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LETTERS

Dating the origin of the Orchidaceae from a fossil orchid with its pollinator

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Since the time of Darwin[1], evolutionary biologists have been fascinated by the spectacular adaptations to insect pollination exhibited by orchids. However, despite being the most diverse plant family on Earth[2], the Orchidaceae lack a definitive fossil record and thus many aspects of their evolutionary history remain obscure. Here we report an exquisitely preserved orchid pollinarium (of *Meliorchis caribea* gen. et sp. nov.) attached to the mesoscutellum of an extinct stingless bee, *Proplebeia dominicana*, recovered from Miocene amber in the Dominican Republic, that is 15–20 million years (Myr) old[3]. This discovery constitutes both the first unambiguous fossil of Orchidaceae[4] and an unprecedented direct fossil observation of a plant–pollinator interaction[5,6]. By applying cladistic methods to a morphological character matrix, we resolve the phylogenetic position of *M. caribea* within the extant subtribe Goodyerinae (subfamily Orchidoideae). We use the ages of other fossil monocots and *M. caribea* to calibrate a molecular phylogenetic tree of the Orchidaceae. Our results indicate that the most recent common ancestor of extant orchids lived in the Late Cretaceous (76–84 Myr ago), and also suggest that the dramatic radiation of orchids began shortly after the mass extinctions at the K/T boundary. These results further support the hypothesis of an ancient origin for Orchidaceae.

Family Orchidaceae Juss., 1789
Subtribe Goodyerinae Klotzsch, 1846
Meliorchis caribea gen. et sp. nov.

Etymology. The generic name alludes to the plant's pollination mode by meliponine bees and incorporates the Greek name of an orchid (orchis: testicle). The specific epithet *caribea* refers to the Caribbean region.

Holotype. Museum of Comparative Zoology (Harvard University), catalogue number MCZ-31141.

Horizon and locality. Specimen was excavated in the year 2000 from a mine located east of Santiago, Cordillera Septentrional, Dominican Republic. Lignite and sandy clay beds, Early to Middle Miocene (15–20 Myr old; ref. 3).

Diagnosis. The species is separated from other members of Goodyerinae by the bent anther, large angular massulae (~100 per pollinarium), and tightly packed pollen units (20 × 20 μm). The amber piece (20 × 14 × 5 mm) contains a single inclusion of *Meliorchis caribea*. Two complete pollinia (each ~1,000 × 500 μm), belonging to a single pollinarium, are firmly attached to the mesoscutellum of a worker bee, *Proplebeia dominicana*[7] (Fig. 1a). The tapering pollinia consist of >100 loosely packed angular massulae (~200 × 100 μm, Fig. 1b), each of which encapsulates several tetrads; obovoid pollen units are tightly packed.

These pollinarium features are found only in the Orchidoideae[8]. A survey of herbarium specimens of all Neotropical genera within this subfamily showed that the size, shape and ornamentation of the fossil closely resemble those of modern members of the subtribe Goodyerinae, particularly the genera *Kreodanthus* and *Microchilus* (Supplementary Table 1). In addition, the position of the pollinarium on the fossilized bee enables us to make inferences about unique aspects of the flowers of *Meliorchis*, even in the absence of fossil flowers. Whereas in living Goodyerinae the pollinarium normally is attached to the mouthparts of pollinating bees[9] (Fig. 2a), the

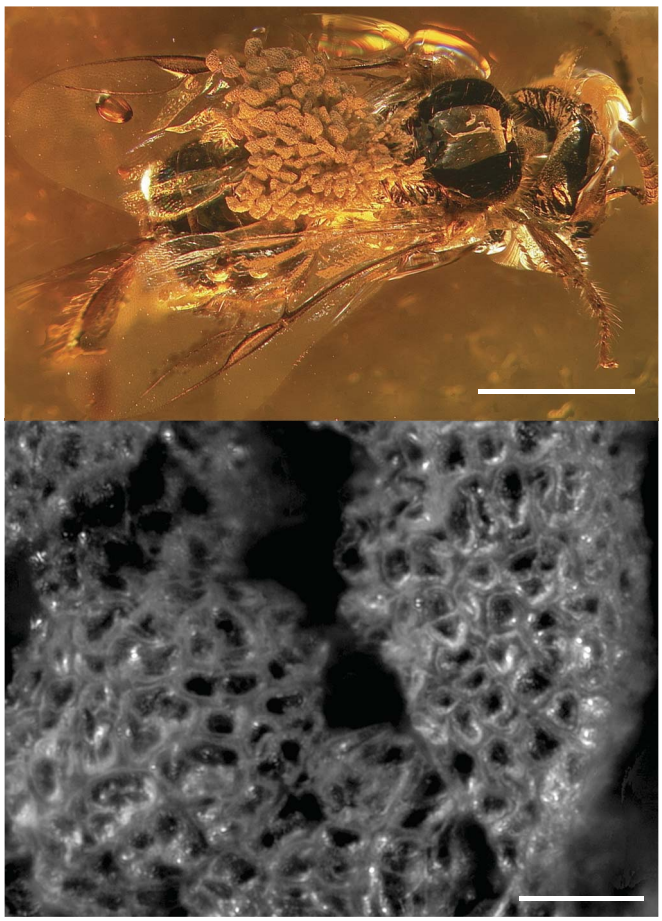

Figure 1 | Holotype of *Meliorchis caribea* gen. et sp. nov. This orchid pollinarium, carried by a worker stingless bee (*Proplebeia dominicana*), is preserved in amber from the Dominican Republic and represents the first definitive fossil record for the family Orchidaceae. **a**, General view of encapsulated specimen (scale bar, 1,000 μm). **b**, Detailed view of the pollinia surface showing pollen units (scale bar, 50 μm).

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pollinarium of *Meliorchis* is attached to the mesoscutellum (dorsal surface of the thorax) of worker bees of *P. dominicana*. This indicates that the flower of *M. caribea* was gullet-shaped, and, rather than the bee probing the lip of the flower with its tongue as in modern Goodyerinae (Fig. 2a), the anterior part of the bee would have had to enter the flower completely (Fig. 2b).

Because evidence of plant–pollinator interactions is exceedingly rare in the fossil record, our current knowledge of ancient pollination is indirectly inferred from specialized morphological features of fossilized insects[10–12] and flowers[13–15]. In addition, records of pollen grains on fossil insects and in coprolites provide circumstantial evidence for ancient insect–flower interactions[5,6,10,12,14], although these observations—with the exception of amber-preserved fig wasps carrying fig pollen[6]—do not exclude the possibility of flower visitation without pollination[5]. In contrast, because in most orchids the staminal filaments are fused to the style, the anatomical match required for a pollinator to remove the pollinarium is nearly identical to that necessary for its subsequent delivery (Fig. 2). Thus, *P. dominicana* bee workers were almost certainly pollinators of flowers of *M. caribea*. Because modern stingless bees pollinate numerous rainforest angiosperms[16], including several tropical orchid species[17], this fossil shows that adaptation by tropical orchids to specialized pollinators occurred at least as far back as the Miocene.

To explore the phylogenetic position of *Meliorchis* in relation to Modern orchid taxa, we constructed a morphological character matrix consisting of 25 characters and 15 taxa adapted from a previous study[18] (see Supplementary Methods for details). Heuristic tree searches optimized by maximum parsimony yielded 129 equally short trees, all of which supported monophyly of both the subfamily Orchidoideae and the subtribe Goodyerinae (Fig. 3). The position of *Meliorchis* within Goodyerinae is supported by a bootstrap of 91%. Of the 129 recovered trees, none supported *Meliorchis* as a sister clade to the rest of the Goodyerinae genera. Together, these results indicate that *Meliorchis* represents a differentiated lineage within extant Goodyerinae. On the basis of estimated ages of Dominican amber[3], a minimum age of 15–20 Myr can be assigned to the subtribe Goodyerinae.

Previously published putative orchid fossils have lacked diagnostic characters that would definitively assign them to Orchidaceae[4,19]. In fact, in a thorough review of all known specimens, it was concluded that Orchidaceae have 'no positive or useful fossil record'[4]. This absence in the fossil record, most likely owing to their non-diagnostic leaves and lack of wind-dispersed pollen, has spurred considerable disagreement regarding orchids' age of origin and timing of diversification. Whereas orchids' highly specialized pollination mechanisms, epiphytism and absence in fossil deposits were cited by early workers in support of a recent age[4,20,21], their worldwide distribution[2] and basal placement in the order Asparagales[22] suggest an older age. Indeed, three recent molecular clock studies that broadly sampled angiosperm clades (including a few orchid representatives) obtained radically different age estimates for the Orchidaceae, ranging from ~26 Myr old[23] and ~40 Myr old[24] to ~110 Myr old[25]. Such age discrepancies are most likely due to under-represented sampling and absence of internal calibration points. We here use both the age and phylogenetic position of *M. caribea* and other fossil monocots to estimate the timing of diversification for Orchidaceae.

We calibrated a molecular phylogenetic tree of Orchidaceae by implementing a relaxed-clock model through penalized likelihood and non-parametric rate smoothing (NPRS). We built a molecular phylogenetic tree of Orchidaceae that was based on plastid DNA sequences obtained from GenBank for 55 orchid genera representing all major lineages in the family, and five basal Asparagales genera as outgroup taxa. Our divergence time estimates using penalized likelihood suggest that extant Orchidaceae shared a most recent common ancestor in the Late Cretaceous, 76 ± 5 to 84 ± 6 Myr ago, depending on whether we use the oldest or youngest estimates of the ages of the fossils used to calibrate the relaxed molecular clock (Fig. 4). Similarly, age estimates obtained using NPRS suggest that crown Orchidaceae shared a common ancestor 76 ± 4 to 83 ± 4 Myr ago. Our results also suggest that stem lineages of all five orchid subfamilies were present early in the evolutionary history of Orchidaceae, before the end of the Cretaceous, ~65 Myr ago (Fig. 4). The extant lineages of the two largest orchid subfamily clades (Orchidoideae and Epidendroideae), which together encompass >95% of the living orchid species, began to diversify early in the Tertiary, although more thorough taxonomic sampling could result in older age estimates of their common ancestor.

The discovery of *Meliorchis caribea* and the internally calibrated molecular clock analyses presented here reject the hypothesis of a relatively recent (Eocene or younger) origin of Orchidaceae[4,21].

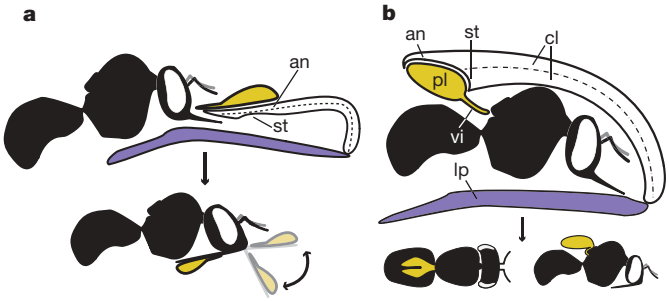

Figure 2 | Morphology and pollinarium placement of modern Goodyerinae and hypothetical reconstruction of floral morphology of *Meliorchis caribea*. **a**, The parallel lip (lp) and column (cl) and the erect anther (an) of extant Goodyerinae typically result in the pollinarium (pl) attachment on the pollinator's mouthparts. **b**, The attachment of the pollinarium to the mesoscutellum (dorsal surface of thorax) of a worker bee is only possible when the lip and column of the flower are parallel but the anther is bent. Under this scenario, the distance between the lip and the column must be ~2.5 mm to enable a *P. dominicana* worker to crawl into the flower and remove the pollinarium with its mesoscutellum as it retreats; st, stigma; vi,viscidium.

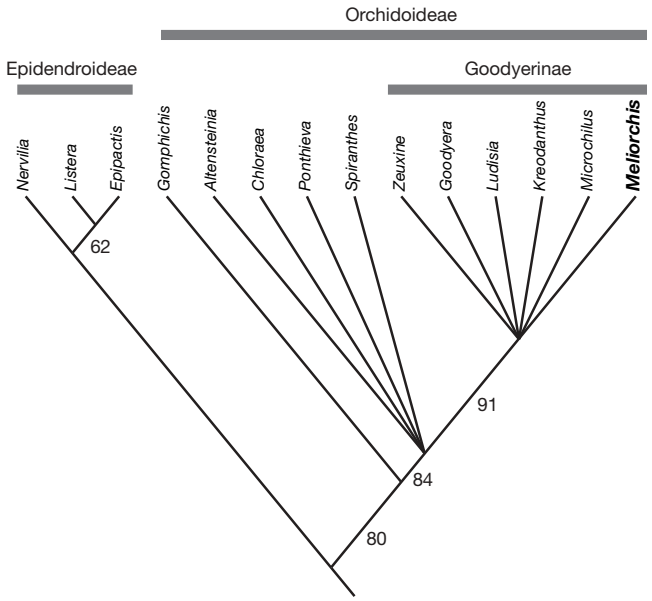

Figure 3 | Cladogram showing the estimated position of *Meliorchis* among modern clades in the orchid subfamily Orchidoideae. A strict consensus of the 129 shortest trees (tree length = 42, consistency index = 0.619, retention index = 0.660) obtained using 25 morphological characters for 15 taxa; values beside nodes correspond to bootstrap percentages (1,000 replicates). None of the shortest trees recovered *Meliorchis* as sister to all the other Goodyerinae included.

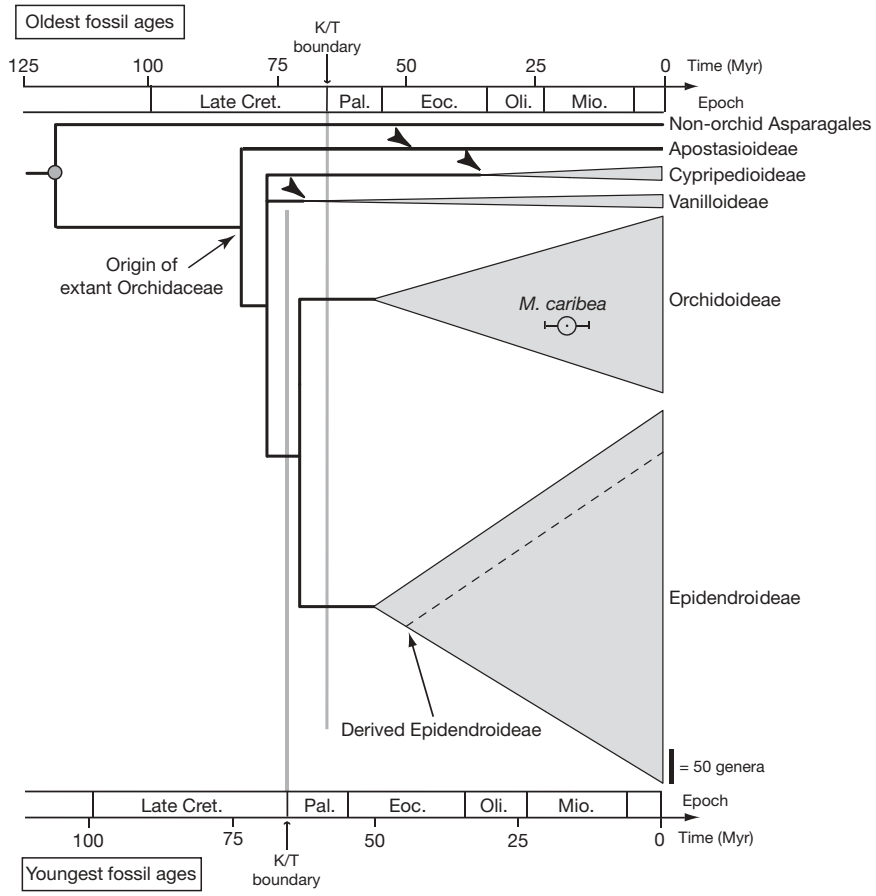

Figure 4 | Fossil-calibrated molecular clock chronogram of the family Orchidaceae, based on ~3 kilobases of plastid DNA (*matK* and *rbcL*). The relative size of each clade is proportional to the number of genera described in each orchid subfamily. Crown ages of small clades are indicated with arrow heads. Two sets of dates were used to calculate orchid divergence times: the oldest and youngest estimates of the ages of the fossils. The age boundaries of *M. caribea* (15–20 Myr old) relative to each timescale correspond to the distance between the circle's centre and the vertical bar. Additional monocot fossil records outside Orchidaceae were used to calibrate the root of the tree (node indicated by filled circle). Branch lengths were optimized under the maximum likelihood model of sequence evolution GTR+Γ+I using a 95% majority-rule consensus tree (see Supplementary Information); node ages were estimated using a penalized likelihood method[29].

Instead, our results favour the hypothesis of an ancient (Late Cretaceous)[22,25] origin of extant Orchidaceae, but at the same time support a Tertiary radiation of the most diverse epiphytic clades. Our age estimates are younger than the oldest proposed for the family by previous studies[25], but we note that our age calculations should be regarded as minimum estimates, which could be pushed back with additional fossil discoveries. Our scenario corresponds to that previously proposed[2,22], is consistent with the observed disjunct pantropical distributions of the subfamily clades and the early-splitting genera (for example, *Vanilla*), and reinforces the possibility of a Late Cretaceous biotic exchange between tropical continents.

METHODS

Colour photomicrographs were taken with a JVC digital camera (KYF75U) mounted on a MZ16 Leica dissecting scope; black and white micrographs were taken with a Retiga EXi digital camera mounted on a Leica Leitz-dmrb compound microscope (objective ×40). In both cases, 10 sequential shots at different focal depths were processed with the Auto-Montage software (Syncroscopy, 2002) to produce a single composite image.

The phylogenetic position of *Meliorchis* was explored using morphological characters from flowers, pollinaria and pollen micro-morphology, all of which were directly observable or inferable from the type specimen of *M. caribea*. We treated all character states as unordered and weighted them equally. Because *Meliorchis* unambiguously belongs to the subfamily Orchidoideae, we only included representative genera from this group. We selected outgroup taxa on the basis of previous studies that used both morphological[18] and molecular[26] data. Heuristic tree searches were performed via maximum parsimony with the TBR algorithm (100 random addition replicates). A total of 1,000 replicates were run to estimate bootstrap support; all analyses were performed in *PAUP** v.4.0b.

Consensus phylogenetic trees of bayesian analyses were obtained with the software *MrBayes* v3.1.1 (for details, see Supplementary Materials). Our topologies agree with those obtained by previous studies[27,28]. Divergence times were calculated by penalized likelihood and NPRS, using the truncated Newton algorithm in the software *r8s* v 1.71[29]. Two sets of dates were used, corresponding to the youngest and oldest estimates of the ages of the fossils used as node age constraints. We applied (1) the age of *Meliorchis* (15–20 Myr old; ref. 3) as a minimum age for the monophyletic Goodyerinae; (2) the age of the oldest known Asparagales (93–105 Myr old, see Supplementary Methods for details) as a minimum age constraint at the root of the tree; and (3) the age of the oldest known fossil monocot as the maximum age at the root of the tree (110–120 Myr old; ref. 30).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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