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ORIGINAL

Differences in the ultrastructure of their large warts allow white cypress pine (*Callitris glaucophylla*) to be distinguished from black cypress pine (*C. endlicheri*)

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Abstract White cypress pine (*Callitris glaucophylla*) wood is durable enough to be used outdoors, but occasionally there are reports of its premature failure in ground contact, which may be due to its substitution by the less durable species, black cypress pine (*C. endlicheri*). It has been difficult to prove this, however, because the woods of both species are very similar in structure and cannot be separated using conventional anatomical features. This study examined whether differences in the size and morphology of warts on tracheid walls in the two species could be used to identify them. There were significant differences in the height, width and shape of warts in the two species, but there was considerable overlap in the distribution of these parameters between specimens. Warts in *C. endlicheri* were more likely to be bent-over near their tops than those in *C. glaucophylla*, and the angle bending of warts was greater in *C. endlicheri*. Quantification of these parameters produced complete separation of multiple specimens of the two species, and could potentially be used to help determine whether premature failure of *C. glaucophylla* heartwood in ground contact is the result of its substitution by *C. endlicheri*. More generally it can be concluded that the morphology of large warts may have taxonomic value in identifying coniferous wood species beyond the generic level.

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Introduction

White cypress pine (*Callitris glaucophylla* Thompson & Johnson) is a medium-sized coniferous tree that is abundant in the drier inland regions of South Eastern Australia where the species sustains a small, but regionally significant, timber processing industry (Anderson 1968; Boland et al. 1984; Salmon 1990). The wood of *C. glaucophylla* is particularly dense and hard for softwood and it is resistant to termite and fungal attack (Rudman 1966; Bootle 1985; Johnson et al. 2006). These characteristics have led to its use as poles and posts (Cummins and Dadswell 1935), and for this "in-ground application" it is classified as a Class 2 species giving 15–25 years service, based on the mean service life of heartwood stakes in soil (Cookson 2004). In recent years, however, there have been isolated reports of the premature failure of house stumps and fence posts made from *C. glaucophylla*, and there has been interest in understanding the reason for this (Ilic 1996). One possible explanation that has been proposed by Ilic (1994, 1996) is that *C. glaucophylla* may have been substituted for by black cypress pine (*Callitris endlicheri* [Parl.] F.M. Bailey), which is less durable than *C. glaucophylla* (Rudman 1966; Johnson et al. 2006). This is plausible as the trees of both species look alike, their geographical distributions overlap and in some areas of New South Wales they occur together as co-dominants in open woodlands (Baur 1965; Bowman and Harris 1995). The veracity of this explanation could simply be established by taking wood samples from posts that had failed prematurely and determining their identity. However, it is difficult to do this because the woods of *C. glaucophylla* and *C. endlicheri* are so similar in structure that they cannot be separated using conventional anatomical features (Dadswell and Eckersley 1935). Hence, attempts have been made to identify and separate the two species using differences in their chemical characteristics (Bootle 1985; Ilic 1996). Bootle (1985) noted that the odour of *C. endlicheri* was not as strong as that of *C. glaucophylla*. He also observed that a match-stick-sized sample of wood from *C. endlicheri* burnt to a grey ash whereas a similar test on *C. glaucophylla* produced a white ash (Bootle 1985). Ilic (1994, 1996), however, performed the same burning splinter test on *C. glaucophylla* and *C. endlicheri* and observed no difference between them. He did, however, observe some differences in the ultrastructure and chemical characteristics of the two species (Ilic 1994, 1996). For example, he noted that the warty layer was more distinct and coarse in *C. glaucophylla* than in *C. endlicheri* and the latter had a distinct sweet smell reminiscent of Japanese cedar [*Cryptomeria japonica* (L.f.) D. Don], whereas *C. glaucophylla* had a more pungent odour (Ilic 1996). The most characteristic difference between the two species observed by Ilic (1994, 1996) was the consistent formation of a crystalline exudate or frost of the sesquiterpene alcohol guaiol on sawn surfaces of *C. endlicheri*, which was absent from the wood of *C. glaucophylla*. Dadswell and Dadswell (1931), however, reported that the wood of *C. glaucophylla* and *C. endlicheri* contained similar quantities of guaiol, and subsequently Dadswell and Eckersley (1935) noted that distinct white needle-sized crystals could form on the surface of *C. glaucophylla*. Therefore this feature may not be completely effective at separating *C. glaucophylla* from *C. endlicheri*. Furthermore, when identifying and separating wood species it is desirable to use more than one

character (Jane 1970). Hence, it would be useful to find additional features that could be used to separate *C. glaucophylla* from *C. endlicheri*.

In our previous study of the warty layer in all species in the genus *Callitris* it was shown that large warts in *C. endlicheri* had a pedestal-like base and a narrow, tubular upper part that was often bent-over (Heady et al. 1994). These morphological features were quite unique and were not seen in other *Callitris* species including *C. glaucophylla*, which had squat conical warts. Hence, it was speculated that the morphology of warts in *C. endlicheri* might be sufficiently distinctive to allow it to be separated from other *Callitris* species, including *C. glaucophylla* (Heady et al. 1994). In this paper this hypothesis has been tested. The aim was simply to develop an effective method of separating *C. glaucophylla* from *C. endlicheri*, which could possibly be used to settle disputes involving claims that premature failure of *C. glaucophylla* in ground contact was the result of its substitution by *C. endlicheri*.

Materials and methods

A permit was obtained from State Forests of New South Wales to sample cypress pine trees (Permit number 05417, 28 October 1999). Wood samples of *C. glaucophylla* and *C. endlicheri* were obtained from 5 mm increment cores taken from trees of unknown age, but of adult appearance, growing in inland areas of New South Wales, Australia where the geographical distributions of the two species overlapped. Samples were obtained from two separate trees at 12 sites for each species (48 trees in total). No trees from geographically distinct populations of either species were sampled. Hence, no comment on variation in the ultrastructure of warts arising from genetic differences between provenances of *C. glaucophylla* and *C. endlicheri* can be made.

Increment core samples were obtained from two separate trees at 12 sites for each species (48 trees in total). A 3 mm long section was cut from each increment core and this section was split down its longitudinal axis using a sharp single-edged razor blade. The resulting samples were placed in small 5 mL plastic cups containing distilled water at 20°C for 3 days to saturate and soften them. These samples were trimmed to a final size of approximately $3 \times 2 \times 2$ mm^3 and clean undamaged radial longitudinal surfaces were prepared as described previously (Heady et al. 1994). Specimens were cleaned of cytoplasmic debris by washing them in several changes of distilled water, dried and coated with a 10 nm layer of gold, as described previously. A Cambridge Instruments S360 scanning electron microscope (SEM) fitted with a high-brightness lanthanum hexaboride (LaB_6) electron source was used to produce high-resolution images of the warty layer for each wood sample. The electron optics system of the SEM was optimised for high resolution by using a 30 μm diameter final aperture, a working distance of 7–9 mm, electron beam current of 5 pA, and an accelerating voltage of 20 kV. Variation in working distance used to view specimens occurred because the heights of wood specimens varied. The SEM was able to compensate for such differences, and variations in height measurements of warts over the working distances used were less than 1%.

Prepared samples were tilted to an angle of 55° and mechanically rotated within the chamber of the SEM so that the longitudinal axes of tracheids were orientated vertically on the SEM screen. Ten separate tracheids in each of the 48 wood samples (24 for each species) were viewed longitudinally at a magnification of 30,000 times. The resulting SEM images provided a clear side-on view of at least five or more individual warts in the warty layer. Sampled areas within the warty layer were selected at random, but areas showing evidence of compression wood or containing pits, rays and resinous extractives were avoided. High-resolution digital images of warts were created directly from the SEM image using an ImageSlave (Meeco Pty. Ltd) digitising system and stored as TIFF files. These were subsequently viewed and analysed on a Macintosh computer using the public domain image analysis program NIH Image (V. 1.62) available at http://www.rsbinfo.nih.gov/nih-image. The width of each of the sampled tracheids was measured and the following information was obtained for five large warts in each sampled area: (1) Height from base to tip; (2) Widths at positions representing 0% (base), 25%, 50% (middle) and 75% of the total height of each wart; (3) Whether the tops of warts were bent-over; (4) Angle made by the part of each wart that was bent-over. Warts were defined as "bent-over" only if the angle of the upper part of the wart was different from the angle of the lower part of the wart. Warts that were continuously inclined from the base upwards were categorised as being "upright". Wart angles were measured using a protractor held against the face of the computer screen, and the position where the wart began to bend was estimated.

Prior to statistical analysis of data it was necessary to develop parameters for wart shape that best discriminated between the two species. Exploratory analysis including detailed examination of the statistical properties of data led to two quantitative measures of wart shape being developed. These were: (1) Wart width ratio defined as natural logarithm of width of an individual wart at 25% of its height divided by its width at 75% of its height; and (2) wart shape defined as natural logarithm of wart width ratio divided by square root of wart height. Analysis of variance was used to examine the differences in wart height, width and shape between *C. glaucophylla* and *C. endlicheri* and also differences between the two species in the positions where warts began to bend and the angle of bending. Variance components analysis using restricted maximum likelihood (REML, Searle et al. 1992) was used to determine the nature of the relationship between wart shape and the width of tracheids and whether the relationship changed with species. All statistical computation was performed using Genstat 5 (Genstat 2000). Significant results ($P < 0.05$) are presented graphically and least significant difference (LSD) bars or 95% confidence intervals ($P < 0.05$) on graphs can be used to compare differences between means for the two species.

A qualitative test was also performed to examine whether differences in wart shape in the two species could be used to identify authentic wood samples of each species whose identity was not known to the observer. This involved randomly selecting 28 prepared SEM samples from different white and black cypress pine trees (above) and re-examining each one using scanning electron microscopy. Ten to 20 tracheids were examined in each specimen and an overall impression was formed of whether warts on the tracheids walls of the specimens were generally

squat and conical or tubular and bent-over. Specimens whose warts had the former characteristics were identified as *C. glaucophylla*, and those with tubular, bent-over warts, were identified as *C. endlicheri*. These derived identities for the specimens were then compared with their true identities.

Results

Scanning electron microscopy confirmed that large warts in *C. glaucophylla* are generally squat and conical with a wide base and a relatively narrow upper part (Fig. 1), whereas those in *C. endlicheri* are longer and more tubular in shape (Fig. 2). These observations were reflected in the measurements and analysis of the size and shape of the large warts in the two species. Warts in *C. glaucophylla* were significantly ($P < 0.001$) shorter and wider than those in *C. endlicheri*, but there was considerable overlap in the average heights and widths of warts in different specimens (Fig. 3). Differences in the widths of warts in the two species were most pronounced at 25% of wart height (Fig. 3). In contrast there was less difference in the width measurements towards the tops of the warts (Fig. 3). Hence, the parameter wart width ratio, which is the ratio of width of warts at 25% of their height to that at 75% of wart height, was better at separating the two species than wart height alone (Fig. 3). Similarly, the shape of warts, which was defined as logarithm of wart width ratio divided by square root of wart height, was also better than wart height at separating *C. glaucophylla* from *C. endlicheri* (Fig. 3). Nevertheless, there was still overlap between the two species in the distribution of these shape parameters at the specimen (tree) level (Fig. 3). It is not known if such variation is due to genetic differences between trees. Variance components analysis of the relationship between tracheid width and wart shape indicated that the shape of warts in both species was unaffected by tracheid width.

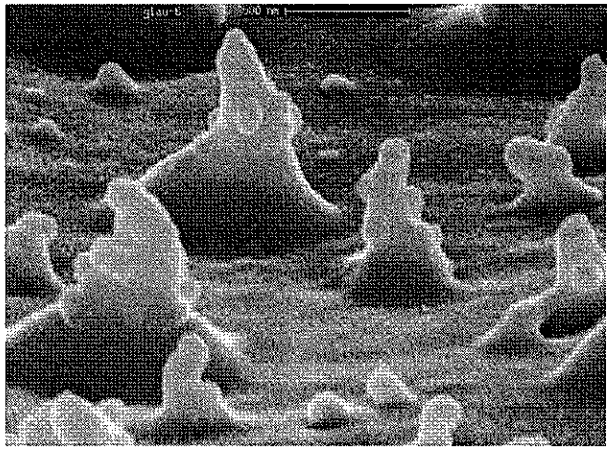

Fig. 1 SEM photomicrograph of warty layer in white cypress pine (*C. glaucophylla*) showing large conical-shaped warts (*lower-left* and *centre-left*)

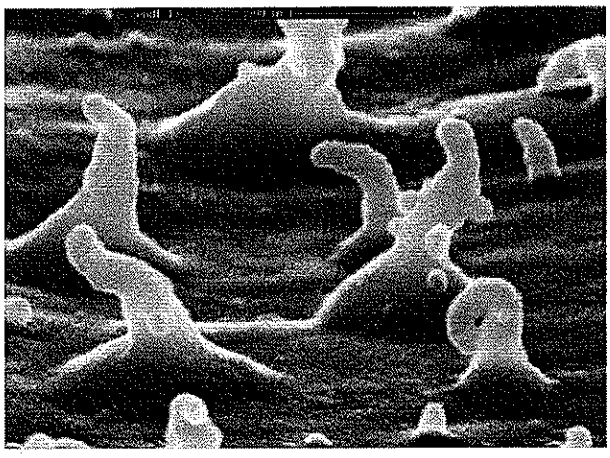

Fig. 2 SEM photomicrograph of warty layer in black cypress pine (*C. endlicheri*) showing five large tubular bent-over warts

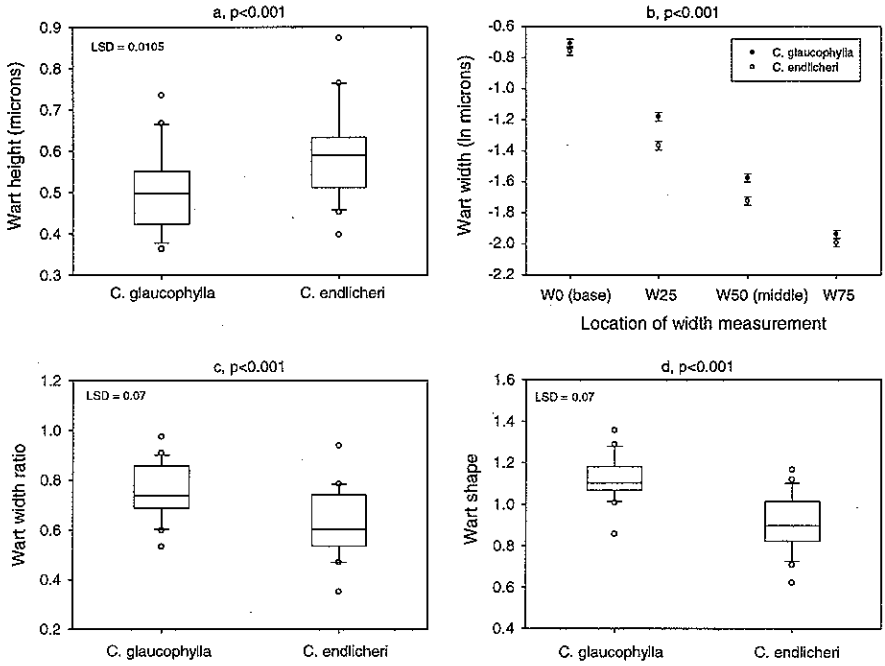

Fig. 3 Difference in the size and shape of warts in white cypress pine (*C. glaucophylla*) and black cypress pine (*C. endlicheri*); (**a**) Wart height; (**b**) Wart width at their base and at 25, 50 and 75% of total wart height; (**c**) Wart width ratio; (**d**) Wart shape

One of the most obvious differences in the morphology of warts in the two species was that warts in *C. endlicheri* tended to be bent-over whereas those in *C. glaucophylla* were more likely to be straight. This was apparent from visual observation of whole

tracheids walls of both species (Figs. 4, 5), and was also reflected in measurements and analysis of the probability of warts being straight or bent along their length (Fig. 6). Hence, the probability of warts being straight in *C. glaucophylla* was 0.67 compared to 0.23 in *C. endlicheri* (Fig. 6). Conversely, warts in *C. glaucophylla* showed a much lower probability of being bent along their length than warts in *C. endlicheri*. The angle of bending of warts, which includes figures for warts that were straight (angle = 0), was significantly lower in *C. glaucophylla* than in *C. endlicheri* (Fig. 6). Furthermore, there was no overlap between the two species in the distribution of these parameters at the specimen level (Fig. 6). Large warts in *C. endlicheri* were also more

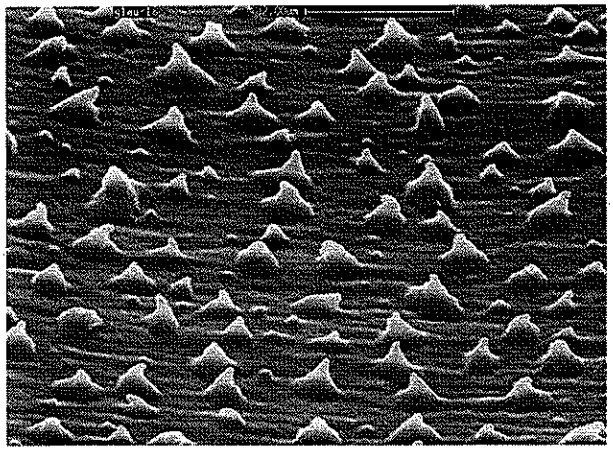

Fig. 4 SEM photomicrograph of warty layer in an individual white cypress pine (*C. glaucophylla*) tracheid showing large numbers of squat conical warts. Note that very few of the warts are bent over

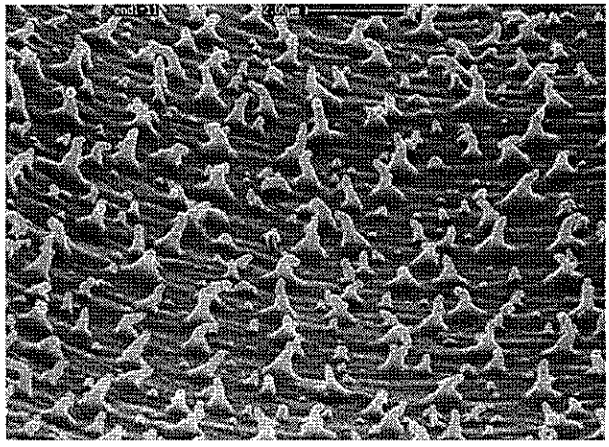

Fig. 5 SEM photomicrograph of warty layer in an individual black cypress pine (*C. endlicheri*) tracheid showing large numbers of tubular warts that are bent-over, and some that are joined together (*top left*). Note the variation in the degree to which warts are bent over

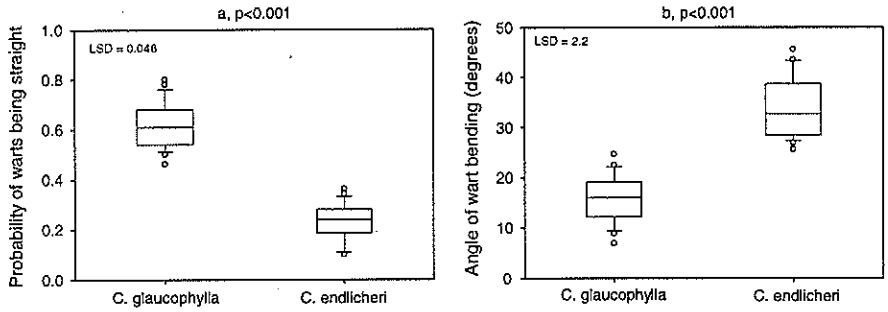

Fig. 6 Differences in the angular orientation of warts in white cypress pine (*C. glaucophylla*) and black cypress pine (*C. endlicheri*); (**a**) Probability of warts being straight (0°) or being bent-over at different angles to the vertical; (**b**) Average angle of large warts

frequently joined together (anastomosed) than those in *C. glaucophylla* (compare Figs. 5 with 4). These morphological characteristics of warts were easier to see in sapwood, but they could also be seen in heartwood whose cell walls were not coated with resinous extractives (Fig. 7). Our test to determine the species identities of 28 unknown white and black cypress pine specimens based on qualitative assessment of the morphological characteristics of their warts produced 24 correct identifications and 4 incorrect ones.

Discussion

Our results have shown that the woods of *C. glaucophylla* and *C. endlicheri* can be separated by quantifying whether their large warts are straight or bent-over and the

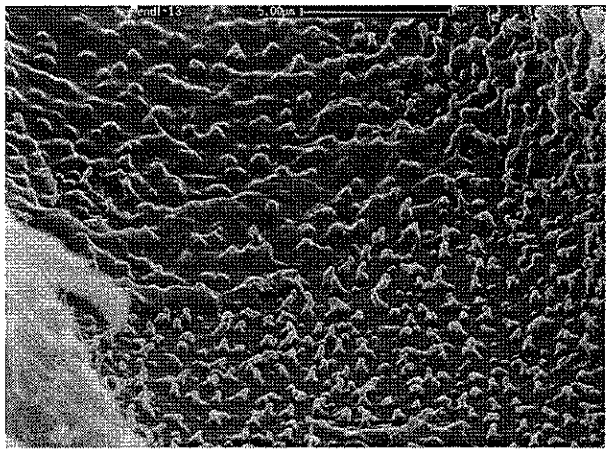

Fig. 7 SEM photomicrograph of a tracheid wall in black cypress pine (*C. endlicheri*) that is partially occluded by resinous extractives. Note that it is still possible to see warts in the lower part of the tracheid that is not covered by extractives

angle of bending of warts in the two species. Warts were significantly shorter in *C. glaucophylla* than in *C. endlicheri*, and statistically significant differences in the width and shape of warts in the two species were also observed, but it was not possible to completely separate the two species using these parameters. It was time consuming to obtain quantitative information on the morphology of warts and therefore it was tested whether qualitative assessment of the distinctive features of warts in *C. glaucophylla* and *C. endlicheri* could be used to separate them. This test was reasonably successful at correctly identifying authentic unmarked specimens of each species (24 out of 28 or 86%), but this level of success is probably not sufficient for cases where the correct identity of the species needs to be established "beyond reasonable doubt". In such situations quantitative analysis of the angular orientation and morphology of warts would be required. This can only be done using electron microscopy since the minimum magnification at which differences in wart morphology became apparent was approximately 10,000 times. Furthermore, the distinctive shapes of warts were only perceptible when SEM samples were viewed obliquely by tilting them at 55°. When samples were viewed from above the differences in the shapes of warts in *C. glaucophylla* and *C. endlicheri* were far less noticeable. Previous studies of the warty layer have mainly observed warts from above and this may explain why they have generally not commented on differences in the morphology of warts between species. Our findings indicate that information on wart morphology could be obtained from both earlywood and latewood since there was no relationship between tracheid width and the shape of large warts. Furthermore, it was possible to observe the morphological features of large warts in heartwood, although they were easier to see in sapwood. Hence, our procedures for the separation of *C. glaucophylla* and *C. endlicheri* could be used to help determine whether premature failure of *C. glaucophylla* heartwood in ground contact was the result of its substitution by *C. endlicheri*.

The warty layer was first described by Liese (1951) and Kobayashi and Utsumi (1951). Liese (1957) was also the first to describe the size of warts in *Callitris*. There have been several studies that have described the appearance of warts in different coniferous wood species and commented upon its taxonomic significance (Harada 1953, 1955; Liese and Johann 1954; Frey-Wyssling et al. 1955, 1956; Liese 1956, 1963; Wardrop and Davies 1962; Ohtani and Fujikawa 1971; Kocoń 1985; Roig 1992). Liese (1963) concluded that some characters such as "the absence of the warty layer in certain species and genera, as well as the big warts of several species" could be useful in combination with other anatomical features for wood identification. For example, the presence of a distinct warty layer in the hard pines (Diploxylon) can be used to distinguish them from soft pines (Haploxylon), which rarely have a pronounced warty layer (Frey-Wyssling et al. 1955, 1956; Liese 1956). Liese (1963) later went on to recommend that anatomical descriptions of wood species should also include observations of the warty layer, if possible. We concur with this recommendation, but would further suggest that these descriptions should include observations on the morphology of large warts within the warty layer. Such information is much easier to obtain now than when many previous studies of the warty layer were done because of the increased resolution and ease of use of scanning electron microscopes and their widespread availability. The distinctive

morphological features of large warts in *C. endlicheri* were clearly of value in separating this species from *C. glaucophylla*. Previously, it was also noted that the unique seal-shape of warts in *C. drummondii* was distinctive enough to identify this species (Heady et al. 1994). Hence, the morphology of warts may have particular taxonomic value in identifying and separating coniferous wood species beyond the generic level (in situations which justify the time and expense involved in using electron microscopy). Further studies of larger numbers of genera and species, however, would be needed to confirm this. One genus whose species might benefit from closer examination of the morphology of their warts is *Juniperus*, which has large warts, and contains species such as Eastern red cedar (*Juniperus virginiana* L.) and Southern red cedar (*J. silicicola* (Small) Bailey) that cannot be separated using conventional anatomical features (Jane 1970; Panshin and de Zeeuw 1980).

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