Early reproductive developmental anatomy in Decaisnea (Lardizabalaceae) and its systematic implications

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• Background and Aims Decaisnea insignis, known as ‘dead man’s fingers’ (Lardizabalaceae), is widely distributed in China and the Himalayan foothill countries. This economically important plant, which is the only species in the genus, has not been the subject of any embryological studies aside from a brief, older paper that lacks micrographs. Data on Decaisnea are also important because its systematic position has been unstable since the genus was established in 1855. Therefore, the objectives of this study were: (a) to use modern microscopy to document early reproductive anatomical development in Decaisnea; and (b) to compare qualitatively these early embryological characters with allied taxa in a systematic context.

• Methods Decaisnea insignis floral buds and inflorescences were regularly collected from Shaanxi Province, China and prepared for light microscopy. The embryological characters studied were qualitatively compared with those of allied taxa via a thorough examination of the existing literature.

• Key Results Early reproductive anatomy in Decaisnea was documented and novel revelations made. It was discovered that the pollen is shed when three-celled (not two-celled, as previously reported), and that endosperm formation is nuclear (not cellular or helobial, as previously reported). These two newly revealed embryological characters are not found in any other members of Lardizabalaceae. Furthermore, neither are persistent antipodals, cells, which we confirmed to be present in Decaisnea.

• Conclusions Decaisnea and other Lardizabalaceae characteristically have tetrasporangiate anthers, a secretory tapetum, simultaneous microsporocyte cytokinesis, primarily bitegmic, crassinucellate ovules, and a Polygonum type embryo sac. However, in the family, persistent antipodals, nuclear endosperm, and pollen shed at the three-celled stage are only found in Decaisnea. These embryological data prompted the suggestion that Decaisnea needs elevation above the level of genus.

Key words: Decaisnea insignis, embryology, endosperm, Lardizabalaceae, microscopy, pollen, reproductive anatomy, systematics.

INTRODUCTION

Decaisnea is a monotypic genus of Lardizabalaceae, with the species, Decaisnea insignis (Griffith) Hook. f. & Thomson (Chen and Tatemi, 2001), widely distributed from central to south-western China, extending into Bhutan, Myanmar, Nepal, Sikkim and north-eastern India. The plant is nicknamed ‘dead man’s fingers’, as it possesses racemes of striking deep purplish-blue elongated fruits (follicles). The plant is economically important, as it is readily cultivated as an ornamental, and its fruits are deemed to be a delicacy. However, in spite of the value of Decaisnea, it has not been the subject of any dedicated embryological studies aside from a solitary and brief older paper by Swamy (1953), which is limited in that it exclusively presents line drawings. As such, a modern examination of the embryology of Decaisnea is needed.

The systematic position of Decaisnea has been unstable since the genus was established in 1855 (Griffith, 1855). Presently, the Lardizabalaceae is generally recognized to have nine genera: Akebia, Boquila, Decaisnea, Holboellia, Lardizabala, Parvataia, Sargentodoxa, Sinofranchetia and Stauntonia (Qin, 1997; Chen and Tatemi, 2001). However, Loconte and Estes (1989) state that Decaisnea could be treated as a subfamily named Decaisneoideae within Lardizabalaceae, as indicated by an outgroup comparison and parsimony analysis of 34 genera within Ranunculales. Further to this, Loconte et al. (1995) conclude that the genus Decaisnea should be a new family within a new order Lardizabalales within ranunculids as implied by a morphologically based cladistic analysis on 116 ingroup taxa and five outgroups coded for 109 characters and 192 apomorphic character states. Thorne (2000, 2007) acknowledges the elevated status of Decaisnea suggested by Loconte and Estes (1989) and Loconte et al. (1995).

Qin (1989, 1997), however, regards Decaisnea as the only genus within the tribe of Decaisneae, which, along with three other tribes (Sinofranchetieae, Lardizalabaeae and Akebieae) comprise Lardizabalaceae. Many authors support Qin’s taxonomic treatment (Qin, 1989). Chen and Tatemi (2001) agree that Decaisnea is a genus within Lardizabalaceae; this view is supported by the Angiosperm Phylogeny Group (APG II, 2003) and Mabberly (2008). Using a cladistic analysis of 43 morphological characters sensu lato, Wang et al. (2002)
provide support for Qin’s view (Qin, 1989), but note that the phylogeny of Lardizabalaceae needs further study. Zhang et al. (2005) also agree with Qin’s taxonomic system (Qin, 1989). Hoot et al. (1995a, b, 1999) constructed a molecular phylogeny based on chloroplast and nuclear DNA sequences, which resolved Decaisnea as a member of Lardizabalaceae, but did not include morphological or developmental characters.

Embryological data have been helpful in inferring relationships among genera and families (Bhojwani and Bhatnagar, 1978). Endress and Igersheim (1999) present an excellent review of gynoecium diversity and systematics of the basal dicots, which includes a useful synopsis of ovular characteristics of the Lardizabalaceae, but does not specifically describe embryology nor focus on the genus Decaisnea. As such, a study of embryological ontogeny in Decaisnea would not only contribute new information regarding reproductive development in this important plant, but would also provide a new suite of characters for systematic studies; Swamy (1953) did not compare his limited embryological data on Decaisnea with those of allied taxa nor discuss their systematic implications. Here, anther, pollen, ovule, embryo sac and early endosperm development in Decaisnea insignis are studied. These early embryological characters are then compared with allied taxa and the systematic implications discussed.

**MATERIALS AND METHODS**

**Collection and photography of whole floral buds and flowers**

About five floral buds or maturing inflorescences of Decaisnea insignis (Griffith) Hook. f. & Thomson were collected every 5 or 6 d from Taibai Mountain in Shaanxi Province, China (altitude 1200–1500 m; voucher: Zhang 20030609, SANU) from 1 March 2005 to 1 May 2006 in order to obtain a range of developmental stages. Samples were fixed in formalin–acetic acid–ethanol (FAA) 2 : 2 : 1 volume/volume/volume (v/v/v). Floral buds and flowers (about three per sampling date) were dissected and photographed using a Nikon DXM 1200 stereo microscope.

**Preparation for sectioning and microscopy**

Fixed male and female flower buds were dehydrated in an ethanol series (70 %, 85 %, 95 %, 100 % and 100 % ethanol, 2 h each), and embedded in paraffin wax. Approximately ten serial sections of individual flowers (at least two flowers) were cut at 6–9 μm, stained for 4 h in 4 % Heidenhain’s iron-alum, washed for 40 min with H2O, stained for 4 h with 0.05 % hematoxylin, washed again with H2O (30 min), and mounted on slides in a gelatin solution (1 g gelatin, 100 mL H2O, 2 g phenol, 15 mL glycerol; Li, 1978). Photographs were taken with an Olympus SP-565UZ digital camera mounted on an Olympus BH-2 photomicroscope equipped with Nomarski optics. The tonal qualities of the images were adjusted, labels were added, and plates assembled with Adobe Photoshop CS2 and CS3.

**RESULTS**

**Decaisnea morphology**

Decaisnea insignis plants used in this study were found to be polygamo-monoecious, as individuals possessed male flowers, female flowers and bisexual flowers; the strictly monoecious condition is apparently rare. For consistency, only unisexual flowers were examined. In early spring, winter buds are ovoid and possess two outer scales (Fig. 1A). The inflorescence is a terminal panicle of racemes (Fig. 1B). Each flower is subtended by a bract (Fig. 1C). Each flower has six subimbricate sepals and lacks petals. Male flowers have six stamens with oblong anthers, and three carpellodes that are small and concealed within the filament tube (Fig. 1D). Female flowers have staminodes that are either free, or, less frequently, connate at the base, and have three cyclically arranged straight carpels (not shown). The mature fruit consists of three follicles.

**Development of the anther wall**

The anther is tetrasporangiate (Fig. 2A). The single-celled archesporium is hypodermal and undergoes a periclinal division, resulting in a primary parietal layer and a primary sporogenous layer. The parietal layer divides periclinally to form two layers: the inner layer contributes to the tapetum (Fig. 2B), while the outer parietal layer undergoes another periclinal division, resulting in an endothecium toward the outside and a middle layer toward the inside (Fig. 2C). The mature anther wall is thus comprised of five or six layers: a single-layered epidermis, a single-layered endothecium, two or three middle layers, and a single-layered tapetum (Fig. 2D), and conforms to the dicotyledonous form of anther wall development.

The tapetum is of dual origin: most of it develops from the primary parietal layer, but a component also arises from the ground tissue of the connective side (not shown). After formation of the anther wall but before microsporogenesis, about half of the tapetal cells undergo mitosis without cytokinesis, becoming binucleate (Fig. 3A). Upon initiation of microsporecyte meiosis, the tapetal cells elongate radially and protrude into the anther locule (Fig. 3B). The tapetal cells degenerate at their original sites following microsporogenesis. Therefore, the tapetum is of the secretory (glandular) type. The epidermis persists at maturity, the endothecium develops fibrous thickenings, and the middle layers are ephemeral, degenerating shortly before the microspores develop into pollen grains.

**Microsporogenesis and microgametogenesis**

After formation of the anther wall, each microsporangium contains numerous sporogenous cells (Fig. 2D). Microsporocytes originate from the primary sporogenous layer as well as from secondary sporogenous cells (Fig. 3A). Individual microsporocytes become enclosed in a thick callose wall when their nuclei enter prophase of meiosis I (Fig. 3B). Meiosis II is followed by simultaneous cytokinesis with centripetally advancing constriction furrows, and results in tetrahedral microspore tetrads, which enlarge and acquire thick callose walls (Fig. 3C). Shortly after callose formation, the callose walls promptly break down, releasing microspores from the tetrad (Fig. 3D). Then, formation of a large vacuole pushes the single nucleus of each freed microspore toward the microspore wall (Fig. 3E). Each microspore divides to form a large tube (vegetative) cell in the vicinity of the large vacuole and a small generative cell...
in the region that housed the original wallward microspore nucleus (Fig. 3F). The generative cell undergoes a further division, resulting in two sperm cells (Fig. 3G). Tricolpate pollen grains are shed at this three-celled stage (Fig. 3H).

Megasporogenesis and megagametogenesis

Numerous ovules are found in two rows on either side of an adaxial carpel suture (Fig. 4A). The archesporium is one-celled (Fig. 4B), and transforms into a megasporocyte by cutting off a parietal cell (Fig. 4C). The parietal cell undergoes further divisions to form the nucellar tissue (Fig. 4D), which causes the megasporocyte to become deep-seated within the ovule. Thus, the ovule is crassinucellate.

Following parietal cell divisions, the megasporocyte undergoes meiosis I, resulting in a dyad (Fig. 4D). Meiosis II produces a T-shaped tetrad of megaspores (Fig. 4E). The three micropylar megaspores of the tetrad degenerate, while the...
chalazal megaspore becomes visibly functional, possessing a prominent nucleus (Fig. 5A). The functional megaspore develops successively into a two-nucleate (Fig. 5B), four-nucleate (Fig. 5C) and, finally, eight-nucleate embryo sac (Fig. 5D, E) by three mitotic divisions. Thus, the mode of embryo sac formation is of the *Polygonum* type.

The three micropylar nuclei become the egg and two synergids, collectively comprising the egg apparatus (Fig. 5D). The unfertilized egg cell is highly vacuolate (Fig. 5D). The two median nuclei become the polar nuclei (Fig. 5D, E), and the chalazal nuclei become the three antipodals (Fig. 5D, E). The polar nuclei fuse in the centre, forming the fusion (secondary) nucleus of the central cell and remain in this central location until fertilization (Fig. 6A).

**Double fertilization and development of endosperm**

Following fertilization of the egg cell, the zygote becomes densely cytoplasmic (Fig. 6B). Soon after, the primary endosperm nucleus of the central cell migrates toward the antipodals, which persist at the chalazal pole of the embryo sac (Fig. 6C). The first division of the primary endosperm nucleus is not accompanied by wall formation (Fig. 6D). Free nuclear endosperm formation ensues within several mitotic divisions (Fig. 6E). As early nuclear endosperm
develops, the two synergids become reduced, while the antipodals persist at the chalazal pole (Fig. 6E).

**Ovule development**

Ovule development is concurrent with events of megasporogenesis and megagametogenesis. The ovule is nearly straight early in development (Fig. 7A), but a slight curvature becomes noticeable when the megasporocyte has completed meiosis II (Fig. 7B), and as the ovule approaches maturity, it gradually becomes anatropous (Fig. 7C), completing curvature when the embryo sac reaches the eight-nucleate stage (Fig. 7D). The ovule is bitegmic. The inner integument is initiated simultaneously with the onset of megasporogenesis (Fig. 7A), while the outer integument is initiated when the embryo sac has become two-nucleate. The integuments do not complete...
development until the embryo sac reaches the eight-nucleate stage, when the endostome micropyle becomes evident (Fig. 7D).

**DISCUSSION**

**Early embryological features of Decaisnea and other Lardizabalaceae**

**Development of the anther wall.** It has been found that development of the anther wall is of the dicotyledonous type in Decaisnea, since the endothecium and middle layers originate from a single layer of cells, and the tapetum, which is secretory rather than amoeboid, does not originate from connective tissue. Most of the present observations on anther development confirm and expand those of Swamy (1953), as wall layer ontogeny is explicitly documented. However, while Swamy (1953) suggests that all tapetal cells are binucleate, it is noted that some remain uninucleate, even late in development.

There are noteworthy differences in early embryological development of Decaisnea when compared with other genera in the Lardizabalaceae. Development of the anther wall is of the basic type in Sargentodoxa (Liu and Sheng, 2003), and of both the dicotyledonous and basic type in Sinofranchetia (Zhang et al., 2005). Like Decaisnea, the tapetum is secretory in Holboellia (Bhatnagar, 1965), Sargentodoxa (Liu and Sheng, 2003) and Sinofranchetia (Zhang et al., 2005). In Stauntonia hexaphylla, the tapetal cell walls appear to break down (Yoshida and Nakajima, 1978), as is typical of an amoeboid tapetum, but the protoplasts remain in situ, which is characteristic of a secretory tapetum. The tapetal cells typically contain no more than two nuclei in Decaisnea and Holboellia angustifolia (Wang, 2001), while they contain two to four nuclei in Holboellia latifolia (Bhatnagar, 1965).

**Microsporogenesis and microgametogenesis.** It has been observed that cytokinesis following microsporocyte meiosis is simultaneous, and results in tetrahedral tetrads in
Decaisnea. The same is true for Holboellia angustifolia (Wang, 2001), Sargentodoxa (Liu and Sheng, 2003) and Sinofranchetia (Zhang et al., 2005). However, tetrads are tetrahedral or decussate in Holboellia latifolia (Bhatnagar, 1965). It has been found that the mature tricolpate pollen grains of Decaisnea are three-celled at the time of shedding, and not two-celled as reported by Swamy (1953). Holboellia latifolia (Bhatnagar, 1965), Holboellia angustifolia (Wang, 2001), Sargentodoxa (Liu and Sheng, 2003) and Sinofranchetia (Zhang et al., 2005) are also reported to have two-celled mature pollen grains when shed.

Megasporegenesis and megagametogenesis. The present results indicate that Decaisnea possesses T-shaped megaspor tetrads. Holboellia latifolia also possesses T-shaped megaspor tetrads (Bhatnagar, 1965), as is found in Sargentodoxa, although the megaspor tetrads in this genus are occasionally linear (Sheng et al., 2005). The megaspor tetrads are linear in Holboellia angustifolia (Wang, 2001) and Sinofranchetia (Zhang et al., 2005). Embryo sac development in Decaisnea conforms to the Polygonum type; this is also the case in Holboellia latifolia (Bhatnagar, 1965), Akebia spp. (Yoshida and Michikawa, 1973), Stauntonia hexaphylla (Yoshida and Nakajima, 1978), Holboellia angustifolia (Wang, 2001), Sargentodoxa (Sheng et al., 2005) and Sinofranchetia (Zhang et al., 2005). The present findings show that the antipodals in D. insignis are persistent and large; these findings are consistent with Swamy’s report (Swamy, 1953). In contrast, the antipodals are small and ephemeral in Akebia spp. (Yoshida and Michikawa, 1973).
Holboellia latifolia (Bhatnagar, 1965) and Stauntonia hexaphylla (Yoshida and Nakajima, 1978). In Decaisnea, the polar nuclei fuse before fertilization. While Swamy (1953) similarly notes that the two polar nuclei of Decaisnea fuse, his report suggests that the unfertilized fusion nucleus migrates toward the antipodals. We maintain that migration toward the antipodals only occurs after double fertilization has taken place, as the large nucleus of the central cell is only found near the antipodals following a visible change in the egg cell to a more cytoplasmic state, indicative of fertilization (Raghavan, 1998).

Double fertilization and development of endosperm. The present findings regarding early endosperm development in Decaisnea do differ somewhat from those of Swamy (1953). Similarly, it is clear that it is the fertilized primary endosperm nucleus that migrates toward the antipodals, not the unfertilized fusion nucleus. Furthermore, we believe that endosperm development in Decaisnea is of the free nuclear type, not the helobial type as described by Swamy (1953). Using line drawings, Swamy (1953) documents a first cellular division of the primary endosperm nucleus that partitions the endosperm into a micropylar and chalazal chamber; while Swamy (1953) did not explicitly use the term ‘helobial’, he had effectively described such a type of endosperm development (Raghavan, 1998). However, no evidence of such a division has been seen, and we maintain that the endosperm in Decaisnea is of the free nuclear type. All members of the Lardizabalaceae are generally believed to have ab initio cellular endosperm (Johri et al., 1992).

Ovule development. In Decaisnea, ovules are anatropous, bitegmic and crassinucellate (see also Endress and Igersheim, 1999). However, we disagree with Swamy’s (1953) report regarding the timing of integumentary development. In the present study it was found that the inner integument is initiated simultaneously with the onset of megasporogenesis, the outer integument is initiated when the embryo sac has become two-nucleate, and that the integuments do not complete development until the embryo sac reaches the eight-nucleate stage. Swamy (1953), on the other hand, suggests both integuments are initiated simultaneously, and that integumentary development is complete at the four-nucleate stage. Akebia quinata (Endress and Igersheim, 1999), Holboellia angustifolia (Wang, 2001) and

**Fig. 6** Events immediately surrounding double fertilization. (A) The two polar nuclei of the central cell fuse to form the fusion nucleus (fn) immediately prior to fertilization. The fusion nucleus resides in a central position until fertilization. (B) Zygote (z) at the micropylar pole. The densely stained cytoplasm of the cell indicates that fertilization has occurred. (C) Soon after fertilization of the fusion nucleus to form the primary endosperm nucleus (pe), it migrates toward the antipodals, which persist at the chalazal pole of the embryo sac (arrowheads). (D) The first division of the primary endosperm nucleus resulting in two endosperm nuclei (e) is not accompanied by wall formation. The antipodals (arrowheads) persist at the chalazal pole. The zygote (z) remains densely cytoplasmic. (E) After several mitotic divisions without cytokinesis, numerous endosperm nuclei (e) are produced. Arrowheads label the persistent antipodals. Scale bars = 20 μm.
**Sinofranchetia** (Endress and Igersheim, 1999; Zhang et al., 2005) also have ovules that are anatropous, bitegmic and crassinucellate. The ovules are hemianatropous in **Sargentodoxa** (Sheng et al., 2005).

**Embryological comparison of Decaisnea with allied groups: systematic implications**

The present study shows that **Decaisnea** and other genera of **Lardizabalaceae** generally share the following embryological characters: tetrasporangiate anthers (Johri et al., 1992), secretory tapetum (Bhatnagar, 1965; Liu and Sheng, 2003; Zhang et al., 2005), simultaneous cytokinesis in the microsporocytes (Wang, 2001; Liu and Sheng, 2003; Zhang et al., 2005), primarily bitegmic, crassinucellate ovules (Bhatnagar, 1965; Wang, 2001; Zhang et al., 2005) and a **Polygonum** type of embryo sac development (Yoshida and Michikawa, 1973; Yoshida and Nakajima, 1978; Wang, 2001; Zhang et al., 2005; Sheng et al., 2005).

However, **Decaisnea** displays three embryological characters that are rarely found in **Lardizabalaceae**, and are thus of

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**Fig. 7** Ovule development in **Decaisnea insignis**. (A) The ovule is nearly straight early in development. The inner integument (i) is initiated simultaneously with the onset of megasporogenesis. fu, Funiculus. The megasporocyte is entering meiosis, and can be better observed in Fig. 4C. (B) A slight ovular curvature becomes noticeable when the megasporocyte has completed meiosis II to form the tetrad (see Fig. 4E for a better view of the megaspore tetrad). i, i, inner integument. (C) As the ovule approaches the stage where the embryo sac is two-nucleate (two-nucleate embryo sac seen here and more clearly in Fig. 5B), the ovule gradually becomes anatropous, and the outer integument (o) is initiated. fu, Funiculus; i, inner integument. (D) Ovule curvature is complete when the embryo sac reaches the eight-nucleate stage (details of eight-nucleate embryo sac in Fig. 5D, E). At this time, the inner (i) and outer (o) integuments have completed development and the endostomic micropyle has become evident. fu, Funiculus. Scale bars: (A) = 30 μm; (B–D) = 20 μm.
substantial systematic implication. First, the antipodals are persistent and relatively large in *Decaisnea*, while they are ephemeral and smaller in the other Lardizabalaceae (Bhatnagar, 1965; Yoshida and Michikawa, 1973; Yoshida and Nakajima, 1978; Wang, 2001; Sheng et al., 2005; Zhang et al., 2005). Secondly, the endosperm of *Decaisnea* is free nuclear, while endosperm is *ab initio* cellular in most Lardizabalaceae (Johri et al., 1992). Thirdly, the pollen grains of *Decaisnea* are shed when they are three-celled, while those of most Lardizabalaceae are shed at the two-celled stage (Johri et al., 1992).

With these family-level incongruencies in mind, it is important to note that two of the three embryological characters that distinguish *Decaisnea* from other Lardizabalaceae are regularly observed in Ranunculales, specifically Ranunculaceae. Like *Decaisnea*, members of Ranunculaceae have persistent antipodals (Williams and Friedman, 2004) and free nuclear endosperm (Johri et al., 1992). While pollen grains in most genera of Ranunculaceae are shed at the two-celled rather than the three-celled stage (Brewbaker, 1967; Johri et al., 1992), Ranunculaceae have a secretory tapetum, simultaneous microsporocyte meiosis, primarily bitegmic, crassinucellate ovules that are numerous in each carpel, and a Polygonum type embryo sac (Jalan, 1963; Johri et al., 1992), like most Lardizabalaceae. We do not conclude that *Decaisnea* is more closely related to the Ranunculaceae; however, the embryological characteristics of *Decaisnea* relative to Lardizabalaceae and other Ranunculaceae may suggest that the systematic position of *Decaisnea* calls for further evaluation and elevation.

Thus, it would be premature to make formal taxonomic conclusions before stronger evidence is obtained and before evolutionary trends of the gross morphology are finally elucidated; however, we believe our embryological results indicate that *Decaisnea* is not a simple ‘genus-level fit’ in the Lardizabalaceae. The presence of persistent antipodals, free nuclear endosperm, and three-nucleate pollen upon shedding are characters unique to *Decaisnea* within Lardizabalaceae. Such normally conservative characters can be used to circumscribe taxa above the generic rank (Tobe, 1989). As mentioned, based on morphological data, Loconte and Estes (1989) suggest that *Decaisnea* could be treated as a subfamily named Decaisneoideae, and Loconte et al. (1995) circumscribe *Decaisnea* as a new family within Lardizabalales. Qin (1989, 1997) has treated *Decaisnea* as a monogenic tribe *Decaisneae*. In light of the present embryological study on *Decaisnea insignis*, we believe such statements deserve serious reconsideration, particularly those of Qin (1989, 1997). Our suggestion neither directly confirms nor refutes a chloroplast and nuclear DNA sequence-based molecular phylogeny wherein *Decaisnea* was resolved as a member of Lardizabalaceae (Hoot et al., 1995a, b, 1999). However, we recommend that our new embryological results be incorporated in future phylogenetic studies.

**Conclusions**

*Decaisnea* and other genera of Lardizabalaceae characteristically have tetrasporangiate anthers, a secretory tapetum, simultaneous microsporocyte cytokinesis, primarily bitegmic, crassinucellate ovules and a Polygonum-type embryo sac. However, in the family, only *Decaisnea* has persistent antipodals, nuclear endosperm, and three-celled pollen upon shedding. Based on these embryological results, we suggest that monospecific *Decaisnea* is in need of taxonomic re-evaluation and circumscription above the genus level.

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**LITERATURE CITED**


