Structure and abnormalities in cones of the Wollemi pine (*Wollemia nobilis*)

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Summary. Reproductive structures of both genders of *Wollemia nobilis* were investigated, including both wild-type and teratological cones. Typically, both pollen cones and seed cones in this species are terminal on first order branches. At maturity, wild-type pollen cones are pendulous and cylindrical; wild-type seed cones are broad and ellipsoidal in shape. The teratological structures consisted of a basal region that resembled a typical fertile seed cone, and an apical proliferation that terminated in a well-developed pollen cone, resulting in a 'bisexual' unit. The proximal seed cone and the distal pollen cone were separated by a sterile region that represents an elongation of the cone axis. Of a total of 14 anomalous bisexual units investigated, all had the same bauplan. Such an arrangement of basal ovulate and distal staminate reproductive structures in a teratological conifer cone has previously not been reported for *Wollemia*. This topology is 'inside-out' with respect to most other reported anomalous bisexual conifer cones, which possess proximal staminate and distal ovulate structures. We discuss these spontaneous abnormalities in the broader context of understanding the homologies of seed-plant reproductive structures. The patterning of conifer cones is apparently highly labile, perhaps related to the extended cone axis and relatively long developmental duration.

Key Words. Araucariaceae, bisexuality, conifers, evolution, morphology, teratology.

Introduction

Wollemi pine (*Wollemia nobilis* W. G. Jones, K. D. Hill & J. M. Allen) is an Australian 'living fossil' that was discovered in 1994 and introduced into cultivation relatively recently (Jones *et al.* 1995; Trueman *et al.* 2007). *Wollemia* is assigned to the conifer family Araucariaceae (e.g. Farjon 2008, 2010; Eckenwalder 2009), which contains only two further genera *Araucaria* and *Agathis*. The family has a long evolutionary history, displaying considerable diversity in the fossil record, so that extinct taxa encompassed more widespread biogeography and a more diverse spectrum of genera and species (Stockey 1982; Gilmore & Hill 1997; Kunzmann *et al.* 2004; Escapa & Catalano 2013). Well-documented fossils of *Wollemia* closely resemble the living *W. nobilis* (Jones *et al.* 1995; Peakall *et al.* 2003; McLoughlin & Vajda 2005).

Wollemia nobilis is a monoecious tree species with staminate and ovulate reproductive structures arranged in compact, unisexual cones borne on the same tree; the male pollen cones are typically located in lower parts of the crown and the female seed cones in the upper parts; both genders are terminal on 3 - 6-year-old lateral first order branches (Jones *et al.* 1995). Phylogenetic studies indicate a sister-group relationship between *Wollemia* and *Agathis* (Escapa & Catalano

2013). In *Agathis* and *Wollemia*, the seeds are winged, ovules and cone scales are ontogenetically free, and the seeds can be shed independently from the cone scale at maturity, all features lacking in *Araucaria* (Jones *et al.* 1995; Chambers *et al.* 1998). In *Araucaria* and *Wollemia*, the cone scales have a long distal spine-like appendage, which is absent in *Agathis*.

By chance, a 12-year-old greenhouse-cultivated Wollemi pine tree produced — in addition to wild-type staminate and ovulate cones — several teratological seed cones with an apical proliferation that terminated in a well-developed pollen-bearing cone, thus forming a unit that is apparently bisexual, both morphologically and functionally. Previously, relatively few teratological cones have been described in Araucariaceae. Rare examples include male cones of *Araucaria* with leaf-like bract scales (Masters 1869), a female cone of *Araucaria excelsa* (Lamb.) W. T. Aiton with an apical proliferation that produced a second female cone (Worsdell 1916), a bisexual cone of *Agathis brownii* (Lem.) L. H. Bailey that was female at the base and male at the tip (Lanner 1966) and a bisexual cone of *Araucaria bidwillii* Hook. with a longitudinal 'feminized zone' along one of its margins (Bateman *et al.* 2011). As far as we know, no teratological reproductive structures have previously

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been described in *Wollemia*. In this paper, we describe the morphology and anatomy of both wild-type and teratological cones of *Wollemia nobilis* and discuss their potential broader significance in understanding the homologies of reproductive structures in seed plants.

Coniferous reproductive structures are typically unisexual, bearing either pollen or ovules/seeds. In rare cases, anomalous bisexual cones are formed that ultimately bear reproductive elements of both genders (e.g. Schooley 1967; Bateman *et al.* 2011; Rudall *et al.* 2011). A second type of spontaneous anomaly that can occur in conifer cones is the proliferation of the cone apex. Both pollen cones and seed cones are compact structures with determinate growth; in typical cones the apex becomes entirely used up during cone formation. In teratological proliferated cones, the apex maintains a more sustained period of growth, usually as a vegetative shoot axis (Tosh & Powell 1986; Caron & Powell 1991; Jagel & Stützel 2001; Farjon 2008; Owens 2008; Bateman *et al.* 2011; Rudall *et al.* 2011). Proliferated cones are most commonly seed cones; comparatively few descriptions exist of proliferated pollen cones (e.g. Dörken & Nimsch 2016).

Such records of naturally occurring abnormalities are significant because they can contribute to our understanding of evolution (e.g. Worsdell 1916; Rudall & Bateman 2003, 2010; Bateman & Rudall 2006; Bateman *et al.* 2011; Dörken 2011; Rudall *et al.* 2011; Dörken & Rozynek 2013; Dörken & Stützel 2011a, b; Dörken & Rudall 2018). In particular, the transition from unisexual to bisexual reproductive units is a crucial aspect of the evolutionary origins of flower-like structures in seed plants (e.g. Bateman *et al.* 2006; Rudall & Bateman 2010; Rudall *et al.* 2011). At the molecular level, current knowledge of developmental genetic mechanisms in seed plants is drawn primarily from angiosperm model species (e.g. *Arabidopsis thaliana*) that are ideally self-fertile and amenable to genetic transformation, possessing the advantageous features of small stature, rapid generation time and a small genome size. Conifers in general do not make good model species as they are long-lived woody perennials with large genome sizes (Uddenberg *et al.* 2015). Thus, spontaneous natural mutants, such as the one described here, are of great interest in studies of conifer evo-devo; they provide a useful resource in developing and testing hypotheses of cone homologies and seed-plant evolution. For example, comparative morphological and gene-expression studies on the *acrocona* mutant of spruce (*Picea abies* (L.) H. Karst. var. *acrocona*), in which vegetative shoots are homeotically transformed into seed cones, allowed insightful comparison with gene expression in wild-type cones of this species (Carlsbecker *et al.* 2013).

Conifer cone morphology

As widely interpreted in modern literature, most coniferous pollen cones consist of a single unbranched axis bearing several pollen-producing microsporangiophores, whereas seed cones are typically branched, polyaxial structures consisting of several bract/seed scale complexes (Coulter & Chamberlain 1917; Florin 1951, 1954). This hypothesis of cone dimorphism is supported by numerous comparative morphological and gene-expression studies on conifer cones (e.g. Page 1990; Farjon 2010; Stützel & Røwekamp 1997, 1999; Mundry 2000; Farjon & Garcia 2003; Eckenwalder 2009; Carlsbecker *et al.* 2013; Dörken 2017). It has important implications for reconstructing the evolution of developmental genetic mechanisms in seed plants (Rudall *et al.* 2009, 2011; Rudall & Bateman 2010). In contrast, the angiosperm flower is widely regarded as a simple (uniaxial) strobilus typically bearing outer/proximal microsporophylls (stamens) and inner/distal distal megasporophylls (carpels) (Bateman *et al.* 2006). The branched (polyaxial) pollen cones that occur in a few conifer genera represent rare cases among living conifers, as in *Cephalotaxus* (Cephalotaxaceae), *Pseudotaxus* (Taxaceae), *Juniperus drupacea* Labill. (Cupressaceae s.str.) and some Podocarpaceae (Lemoine-Sebastian 1967; Wilde 1975; Mundry & Mundry 2001; Dörken *et al.* 2011; Schulz *et al.* 2014; Dörken & Nimsch 2016).

In seed cones, the bract scale represents a leaf that bears an axillary ovuliferous short shoot, the seed scale. Among conifers, leaf-like structures bearing ovules (i.e., macrosporophylls) are always absent (Takaso & Tomlinson 1992; Jagel & Stützel 2001, 2003; Jagel 2002; Farjon & Garcia 2003; Schulz & Stützel 2007; Dörken 2011). In seed cones of Pinaceae, the bract scale and the seed scale can be clearly recognised as two distinct structures, but in other coniferous families, the bract/seed scale complex is strongly reduced and modified. For example, in Cupressaceae s.l. the seed scale is reduced to its ovules (e.g. Page 1990; Gadek *et al.* 2000; Jagel & Stützel 2001; Schulz & Stützel 2007; Jagel & Dörken 2014, 2015a, b; Dörken 2011; Dörken & Jagel 2014; Dörken *et al.* 2017). In Araucariaceae, including *Wollemia nobilis* (Farjon 2008, 2010; Eckenwalder 2009), the bract and seed scale are congenitally fused to each other, so that only one type of cone scale is formed; tissues of the upper part of the cone scale belong to the seed scale, whereas those of the lower part belong to the bract scale (e.g. Coulter & Chamberlain 1917; Eckenwalder 2009; Farjon 2010).

Material and Methods

Three types of cone of *Wollemia nobilis* were investigated in this study: (1) ten wild-type pollen cones; (2) ten

wild-type seed cones; (3) 14 teratological bisexual units. All cones were collected by one of us (VD) from a single 12-year-old tree cultivated in the Botanic Garden of the University of Konstanz, Germany. Another similar tree grown nearby did not produce anomalous cones. The trees are grown in pots and kept outdoors in summer and stored in a temperate house at 6–7° C in winter.

Freshly collected material was photographed, then fixed in FAA (100 ml FAA = 90 ml 70% ethanol + 5 ml acetic acid 96% + 5 ml formaldehyde solution 37%) before being stored in 70% ethanol. Anatomy was studied from serial sections using the classical paraffin technique and subsequent astrablue/safranin staining (Gerlach 1984). Macro-photography was undertaken using a digital camera (Canon PowerShot IS2) and microphotography with a digital photomicroscope (Keyence VHX 500F) equipped with a high-precision VH mounting stand with X-Y stage and bright-field illumination (Keyence VH-S5).

For SEM analysis, FAA-fixed material was dehydrated in formaldehyde dimethyl acetal (FDA) for at least 24 hours (Gerstberger & Leins 1978) and critical-point dried, then mounted onto SEM stubs and sputter-coated using a sputter coater SCD 50 Baltec (Balzers). Specimens were examined using an Auriga Zeiss TM.

Results

Wild-type pollen cones

Typical pollen cones are terminal on plagiotropic to slightly pendulous, 2–6-year-old lateral first-order branches (Fig. 1A). The pollen cones lack a distinct stalk. They are subtended by six to eight small leaves, which form a cup-like ‘involucrum’ (Fig. 1C). The cone consists of several hundred helically arranged hyposporangiate microsporangiophores (Fig. 1C). Each microsporangiophore has a shield-like flattened terminal scutellum and a central stalk (Fig. 1E, F). Abaxially, four to nine strongly elongated microsporangia occur parallel to the central stalk (Fig. 1E, F). The pollen cones are monoaxial (unbranched) structures. Immature pollen cones are more-or-less plagiotropic; they are pendulous at maturity. At the stage of pollination, they are cylindrical, about 10–15 cm long and 1–3 cm wide (Fig. 1A, C). By the time they release the pollen, the dark-green to reddish microsporangiophores spread distantly from each other (Fig. 1C, D) due to a significant elongation of the cone axis that leads to an expansion of internode length. After releasing the pollen, the pollen cones dry and are soon abscised, and leave a roundish abscission scar at the shoot axis (Fig. 1B).

Wild-type seed cones

Typical seed cones are terminal on plagiotropic to slightly ascending, 3–6-year-old lateral first order branches. The seed cones are in a plagiotropic to slightly downward position at pollination time and remain in this position even at maturity (Fig. 1G). The seed cone consists of over 100 bract/seed scale complexes. The seed scale is entirely fused to the bract scale, so that only one type of cone scale is formed (Fig. 1L, M). The cone scales are helically arranged (Fig. 1H); they are rhombic to triangular with a narrowed base and a lateral winged margin (Fig. 1L). The cone scales are c. 30–40 mm long, 20–35 mm in diameter and 2–5 mm thick. Each cone scale has a distal appendage that is 5–15 mm long, 2–5 mm wide and strongly incurved (Fig. 1G, H, J). Each cone scale bears a single ovule in its basal part (Fig. 1L, M), with the micropyle located towards the cone axis. No leaf-like structure can be observed, in the sense of a macrosporophyll that bears the ovule. The basalmost and distalmost cone scales are sterile (Fig. 1J); only the cone scales in the middle region of the cone are fertile. The pollinated seed cones mature in two to three years. At maturity, they have a broad, ellipsoidal to spherical shape and are c. 8–10 cm long and 6–10 cm wide (Fig. 1H). Mature seed cones shed individual cone scales so that ultimately only the cone axis and some of the basalmost sterile cone scales remain on the tree. Abscission of the cone scales is accompanied by drying and shrinking of the cone axis. The seeds are 8–12 mm long, 6–10 mm wide, and brownish with an encircling wing.

Bisexual teratological units

On the plant investigated, a total of 14 bisexual structures were found, each occurring at the tip of a well-grown lateral first-order branch (3–6-years old) in the distalmost parts of the tree crown (Fig. 2). The basal region of these anomalous bisexual units resembles a typical fertile seed cone. However, the apex maintains growth into an apical proliferation that terminates in a fully developed fertile pollen cone (Figs 2A, C, E, G, J). Formation of the basal seed cone and the apical proliferation is a successive process. The seed cone develops first, then approximately 2–3 months after the time of pollination, the apical meristem of the seed-cone axis commences regrowth in the form of an apical foliar shoot axis. This proliferated shoot axis is about 3–10 cm long and bears 5–27 normal sterile leaves (Figs 2A, C, E, G, J); it is of determinate growth, and its apex becomes completely consumed by formation of a terminal pollen cone. Both the basal ovulate and the terminal staminate structure are fertile and show the typical morphology and anatomy of their respective cone types (Fig. 1). At seed-cone maturity, two to three years

Fig. 1. *Wollemia nobilis*, morphology and anatomy of wild-type cones (A–F pollen cones, G–M seed cones). A mature pollen cones on a tree; B abscission scar of a pollen cone on a lateral short branch; C mature pollen cone at pollination time; D detail of C, showing microsporangiophores spreading; E, F details of a single hyposporangiate microsporangiophore (SEM); G mature seed cone in its natural position on a tree; H mature seed cone; J detail of the distal part of the seed cone illustrated in H, with sterile terminal cone scales (arrow); K longitudinal section of a seed cone; L detail of a fertile cone scale bearing a single ovule, left adaxial view, right abaxial view; M longitudinal section of the basal part of a cone scale through the single ovule.

after pollination, the seed-cone axis dries and shrinks to shed the cone scales and seeds. Subsequently, the apical proliferation also dries out and is abscised.

Discussion

Teratological cones with apical and lateral proliferations

In the teratological seed cones of *Wollemia nobilis* investigated here, the proliferated axis terminates in a fully developed pollen cone. Occasional apical proliferations are not uncommon in seed cones; they are reported in several coniferous families, but the apical proliferation is typically entirely vegetative (e.g. Tosh & Powell 1986; Caron & Powell 1991; Jagel & Stützel 2001; Farjon 2008; Owens 2008; Bateman *et al.* 2011; Dörken *et al.* 2011; Rudall *et al.* 2011). Such vegetative apical proliferations represent a sterile elongation of the cone axis, characterised by a dramatic change in internode length and the size and shape of the inserted leaves. The apex of the cone axis returns to/retains its vegetative state and maintains growth for a limited period. In typical wild-type seed cones, the apex is ultimately consumed by development of the distal-most cone scales, as in *Wollemia nobilis* (Fig. 1J, K). The duration of the apical proliferation is always closely correlated with the life-span of the seed cone.

In contrast, proliferated pollen cones are relatively rare (e.g. Dörken & Nimsch 2016). This gender disparity could be explained by the relative difference in cone longevity. Depending on the particular species, seed cones can take up to three or four years to develop to maturity, but pollen cones of all conifers rapidly dry and are abscised soon after pollen release.

In contrast with apical vegetative proliferations of seed cones, lateral proliferations inserted in the axil of a cone scale have been described for several conifers (Worsdell 1916; Neubauer 1976; Dörken 2011). For example, in laterally proliferated seed cones of *Metasequoia glyptostroboides* Hu & W. C. Cheng, a vegetative, foliar short shoot is inserted in the axil of a cone scale instead of the typical single row of axillary ovules (Neubauer 1976; Dörken 2011). Both the cone scale and the axillary proliferation have their own vascular supply. Thus, the axillary proliferation precisely replaces the single row of ovules. Dörken (2011) interpreted the sterile leaves inserted at the proliferated axillary short shoot as transformed, leaf-shaped, sterile ovules. This hypothesis is strongly supported by the developmental pattern of the leaves inserted at the proliferated shoot axis and the axillary row of ovules in typical cone scales; in both cases, development is centripetal/acropetal (Jagel & Stützel 2001, 2003; Jagel 2002; Dörken 2011; Dörken & Jagel 2014; Jagel & Dörken 2015a, b). In particular, the

morphology, anatomy and vasculature of these axillary proliferations support the hypothesis of a homologous replacement of an axillary row of ovules by a vegetative short shoot, so that ovules can occupy the position of entire leaves in the bauplan (Dörken 2011). This hypothesis could explain the non-axillary position of ovules that occurs in other Cupressaceae (Jagel & Stützel 2001, 2003; Jagel 2002; Schulz *et al.* 2003; Schulz & Stützel 2007). Thus, these axillary proliferations could be regarded as an atavism that potentially elucidate scenarios developed to explain seed-cone evolution in conifers.

Bisexual teratological units

There are several different ways to produce teratological bisexual units within conifers (Rudall *et al.* 2011). Those of *Wollemia nobilis* described here represent a relatively rare type. In addition to changes in internode length and the size and shape of the inserted leaves, an abrupt gender transition has occurred, from proximal ovulate to distal staminate reproductive structures, resulting in a short bisexual axis with two clearly demarcated zones of contrasting genders. The duration of the proliferated staminate unit is closely correlated with the life span of the proximal ovulate unit. As is usual for the two genders in conifers, the terminal pollen cone matures before the basal seed cone, so that pollen can be released long before the seed cone axis begins to dry and shrink.

This *Wollemia* seed-cone abnormality partly resembles a recurring teratological seed-cone type that was illustrated and described by Rudall *et al.* (2011) and Jagel & Dörken (2014) in *Cryptomeria japonica* D. Don (Cupressaceae). Within these cones, an apical fertile proliferation is developed that bears several fertile pollen cones, forming a bisexual unit. However, the bisexual unit of *Cryptomeria japonica* differs from *Wollemia nobilis* in one important feature: it bears a cluster of several pollen cones, mostly in an axillary position, so that the apex of the proliferation remains vegetative. In contrast, in *Wollemia nobilis*, a single terminal pollen cone formed at the proliferated shoot axis, making shoot growth determinate.

Rudall *et al.* (2011) and Bateman *et al.* (2011) described several other types of reproductive proliferation in extant and extinct conifers, including relatively compact bisexual cones with a transition from proximal staminate to distal ovulate reproductive structures (e.g. in *Tsuga*: Pinaceae). Crucially, in contrast with the bisexual units of *Wollemia nobilis*, these teratological cones lacked a sterile vegetative extended shoot separating the reproductive structures of the two genders. Most reports of bisexual coniferous cones describe the topology of the bisexual cones of *Tsuga*. A single description for *Abies balsamea* (L.) Mill. (Pinaceae) (Schooley 1967) and another for

Fig. 2. *Wollemia nobilis*, bisexual units bearing proximal (basal) ovulate and distal staminate reproductive structures, separated by a sterile region. A–F morphology of a bisexual unit; A, C, E overview; B, D, F details of sterile region showing transition from ovulate to staminate reproductive structures; G–M bisexual unit; G proliferated seed cone terminating in a fully developed pollen cone; both the ovulate and staminate structures are fertile; H transitional region between the proximal seed cone and the apical proliferation; J longitudinal section of a bisexual unit; K detail of the basal fertile ovulate region of the seed cone illustrated in J; L detail of the seed cone illustrated in J, showing the base of the distal proliferation; M detail of the distal fertile pollen cone shown in J; the pollen cone apex is consumed by formation of the distalmost microsporangioophores.

Agathis brownii (Lanner 1966) show a comparable arrangement of reproductive structures to that described here for *Wollemia nobilis*, with apical staminate and basal ovulate structures.

The relative organisation of sterile and fertile elements in the *Tsuga* type of bisexual cone corresponds fairly closely to that prevailing in bisexual angiospermous flowers, in which the outer/proximal androecium consists of pollen-producing organs and the inner/distal gynoecium consists of ovuliferous organs. This type of organisation is almost ubiquitous in angiosperms, although some diversity exists among early-divergent taxa (Rudall *et al.* 2009). By contrast, in the case of *Wollemia nobilis* described here, the arrangement of distal staminate and proximal ovulate structures represents the inverse condition to that of bisexual angiospermous flowers, in which the arrangement of proximal staminate and distal ovulate organs is strictly constrained. A further distinct difference is the temporally staggered development of ovulate and staminate structures, which in *Wollemia* occurs about two or three months apart, compared with a maximum of a few days in bisexual angiospermous flowers. Furthermore, in bisexual angiospermous flowers, staminate and ovulate elements are never separated by a sterile shoot axis, as is the case in the bisexual units of *Wollemia*.

Conclusions

Regardless of whether the cone is interpreted as uniaxial or polyaxial, comparative gene-expression studies can potentially help to evaluate interlinked hypotheses regarding the homologies and evolution of seed-plant reproductive structures. To date, most such studies have been carried out on model angiosperms (especially *Arabidopsis*), in which the identity and relative arrangements of the reproductive organs are controlled by a combination of B- and C-class MADS-box genes (Coen & Meyerowitz 1991). A subtly different expression pattern occurs in extant gymnosperms, in which orthologous AG-like (C-class) genes are expressed in both male and female reproductive organs, whereas AP3/PI-like (B-class) genes are expressed in male reproductive organs alone, indicating a regulatory change at some stage in seed-plant evolution (e.g. Theissen & Becker 2004; Zhang *et al.* 2004; Tavares *et al.* 2010; Groth *et al.* 2013). Upstream of floral organ expression, the gene *LEAFY/FLORICAULA* (*LFY/FLO*) encodes a transcription factor that regulates the floral B- and C-class genes in angiosperms (Moyroud *et al.* 2010) and the orthologous B-genes in extant gymnosperms (Moyroud *et al.* 2017). Similarly, *LFY*-like genes are expressed in gymnosperm reproductive structures; for example, Carlsbecker *et al.* (2013) showed that the

transition from vegetative to reproductive structures in the *Acrocona* mutant of spruce is correlated with an upregulation of *LFY*-like genes.

Most spontaneous abnormal bisexual cones appear to be modified seed cones rather than modified pollen cones (Rudall *et al.* 2011), as in the example shown here in *Wollemia*. Changes in expression of *LFY*-like expression along the cone axis could be responsible for the abnormalities that we describe, although this hypothesis requires testing. The apical proliferation in the teratological *Wollemia nobilis* seed cones investigated here is a pollen-bearing structure, and hence the entire bisexual unit is ‘inside-out’ with respect to many other conifer cone abnormalities. Conifer seed cones with vegetative apical proliferations could result from loss of C-class gene expression causing a reversion to vegetative structures and loss of determinacy at the cone apex. In the case of *Wollemia nobilis*, there is a relatively extended intervening vegetative axis between the two reproductive regions, indicating that this reversion was subsequently followed by expression of B-class related genes. We conclude that the patterning of conifer cones is relatively labile, probably due to their extended axis and long developmental duration.

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