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Morphological study of floral nectaries in *Euonymus* and the probable origin of the echinate fruit surface

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ABSTRACT

A conspicuous nectary disk is common but has a distinguishing morphology in the cosmopolitan genus *Euonymus*. Our study focuses on the morphology of floral nectaries in 21 representatives of *Euonymus* and *Glyptopetalum*. Two main types of nectaries were documented: a mix of inter- and extrastaminal nectaries existed between the corolla and the stigma, while the intrastaminal nectaries were distributed between the stigma and the stamen bases. The main route of nectar release in *Euonymus* is via modified stomata, and different nectarostomata locations were observed: in depressions, slightly raised above the epidermal surface or at the same level as the epidermis. Floral nectaries in *E.* sect. *Echinococcus* species developed into the protrusions on the fruit surface at the later stage. The development of nectaries not only explained the mystery of the origin of the echinate fruit surface, but also showed that differences in fruit surface might be inappropriate for use in infrageneric classification. These discoveries inform morphological observations of floral nectaries in *Euonymus*.

1. Introduction

Nectaries are specialized structures that produce and secrete nectar (Fahn, 1979; Nepi et al., 1996; Nicolson et al., 2007). Generally, the nectary is composed of three tissue types: nectary epidermis, nectary parenchyma and subnectary parenchyma (Nicolson et al., 2007). Nectary epidermis may have trichomes or modified stomata as secretory structures. Extrafloral nectaries may provide rewards for insects to defend fruit and seeds from predators; floral nectaries may also attract agents of pollination (Pacini et al., 2003; Thornburg et al., 2003). In response to these interactions, animals and flowers have co-evolved, leading to a diversity of floral nectary types which may play a crucial role in driving floral evolution and diversity in flowering plants (Thornburg et al., 2003). This variation in floral nectaries (e.g. shape, structure, position) has frequently been used to classify various plant genera in taxonomic and phylogenetic studies (Solereder et al., 1908; Fahn, 1953; Ancibor, 1969; Smets, 1986; Galetto, 1997; Galetto and Bernardello, 2004; Bernardello, 2007).

Euonymus L. (Celastraceae) consists of approximately 129 species which are mainly distributed in East Asia to the Himalayan Region as well as South Asia and Southeast Asia (Ma, 2001). The habitats of *Euonymus* species are highly diverse; flowers and fruits of this genus are inconspicuous and caducous. Intensive taxonomic studies of most species are limited by the number of specimens available after species descriptions. Infrageneric classification of *Euonymus* is mainly based on whether the capsules are angular, smooth, echinate or dehiscent (Blume, 1825; Maximowicz, 1881; Sprague, 1908; Wang, 1939; Nakai, 1941; Loesener, 1942; Blakelock, 1951; Ma, 2001; Ma and Funston, 2008). Fruit characters were first used in grouping of *Euonymus* in 1825 (Blume, 1825). Then, species with echinate capsules were included in *Euonymus* for the first time in 1908 (Sprague, 1908). The comprehensive revision of Celastraceae in 1942 recorded more than 100 *Euonymus* species in the world, which established the foundation for further studies on *Euonymus* (Loesener, 1942). Blakelock's monograph which comprised 176 species was the most important work for the taxonomy of *Euonymus* (Blakelock, 1951). This genus is divided into five sections in the most recent classification: *E.* sect. *Uniloculares*, *E.* sect. *Echinococcus*, *E.* sect. *Ilicifolia*, *E.* sect. *Melanocarya* and *E.* sect. *Euonymus* (Ma, 2001; Ma and Funston, 2008). *Glyptopetalum* Thw. (Celastraceae) is a small genus containing about 20 species in

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tropical and subtropical Asia (Liu and Funston, 2008). There are many significant similarities between *Glyptopetalum* and *Euonymus*. *Glyptopetalum* is treated as a section of *Euonymus* in some studies, but others believe that it is independent though closely related with *Euonymus* (Thwaites, 1856; Baillon, 1880; Hou, 1963). The molecular phylogenetic relationships of *Euonymus* are controversial. It has therefore been suggested that *Euonymus* be considered paraphyletic and the infrageneric classification unnatural, whereas *Euonymus* and *Glyptopetalum* form a monophyletic group together (Meng et al., 2011; Simmons et al., 2012; Simmons and Kappa, 2013; Li et al., 2014).

Flowers of *Euonymus* have a broad, flat and well-exposed disk surrounding the ovary. The blooming stage of the flowers is from March to August, and the lifespan of a single flower is approximately three days (Ma, 2001). When the flower is fully open, certain areas of the disk are sharply outlined by a great abundance of nectar, which attracts a variety of insects, including bees, ants, beetles, and flies (Berkeley, 1953; Konarska, 2015). In spite of the availability of comprehensive publications dealing with anatomical and morphological aspects of floral nectaries (Pacini et al., 2003), studies focused on floral nectaries in *Euonymus* are rare. To date, the nectary structure and nectar secretions have only been studied in five species from this genus (Berkeley, 1953; Matthews and Endress, 2005; Konarska, 2015). The nectaries of *E. americanus* L. and *E. japonicus* Thunb. are the first to be described: the nectariferous area is usually rose colored and is thus set off from the ovary and the regions around the base of each filament (Berkeley, 1953). Nectaries of *E. latifolius* (L.) Mill appear between the corolla and the stigma, with stomata sunken in pits (Matthews and Endress, 2005). According to Konarska (2015), the nectaries of *Euonymus fortunei* (Turcz.) Hand.-Mazz. and *E. europaeus* L. differ in size, location, abundance of stomata, and nectar content. The nectary disk is typical of Celastraceae, and it is one type of receptacular nectary (Fahn, 1979). Observations in other genera in this family show that floral nectaries are diverse in morphology, anatomy and histology (Berkeley, 1953; Matthews and Endress, 2005; Tan et al., 2007; Gomes and Lombardi, 2013). Clearly, nectary types have evolved in Celastraceae and display an array of adaptations.

Although floral nectaries have aroused interest in *Euonymus*, there is a paucity of data with regard to nectary morphology. Accordingly, in the present study, we investigate whether nectary features, such as form type and nectarostomata location, are correlated with the most recent classification of *Euonymus*. For this purpose, we characterized the diversity and development of nectaries in this genus. We also provide a detailed analysis of the origin of the echinate fruit surface.

2. Materials and methods

Floral nectaries were surveyed from flowers field-collected in China. In order to reduce sampling error, 10 flowers from each species were sampled at different developmental stages (before, during, and after anthesis). Twenty-one species of flowers from *Euonymus* (19 species) and *Glyptopetalum* (2 species), were randomly selected and fixed in FAA (formalin: acetic acid: 50% ethanol = 5: 5: 90). The source of the species studied for each analysis is given in Table 1. All *Euonymus* voucher specimens have been stored at the Shanghai Chenshan Herbarium (CSH), and the two *Glyptopetalum* voucher specimens have been deposited at the Beijing Normal University Herbarium (BNU). The scientific names of species in this study generally follow the recent taxonomic revisions (Ma, 2001; Ma and Funston, 2008).

The presence and position of nectaries on the flower disk were examined under a dissecting microscope (Leica S8APO) after the calyx and corolla were removed. The characteristics of the nectary gland were observed under scanning electron microscope and light microscope. Tissue specimens were first dehydrated in an ethanol series of 50, 60, 70, 80, 95, and 100% (two changes); subsequently, specimens were passed through a mixture of 100% ethanol and t-Butanol in following the proportions, ethanol: t-Butanol (3: 1, 2: 2, 1: 3 v/v) and 100% t-Butanol for 10 min intervals at each step; then the samples were dried in a t-Butanol Freeze Drying Device (VFD-21S). The dried materials were carefully mounted on aluminum stubs under a stereomicroscope (Olympus SZ61). After being coated with gold, the samples were viewed using a scanning electron microscope (FEI Quanta 250) at an accelerating voltage of 12.5 KV.

3. Results

3.1. Floral morphology and form of nectaries

Euonymus flowers are tetramerous or pentamerous, actinomorphic, and 5–25 mm in diameter, with white green or purple petals and inconspicuous calyx. Stamens are inserted on a fleshly disk with yellow or purple anthers. The ovary is inferior, and the base of the style is surrounded by the disk.

All examined species had prominent nectaries on the disk (Fig. 1). Organ initiation of the nectary occurred at a primordial floral stage when the flower bud was still quite small. According to the classification of receptacular nectaries developed by Schmid (1988), which was based on the location of the gland relative to stamens, there are two major types of nectaries in *Euonymus*: a mix of inter- and extrastaminal types, and the intrastaminal type. The nectary disk of inter- and extrastaminal types is located between the corolla and the stigma, and thus also encompasses the stamen bases like a congenitally united collar (*Euonymus sanguineus*, *Euonymus schensianus*, *Euonymus verrucocarpus*, *Euonymus wilsonii*, *Euonymus actinocarpus*, *Euonymus balansae*, *Euonymus alatus*, *Euonymus hukuangensis*. *Euonymus yunnanensis*, *Euonymus wui*, *Euonymus nitidus*, *Euonymus oblongifolius*, *Glyptopetalum longipedicellatum*, and *Glyptopetalum longepedunculatum*) (Fig. 1: a, g). The nectary disk of the intrastaminal type is located between the stamen and the stigma, and the stamen bases are at the edge (*Euonymus acanthoxanthus*, *Euonymus pseudovagans*, *Euonymus theifolius*, *E. fortunei*, *Euonymus centidens*, *Euonymus tingens*, and *Euonymus microcarpus*) (Fig. 1: d).

3.2. Location of nectarostomata

In *Euonymus*, nectar is secreted by modified stomata that have lost the capacity to open and close (Fig. 1). Variations in the location of nectarostomata were found on the apex of nectaries. In *E. sanguineus*, *E. centidens*, *E. alatus*, *E. hukuangensis*, *E. nitidus* and *E. oblongifolius*, the nectarostomata are sunken in pits (Fig. 1: b, c). In all species of *E.* sect. *Echinococcus* and *E.* sect. *Ilicifolia*, as well as *E. tingens* (*E.* sect. *Euonymus*), the nectarostomata are located on distinct convexities, forming structures resembling chimneys or volcanoes (Fig. 1: e, f; Fig. 2). In *E. schensianus*, *E. yunnanensis*, *E. wui*, and *E. microcarpus*, as well as all species of *Glyptopetalum*, the nectarostomata are level with adjacent epidermal cells (Fig. 1: h, i).

4. Discussion

4.1. Variation of nectary morphology among sections

Nectaries are commonly similar throughout some families, such as Lamiaceae, Brassicaceae, and Asteraceae (Kumari, 1986; Davis et al., 1998; Mani and Saravanan, 1999). At least two types of floral nectaries exist in Cucurbitaceae (Pacini et al., 2003). A conspicuous nectary disc is common in Celastraceae: the disc extends