JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2005, p. 551–555 Vol. 43, No. 2
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.2.551–555.2005

Multiple Genetically Distinct Groups Revealed among Clinical Isolates Identified as Atypical *Aspergillus fumigatus*

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Received 21 January 2004/Returned for modification 8 March 2004/Accepted 16 September 2004

To investigate whether genetic variants of *A. fumigatus* are found among clinical isolates, four isolates that were originally identified as poorly sporulating strains of *Aspergillus fumigatus* were subjected to molecular analysis. DNA sequence analysis of the alkaline protease genes of these isolates showed that each is genetically distinct and each shows substantial variation (7 to 11%) from the *A. fumigatus* nucleotide sequence. Subsequent morphological examination suggested that all of the isolates could be classified as *Aspergillus viridinutans*. To clarify the taxonomic status of these four clinical isolates and of two previously identified as atypical *A. fumigatus* isolates, partial β-tubulin and 18S rRNA gene sequences were determined. Each of the six atypical strains had a unique β-tubulin sequence, whereas the sequences of three standard isolates of *A. fumigatus*, which were included as controls, were identical to the published *A. fumigatus* β-tubulin sequence. The very low level of DNA sequence variation detected in standard isolates of *A. fumigatus* compared with other isolates from members of *Aspergillus* section *Fumigati* suggests that it may be a relatively recently evolved species. The 18S rRNA gene of two of the atypical isolates differed from that of *A. fumigatus* at a single nucleotide position. Phylogenetic analyses do not support the classification of all of these isolates as *A. viridinutans*. Thus, some of these isolates represent new species which are potential opportunistic pathogens.

Aspergillus fumigatus is the species most commonly associated with aspergillosis in immunocompromised patients. A number of DNA-based methods have been developed to detect *A. fumigatus* for diagnostic purposes and to type strains to study the origin and transmission of nosocomial infections. Diagnostic methods using PCR rely on amplification of specific DNA sequences, which could vary between strains. Sequence analysis of protein coding genes such as the cytochrome *b* gene (21) has revealed little or no sequence variation between standard isolates of *A. fumigatus*. In contrast, seven isolates of *Aspergillus viridinutans*, another asexual species in the section *Fumigati*, showed considerable genetic variability in β-tubulin gene sequences (19).

Members of the *Aspergillus* section *Fumigati* are distinguished by the profiles of mycotoxins and secondary metabolites that they produce (2) and, in the case of species with known sexual states (genus *Neosartorya*), the morphology of the ascospores (17). The evolutionary relationship of *A. fumigatus* to other species from *Aspergillus* section *Fumigati* has been investigated using DNA sequence data obtained from the β-tubulin gene (3), the hydrophobin gene (3), and the mitochondrial cytochrome *b* gene (21). These analyses have shown that morphological variation in isolates of *A. fumigatus* is not necessarily accompanied by variation in chemical profiles or DNA sequences (2, 21).

In a previous study, DNA sequence analysis of the alkaline protease gene (Alp) was used to investigate two atypical isolates of *A. fumigatus* (NSW3 and FRR 1266) (8). The sequence obtained from an isolate with standard morphology (QLD1) showed greater than 99% identity with published sequences for three human isolates. However, the two atypical isolates differed by more than 6 and 10% of the nucleotides, respectively (8). Subsequent phylogenetic analysis of β-tubulin sequences by Varga et al. (20) indicated that FRR 1266 is closely related to *A. viridinutans*. In this study, we use DNA sequence analysis of the Alp, β-tubulin, and 18S rRNA genes to clarify the taxonomic status of these isolates and four additional clinical isolates with similar characteristics.

MATERIALS AND METHODS

Fungal strains. The strains used in this study are listed in Table 1. Isolates MK245, MK246, MK284, and MK285 were obtained from the Mycology Reference Laboratory, Royal North Shore Hospital, Sydney, New South Wales, Australia, for identification. Strains NSW3 and QLD1 were obtained from ostriches suffering from aspergillosis (7). These six isolates were considered to be the causative agent of aspergillus infections. FRR 581, FRR 582, and FRR 1266 were provided by Ailsa Hocking and John Pitt of the Commonwealth Scientific and Industrial Research Organisation, Food Science Australia, North Ryde, New South Wales, Australia. The six atypical isolates described in this paper have been deposited in the Commonwealth Scientific and Industrial Research Organisation FRR Culture Collection, North Ryde, New South Wales, 1670 Australia.

PCR amplification. The PCR primers that were used in this study are listed in Table 2.

DNA sequencing. The DNA sequences of the PCR products were determined by a combination of direct sequencing of the PCR product and sequencing of the cloned PCR products and subclones derived thereof by standard molecular techniques (16). The sequences of both strands of the Alp gene PCR products were determined. The 18S rRNA gene PCR products were sequenced on one strand and compared to the sequence of the 18S rRNA gene of *A. fumigatus*. When nucleotide sequence differences were detected, they were confirmed by sequencing both strands. The β-tubulin PCR products were sequenced with the benA1 and benA2 primers as described in Geiser et al. (3).

Fungal morphology. The morphology of strains MK245, MK246, MK284, MK285, NSW3, and FRR 1266 was examined by John Pitt and Ailsa Hocking at Food Science Australia, North Ryde, New South Wales, Australia.

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TABLE 1. Source of fungal strains

Strain	Source	Country of origin	Morphology[a]	FRR no.[b]
MK245	Human transplant tissue Recipient, lung	Australia	Atypical	5678
MK246	Cat, thoracic mass	Australia	Atypical	5679
MK284	Cat, retrobulbar abscess	Australia	Atypical	5680
MK285	Cat, respiratory tract	Australia	Atypical	5681
NSW3	Ostrich, air sac-lungs	Australia	Atypical	5677
FRR 1266	Soil	Australia	Atypical	1266
QLD1	Ostrich, air sac-lungs	Australia	Standard	
FRR 581	City refuse	Indonesia	Standard	581
FRR 582	Soil	Indonesia	Standard	582

[a] Strains exhibiting colony morphology typical of *A. fumigatus* are listed as standard.
[b] FRR Culture Collection, Food Science Australia, P.O. Box 52, North Ryde, NSW 1670, Australia.

RESULTS

Alkaline protease gene amplification. Genetic variation in four clinical isolates (MK245, MK246, MK284, and MK285) was initially assessed by DNA sequence analysis of the Alp gene. Primers alp1 and alp2, which were used previously to amplify part of the Alp gene of *A. fumigatus* and genetic variants (7, 8), failed to yield amplification products from two of the isolates, MK246 and MK284. Longer primers (alp3 and alp4), based on regions of high similarity in the Alp genes of *A. fumigatus*, *Aspergillus oryzae*, *Aspergillus nidulans*, and *Aspergillus flavus*, were synthesized (Table 2) and were successfully used to amplify a 1.3-kb section of the Alp gene.

Phylogenetic analysis of alkaline protease gene sequences. The sequences of the Alp PCR products from the four clinical isolates were compared to the sequences from two strains which we had analyzed previously (NSW3 and FRR 1266) and published sequences for *A. fumigatus* and other *Aspergillus* species (Table 3). The isolates fell into two classes. The Alp gene sequence of MK245 showed the highest similarity to NSW3, and the Alp gene of both of these isolates showed a similar degree of nucleotide similarity to the Alp of *A. fumigatus* (93%) (Table 4). MK246, MK284, and MK285 showed the highest similarity to FRR 1266, and the Alp genes from this group of strain showed 88 to 89% identity to the *A. fumigatus* Alp gene. The Alp gene sequences of the four clinical isolates were each unique, but the MK246 and MK284 sequences differed by only 2%. Phylogenetic analysis of the Alp sequences supports the division of the six isolates into two groups (Fig. 1). MK245 and NSW3 form a cluster with *A. fumigatus* (cluster 1), whereas MK246, MK284, MK285, and FRR 1266 form a separate cluster in this tree (cluster 2).

TABLE 2. PCR primers used in this study

Name[a]	Target gene	Sequence
rRNA1	18S rRNA	5′-GTGAAACTGCGAATGGCTCA-3′
rRNA2	18S rRNA	5′-CCAACTTTCCGGCTCTGGGG-3′
alp1	Alkaline protease	5′-AAACGCAATCTGGAGCGTCG-3′
alp2	Alkaline protease	5′-CATTGCCATTGTAGGCAAGC-3′
alp3	Alkaline protease	5′-ATTCCTGGCAAGTACATCGTGAC CTTCAAG-3′
alp4	Alkaline protease	5′-ATTGCCATTGTAGGCAAGCTTGT TGGGGCT-3′
benA1	β-Tubulin	5′-AATAGGTGCCGCTTTCTGG-3′
benA2	β-Tubulin	5′-AGTTGTCGGGACGGAAGAG-3′

[a] Primer rRNA1 corresponds to nucleotides 43 to 62 and primer rRNA2 is complementary to nucleotides 1669 to 1688 in the 18S rRNA gene of *A. fumigatus* (11). Primer alp1 corresponds to nucleotides 480 to 499 and primer alp2 is complementary to nucleotides 1676 to 1695 in the Alp gene of *A. fumigatus* (6). Primer alp3 corresponds to nucleotides 393 in the Alp protease gene of *A. fumigatus* (6). Primers benA1 and benA2 are described in Geiser et al. (3).

Morphology. To determine whether the two groups of isolates were distinguished by morphological differences, the isolates were reexamined by John Pitt and Ailsa Hocking at Food Science Australia without prior knowledge of the results of the phylogenetic analysis. They concluded that all the isolates could be classified as *A. viridinutans*. Colonies of *A. viridinutans* resemble those of *A. fumigatus*, except that sporulation is reduced, so colonies are pale green blue rather than deep blue, and growth at 37°C is slower with a colony diameter of 50 mm after 7 days on Czapek yeast agar, rather than >70 mm. *A. viridinutans* is distinct microscopically in having stipes that are short, <50 μm long, and often bent just below the vesicle to give an asymmetrical appearance, termed nodding by Raper and Fennell (15). Stipes of *A. fumigatus* are straight and often 300 μm long. Vesicles are small and up to 15 μm wide, while those of *A. fumigatus* are up to 30 μm in diameter. *A. viridinutans* appears to be endemic to Australia (J. Pitt, personal communication).

Analysis of β-tubulin gene sequences. Partial β-tubulin gene sequences were available for a number of *A. viridinutans* isolates (19) and other species in *Aspergillus* section *Fumigati* (3, 20). To allow comparison of the six atypical isolates with other members of *Aspergillus* section *Fumigati*, the same section of the β-tubulin gene used in previous analyses was amplified with the primers designed by Geiser et al. (3), and the DNA sequences of the PCR products were determined.

Phylogenetic analysis of the β-tubulin gene sequences from the six atypical isolates, six isolates of *A. viridinutans* (19), and other species from *Aspergillus* section *Fumigati* is shown in Fig. 2. The phylogenetic tree generated by the DNA sequence parsimony method is similar to that shown in Fig. 1 in that it places the cluster 1 and 2 isolates in separate lineages. However, the topology within cluster 1 differs with respect to the position of MK285 (cluster 1b). The cluster 1a isolates (MK246, MK284, and FRR 1266) are grouped with four of the six *A. viridinutans* isolates, including the type strain IMI 062875. The tree also indicates that MK285 is closely related to *Neosartorya aureola* (cluster 1b), NSW3 to *Neosartorya spinosa* (cluster 2c), and MK245 to *A. viridinutans* IMI 306135 (cluster 2b). The neighbor-joining method gave a tree with identical topology, except that the positions of *Aspergillus brevipes* and *Neosartorya pseudofischeri* were exchanged (data not shown).

Partial β-tubulin gene sequences were obtained from three standard isolates of *A. fumigatus* (Table 1). All sequences were identical to the *A. fumigatus* sequence reported by Geiser et al. (3). In contrast, a maximum of 98% similarity was observed in the β-tubulin gene sequences of the other isolates that were analyzed (Table 5).

Sequence analysis of the 18S rRNA gene. The DNA sequence of a 347-bp section of the 18S rRNA gene of NSW3 and FRR 1266 had previously been shown to be identical to the sequence of the 18S rRNA gene of *A. fumigatus* (8). To further investigate the taxonomic status of these two strains and the

TABLE 3. Sequences used in phylogenetic analyses

Species	Strain	Gene	Accession no.[a]	Reference
Aspergillus sp.	MK245	Alkaline protease	AY590134 (G)	This study
		β-Tubulin	AY590128 (G)	This study
Aspergillus sp.	MK246	Alkaline protease	AY590135 (G)	This study
		β-Tubulin	AY590129 (G)	This study
Aspergillus sp.	MK284	Alkaline protease	AY590136 (G)	This study
		β-Tubulin	AY590130 (G)	This study
Aspergillus sp.	MK285	Alkaline protease	AY590137 (G)	This study
		β-Tubulin	AY590133 (G)	This study
Aspergillus sp.	NSW3	Alkaline protease	Y15871 (E)	8
		β-Tubulin	AY590132 (G)	This study
Aspergillus sp.	FRR 1266	Alkaline protease	Y15873 (E)	8
		β-Tubulin	AY590131 (G)	This study
A. brevipes	NRRL 2439	β-Tubulin	AF057311 (G)	3
Aspergillus clavatus	H522	β-Tubulin	AF057312 (G)	3
A. flavus	28	Alkaline protease	S67840 (E)	14
Aspergillus fumigatus var. *ellipticus*	NRRL 5109	β-Tubulin	AF057314 (G)	3
A. fumigatus	HD133	β-Tubulin	AF057315 (G)	3
	CHUV-192-88	Alkaline protease	Z11580 (Y)	6
A. nidulans	MH2	Alkaline protease	L31778 (G)	9
A. oryzae	ATCC 20386	Alkaline protease	S79617 (G)	10
Aspergillus niger	N400	Alkaline protease	L19059 (G)	5
A. viridinutans	IMI 062875	β-Tubulin	AF134779 (G)	19
A. viridinutans	IMI 133982	β-Tubulin	AF134775 (G)	19
A. viridinutans	IMI 182127	β-Tubulin	AF134777 (G)	19
A. viridinutans	IMI 280490	β-Tubulin	AF134780 (G)	19
A. viridinutans	NRRL 6106	β-Tubulin	AF134778 (G)	19
N. aureola	NRRL 2244	β-Tubulin	AF057319 (G)	3
N. fischeri	NRRL 181	β-Tubulin	AF057322 (G)	3
N. pseudofischeri	NRRL 20748	β-Tubulin	AF057325 (G)	3
N. spinosa	NRRL 5034	β-Tubulin	AF057329 (G)	3
Trichoderma harzianum	IMI 206040	Alkaline protease	M87518 (G)	4

[a] Sequences were obtained from GenBank (G) or EMBL (E) DNA databases through the Australian National Genome Information Service (ANGIS).

four atypical clinical isolates, 1.6 kb of the 18S rRNA gene from each isolate was determined. The DNA sequence of the PCR product generated with primers rRNA1 and rRNA2 (Table 2) from the cluster 1a isolates MK246 and MK285 contained a single nucleotide difference, T instead of C at position 483 of the *A. fumigatus* 18S rRNA gene sequence reported by Nikkuni et al. (11). The sequences of the remaining four atypical isolates in clusters 1 and 2 were identical to the *A. fumigatus* 18S rRNA gene.

TABLE 4. DNA sequence similarity in the alkaline protease genes of *Aspergillus*[a]

Organism[b]	1	2	3	4	5	6	7	8	9	10	11	12
1 *A. fumigatus*	100											
2 NSW3	93	100										
3 MK245	93	95	100									
4 FRR 1266	88	92	90	100								
5 MK285	89	91	90	94	100							
6 MK246	89	91	91	94	94	100						
7 MK284	89	91	91	94	95	98	100					
8 *A. oryzae*	72	73	72	70	71	71	71	100				
9 *A. nidulans*	68	69	69	69	69	69	69	65	100			
10 *A. flavus*	67	67	67	66	66	66	66	67	64	100		
11 *A. niger*	61	62	62	61	61	61	61	60	59	62	100	
12 *T. harzianum*	47	48	48	48	47	47	47	47	47	49	45	100

[a] Percent identical nucleotides are given for the sequences corresponding to nucleotides 480 to 1664 in the Alp gene of *A. fumigatus* (6).
[b] The source of the Alp gene sequences is given in Table 3.

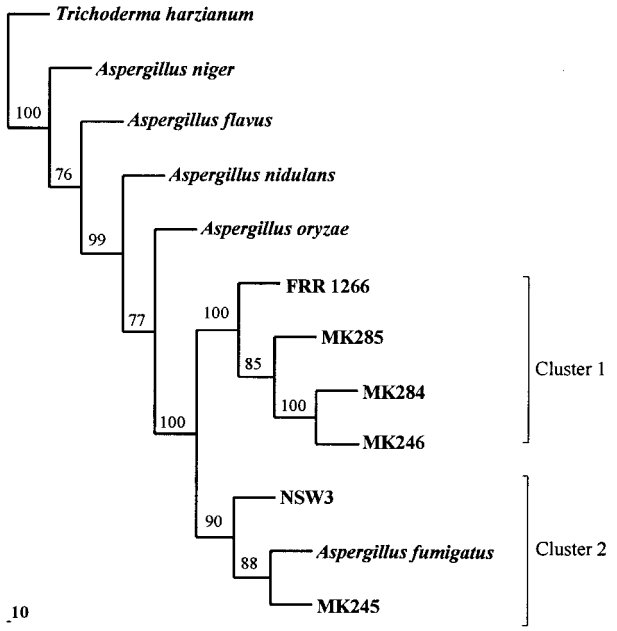

FIG. 1. Phylogenetic tree based on the DNA sequences corresponding to nucleotides 480 to 1664 in the alkaline protease gene of *A. fumigatus* (6). The sources of the DNA sequences used in the analysis are given in Table 3. The tree was constructed using the PILEUP, ESEQBOOT, EDNAPARS, and ECONSENSE programs in the PHYLIP computer package (1) through the Australian National Genome Information Service (ANGIS). Only bootstrap values of >60% are shown.

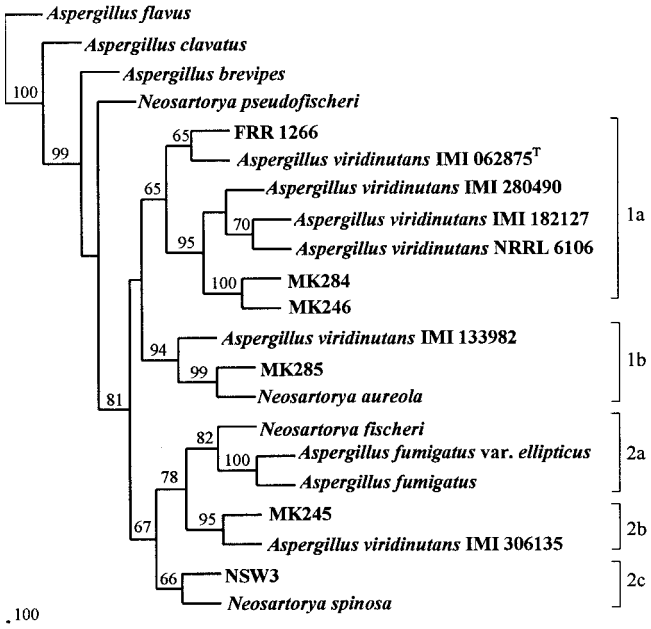

FIG. 2. Phylogenetic tree based on the DNA sequences corresponding to nucleotides 1 to 453 in the partial β-tubulin gene sequence of *A. fumigatus* (3). The sources of the DNA sequences used in the analysis are given in Table 3. The tree was constructed using the PILEUP, ESEQBOOT, EDNAPARS, and ECONSENSE programs in the PHYLIP computer package (1) available through ANGIS. Only bootstrap values of >60% are shown. The type strain (T) of *A. viridinutans* is indicated.

DISCUSSION

Though morphological examination of the two isolates from cluster 2 (NSW3 and MK245) indicated that they could be classified as *A. viridinutans*, phylogenetic analysis did not. It has been shown that species recognition based on morphological differences often leads to the inclusion of two or more species, as defined by phylogenetic analyses or mating tests, in a single morphological species (18). For example, nine species were recognized within *Fusarium graminearum*, based on the phylogenetic analysis of 11 genes (12). Morphological characters, which could be used to distinguish some of these phylogenetically distinct species, were subsequently identified. It may be that further phenotypic examination of the isolates will reveal characteristics which distinguish the atypical isolates in cluster 1 from cluster 2.

TABLE 5. DNA sequence similarity in the β-tubulin genes of *Aspergillus*[a]

Organism[b]	1	2	3	4	5	6	7	8	9	10	11
1 *A. fumigatus*	100										
2 *A. viridinutans* IMI 062875	90	100									
3 NSW3	92	96	100								
4 MK245	91	94	95	100							
5 MK285	90	96	95	93	100						
6 FRR 1266	90	96	96	93	95	100					
7 MK246	89	95	94	92	94	94	100				
8 MK284	89	94	95	92	93	94	98	100			
9 *N. fischeri*	94	93	95	94	93	93	92	91	100		
10 *N. aureola*	88	93	95	93	98	94	93	92	92	100	
11 *N. spinosa*	92	97	98	95	96	96	95	94	96	95	100

[a] Percent identical nucleotides are given for the sequences corresponding to nucleotides 1 to 453 in the partial β-tubulin gene sequence of *A. fumigatus* (3). For each pair of sequences, the shorter sequence was used in calculating percent identical nucleotides.

[b] The source of the β-tubulin gene sequences is given in Table 3.

The partial β-tubulin gene sequences of two of the atypical isolates show 98% identity with *Neosartorya* species, NSW3 with *N. spinosa* (cluster 2c) and MK285 with *N. aureola* (cluster 1b). These results suggest that these two isolates may represent asexual mutants derived from *Neosartorya* species or may be closely related asexual species.

MK245 (cluster 2b) grouped with *A. viridinutans* IMI 306135 (isolated from soil in western Australia) which Varga suggested may represent a new species or a highly unusual isolate of *A. fumigatus* based on toxin profiles, mitochondrial DNA, restriction fragment length polymorphism analyses, and partial β-tubulin sequences (19). Thus, cluster 2b represents an undescribed species of *Aspergillus* that is capable of infecting immunocompromised patients.

The partial β-tubulin gene sequences of all the atypical isolates and strains classified as *A. viridinutans* were unique. In contrast, the partial β-tubulin gene sequences of three standard *A. fumigatus* isolates from Australia and Indonesia are identical to the sequence obtained from *A. fumigatus* strain HD133 from the Institut Pasteur in Paris, France. The very low level of DNA sequence variation detected in *A. fumigatus* compared with other isolates from members of *Aspergillus* section *Fumigati* suggests that it may be a relatively recently evolved species.

DNA sequence analysis of two protein-encoding genes of the cluster 1a isolates revealed a considerable degree of genetic variation in this group of strains (from 2 to 8% in pairwise comparisons) (Table 5 and data not shown). The high level of DNA sequence variation and phylogenetic analyses indicate that this group may contain several cryptic species. However, the separate clades within cluster 1a are not all strongly supported. Furthermore, the phylogenetic tree of Alp sequences includes MK285 within this group of atypical isolates, whereas the tree of β-tubulin sequences does not. The phylogenetic species recognition approach proposed by Taylor et al. (18) is based on concordance of multiple gene genealogies. As only two polymorphic genes have been analyzed and the resulting two trees are not concordant, the asexual strains within cluster 1 could be considered a single species (*A. viridinutans*) by this method. Analysis of additional genes will be required to clarify the taxonomic status of this group.

Analysis of the 18S rRNA gene showed that four of the isolates had a sequence identical to *A. fumigatus*, and the others (MK246 and MK284) differed at only one position. Thus, some of the isolates in clusters 1 and 2 share an identical 18S rRNA gene sequence. The sequence of the *Neosartorya fischeri* 18S rRNA gene (GenBank accession no. NFU21299) contains a single-base-pair deletion corresponding to position 1327 of the *A. fumigatus* sequence (8). Based on our experience with sequencing the 18S rRNA gene, this difference is likely to be due to a sequencing error, as we have observed sequencing anomalies at exactly that position. The sequences of the *A. viridinutans*, *N. aureola*, and *N. spinosa* 18S rRNA genes have

not been determined. Sequence analysis of the 28S rRNA genes of *A. fumigatus*, *N. fischeri*, *N. aureola*, and *N. spinosa* strains has revealed sequence differences between these four species (13). There is no accepted rule on the number of nucleotide differences in rRNA genes which define different species or genera.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Ron Wicks and Karen Gray. We thank John Pitt and Ailsa Hocking for morphological examinations and David Backhouse for helpful discussions.

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