



Phylogenetic relationships within Orchidaceae based on a low-copy nuclear coding gene, *Xdh*: Congruence with organellar and nuclear ribosomal DNA results

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ABSTRACT

Using parsimony and Bayesian analyses, we estimated higher-level relationships within Orchidaceae, focusing on subfamilies and tribes. DNA sequences of part of the low-copy nuclear protein gene *Xdh* were obtained for 154 taxa including 126 genera of Orchidaceae and outgroup families of Asparagales. The general topology of the *Xdh* trees is congruent with those published previously based on plastid protein-coding genes and non-coding nuclear ribosomal DNA. The five subfamilies previously recognized are monophyletic and well supported. The results indicate that monandrous condition evolved independently in Vanilloideae and Epidendroideae/Orchidoideae. The analysis clarifies relationships between tribes of Epidendroideae such as Vandeae *sensu lato* to Collabieae, Epidendreae to Calypsoeae and Malaxideae to Dendrobieae. Also relationships of *Bromheadia*, *Imerinaea*, *Sirhookera*, and achlorophyllous species of *Corallorhiza*, *Gastrodia*, *Limodorum*, *Neottia*, *Wulfschlaegelia* are for the first time evaluated in a broad molecular phylogenetic framework.

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1. Introduction

All pre-DNA era classifications for Orchidaceae were based on a relatively small set of morphological and anatomical features, particularly aspects of the gymnostemium (column) such as degree of pollen aggregation (i.e., pollinium formation) and anther-associated structures (i.e., pollinaria). Most morphological traits probably represent expression suites of many genes, and, thus, their distribution among genera should be reflected in phylogenetic relationships. However, the adaptive nature of many phenotypic features, particularly floral traits in a family for which we suspect pollinator relationships have played a major role, interferes with our ability to accurately interpret many morphological characters, and their distribution may not therefore always reflect phylogeny. These problems are further complicated by the intuitive reliance of most previous authors on certain traits to the exclusion of others. As a consequence, there have been many reciprocally conflicting systems of orchid classification (Burns-Balogh and Funk, 1986; Dressler, 1981, 1993; Rasmussen, 1985; Szlachetko, 1995). Only that of Burns-Balogh and Funk was based on phylogenetic analyses

of morphological data; that of Dressler (1981, 1993), even though presented in a phylogenetic tree format with synapomorphies indicated, used only “cladistic reasoning” in the development of his ideas of orchid evolution and classification. The history of pre-DNA era classification has been reviewed in Cameron (2007), and we will only discuss here the major features of these systems because this is the major focus of this study. Dressler (1979) divided Orchidaceae into six subfamilies: Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae, Vandoideae, and a then new subfamily, Spiranthoideae. The features on which Dressler (1979) based this division were: position of the anther (straight or bent), structure of the rostellum, shape of the subsidiary cells in leaves and aggregation of pollen grains into pollinia. In his 1981 system, he kept these same subfamilial divisions. In 1983, Dressler revised his system based on new studies on the structure of the orchid seeds, which, although simple and dust-like, exhibit a series of what appeared to be conservative characters. Dressler (1983) in his new system included Vandoideae in Epidendroideae, thus leaving in Orchidaceae four other subfamilies: Apostasioideae, Cypripedioideae, Orchidoideae and Spiranthoideae. In the subsequent years Dressler produced more detailed treatments of several tribes and subfamilies: Vandeae (Dressler, 1989), Neottieae (Dressler, 1990a), Spiranthoideae (Dressler, 1990b) and Epidendroideae (Dressler, 1990c). These formed the foundation for yet another

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orchid classification, which Dressler published in 1993. The most important change in this classification was the shift of Neottieae s.s. to Epidendroideae, in which he distinguished two further evolutionary lineages, termed the “cymbidioid” and “epidendroid phylads”. This classification relied on the previous set of features overlaid with the results of recent studies on seed morphology, leaf subsidiary cells and chromosome numbers.

Rasmussen (1985) based his division of Orchidaceae mainly on Dressler's classification from 1981. The main difference that Rasmussen (1985) proposed was the division of the order Orchidales into three families, similar to Vermuelen's system (1966): Apostasiaceae, Cypridiaceae and Orchidaceae. In this system, Apostasiaceae and Cypridiaceae are recognized as separate families because they have more than one anther (2–3), which is the hallmark trait of most orchids. Szlachetko in 1995 published a classification of Orchidales based mainly on the structure of the gynostemium (column), but he also included a wide spectrum of data on vegetative structures (leaves, inflorescences, seeds, velamen, and micromorphology) as secondary traits. Szlachetko (1995), like Vermuelen (1966) and Rasmussen (1985), divided Orchidales into three families: Apostasiaceae, Cypridiaceae and Orchidaceae. The last he divided into eight subfamilies (with 47 tribes and 140 subtribes): Thelymitroideae, Orchidoideae, Tropidioideae, Spiranthoideae, Neottioideae, Vanilloideae, Epidendroideae and Vandoideae. A noteworthy aspect of this classification is recognition of Vanilloideae, which before this had routinely been considered members of Epidendroideae. Morphology of *Vanilla* and its relatives was noted by Szlachetko (1995) to be different in several aspects from that of other epidendroids, and their elevation to subfamily level presaged the DNA studies (although not with exactly the same component tribes; see below) that likewise identified them as a group misplaced in Epidendroideae.

In the recent years, new information has been added to anatomical and morphological data from other fields, such as genetics and molecular biology. The use of molecular techniques has made it possible to define variables between organisms at the level of DNA sequence. Classifications based largely on molecular phylogenetics (Chase et al., 2003) differ in modest ways from the classical systems based on morphology, although the DNA trees are not hugely different from those produced by cladistic analyses of morphological data (Freudenstein and Rasmussen, 1999).

Current research made on the basis of DNA sequence analysis in Orchidaceae has taken two approaches. At lower taxonomic level (tribes and below), studies have usually studied non-coding plastid markers (often the *trnL* intron and the *trnL-F* intergenic spacer) and ITS nuclear ribosomal DNA spacer (ITS1-5.8S-ITS2). Conversely, at the family level plastid protein-coding genes have been the primary focus: *rbcl* (Cameron et al., 1999; Chase et al., 1994), *matK* (Freudenstein et al., 2004), *psaB* (Cameron, 2004) and *ycf1* (Nuebig et al., 2009). In addition, studies using nuclear ribosomal 18S rDNA (Cameron and Chase, 2000) and the mitochondrial *nad1* b-c intron (Freudenstein et al., 2000; Freudenstein and Chase, 2001) have been published for Orchidaceae as a whole in recent years. However, the level of sequence variation in the last two DNA regions was insufficient for resolving relationships below the rank of tribe (but 18S rDNA performed well in the vanilloid orchids; Cameron, 2009), and this is why most researchers have preferred to use genes from the plastid genome in such studies. The first molecular phylogenetic analysis attempting to examine higher-level relationships of Orchidaceae using plastid sequences (*rbcl*) was Chase et al. (1994). They used 33 orchid sequences and 62 other lilioid monocots to look at the placement of orchids among monocots. In spite of sparse taxon sampling, results of this study were compatible with division of the orchids into five subfamilies: Apostasioideae, Cypridiaceae, Epidendroideae, Orchidoideae, and Vanilloideae. The apostasioids and cypridioids were clearly more closely related to other orchids

than to any other family, but their recognition as three families versus one could not be clearly resolved by this study. The recognition of five subfamilies was confirmed by Cameron et al. (1999), who expanded the dataset used by Chase et al. (1994) to 171 taxa to get better representation of the tribes and subtribes of Orchidaceae. Several previously recognized subfamilies based on morphological characters (Neottioideae, Spiranthoideae and Vandoideae) were clearly rejected by the results of these studies. The spiranthoid orchids were deeply embedded in Orchidoideae, and Vandoideae were sister to a single tribe, Epidendreae, of Epidendroideae. Neottioideae appeared polyphyletic or at least were a grade (paraphyletic) relative to Epidendroideae. Separation into three families was still possible, but it was clear that all species considered to be orchids formed a clade; the apostasioid orchids were sister to the rest (although not strongly supported as such), so recognition of Apostasiaceae was possible, whereas the slipper orchids (previously Cypridiaceae) appeared to be embedded within the family (although again not strongly supported in this position). Vanilloideae appeared to be supported by the early molecular results; this represents one of the major changes from most previously published classifications for Orchidaceae, although not that of Szlachetko (1995), but he included some taxa in Vanilloideae that have been shown to be members of Epidendroideae (e.g., Arethuseae and Triphoreae), so in fact the circumscription based on DNA analyses is unique. Chase et al. (2003) proposed a new phylogenetic classification of Orchidaceae using previously published data. The new system was largely based on the combined analysis of many molecular markers from different genomes: *atpB*, *rbcl*, *matK*, *psaB*, *trnL-F* (all plastid), 18S rDNA (nuclear) and *nad1* intron (mitochondrial). The morphological cladistic work of Freudenstein and Rasmussen (1999) was also considered in this classification, particularly from the standpoint of the distribution of morphological characters that were congruent with the molecular results. Chase et al. (2003) divided Orchidaceae into five subfamilies, 17 tribes and 42 subtribes. The analyses collectively supported the following set of relationships: [Apostasioideae [Vanilloideae [Cypridiaceae [Epidendroideae [Orchidoideae]]]]. All results suggested that monandry in Vanilloideae and Epidendroideae/Orchidoideae evolved independently. The analyses also recovered a group that consists of Vandeae plus Cymbidiaceae, which is more or less congruent with Vandoideae *sensu* Dressler (1981), but this clade was nonetheless embedded within Epidendroideae. There were three unplaced subtribes within Epidendroideae in this classification: Agrostophyllinae, Dendrobiinae and Collabiinae. Clearly, recognition of Cypridiaceae is inappropriate, whereas although compatible with the molecular results recognition of Apostasiaceae seemed unnecessary to these authors. The apostasioids are clearly orchid-like in morphological characters and phylogenetic position, and recognizing them as a separate family destroyed evidence of their higher-level relationships. They also uniquely share with all other taxa recognized as orchids the mycotrophic protocorm seedling stage (Kristiansen et al., 2001), which can thus be seen as a synapomorphy for Orchidaceae *sensu lato*.

Low-copy nuclear genes have seen relatively little application in higher-level angiosperm phylogenetics, but they hold the potential to be more informative than plastid genes because of expected higher rates of sequence divergence (Wolfe et al., 1987). Due to their biparental inheritance, nuclear DNA regions give also information about hybridization, a phenomenon of major importance in the evolution of angiosperms (Paun et al., 2009). These possible benefits are, however, counter-balanced by other phenomena such as recombination that can blur the pattern of relationships (Clarkson et al., 2010; Kelly et al., 2009). In the present study, we used a low-copy nuclear gene, *Xdh*, to estimate phylogenetic relationships within Orchidaceae. *Xdh* codes for xanthine dehydrogenase (XDH), which belongs to the molybdenum cofactor dependent hydroxylase class of enzymes. XDH is involved in nucleic acid degradation in bacteria,

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