Pollen morphology of Nothofagus (Nothofagaceae, Fagales) and its phylogenetic significance

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ABSTRACT. Nothofagaceae (southern beeches) are a relatively small flowering plant family of trees confined to the Southern Hemisphere. The fossil record of the family is abundant and it has been widely used as a test case for the classic hypothesis that Antarctica, Patagonia, Australia and New Zealand were once joined together. Although the phylogenetic relationships in Nothofagus appear to be well supported, the evolution of some pollen morphological traits remains elusive, largely because of the lack of ultrastructural analyses. Here we describe the pollen morphology of all extant South American species of Nothofagus, using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy (LM), and reconstruct ancestral character states using a well-supported phylogenetic tree of the family. Our results indicate that the main differences between pollen of subgenera Fuscospora (pollen type fusca a) and Nothofagus (pollen type fusca b) are related to the size of microspines (distinguishable or not in optical section), and the thickening of colpi margins (thickened inwards, or thickened both inwards and outwards). In particular, Nothofagus alessandrii, the only extant South American species of subgenus Fuscospora, presents distinctive pollen features that have not been observed in any other species of the genus (i.e. a large granular infratectum and spongy apertural endexine). Species of subgenus Lophozonia are characterized by having the largest pollen grains, with polygonal outline in polar view, microspines distinguishable in optical section, long and non-thickened colpi, and a thin endexine. The reconstruction of character states for the node corresponding to the common ancestor to genus Nothofagus leads us to conclude that the ancestral form of Nothofagaceae should have had: equatorial diameter < 40 μm, circular outline in polar view, microspines distinguishable in optical section, long and non-thickened colpi, and a thin endexine. These features are fully consistent with those present in Nothofagidites senectus Dettmann & Playford, the oldest fossil species of Nothofagaceae recorded in Campanian-Maastrichtian sediments of Gondwana.

KEYWORDS: Nothofagus, South America, pollen morphology, exine structure, character evolution

INTRODUCTION

Nothofagus Blume, the only member of the family Nothofagaceae (Kuprianova 1962), comprises ca 42 species of prominent trees of the Southern Hemisphere. It grows in forests of southeastern Australia including Tasmania, New Caledonia, New Guinea, New Zealand, and southwestern South America (Romero 1977). It comprises four subgenera, Lophozonia, Fuscospora, Nothofagus and, Brassospora recognized on the basis of cupule morphology, leaf architecture and cuticular morphology (Hill & Read 1991). Nothofagus has been a test case for the hypothesis that Gondwanan vicariance can explain major biogeographic patterns; for that reason its evolutionary history has motivated major studies based on its pollen morphology, fossil record and cladistic biogeography (Sauquet et al. 2012).

Morphological features of *Nothofagus* pollen have been studied in some detail. For example, Praglowski (1980, 1982) examined the pollen of seven species of *Nothofagus* and defined three types named fusca, menziesii and brassii, which differ mainly in features of the colpi.

Dettmann et al. (1990) studied extant and fossil pollen of *Nothofagus* and found larger diversity in fossil pollen grains, distinguishing four subtypes in addition to those defined by Praglowski (1982). These subtypes were circumscribed on the basis of characters of the colpi, outline of the pollen, and the ratio between length of the colpi and equatorial diameter. Wang et al. (2000) confirmed the division into three pollen types proposed by Praglowski (1982) from study of pollen of 26 species using LM, SEM and MET.

The correspondence between the *Nothofagus* subgenera and their pollen morphology has been explored using a widely accepted phylogeny of the family (Manos 1997). However, an ultrastructural analysis of the pollen grains has never been taken into account. The main objectives of the present study are to (1) provide more extensive knowledge of pollen morphology of South American species of *Nothofagus* using light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM), including species of subgenus *Lophozonia* which have never been observed under SEM and TEM and (2) sketch the evolutionary trends of pollen features on the maximum likelihood tree provided by Sauquet et al. (2012).

**MATERIAL AND METHODS**

Pollen grains from all South American species of *Nothofagus* (*N. alessandrii*, *N. alpina*, *N. glauca*, *N. obliqua*, *N. antarctica*, *N. betuloides*, *N. dombeyi* and *N. nitida*) and *Betula platyphylla* were removed from anthers of herbarium specimens from CONC, LP and SI (herbarium acronyms follow Index Herbariorum [http://sciweb.nybg.org/science2/IndexHerbariorum.asp](http://sciweb.nybg.org/science2/IndexHerbariorum.asp)).

Specimens studied:

- *Nothofagus alessandrii* Espinosa: NN (CONC 155232).
- *Nothofagus alpina* (Poepp. and Endl.) Oerst.: Schajavskoy (LP).
- *Nothofagus antarctica* (G. Forst.) Oerst.: Delucchi 591 (LP).
- *Nothofagus betuloides* (Mirb.) Oerst.: Guerrido et al. 626 (SI).
- *Nothofagus dombeyi* (Mirb.) Oerst.: Delucchi 607 (LP).
- *Nothofagus obliqua* (Mirb.) Oerst.: Schajavskoy (LP).

For light microscopy (LM), pollen was acetolysed according to Erdtman (1960); the slides were prepared by mounting the pollen in glycerol jelly and sealing with paraffin. For SEM, acetolysed and non-acetolysed pollen grains were suspended in 90% ethanol, mounted on stubs and examined using a Philips XL30 TMP SEM at the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”. For TEM, the fresh pollen grains were fixed in 1.5% glutaraldehyde and then buffered in 2% OsO<sub>4</sub> for 2 hours at room temperature; they were washed for 30 minutes in distilled water, dehydrated in an ethanol series and finally embedded in acetone-Spurr 3:1 for 6 h and twice in Spurr for 24 h. Ultrathin sections were cut using a diamond knife fitted into a Sorvall Porter-Blum MT2-B ultramicrotome. Sections were mounted in single grids and stained with lead citrate (1 min) and uranyl acetate (10 min). The observations were made with a Jeol JEM 1200 EX II transmission electron microscope from the Servicio Central de Microscopía Electrónica of the Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata.

The classical taxonomy of genus *Nothofagus* (*Nothofagaceae=Nothofagus*) is followed in this contribution (Hill & Read 1991). Terminology used for pollen description follows Punt et al. (2007). We selected six characters that were found to be variable among *Nothofagus* taxa: equatorial diameter, outline in polar view, aperture length, microspines (distinguishable or not in optical section), endexine thickening at the colpi level, and ratio of endexine/ectexine thickness. We avoided using the polar and equatorial diameter ratio because *Nothofagus* pollen grains are oblate to per-oblate and are usually observed only in polar view. We also avoided using the character “shape of aperture ends” of Dettmann (1990); we instead used the character “endexine thickening at the apertures”. In fact, if the endexine is thickened at the apertural level the ends of the colpi are well delimited, whereas if the endexine is not thickened at the apertural level, the ends of the colpi appear as fissures.

The definition and states of each character together with the data matrix are given in Table 1. Equatorial diameter, outline in polar view, aperture length, microspines and endexine thickening at aperture level were measured on 25 pollen grains under LM (Appendix A); and the ratio of endexine/ectexine thickness was measured on 10 grains under TEM.
We reconstructed ancestral character states of *Nothofagus* pollen using Mesquite 3.10 (Maddison & Maddison 2016) functions Likelihood Ancestral States (ML), under the Mk1 model of evolution, and Parsimony Ancestral States (MP) on the published phylogenetic tree of Sauquet et al. (2012).

Data for species of subgenus *Brassospora* were taken from Praglowski (1980, 1982) and the Austral-Asian Pollen and Spore Atlas (APSA Members 2007). We use the outgroups selected by Sauquet et al. (2012), *Betula platyphylla* Roth. and *Fagus grandiflora* Ehrh. *Betula platyphylla* ultrastructure only data were taken from El-Ghazaly (1999), El-Ghazaly and Huysmans (2001) and Blackmore et al. (2003), and *Fagus grandiflora* data from Praglowski (1982) and Heenan and Smissen (2013).

### RESULTS AND DISCUSSION

*Nothofagus* pollen grains appear to be similar among species, but they show some variation in size, shape, apertural features, ornamentation and exine structure. These characters are distinguishable under LM, which is useful when comparing with fossil pollen grains. Additionally, details of exine structure revealed under TEM reinforce the characterization of the features optimized in the phylogenetic tree of Sauquet et al. (2012).

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**Table 1. Data matrix of exine characters**

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Exine characters used in this analysis.
1 – Equatorial diameter < 40 μm (0), > 40 μm (1).
2 – Outline in polar view (amb) polygonal (0), circular (1), star-like (2).
3 – Aperture length < 11 μm (0), 14–17 μm (1), 25–29 μm (2).
4 – Microspines not distinguishable in optical section (0), distinguishable in optical section (1).
5 – Endexine thickening at aperture level present inwards and outwards (0), present inwards (1), absent (2).
6 – Endexine/ectexine thickness ratio ca 1:10 (0), ca 1:2 (1).
GENERAL POLLEN MORPHOLOGY

LM

Pollen grains are isopolar, radially symmetrical, oblate to peroblate, subcircular to elliptic in equatorial view (Pl. 1, fig. 9), with convex or nearly straight mesocolpia in polar view (Pl. 1, figs 1–8). The equatorial diameter range of the analysed species is 26–57 μm; *Nothofagus alessandrii* is the smallest and *N. alpina* the largest (Appendix A). The apertures are 4–7 stephanocolpate, colpi are narrow and marginate with rounded ends and parallel edges, or have a fissurate aspect (as a subtle interruption of the exine) (Pl. 1, figs 1–3). Pollen grains with marginate colpi have the exine thickened inwards at the aperture level (Pl. 1, fig. 4) or have both inwards- and outwards-thickened margins (i.e. protruding above the surface) (Pl. 1, figs 5–9). The exine is microechinate and the surface is psilate between microspines in all species. Sexine and nexine are not distinguishable under LM.

SEM

The sculpture consists of minute and uneven-sized microspines (Pl. 2–4, figs 2, 6, 10) which are mostly conical. In *Nothofagus glauca* and *N. alessandrii*, the microspines appear to be connected with delicate strands of sporopollenin (Pl. 2, fig. 6; Pl. 3, fig. 2). In *N. alessandrii* the microspines are conspicuous (Pl. 1, fig. 4).

TEM

The ectexine is 0.5–1 μm thick; it consists of a compact tectum 0.2–0.4 μm thick, a thin granular infratectum and a continuous foot layer as thick as the tectum. The microspines are compact and appear to be deep-rooted in the endexine (Pl. 2, fig. 7).

The endexine is 0.1–0.2 μm thick (Pl. 2–4, figs 3, 7, 11). In the species of subgenus *Nothofagus* it is thickened both inwards and outwards at the colpi level, forming a margin (Pl. 3, figs 8, 12; Pl. 4, figs 4, 8, 12).
Remarks: in the species of subgenus *Nothofagus* the intine is notably thickened beneath the aperture, forming an oncus (Pl. 3, figs 8, 12; Pl. 4, fig. 4). In *Nothofagus glauca*, *N. antarctica*, *N. dombeyi* and *N. nitida* a lamellate layer is situated between the endexine and the intine (Pl. 2, fig. 8; Pl. 3, figs 8, 12; Pl. 4, fig. 4). This layer is comparable with the MGL (membranous granular layer) defined by El-Ghazaly and Huysmans (2001).

**DIAGNOSTIC FEATURES OF SUBGENERA**

Subgenus *Lophozonia* (menziesii type)

Pl. 1, figs 1–3; Pl. 2

The pollen has straight or more or less convex mesocolpia; the outline in polar view is circular to polygonal (Pl. 1, figs 1–3). The pollen grains are the largest within the genus (Appendix A). Colpi (4–8) are long, reaching

**Fig. 2.** Equatorial diameter (a) and outline in polar view (b), of pollen grains of *Fagus grandifolia*, *Betula platyphylla* and *Nothofagus* sp. div., optimized onto the ML tree of Sauquet et al. (2012) using the Mesquite program, version 3.10 (Maddison & Maddison 2016). Circles at the tips represent the observed character states in each taxon, while the circles of the nodes are pie diagrams that indicate the likelihood of each state. The likelihood reconstruction finds the state that maximizes the probability of arriving at the observed states in the terminal taxa, given the Mk1 model of evolution, and considering all possible assignments to the other ancestral states.
15 µm, and have a fissurate appearance. The exine is thin (1 µm thick or less). Microspines are usually uneven-sized and distinguishable in optical section. The endexine/ectexine thickness ratio is ca 1:10 – 1:5 at the mesocolpia (equatorial section) (Pl. 2, figs 3, 7, 11). The granular infratectum is ca 0.1 µm thick.

Remarks and comparisons. *N. alpina* (Pl. 1, fig. 1; Pl. 2, figs 1–4): the foot layer is lacking at colpi margins and the endexine is disrupted defining a concave surface (Pl. 2, fig. 4; Fig. 1a).

*N. glauca* (Pl. 1, fig. 2; Pl. 2, figs 5–8): the colpi appear as an abrupt gap at the ectexine level. The colpi margins are poorly defined. The microspines have rounded tips and are connected by delicate strands; the tectum surface between microspines is scabrate (Pl. 2, fig. 6). The endexine is slightly thickened at the colpi margins. A lamellate layer is situated between the endexine and the intine (Pl. 2, fig. 8; Fig. 1b). This layer is comparable with the MGL.

*N. obliqua* (Pl. 1, fig. 3; Pl. 2, figs 9–12): an abrupt gap in the ectexine is observed at the aperture level. The endexine persists discontinuously in the aperture (Pl. 2, fig. 12; Fig. 1c). The intine is 1.4 µm thick, with two layers, the external (ca 0.7 µm thick) denser and darker than the internal. Both layers have lamellar texture (Pl. 2, fig. 12; Fig. 1c).

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**Fig. 3.** Pollen grains of *Fagus grandifolia, Betula platyphylla* and *Nothofagus* sp. div. Aperture length (a) and microspines (distinguishable or not in optical section) (b), optimized onto the ML tree of Sauquet et al. (2012) using Mesquite program.
Subgenus *Fuscospora* (*fusca* a type)

Pl. 1, fig. 4; Pl. 3, figs 1–4

The pollen has slightly convex or sometimes straight mesocolpia; the outline in polar view is circular to subcircular (Pl. 1, fig. 4). The pollen grains are the smallest within the genus (Appendix A). The colpi (5–7) are short, with rounded ends, with inwards-thickened colpi margins (Pl. 1, figs 5–9; Pl. 3, fig. 4; Fig. 1d). The microspines are conspicuous, connected by delicate sinuous strands distinguishable under SEM (Pl. 3, figs 5, 8, 9, 12; Pl. 4, figs 1, 4, 5, 8, 9, 12; Fig. 1e). The exine is thicker than that observed in subgenera *Nothofagus* and *Lophozonia* (Pl. 3, figs 3, 4). The endexine has a spongy appearance at the level of colpi margins (Pl. 3, fig. 4).

Subgenus *Nothofagus* (*fusca* b type)

Pl. 1, figs 5–9; Pl. 3, figs 5–12; Pl. 4, figs 1–12

The pollen has slightly convex mesocolpia; the outline in polar view is subcircular. The equatorial diameter range is 26–40 μm. The colpi (4–7) are short, with rounded ends and parallel edges, and marginate (Pl. 1, figs 5–9). The margins are formed by both inwards- and outwards-thickened endexine (Pl. 3, figs 5, 8, 9, 12; Pl. 4, figs 1, 4, 5, 8, 9, 12; Fig. 1e). The microspines are not distinguishable in optical section (Pl. 1, figs 5–9). The exine is thicker than that
observed in the menziesii type (Appendix A; Pl. 3, figs 7, 11; Pl. 4, figs 3, 7, 11; Fig. 1e). The endexine/ectexine thickness ratio is ca 1:10 – 1:5 at the equatorial mesocolpia (Appendix A). The granular infractectum is ca 0.1 μm thick (Pl. 3, figs 7, 11; Pl. 4, figs 3, 7, 11; Fig. 1e). The endexine at colpi level is more compact than that in the fusca type (a), and 1–1.5 μm thick (Pl. 3, figs 8, 12; Pl. 4, figs 4, 8, 12; Fig. 1e).

Remarks and comparisons. N. antarctica (Pl. 1, fig. 5; Pl. 3, figs 5–8), N. dombei (Pl. 1, fig. 6; Pl. 3, figs 9–12) and N. nitida (Pl. 1, fig. 7; Pl. 4, figs 1–4) show a lamellate layer between the endexine and the intine comparable with the MGL (Pl. 3, figs 8, 12; Pl. 4, fig. 4). In particular, N. nitida has an exine less than 1 μm thick (Appendix A).

N. pumilio (Pl. 1, fig. 8; Pl. 4, figs 5–8): exine less than 1 μm thick (Appendix A).

N. betuloides (Pl. 1, fig. 9; Pl. 4, figs 9–12): with microspines stronger than in the other species of the subgenus Nothofagus. The endexine is sometimes barely distinguishable from the intine (Pl. 4, fig. 11).

PHYLOGENETIC SIGNIFICANCE OF EXINE CHARACTERS

The results of optimizing selected pollen features onto the ML tree of Sauquet et al. (2012) (Figs 2–4) allow us to formulate hypotheses on their evolution across the family. It is worth noting that the results obtained with the ML method agree with those obtained using MP (see supplementary figures).

Equatorial diameter (Fig. 2a): small size (less than 40 μm) is the ancestral (plesiomorphic) character state (p = 0.82) present in the outgroups (Betula and Fagus) and retained in subgenera Fuscospora, Nothofagus and Brassospora. Equatorial diameter larger than 40 μm is a synapomorphy of subgenus Lophozonia.

Outline in polar view (amb) (Fig. 2b): circular outline is reconstructed as the ancestral character state shared by Betula, Fagus, basal species of subgenus Fuscospora (Nothofagus alessandrii and N. gunnii), and Nothofagus (p = 0.57). Polygonal outline is a derived state that appears as parallelisms in Lophozonia (with a reversal to circular in N. glauca) and in Fuscospora (N. truncata, N. fusca and N. solandri). Subgenus Brassospora has a derived star-like outline that is a synapomorphy of this clade.

Aperture length (Fig. 3a): the external aperture of Fagus is a relatively long colpus (l = 25–29 μm). Betula presents a pore (diameter = 1–5 μm). A short aperture (l = 1–11 μm) is reconstructed as an ancestral character state shared by Betula, Fuscospora, Nothofagus and Brassospora (p = 0.70). Medium-sized colpus (l = 14–17 μm) is a synapomorphy of subgenus Lophozonia.

Microspines (Fig. 3b): microspines distinguishable in optical section is the inferred ancestral state in the genus (p = 0.97). Only subgenus Nothofagus has microspines not distinguishable in optical section, which is derived within genus Nothofagus and homoplastic with the same state in Betula. Fagus is psilate; using SEM the surface is verrucar-rugulose (Praglowski 1982).

Endexine thickenings at the apertures (Fig. 4a): endexine thickenings (of two types) are shared by Betula, Fagus and all the ingroup with the exception of subgenus Lophozonia (p = 0.59), which seems to have lost them during the early evolution of the family. This loss appears to be a synapomorphy of Lophozonia. The apertural endexine is thickened inwards in Fagus, Fuscospora and Brassospora, while Betula and subgenus Nothofagus shared, as a parallelism, apertural endexine thickened outwards, protruding above the surface.

Endexine/ectexine thickness ratio (Fig. 4b): a thin endexine (endexine/ectexine thickness ratio ca 1:10) is reconstructed as an ancestral character state present in the outgroups and retained in subgenera Fuscospora, Nothofagus and Lophozonia (p = 0.99). A thicker endexine (endexine/ectexine thickness ratio ca 1:2) is a synapomorphy of subgenus Brassospora, this may be functionally or developmentally related to the characteristic star-like, concave polygonal pollen grains of this subgenus.

CONCLUSION

Our study of the pollen morphology of species of Nothofagus – some of them previously poorly studied, such as N. alpina, N. glauca and N. nitida – under LM, SEM and TEM, and the optimization of pollen characters on a well supported phylogenetic tree, provide a better understanding of their evolution within
the genus, and also further evidence that the South American species cannot be recognized to a lower taxonomic level than subgenus on the basis of pollen morphology. In general, the morphology is in line with that reported in previous contributions, such as Praglowski (1980, 1982), Zheng et al. (1999) and Wang et al. (2000). Equatorial diameter, outline in polar view, ornamentation and apertural morphology are the most distinctive features. Concerning the pollen of subgenera Fuscospora and Nothofagus, regarded as indistinguishable by Manos (1997), our examination showed that pollen of Fuscospora have microspines distinguishable in optical section and endexine thickened inwards at colpi level, whereas pollen of species of subgenus Nothofagus has microspines not distinguishable in optical section and the endexine thickened both inwards and, outwards at colpi level. N. alessandrii, the only extant South American species of subgenus Fuscospora, presents distinctive ultrastructural features of the exine that have not been observed in any other species of the genus (e.g. thick granular infratectum, spongy apertural endexine) (Appendix A; Pl. 1, fig. 4; Pl. 3, figs 1–4).

Morphological differences between the pollen grains of the subgenus Lophozonia and the rest of the family are noticeable. This subgenus has larger pollen grains, a polygonal outline in polar view, microspines distinguishable in optical section, colpi long, and without endexine thickening and a thin endexine (Appendix A; Pl. 1, figs 1–3; Pl. 2, figs 1–12).

Reconstruction of character states for the node corresponding to the common ancestor of genus Nothofagus leads us to conclude that it had a small equatorial diameter, circular amb, microspines distinguishable in optical section, short apertures thickened inwards and a thin endexine. These features are fully consistent with those recorded in Nothofagidites senectus Dettmann & Playford, the oldest fossil species of the family, recorded from the Campanian and Maastrichtian over wide areas of southern Gondwana (Dettmann et al. 1990). This congruence increases confidence in both the phylogeny and the fossils as reliable records of diversification of the genus.

Future work on fossils of extinct pollen types of Nothofagus, including SEM and TEM analyses, would provide further evidence to explore the evolutionary significance of the here analysed pollen characters and to test palearctic biogeographic hypotheses related to the presence of the subgenus Brassospora in Cenozoic sediments from Patagonia (Dettmann et al. 1990).

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PLATES
Plate 1

Pollen of genus *Nothofagus* under LM. Most grains are in polar view

1. *Nothofagus alpina* (Schajavskoy, LP)
2. *Nothofagus glauca* (Picca 160, SI)
3. *Nothofagus obliqua* (Schajavskoy, LP)
4. *Nothofagus alessandrii* (NN, CONC 155232)
5. *Nothofagus antarctica* (Delucchi 591, LP)
6. *Nothofagus dombeyi* (Delucchi 607, LP)
7. *Nothofagus nitida* (Picca 220, SI)
8. *Nothofagus pumilio* (Gentili, LP)
9. *Nothofagus betuloides* (Guerrido et al. 626, SI). Subequatorial view

Scale bar: 10 μm
Plate 2

Pollen of subgenus *Lophozonia*

1–4. *Nothofagus alpina* (Schajavskoy, LP)
   1. SEM, polar view showing apertures (arrows)
   2. SEM, detail of microspines
   3. TEM, exine section of mesocolpium showing the thin granular infratectum and the microspines deep-rooted in the endexine
   4. SEM, aperture showing the disrupted endexine (arrows)

   5. SEM, subequatorial view showing apertures (arrows)
   6. SEM, microspines are connected by delicate strands (arrows)
   7. TEM, exine section of mesocolpium showing the thin granular infratectum and the microspines deep-rooted in the endexine
   8. SEM, aperture showing the lamellate layer (arrows)

9–12. *Nothofagus obliqua* (Schajavskoy, LP)
   9. SEM, polar view
   10. SEM, detail of microspines
   11. TEM, exine section of mesocolpium showing the thin granular infratectum and the microspines deep-rooted in the endexine
   12. SEM, aperture showing endexine remains (arrows)

Abbreviations: In – intine; E – endexine; F – foot layer; G – granular infratectum; T – tectum; Ms – microspines.

Scale bars of 1, 5 and 9: 10 µm; of 2–4, 6–8 and 10–12: 2 µm
Plate 3

Pollen of subgenus *Fuscospora* and *Nothofagus*

1–4. *Nothofagus alessandrii* (NN, CONC 155232)
   1. SEM, subequatorial view
   2. SEM, microspines are connected by delicate strands (arrows)
   3. TEM, exine section of mesocolpium showing the relatively thick granular infratectum. Section is not perpendicular to the surface
   4. SEM, aperture showing the endexine with spongy appearance (arrows)

5–8. *Nothofagus antarctica* (Delucchi 591, LP)
   5. SEM, subequatorial view
   6. SEM, detail of microspines
   7. TEM, exine section of mesocolpium
   8. SEM, aperture showing the endexine thickenings and the lamellate layer between the endexine and the intine (arrows)

9–12. *Nothofagus dombeyi* (Delucchi 607, LP)
   9. SEM, subpolar view
  10. SEM, detail of microspines
  11. TEM, exine section of mesocolpium
  12. SEM, aperture showing the endexine thickenings and the lamellate layer between the endexine and the intine (arrows)

Abbreviations: In – intine; E – endexine; F – foot layer; G – granular infratectum; T – tectum; Ms – microspines.

Scale bars of 1, 5 and 9: 5 µm; of 2, 6 and 10: 2 µm; of 3, 4, 7, 8, 11 and 12: 1 µm
Plate 4

Pollen of subgenus *Nothofagus*

1–4. *Nothofagus nitida* (Picca 220, SI)
   1. SEM, polar view
   2. SEM, detail of microspines
   3. TEM, exine section of mesocolpium
   4. SEM, aperture showing the endexine thickenings and the lamellate layer between the endexine and the intine (arrows)

5–8. *Nothofagus pumilio* (Gentili, LP)
   5. SEM, subpolar view
   6. SEM, detail of microspines
   7. TEM, exine section of mesocolpium
   8. SEM, aperture showing the endexine thickenings (arrows)

9–12. *Nothofagus betuloides* (Guerrido et al. 626, SI)
   9. SEM, subequatorial view
   10. SEM, detail of microspines
   11. TEM, exine section of mesocolpium showing the endexine barely distinguishable from the intine (arrows)
   12. SEM, aperture showing the endexine thickenings

Abbreviations: In – intine; E – endexine; F – foot layer; G – granular infratectum; T – tectum; Ms – microspines.

Scale bars of 1 and 5: 10 µm; of 9: 5 µm; of 2–4, 6, 8, 10 and 12: 2 µm; of 7 and 11: 1 µm
APPENDIX A

<table>
<thead>
<tr>
<th>Subgenus (pollen type)</th>
<th>Nothofagus (fusca b)</th>
<th>Fuscospora (fusca a)</th>
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<th>Outgroup</th>
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Optimization using MP method

SFig. 1. Pollen grains of *Fagus grandifolia*, *Betula platyphylla* and *Nothofagus* sp. div. Equatorial diameter (a) and outline in polar view (b) optimized onto the ML tree of Sauquet et al. (2012) using Mesquite program.
SFig. 2. Pollen grains of *Fagus* grandifolia, *Betula platyphylla* and *Nothofagus* sp. div. Aperture length (a) and microspines (distinguishable or not in optical section) (b), optimized onto the ML tree of Sauquet et al. (2012) using Mesquite program.
SFig. 3. Pollen grains of *Fagus grandifolia*, *Betula platyphylla* and *Nothofagus* sp. div. Endexine thickening at aperture level (a) and endexine/ectexine thickness ratio (b), optimized onto the ML tree of Sauquet et al. (2012) using Mesquite program.