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Frequency and distribution of cyanogenic glycosides in *Eucalyptus* L'Hérit

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ABSTRACT

In this study approximately 420 of the described species of *Eucalyptus* were examined for cyanogenesis. Our work has identified an additional 18 cyanogenic species, 12 from living tissues and a further six from herbarium samples. This brings the total of known cyanogenic species to 23, representing approximately 4% of the genus. The taxonomic distribution of the species within the genus is restricted to the subgenus *Symphyomyrtus*, with only two exceptions. Within *Symphyomyrtus*, the species are in three closely related sections. The cyanogenic glycoside was found to be predominantly prunasin (**1**) in the 11 species where this was examined. We conclude that cyanogenesis is plesiomorphic in *Symphyomyrtus* (i.e. a common basal trait) but has probably arisen independently in the other two subgenera, consistent with recent phylogenetic treatments of the genus. The results of this study have important implications for the selection of trees for plantations to support wildlife, and to preserve genetic diversity.

1. Introduction

Eucalyptus is a large genus, with over 500 species (Pryor and Johnson, 1971; Chippnedale, 1988), although recent revisions would put that figure higher at around 700–800 (Brooker, 2000). The genus is indigenous to Australia and some neighbouring islands, but is used extensively in plantations around the world. The replacement of indigenous forests can have a significant effect on the local fauna and the choice of species is vital in order to limit the negative effects (Hartley, 2002). A number of physical and chemical characteristics of *Eucalyptus* foliage make them less palatable to herbivores such as toughness, low leaf protein, phenolics, sideroxylonals and various monoterpenes (Moore et al., 2004; Loney et al., 2006). In addition, a number of species have been reported to be cyanogenic, capable of producing toxic cyanide when the leaf tissue is disrupted by chewing (Goodger et al., 2006). There have been numerous cases of livestock poisoning from eating *E. cladocalyx* F. Muell. (Everist, 1981) and there are reports of koalas dying after eating cyanogenic *E. viminalis* Labill. (Morris, 1944).

Cyanogenic glycosides are found in a wide range of taxa including ferns, palms, woody and herbaceous plants, as well as some bacteria and insects (Conn, 1980; Lechtenberg and Nahrstedt, 1999; Zagrobelny et al., 2004). It has been estimated that 11% of all plant species contain cyanogenic individuals (Jones, 1998), although this figure seems high in the view of recent extensive testing of non-commercial species (Miller et al., 2006). All of the six species of *Eucalyptus* from which cyanogenic compounds have so far been identified are members of the same subgenus, *Symphyomyrtus* (see Table 1). Moreover, the aromatic-derived glycoside (*R*)-prunasin (**1**) is the only cyanogen in five species, and the primary one in *E. camphora* subsp. *humeana* Johnson & Hill (Neilson et al., 2006; see Fig. 1). The existence of other cyanogenic species of *Eucalyptus* seemed likely, and therefore we initiated an extensive survey of living plants and herbarium specimens. Few of these species had been tested before. Our aim was to (1) determine the prevalence of cyanogenesis in *Eucalyptus* and its taxonomic associations; and (2) to ascertain whether prunasin (**1**) was the primary cyanogenic glycoside. In order to do this leaf samples were collected from extant individuals growing in arboretums in Australia and California and the cyanogenic glycosides identified. This was supplemented by extensive testing of herbarium specimens.

2. Results and discussion

2.1. Frequency of cyanogenesis in Eucalyptus

Approximately 2200 individual plants representing nearly 400 species were tested for the presence and absence of cyanogenic glycosides using the qualitative Feigl–Anger procedure (Brinker and Seigler, 1992). We identified 18 new cyanogenic species and one subspecies (see Tables 2 and 3). In addition we confirmed

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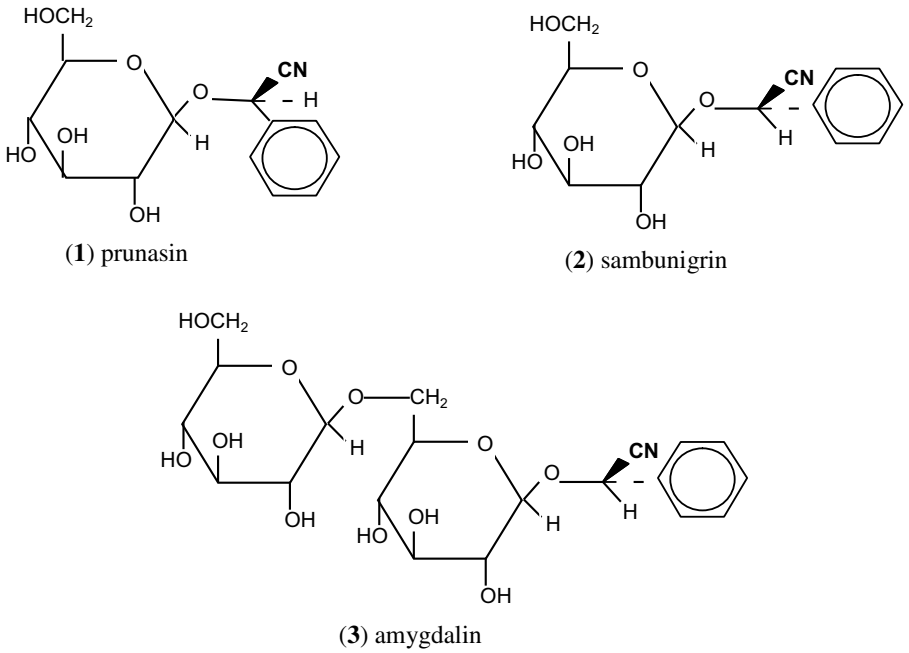

Fig. 1. Structures of three cyanogenic glycosides found in *Eucalyptus*. All are derived from phenylalanine. Prunasin (**1**) is ubiquitous, the epimer sambunigrin (**2**) is rare and the diglycoside amygdalin (**3**) has only been detected in one species to date.

Table 1
Species of *Eucalyptus* known to be cyanogenic. In all species prunasin (**1**) was identified as the primary cyanogenic agent

Species	Section	Series
E. camphora subsp. *humeana* Johnson & Hill[a]	*Maidenaria*	*Foveolatae*
E. cladocalyx F. Muell.[b]	*Sejunctae*	n/a
E. cladocalyx var. *nana*[c]		
E. nobilis Johnson & Hill[d]	*Maidenaria*	*Viminales*
E. polyanthemos Schauer subsp. *vestita* Johnson & Hill[e]	*Adnataria*	*Heterophloiae*
E. viminalis Labill.[f]	*Maidenaria*	*Viminales*
E. yarraensis Maiden & Cambage[g]	*Maidenaria*	*Foveolatae*

All were confirmed as cyanogenic in this study (see Table 2). Accession numbers can be found in Appendix I (supplementary data).
[a] Neilson et al., 2006.
[b] Finnemore and Cox, 1928; Gleadow et al., 1998.
[c] Burns et al., 2002.
[d] Gleadow et al., 2003.
[e] Goodger and Woodrow, 2002.
[f] Finnemore et al., 1935.
[g] Goodger et al., 2002.

the cyanogenic status of four of the six species known to be cyanogenic (Tables 1–3). This brings the total known number of cyanogenic *Eucalyptus* to 23 and represents approx 4% of the species tested. Jones (1998) estimated that approximately 11% of all plants were cyanogenic. This is likely to be an overestimate, as much of the testing until this century has been on crop plants, and two-thirds of crop plants appear to have at least some cyanogenic parts (Jones, 1998). A figure of 5% is perhaps more realistic in natural communities (Adsersen et al., 1988; Adsersen and Adsersen, 1993; Thomsen and Brimer, 1997; Miller et al., 2006) and is similar to the approximately 5% of *Acacia* species were found to be cyanogenic in an extensive survey by Conn and co-workers (Conn et al., 1989).

2.2. Identification of cyanogen

We identified (*R*)-prunasin (**1**) as the cyanogen in 12 of the 18 species found to be cyanogenic (Table 4). The NMR spectroscopic data presented here shows in most cases that the plants contained only prunasin (**1**), although sambunigrin (**2**) was detected in some (see Fig. 1). Traces of sambunigrin (**2**) have been found in *Eucalyptus* before, but this was attributed to racemisation during extraction (Conn, 1980; Gleadow et al., 2003). The presence of >10% sambunigrin (**2**) found in our study raised the question of whether mixtures of prunasin (**1**) and sambunigrin (**2**) occur naturally in *Eucalyptus*, as Maslin et al. (1988) found in some species of *Acacia*. In that paper it was concluded that non-enzymatic racemization could not account for the mixtures of the enantiomers observed. Recent work by Neilson et al. (2006) found that *E. camphora* contained several cyanogenic glycosides in addition to (*R*)-prunasin (**1**), including its epimer (sambunigrin (**2**)) and the diglycoside (amygdalin (**3**)). In two of the species for which we present data here (*E. leucoxylon* and *E. orgadophila*), the proportion of sambunigrin (**2**) differed depending on the analytical method (NMR and GLC). This was almost certainly the result of racemisation of one of the sample preparations (see Table 4).

2.3. Taxonomic considerations

Aromatic cyanogenic glycosides appear to have evolved earlier than the aliphatic forms (Bak et al., 2006). Moreover, the only occasions when aliphatic and aromatic cyanogenic glycosides occur in the same species is the result of alloploidy. Our finding that eucalypts appear to have only phenylalanine-derived cyanogenic glycosides is consistent with the position of *Eucalyptus* at the base of the Rosids clade within higher plants (Ladiges and Udovicic, 2000; Bak et al., 2006).

Extensive sampling meant that the survey of the genus was comprehensive. Tables 2 and 3 list the cyanogenic species together with the taxonomic groups to which they are assigned. The proportion of species tested in each taxonomic grouping was estimated, although exact numbers in each taxon are currently under revision (cf. Pryor and Johnson, 1971; Brooker, 2000; Ladiges and Udovicic, 2000; Bohte and Drinnan, 2005). Overall, we estimate that we sampled 60% of all species from 10 of the 13 currently recognised subgenera. Within the two largest subgenera (*Symphyomyrtus*,

Table 2
Species in *Eucalyptus* identified as cyanogenic in living plants in this study

Species	Section	Series	Accession #
E. acaciiformis Deane & Maiden	*Maidenaria*	*Acaciiformes*	PERTH 1259032
E. burdettiana Blakely & H. Steedman	*Bisectae*	*Lehmannianae*	AD19827
E. caleyi Maiden	*Adnataria*	*Rhodoxylon*	AD98560154
E. cladocalyx F.Muell.[a]	*Sejunctae*	n/a	UCD 53739
E. cylindriflora Maiden & Blakely	*Bisectae*	*Elongatae*	AD56509
E. leptophleba F. Muell.	*Adnataria*	*Aquilonares*	AD98562371
*E. leucoxylon*F. Muell.	*Adnataria*	*Melliodorae*	UCB 1422038
E. leucoxylon ssp. megalocarpa			WAIARB 1615
E. megacornuta C. Gardner	*Bisectae*	*Lehmannianae*	AD98562522
E. orgadophila Maiden & Blakely	*Adnataria*	*Buxeales*	AD98562732
E. ovata Labill.	*Maidenaria*	Foveolatae	ADW 56653
*E. polyanthemos*Schauer.[a]	*Adnataria*	*Heterophloiae*	UCB 302591
E. patellaris F. Muell.	*Adnataria*	Aquilonares	PERTH 1168150
E. steedmanii C. Gardner	*Bisectae*	*Erectae*	AD98563269
E. viminalis Labill.[a]	*Maidenaria*	*Viminales*	CBG 85007
E. yarraensis Maiden & Cambage[a]	*Maidenaria*	*Foveolatae*	CANB 424515

All belong to the subgenus *Symphyomyrtus*. In this study, cyanogenesis was measured using Feigl–Anger papers in the presence of β-glucosidase. Five of these species have already been identified as cyanogenic (see Table 1).
[a] Previously identified as cyanogenic (see Table 1).

Table 3
Cyanogenesis in herbarium specimens of *Eucalyptus*

Species	Subgenus	Section	Accession #
E. diversifolia Bonpl.	*Eucalyptus*	Longistylus	MELU 103697
E. eximia Schauer[a]	*Corymbia*	Septentrionales	UCB 602436
E. microtheca F. Muell.	*Symphyomyrtus*	Adnataria	UCB 489558
E. pilligaensis Maiden	*Symphyomyrtus*	Adnataria	UCB M093441
E. rudderi Maiden	*Symphyomyrtus*	Adnataria	UCB 1367687
E. tectifica *F. Muell.*	Symphyomyrtus	*Adnataria*	UCB 967765

Fresh material was tested from living plants of each species but all were negative. A list of all species that tested negative is included in the supplementary data.
[a] This species is also known as *Corymbia eximia* (Schauer) K.D. Hill & L.A.S. Johnson, subtribe *Angophorinae*, section *Ochraria*, series *Eximia* in the informal 'eucalypt' group (Ladiges and Udovicic, 2000).

Table 4
Identification and quantification of cyanogen from 11 species of *Eucalyptus*

Species	Major cyanogen	S:P GLC	S:P NMR
E.burdettiana	prunasin (**1**)	0:100	
E.caleyi	prunasin (**1**)	8:92	9:91
E.cylindriflora	prunasin (**1**)	2:98	2:98
E.cylindriflora	prunasin (**1**)	16:84[c]	15:85[c]
E.leptophleba	prunasin (**1**)	0:100	
E.leucoxylon	prunasin (**1**)	0:100	22:78[c]
E.leucoxylon[a]	prunasin (**1**)	0:100	0:100
E.leucoxylon	prunasin (**1**)	0:100	0:100
E.megacornuta	prunasin (**1**)	0:100	0:100
E.megacornuta	prunasin (**1**)/sambunigrin (**2**)[b]	47:53[c]	46:54[c]
E.orgadophila	prunasin (**1**)	0:100	0:100
E.orgadophila	prunasin (**1**)	19:81	
E.orgadophila	prunasin (**1**)	24:76[c]	7:93
E.ovata	prunasin (**1**)	0:100	
E.patellaris	prunasin (**1**)		0:100
E.steedmanii	prunasin (**1**)		0:100
E.yarraensis	prunasin (**1**)	0:100	0:100

The ratio of sambunigrin (**2**): prunasin (**1**) (S:P) was calculated by spectra from GLC and/or NMR.
[a] Forma rosea.
[b] An approximately equal mix of sambunigrin (**2**) and prunasin (**1**) (see Fig. 1).
[c] Racemisation of samples almost certainly occurred (EEC).

Eucalyptus), we examined ca. 75% and 60% of species, and a similar proportion from most sections with those genera. The two exceptions were the subgenera *Blakella* and *Corymbia* where we tested approximately 15% and 35% of species. All the species tested are listed in the Appendix (see supplementary data).

Until now all cyanogenic *Eucalyptus* were thought to belong to the subgenus *Symphyomyrtus*, suggesting cyanogenesis may have only arisen once within the genus. Here we report cyanogenesis in two other subgenera: *Eucalyptus*[1] (*E. diversifolia*) and *Corymiba* (*E. exemia* syn.*Corymbia eximia*) (Table 3). The data are consistent with cyanogenesis being a plesiomorphic (i.e. basal) trait in *Symphyomyrtus*, in that the 21 of the 23 cyanogenic eucalypts are in this one group. Moreover, nine of these species are in the same section of the subgenus (*Maidenaria*), with a further 11 species belonging to the related sections *Adnataria* and *Bisectae*. Cyanogenesis is more likely to have arisen independently in the other two subgenera (*Corymbia* and *Eucalyptus*) as they are taxonomically distant from the *Symphyomyrtus* (Brooker, 2000) with *Corymbia* considered by some to be a separate genus (Ladiges and Udovicic, 2000) Interestingly, no living specimens of *E. diversifolia* and *E. eximia*, or any others identified through the herbarium sampling, were found to be cyanogenic.

2.4. Polymorphism and ontogenetic effects

Given that polymorphism is often encountered in cyanogenic species (Gleadow and Woodrow, 2000a; Gleadow et al., 2003), some of the species not identified as cyanogenic in this study may have some cyanogenic individuals. Further, not all plant parts were found to be equally cyanogenic (data not shown). This may reflect polymorphism at either the whole plant or ontogenetic level (Jørgensen et al., 2005; Goodger et al., 2006) and as a result, further testing may reveal cyanogenic individuals.

2.5. Implications for conservation and forestry

Koalas (*Phascolarctos cinereus* Goldfuss) select trees on the relative concentrations of secondary metabolites and protein (Moore and Foley, 2005). Koalas have been known to die after eating cyanogenic forms of *E. viminalis* (Morris, 1944) and when choice is available, feed exclusively on noncyanogenic forms (Pahl, 1985). It is, therefore, very important that in order to support confined populations of koalas (both in zoos and in conservation parks) that the right chemotype is planted. A large number of *E. polyanthemos* and *E. ovata* surveyed here were from trees planted to provide food for koalas at the San Diego Zoo. All were found to be cyanogenic, although both these species are thought to be polymorphic (Goodger et al., 2002; W. Foley & I. Lawler, pers. comm.). Fortunately

[1] Previously known as *Monocalyptus* (Pryor and Johnson, 1971).

E viminalis is also polymorphic for this trait, and all the foliage of species collected from the San Diego Zoo for koala feed was acyanogenic (see supplementary data).

Propagating cyanogenic species, or chemotypes. could be advantageous where trees are to be planted for timber production in order to limit losses by mammalian and insect herbivores (Gleadow and Woodrow, 2002). Surprisingly, there seems to be only a limited growth sacrifice for synthesising of cyanogenic glycosides (Goodger et al., 2006), although their synthesis may be more costly under conditions of limited nitrogen. With the relative concentration of cyanogenic glycosides relative to leaf protein expected to increase with rising atmospheric CO_2 (Gleadow et al., 1998), the results presented here should assist in the choice of species for plantations for timber production (i.e. resistance to herbivores) and forest rehabilitation (i.e. suitable for feeding by arboreal marsupials such as koalas).

3. Experimental

3.1. Plant material

In order to examine the extent of cyanogenesis in *Eucalyptus* species, foliage from both herbarium and living plants was tested for its ability to release HCN. Plants were from the Waite Arboretum South Australia, the Adelaide Botanic Garden, the National Royal Botanic Gardens Canberra, the Royal Botanic Gardens Melbourne, San Diego Zoo (feed collection), and UC Davis arboretum. Approximately 1400 individual living plants representing ca. 330 species were tested for the presence and absence of cyanogenic glycosides using the qualitative Feigl–Anger procedure (Brinker and Seigler, 1992). Replicates of each species were tested. If available, young vigorously growing leaf tissue was tested as such tissue is typically more cyanogenic (Gleadow and Woodrow, 2000). A full list of all species is given in the Appendix (see supplementary data). Voucher specimens were lodged for all plants that tested positive (see Table 4). Samples of approximately 800 herbarium specimens, representing an additional 54 species not tested as living plants were also examined. Samples of *E. diversifolia*, and additional specimens of *E. citriodora* and *E. maculata* were tested using a similar procedure by R. Gleadow in an independent study.

3.2. Testing procedure

Cyanogenesis was tested using a method similar to that of Butler and Conn (1964). Small samples (approx 10–25 mg) of foliage were removed from herbarium specimens, ground in a glass vial and moistened with water. A few drops (*c.* 0.25 mL) of 0.05% almond emulsin (Sigma G-8625) and flax seed linamerase (0.1 units per mL) in 0.1 M phosphate buffer (pH 5) was added to the homogenate. Feigl–Anger test papers were added to detect any HCN released. For testing live plants in the field, a modification was introduced in order to provide an enzyme solution that could be conveniently prepared in the field by simply adding water to dry commercial reagents. An almond emulsin powder that hydrolysed both aliphatic cyanogenic glycosides as well as the aromatic prunasin (**1**), sambunigrin (**2**) and amygdalin (**3**) was used.

3.3. Identification of cyanogen

Cyanogenic glycosides were extracted using the procedure described in detail in Maslin et al. (1985). In brief, leaf material (ca. 150 g) was extracted twice in boiling 95% EtOH, reduced in vacuo (<40 °C) and redissolved in MeOH–$CHCl_3$-H_20 (12:5:1) The $CHCl_3$ phase was removed and discarded. Flavonoids were removed from the aq. phase by the addition of 10% lead acetate ($Pb(CH_3COO)_2 \cdot 3H_2O$) and centrifuged. The resulting supernatant was taken to dryness in vacuo (<40 °C) and partially purified on a polyamide column. The cyanogenic fraction from multiple runs on a C-18 column using HPLC were combined, taken to dryness by lyophilization and redissolved in hexadeutero acetone Me_2CO-d_6 for NMR (Maslin et al., 1988). Identification was based on similarity of NMR spectra with authentic prunasin (**1**) (Seigler, 1975; Secor et al., 1976; Conn et al., 1989). Some samples were purified further by HPLC on a C-18 column with (CH_3CN:H_2O; 16:84, v/v) for assessment by GLC (Conn et al., 1989).

3.4. Nomenclature

Species names and general taxonomy follow are the most recently published classification (Brooker, 2000) which is based conceptually on Pryor and Johnson (1971). Thirteen subgenera are currently recognised, with six being monospecific. Some of the subgenera of *Eucalyptus* are now considered genera, and together are known as 'the eucalypt group', or Eucalypteae (Bohte and Drinnan, 2005). Where species are considered by some to belong to *Corymbia*, rather than as a subgenus of *Eucalyptus* (Hill and Johnson, 1995; Ladiges and Udovicic, 2000), both names are given. Accession numbers for newly identified cyanogenic taxa are included in Tables 2 and 3.

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Appendix I. Supplementary data

Eucalyptus species examined for cyanogenesis with Feigl–Anger papers. Species that tested positive are listed in Tables 2 and 3 with the accession numbers of the voucher specimens. Individuals that tested positive are denoted by the initials **cg** in the first column (Cgluc). Species are listed in order by subgenus. Authorities of species are given. Alternative names are given in the last column, as well as any comments on the certainty or otherwise of the identification. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.03.018.

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