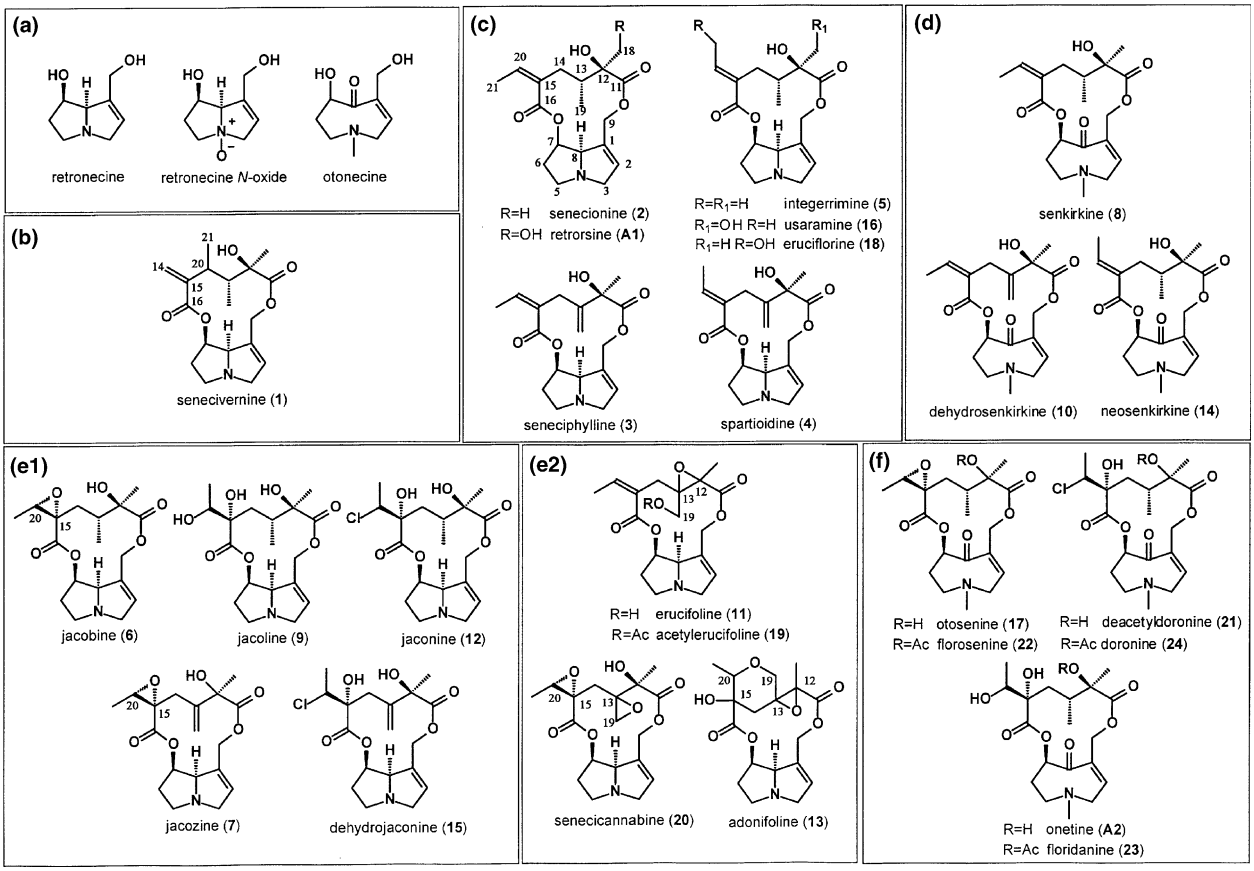

Fig. 1. Structures of the 24 pyrrolizidine alkaloids (PAs) found in sect. *Jacobaea*. (a) = Retronecine and otonecine the two necine bases; only retronecine derivatives form *N*-oxides; (b) = senecivernine, the only PA that cannot be formed from senecionine; (c) = simple senecionine derivatives; (d) = simple senkirkine derivatives; (e1) = jacobine-related epoxides and derivatives; (e2) = erucifoline related expoxides and derivatives; (f) = otosenine-related epoxides and derivatives.

the respective species (Table 1). They were, therefore, included in the phylogenetic studies. There are, however, two significant inconsistencies between our findings and previous reports on PA composition in sect. *Jacobaea* : we did not detect doronenine and bulgarsenine previously isolated from *S. abrotanifolius* (Röder et al., 1984). These two 13-membered macrocyclic PAs are structurally very different from the type of PAs found in sect. *Jacobaea*; they are characteristic for *S. doronicum* and *S. nemorensis* (frequently assigned to the distantly related *Senecio* sect. *Crociseris* Rchb.). The reason for this discrepancy remains unclear and these two PAs were therefore omitted from our phylogenetic analyses.

Generally, the PA profiles established in our study are composed of a higher number of individual PAs per species than those reported previously. This is mainly due to the higher resolution and sensitivity of today's analytical methods. The consistency between available reference data and the diverse PA profiles established in our analyses indicates a stable preservation of the PAs in samples taken from herbarium specimens. Moreover, the analyses revealed no evidence for possible artificial degradation or decay of PAs, e.g. free necine bases or partially hydrolyzed or oxidized derivatives. Most PA profiles are characterized by a few major compounds accompanied by minor constituents. Often, some of these minor compounds account for trace amounts that were close to the lower detection limit of our sensitive analytical methods. Most conspicuous is the large intraspecific qualitative variation often observed between the PA profiles of different populations. In several cases PAs identified from sample I were not found in sample II of the same species and vice versa. Great intraspecific variation in PA profiles is well known from various *Senecio* species. Examples are the highly variable PA profiles of different chemotypes reported for *S. jacobaea* and *S. erucifolius* (Witte et al., 1992a,b; Macel et al., 2004) as well as the variable PA profiles established for populations of *S. vulgaris* and *S. vernalis* (sect. *Senecio*) (von Borstel et al., 1989). From a quantitative point of view, the amount of total PAs established for the individual species, although low in some cases (ranging from 0.01% to 0.8%), was substantial enough to establish the individual profiles. In spite of their great variability, the data provide a representative and reliable

basis for comparative biosynthetic and evolutionary considerations.

2.2. Biogenetic interpretation of the PA profiles found in section Jacobaea

The 26 different PAs (i.e. 24 identified in this study and two additional reported in the literature) found to occur in the 24 species of sect. *Jacobaea* are biosynthetically closely related. With the exception of senecivernine (**1**) all PAs are ultimately derived from senecionine (**2**). Senecivernine (**1**) (Fig. 1(b)) is formed as a dead-end-product in parallel to senecionine (**2**) (Fig. 1(c)). Its necic acid moiety contains, in comparison to that of senecionine (**2**), one of the two isoleucine-derived C_5-units (Stirling et al., 1997) in a different orientation (Fig. 1(b and (c) numbered carbons). A transformation of senecivernine (**1**) into senecionine (**2**) or one of its derivatives can be excluded.

Although none of the biosynthetic reactions in the PA pathway has yet been characterized on the enzymatic level, previous feeding experiments with radioactively labeled senecionine (**2**) confirmed the role of this PA as precursor of most of the structures in consideration (Toppel et al., 1987; Kelly et al., 1989; Hartmann and Dierich, 1998). Senecionine (**2**) was proved to be incorporated into: (i) simple retronecine esters as seneciphylline (**3**), usaramine (**16**), retrorsine (**A1**), and eruciflorine (**18**); (ii) simple otonecine derivatives as senkirkine (**8**); (iii) epoxides of retronecine esters as jacobine (**6**) including jaconine (**12**) its product of chlorolysis, jacozine (**7**), erucifoline (**11**), and acetylerucifoline (**19**); and (iv) epoxides of otonecine esters as otosenine (**17**) and florosenine (**22**). Based on these results and due to the fact that the exact sequences of the transformations remain ambiguous, two hypothetical biogenetic scenarios are illustrated in Fig. 2. The first scenario (Fig. 2(a)) assumes that senecionine (**2**) is specifically converted into its otonecine derivative senkirkine (**8**) which functions as precursor of all other otonecine derivatives, whereas senecionine (**2**) is the specific precursor of all retronecine derivatives. The second scenario (Fig. 2(b)) assumes that the various otonecine derivatives originate independently from each other from their respective retronecine analogs.

In both scenarios, there are two basic reactions involved: the conversion of necine esters into otonecine esters (Fig. 2, reactions 1) and the formation of epoxides (Fig. 2, reactions 2). With the exception of adonifoline (**13**) the formation of all other structures can be deduced by action of six simple single-step reactions: (*Z*/*E*)-isomerization (Fig. 2, reaction a), 13,19-dehydrogenation (reaction b), site specific hydroxylation (reaction c), hydrolysis of 15,20-epoxides (reaction d), chlorolysis of 15,20-epoxides (reaction e), and site-specific *O*-acetylation (reaction f). With the exception of the hydrolytic epoxide degradations (reaction d) all types of single-step transformations have been demonstrated in tracer feeding experiments (Hartmann and Dierich, 1998). The only metabolite that represents a more complex structure is adonifoline (**13**). Together with senecicannabine (**20**), which so far has not been detected in *Senecio* species outside sect. *Jacobaea*, adonifoline is the only rare PA, assumed to occur also in *S. dolichodoryius* (Witte et al., 1992b), a species currently included in the genus *Dendrophorbium* (Senecioneae). All the other 24 PAs are more or less commonly found in *Senecio* species outside sect. *Jacobaea* and can easily be deduced biosynthetically.

2.3. Evolutionary interpretation of the PA profiles found in sect. Jacobaea

We studied the evolutionary history of PA formation by examining PA data of 24 species of sect. *Jacobaea* in the context of the phylogeny of this section. Because we were mainly interested in the evolutionary history of the gain and loss of individual PAs in the PA biosynthetic pathway rather than the phylogenetic component in PA concentration, we studied the qualitative aspects of PA composition (presence/absence of PAs), but did not apply our phylogenetic studies to quantitative PA data.

Optimizations of individual PAs under both biosynthetic scenarios (Fig. 3) show a very large amount of homoplasy, meaning that 'on/off' changes in the production of individual PAs in the evolutionary history of *Senecio* sect. *Jacobaea* have been frequent. In fact, many branches in the cladogram show multiple 'losses'' or 'gains'' of PAs without noticeable evolutionary direction. This is also illustrated by the fact that for most of the more common PAs, all three main clades of sect. *Jacobaea* (the *Incani* s.l.-group, the *S. paludosus*-group, and the *S. jacobaea* s.l.-group (Table 1 and Fig. 3) contain both species in which these PAs are present, as well as species that do not produce these PAs. Acetylerucifoline (**19**), neosenkirkine (**14**), and retrorsine (**A1**) have only been identified in single species (*S. aquaticus*, *S. delphinifolius*, and *S. jacobaea*, respectively) and are therefore, by definition, free of homoplasy. Adonifoline (**13**) is the only PA identified in our studies that is present in more than one species and does not show homoplasy: the potential to produce this PA has only evolved in the common ancestor of *S. abrotanifolius* and *S. adonidifolius* and the formation of adonifoline (**13**) is continued in both species. Senecionine (**2**) and seneciphylline (**3**) are produced by all species in the section. The occurrence of all but one of the PAs found in sect. *Jacobaea* in relatively distantly related Senecioneae species outside of this section shows that the homoplasy in PA formation extends beyond sect. *Jacobaea* and may, therefore, be a general characteristic of PA evolution in Senecioneae.

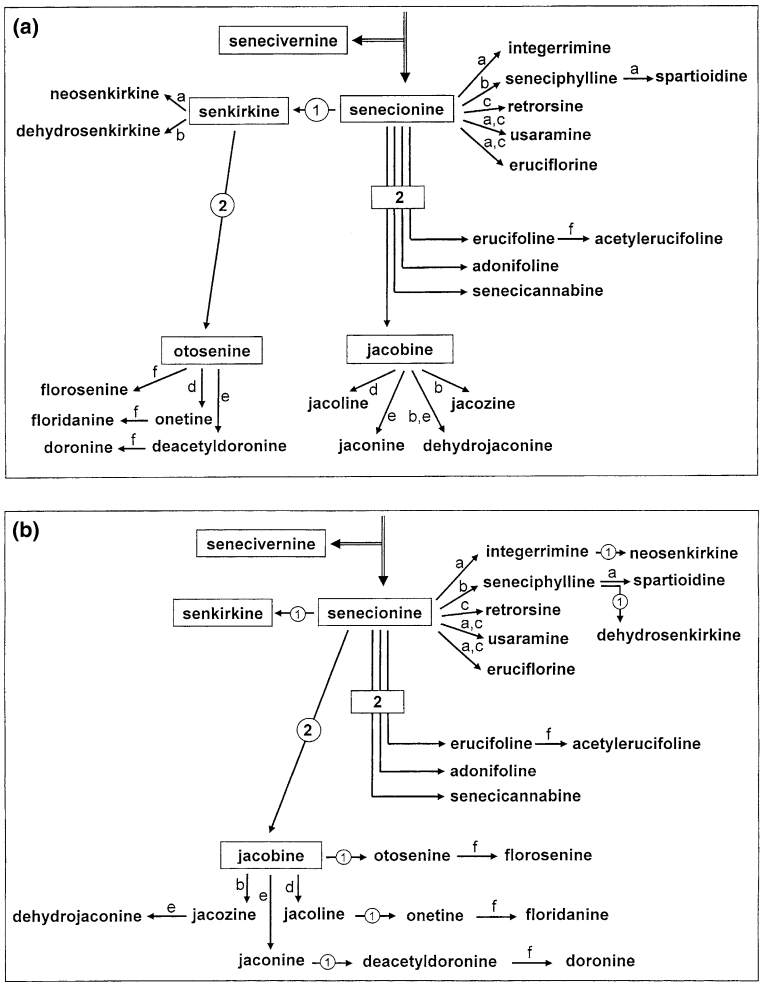

Fig. 2. Biosynthetic sequences deduced from previous tracer studies (Hartmann and Dierich, 1998). With the exception of senecivernine, senecionine is the common precursor of all other PAs. Since the substrate specificity of the enzymes involved is not known, two scenarios are illustrated: (a) = senkirkine is assumed as common precursor of all otonecine derivatives; (b) = the otonecine derivatives originate independently from the respective retronecine derivatives. Two main reactions exist: conversion of retronecine to otonecine (reaction 1) and site-specific epoxide formation (reaction 2). Further structural diversification requires six simple one-step-reactions marked by letters a–f: a = *Z*/*E*-isomerization at C20; b = 13,19-dehydrogenation; c = site-specific hydroxylations; d = hydrolysis of 15,20-epoxide; e = chlorolysis of 15,20-epoxide; f = site-specific *O*-acetylations.

Character optimization of the presence/absence of two basic enzymatic reactions on the phylogeny of sect. *Jacobaea* (Fig. 4(a), the conversion of necine esters into otonecine esters, i.e. reactions 1 in Fig. 2) and the formation of epoxides (Fig. 4(b), reactions 2 in Fig. 2) indicates that not only PAs, but also enzymatic conversions are highly homoplasious. All three main clades of sect. *Jacobaea* contain both species that exhibit these enzymatic conversions and those that do not perform these reactions. The potential to perform the two basic reactions was inferred to be already present in the ancestral species of sect. *Jacobaea*. In most species this potential has been preserved (eight out of 24 for reaction 1; six out of 24 for reaction 2). *S. giganteus* and *S. pancicii* are the only two species in which both basic enzymatic conversions are no longer carried out. The six single-step transformations also showed high levels of homoplasy, except for 13,19-dehydrogenation (Fig. 2, reaction b; results not shown).

It is hard to assume that all the losses and gains of PAs and enzymatic reactions may be interpreted in classical genetic terms as 'birth' or 'death birth' of genes. Based on the genetic control of the intraspecific phenotypic variation of the PA profiles one would assume a transient switch-off and switch-on of the expression of single enzymes or their encoding genes. Since in many species certain PAs are only present in trace amounts and thus almost absent, 'switch-off' may be interpreted as an extreme down-regulation of the respective gene activity. A down-regulated gene is still present and, as a future event, may be up-regulated again. The regulatory mechanisms involved are unknown, transcriptional control is probable but surely not the only possibility. This hypothesis would explain the erratic 'losses' and 'gains' of PAs and enzymatic reactions in the species

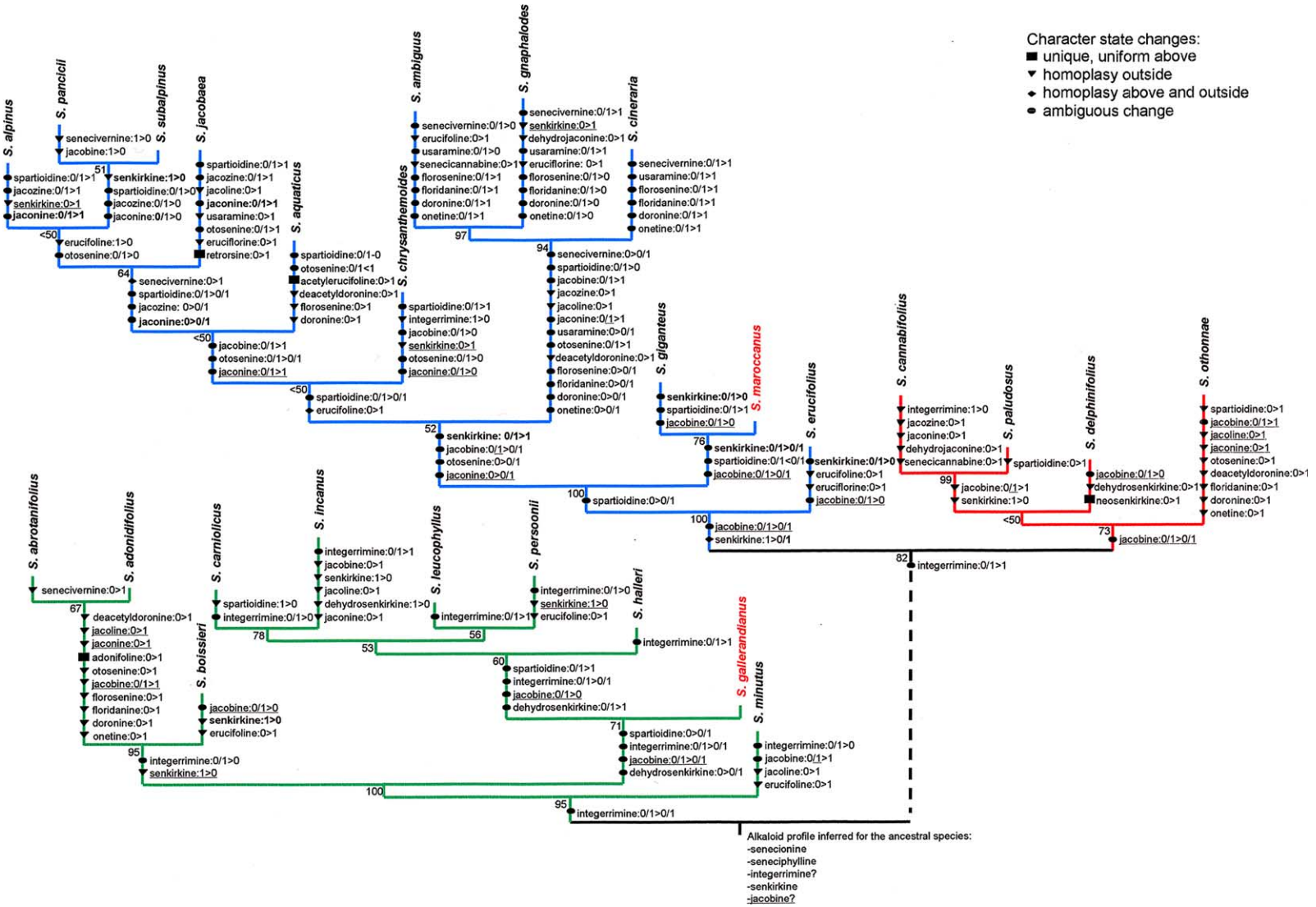

Fig. 3. Character optimization of additive and binary coded presence/absence data of the 26 PAs found in sect. *Jacobaea* on the DNA sequence/morphological strict consensus cladogram adopted from Pelser et al. (2004). All most parsimonious reconstructions under ACCTRAN and DELTRAN settings are shown for each node. The first character state indicates the plesiomorphic (ancestral) state and the second character state presents the apomorphic (derived) state. Equivocal character state changes are indicated with a slash. Character state changes present under biosynthetic scenario A, but absent under B (Fig. 2) are indicated in bold. Character state changes present under B, but absent under A are underlined. PA data were missing for *S. gallerandianus* and *S. maroccanus*. Numbers aside internal nodes are bootstrap support values obtained from Pelser et al. (2004). The three main clades of sect. *Jacobaea* are indicated with different colors: the *Incani* s.l.-group (green), the *S. paludosus*-group (red), and the *S. jacobaea* s.l.-group (blue).

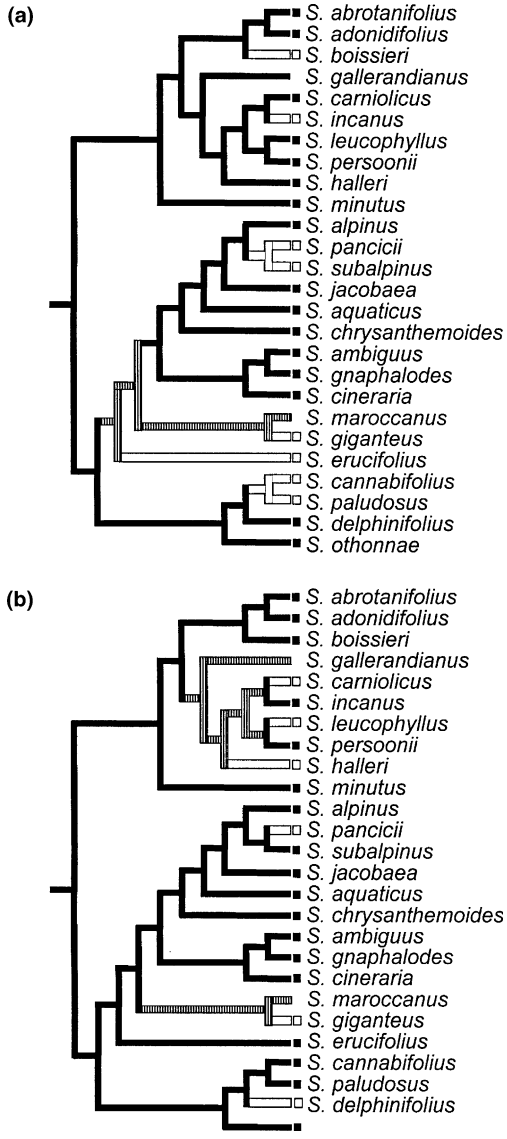

Fig. 4. Character optimization of additive and binary coded presence/absence data of two main enzymatic conversions in the PA biosynthetic pathways on the DNA sequence/morphological strict consensus cladogram of sect. *Jacobaea* adopted from Pelser et al. (2004). Biosynthetic scenarios A and B (Fig. 2) resulted in identical optimizations. (a) = conversion of retronecine to otonecine (reaction 1; Fig. 2); (b) = site-specific epoxide formation (reaction 2; Fig. 2). Branches on which enzymatic conversions were inferred to be present are black, branches on which these conversions were inferred to be absent are white. On some branches ACCTRAN and DELTRAN optimizations give different results. This uncertainty with respect to the presence or absence of an enzymatic conversion is indicated with a hatched pattern. PA data were missing for *S. gallerandianus* and *S. maroccanus*.

of sect. *Jacobaea* creating the divers and variable species-specific PA profiles.

The differences between optimizations under the two hypothetical biosynthetic scenarios are only shown in the optimization of integerrimine (**5**), jacobine (**6**), jacoline (**9**), jaconine (**12**), and senkirkine (**8**) (Fig. 3). Our phylogenetic approach to the evolutionary history of PA formation indicates that scenario A (Fig. 2(a)) fits our phylogenetic reconstruction of the relationships between the species of sect. *Jacobaea* better than scenario B (Fig. 2(b)). Scenario A requires the assumption of 94 'on/off' changes and is therefore more parsimonious than scenario B, which needs 102 'on/off' changes to be optimized on the cladogram. In addition, accepting scenario A as the 'true' representation of the biosynthetic pathways required us to assume that we were unsuccessful in detecting PAs with GC-MS in 15 cases (undetected precursors of PAs that have been detected, and therefore must have been produced by the specimens examined). Whereas 18 undetected PAs have to be assumed for scenario B. These results mean that our GC-MS data provides slightly more support for scenario A than for scenario B.

3. Conclusions

All analyzed species of sect *Jacobaea* contain with great intraspecific qualitative and quantitative variation a varying number of PAs ranging from three to 14 different compounds. A substantial proportion of the observed phenotypic variation in PAs appears to be genetically controlled in *Senecio jacobaea* (Vrieling et al., 1993). In addition to the large inter- and intraspecific differences in PA profiles, we found that PA distribution appears to be largely incidentally within the whole clade. Even closely related species can have widely different PA compositions (e.g., *S. ambiguus* and *S. gnaphalodes*). *Senecio* populations randomly endowed with largely varying PA patterns should be well adapted to hold off non-adapted generalists. A complex alkaloid profile that shows high structural variation between and within species and changes rapidly throughout the course of evolution should reduce the chance of generalist herbivores to overcome the defense barrier since the herbivore has to cope with quite different structures (e.g., retronecine esters, otonecine esters, epoxides etc.). This structural diversification of the PAs apparently does not affect their intrinsic toxicity: all 24 PAs share the common structural features needed to be converted into toxins by cellular cytochrome P450 enzymes. These features are: (i) 1,2-double bond, (ii) an esterified allylic hydroxyl group at C-9, and (iii) a second esterified alcoholic hydroxyl group at C-7 (Fu et al., 2004; Cheeke, 1998; Frei et al., 1992). First studies revealed that herbivory by a few selected generalist herbivores is not influenced by qualitative differences of the PA profile but more by total PA concentrations (Macel, 2003). However, Macel (2003) presented some evidence that the activity of individual PAs against some generalist herbivores are slightly different and that there may be synergistic effects.

Since, without exception, all species of sect. *Jacobaea* retained the ability to synthesize and accumulate PAs, these must be essential for the survival of these species

in their environment. Apparently the species of sect. *Jacobaea* are able to randomly shuffle patterns of senecionine-derived derivatives from the 'genetic pool' of the clade. The potential selective forces causing the great diversity in PA composition within and between species remain unclear.

4. Experimental

4.1. Plant materials

Sources of plant material are: BM, C, E, K, L, RNG, WAG, and WU (herbarium acronyms follow Holmgren et al., 1990). Samples for analysis accounted for approximately 0.3–0.5 g dry weight of flower-heads. If less material was available samples from two or more individuals (number given in parentheses) were pooled prior to analysis. Country names indicate the geographic origin of the samples. For all species except *S. boissieri* two samples were analyzed independently marked I and II. The following taxa were studied: *S. abrotanifolius* L. I: Slovenia (2); II: Switzerland and Austria (2). *S. adonidifolius* Loisel. I: France (1); II: Spain (1). *S. alpinus* (L.) Scop. I: Switzerland (1); II: Austria (1). *S. ambiguus* DC. I: Italy (2); II: Italy (1). *S. aquaticus* Hill I: The Netherlands (1); II: Austria (2). *S. boissieri* DC. Spain (4). *S. cannabifolius* Less. I: Russia (1); II: unknown (1). *S. carniolicus* Willd. I: Italy and Austria (3); II: Austria (1). *S. chrysanthemoides* DC. I: Cultivated (1); II: Nepal Pakistan (3). *S. cineraria* DC. I: Italy (1); II: France (1). *S. delphinifolius* Vahl I: Tunisia (1); II: Tunisia (1). *S. erucifolius* L. I: France (1); II: The Netherlands (1). *S. giganteus* Desf. I: Marocco (1); II: Marocco (1). *S. gnaphalodes* Sieber I: Greece (1); II: Greece (1). *S. halleri* Dandy I: Italy and Switzerland (4); II: Italy (1). *S. incanus* L. I: France, Italy and Switzerland (3); II: Switzerland (3). *S. jacobaea* L. I: unknown (1); II: unknown (1). *S. leucophyllus* DC. I: France (1); II: France (2). *S. minutus* DC. I: Spain (1); II: Spain (5). *S. othonnae* M. Bieb. I: Turkey (1); II: Iran (1). *S. paludosus* L. I: The Netherlands (1); II: The Netherlands (1). *S. pancicii* Degen I: Bulgaria (1); II: Bulgaria (1). *S. persoonii* De Not. I: Italy (1); II: Italy (1). *S. subalpinus Koch* I: unknown (1); II: Slovakia (2).

4.2. Extraction and analysis of pyrrolizidine alkaloids

Dried flower heads were ground and 0.3–0.5 g were extracted in 5 ml of 1 M HCl for 2–3 h and centrifuged; the pellet was dissolved in 1 ml of 1 M HCl and again extracted. The combined supernatants were extracted with 2 ml dichloromethane, the aqueous phase was saved and mixed with an excess of Zn dust and stirred for 3 h at room temperature for complete reduction of PA *N*-oxides. Subsequently the solution was made basic with 25% ammonia and applied to an Extrelut (Merck) column-3 (1.4 ml solution/g Extrelut). The PAs were eluted with dichloromethane (6 ml/g Extrelut). The solvent was evaporated and the residue dissolved in 10–100 μl methanol prior to GC or GC-MS.

The GC-MS data were obtained with a Hewlett Packard 5890A gas chromatograph equipped with a 2 m fused silica guard column (deactivated, ID 0.32 mm) and a 30 m × 0.32 mm analytical column (ZB1 and ZB5, Phenomenex). The capillary column was directly coupled to a triple quadrupole mass spectrometer (TSQ 700, Finnigan). Injector and transfer line were set at 280 °C. The temperature program used was 100 °C (3 min)–300 °C at 6 °C min^{-1} for PA separation. The injection volume was 1 μl. Depending on the concentration, the split ratio was 1:20 or splitless, the carrier gas flow was 1.6 ml min^{-1} He, and the mass spectra were recorded at 70 eV. The retention index (RI) was calculated by a set of hydrocarbons (even numbered C_{12}–C_{28}) by linear interpolation.

Routine GC was performed using a capillary column (15 m × 0.25 mm fused-silica; DB-1, J & W Scientific) (Witte et al., 1993). All other GC conditions were the same as given for GC-MS; detectors: FID and PND. Quantitative analyses were performed via the FID signals using heliotrine as internal standard.

4.3. Phylogenetic analyses

PA profiles for each of the 24 species of sect. *Jacobaea* included in our studies (Table 1) were converted into a data matrix in which PAs were binary coded for their presence (1) or absence (0) for each species. In cases where only one of the two samples of a species was found to contain a given PA, we coded the occurrence of this PA as present. We chose to do this, because such findings show that the potential to produce this PA is present in the species, although it is not produced or identified in all of its specimens. After this matrix was compiled, we used the two hypothetical biosynthetic scenarios (Fig. 2(a) and (b)) to refine our data matrix. For both scenarios a single data matrix was constructed using additive binary coding (Farris et al., 1970): we coded intermediate PAs in a pathway as present in cases when derivatives of these PAs were identified, but the intermediates themselves were not retrieved.

The two data sets were subsequently individually optimized on a maximum parsimony cladogram representing the evolutionary relationships between all 26 species of sect. *Jacobaea* (Fig. 3). This so-called 'character optimization' (also frequently called 'mapping') is a technique that uses information about the PA profiles of extant species of sect. *Jacobaea* (terminal nodes in the cladogram) to reconstruct the profiles of ancestral species of the section (internal nodes) under the optimality criterion of parsimony. The cladogram used

for PA optimization was adopted from Pelser et al. (2004) and is the result of a parsimony analysis of a data set composed of both morphological data and DNA sequence data (the *trnT-L* intergenic spacer (IGS), the *trnL* intron, two regions of the *trnK* intron that flank *matK*, the *psbA-trnH* IGS, and ITS1-5.8S-ITS2). Unfortunately, not all relationships between the species of sect. *Jacobaea* are well supported by the morphological and molecular characters used. This is indicated by low bootstrap values (<70%; Fig. 3; Felsenstein, 1985). This especially concerns the clade (= a group of species that includes their most recent common ancestor and all its descendants) that consists of *S. alpinus*, *S. aquaticus*, *S. chrysanthemoides*, *S. jacobaea*, *S. pancicii*, and *S. subalpinus*, the relationships between these species and the *S. ambiguus–S. cinararia–S. gnaphalodes*–clade, and several relationships within the *S. carniolicus–S. halleri–S. incanus–S. leucophyllus–S. persoonii*-clade. Because the cladogram of sect. *Jacobaea* is used as a 'roadmap' to trace the evolution of PAs in the section, these uncertainties in the patterns of evolutionary relationships should be taken into account when drawing conclusions about PA evolution. We did this by limiting our interpretation of the patterns of PA optimization to clades that have moderate (70–80%) to high (>80%) bootstrap support. To facilitate an easier interpretation of our results in this respect, we discuss our findings on basis of the three main clades of sect. *Jacobaea* as defined by Pelser et al. (2003, 2004): the *Incani* s.l.-group (marked in green in Fig. 3), the *S. paludosus*-group (red), and the *S. jacobaea* s.l.-group (blue).

Because not sufficient material for GC-MS studies could be obtained for *S. gallerandianus* and *S. maroccanus*, PA profiles of these species were not available. PAs in *S. gallerandianus* and *S. maroccanus* were therefore coded as missing data ('?') in the data matrices. PA characters were optimized on the cladogram using both the Accelerated Transformation (ACCTRAN) and Delayed Transformation (DELTRAN) optimization methods (Swofford and Maddison, 1987) in MacClade 4.05 OS X. In situations in which PAs can be optimized in multiple equally parsimonious ways, these different optimization methods give different results. Because there are no biological reasons to prefer one method to the other, we presented both optimizations in Fig. 3. These uncertainties are indicated in Fig. 3 as 'ambiguous changes'.

In addition to optimizing individual PAs on the cladogram, we also optimized the presence/absence of enzymatic reactions (Fig. 2 reactions 1, 2, and a–f) on the phylogeny of sect. *Jacobaea* (Fig. 4) using the same procedure as described in the previous paragraph. This allows us to examine the evolutionary history of different types of enzymatic reactions in the biosynthetic pathways.

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