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# MORPHOMETRIC ANALYSIS OF *VANILLA* SEEDS (ORCHIDACEAE) BY MICROSCOPIC TECHNIQUES

Yasmín A. Alomía[1,2,5], Efrén Muñoz[1], Aleyda M. Acosta-Rangel[3] & J. Tupac Otero[1,2,4]

[1] Departamento de Ciencias Biológicas, Universidad Nacional de Colombia Sede Palmira Calle 32 N° 12-00, Palmira – Valle del Cauca – Colombia

[2] Grupo de Investigación en Orquídeas, Ecología y Sistemática Vegetal

[3] Departamento de Biología, Universidad del Valle, Calle 13 N° 100-00, Cali – Valle del Cauca – Colombia

[4] Instituto de Ciencias Ambientales IDEA, Universidad Nacional de Colombia Sede Palmira.

[5] Corresponding author current address: Departamento de Ciencias Biológicas, Universidad de los Andes, Carrera 1 N° 18ª-10, Bogotá D.C. Colombia. ya.alomia10@uniandes.edu.co

Abstract. Seed morphometric characters of four Colombian *Vanilla* species (*Vanilla odorata*, *Vanilla calyculata*, *Vanilla oroana* and *Vanilla rivasii*) were compared by using both light and electron microscopy. Seeds collected from mature fruits were studied by optical microscopy to characterize coat color, length and width of seeds in *Vanilla* species. Scanning electron microscopy (SEM) was used to characterize the seed surface and cell shape in these species. There were significant differences in length, width and length/width ratio. *Vanilla oronana* had the largest seeds (431 ± 17 µm long and 312 ± 27 µm wide) and differed from the other species by its dark brown seed coat and the presence of protrusions. *Vanilla odorata* was the species with smallest seeds (310 ± 15 µm long and 222 ± 18 µm wide) and light brown seed coat. Seeds of all studied species had smooth coat surfaces and were of oval to ellipsoid in shape. The characters evaluated in this work could serve as taxonomic diagnostic markers in *Vanilla* and these could explain important aspects of the seed biology of this genus.

Resumen. Se caracterizó y se comparó la morfometría de las semillas de cuatro especies de vainillas colombianas (*Vanilla odorata*, *Vanilla calyculata*, *Vanilla oroana* y *Vanilla rivasii*). Las semillas colectadas a partir de frutos maduros fueron estudiadas por microscopía óptica para caracterizar el color, la longitud y el ancho de las semillas. Además, por medio de microscopía electrónica de barrido (MEB) se identificaron las características de la superficie de la testa. Existieron diferencias significativas en la longitud, el ancho y en la relación longitud/ancho entre las especies. *Vanilla oronana* tuvo las semillas más grandes (431 ± 17 µm de largo y 312 ± 27 µm de ancho) y difirió de las otras especies por presentar la coloración parda en tono más oscuro y por la presencia de protuberancias en la testa. *Vanilla odorata* fue la especie con semillas más pequeñas (310 ± 15 µm de longitud y 222 ± 18 µm de ancho) de color pardo claro. En general, las semillas presentaron la testa lisa y una forma elipsoide. Los caracteres evaluados en este trabajo podrían servir como marcadores taxonómicos en *Vanilla* y podrían explicar aspectos importantes de la biología de las semillas de este género.

Key words: Colombia, morphology, traits, orchids, *Vanilla*, SEM

**Introduction.** Most orchid seeds are smaller than 2.0 mm in length with small embryos and have no endosperm (Baskin & Baskin 1998). Orchid seed length varies from 0.05 to 6.0 mm and there is a broad variation in the seed coat reticulation patterns, shape, size and color (Arditti & Ghani 2000). In many species of Orchidaceae, seeds have hyaline seed-coat with visible embryos at the dissection microscope resolution without staining. Nevertheless, some genera such as *Apostasia, Cyrtosia, Epistephium, Galeola, Neuwiedia, Palmorchis, Selenipedium* and *Vanilla* have sclerotized seed coat which does not allow embryo visualization (Molvray & Kores 1995).

Several studies has been performed on the

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Table 1. Source localities of *Vanilla* spp. seeds.

| Species | Locality | Life Zone* | Elevation m.a.s.l | Department |
|---|---|---|---|---|
| *Vanilla oroana* | El Charco | Tropical wet forest | 2 | Nariño |
| *Vanilla rivasii* | Buenaventura | Tropical wet forest | 7 | Valle del Cauca |
| *Vanilla calyculata* | Atuncela | Tropical dry forest | 800 | Valle del Cauca |
| *Vanilla odorata* | Ríofrío | Tropical dry forest | 1122 | Valle del Cauca |

* According to the classification by Holdridge (1947)

morphology of native Californian orchids including *Goodyera, Piperia, Platanthera* and *Spiranthes* species (Healey, Michaud & Arditti, 1980), *Paphiopedilum* and *Cypripedium* (Arditti *et al.* 1979), and *Calypso, Cephalanthera, Corallorhiza* and *Epipactis* (Arditti, Michaud & Healey, 1980). These studies suggest that variations in size seed, shape, color and patterns of the seed coat cells have served as taxonomic and/or phylogenetic markers on seeds of native California orchids and related species (Barthlott 1976, Arditti *et al*. 1979, Arditti *et al*. 1980, Healey *et al.* 1980).

Additionally, seed morphology may affect important biological and ecological aspects such as seed dispersion mechanisms. Most orchid seeds have traits that favor long distance wind dispersal due to their small size, shape and large air space (Arditti & Ghani 2000). On the other hand, the genera with lignified seed coats have contrasting dispersal mechanisms associated with endozoochory. For example, avian seed dispersal was reported in a mycoheterotrophic orchid *Cyrtosia septentrionalis* (Rchb.f.) Garay (Nakamura & Hamada 1978, Suetsugu *et al.* 2015). Additionally, it have been suggested that seeds of *Vanilla planifolia* Andrews are dispersed by birds (Arditti & Ghani 2000). Also, Schlüter, Soto-Arenas & Harris (2007) suggested that *Vanilla* fruits show a typical bat dispersal syndrome, being darkly colored, pendulous, and strongly scented. In all these cases, the lignified seed coat probably protects the seeds as they pass through the digestive tracts of dispersal animals.

*Vanilla* Mill is a genus with nearly 110 species with pantropical distribution (Purseglove *et al*. 1981). *Vanilla* extract is an important fragrant and flavor natural product, obtained from the curated fruit of the hemiepiphyte tropical orchid *Vanilla planifolia* and produced mainly in Madagascar and Indonesia. In Colombia, *Vanilla* species are distributed below 2000 m in all natural regions (Caribbean, Andean, Pacific, West plains and Amazon; Ordoñez-Osorio *et al.* 2011). Soto Arenas & Dressler (2010) reported 11 species; but lately, Molineros-Hurtado, González, Flanagan and Otero (2014) described a new species from the Pacific coast. More recently, the Colombian Catalog of Plants and Lichens reported 15 *Vanilla* species (Bernal *et al.* 2015).

Despite the growing interest about *Vanilla* cultivation, little is known about their ecology, and the seed morphometric studies of *Vanilla* species are scarce (Table 1). Fruit and seed trades of four *Vanilla* species from Mexico using light microscopy were reported showing morphological variation among species and even clones (Reyes-López *et al.* 2014). One species (*V. planifolia*) was studied using scanning electron microscopy (Barthlott *et al.* 2014). Developing seed morphometric studies of *Vanilla* species could be useful for dispersal studies, helping in the seed identification on fecal samples of the disperser potentials.

The potential use of seed morphology to support taxonomic studies has not been taken into account in the taxonomic treatment of *Vanilla* species. A recent revision of *Vanilla* for Central America includes 15 species, but only two species have information about their seeds: *V. martinezii* Soto-Arenas, and *V. trigonocarpa* Hoehne (Soto-Arenas & Dressler, 2010). The aim of this study was to characterize the seed morphometry of four Colombian *Vanilla* species, using light microscopy and scanning electron microscopy.

**Materials and Methods.** Seeds from mature fruits of four Colombian *Vanilla* species: *Vanilla calyculata* Schltr; *V. odorata* C.Presl; *V. oroana* Dodson; and *V. rivasii* Molineros *et al.* were collected from various locations of Colombia (Table 1). Seeds at the

TABLE 2. Length and width average of *Vanilla* seeds.

| Species | Mean Lenght (µm)* | Mean width (µm)* | Lenght/width Ratio |
|---|---|---|---|
| *V. oroana* | 431.24 ± 17.24 a | 312.12 ± 27.30 a | 1.39±0.12 b |
| *V. calyculata* | 393.41 ± 18.30 b | 320.82 ± 14.78 a | 1.23±0.06 a |
| *V. rivasii* | 339.56 ± 15.97 c | 228.21 ± 14.26 b | 1.49±0.10 c |
| *V. odorata* | 310.74 ± 15.62 d | 222.10 ± 17.92 b | 1.41±0.12 b |

*The mean value ± standard deviation (±SD) is reported. Different letters indicate significant differences (P≤0.05) among species after a Tukey test.

TABLE 3. Reported seed size in others studies for *Vanilla* spp.

| Specie | Length (µm) | Width (µm) | Reference |
|---|---|---|---|
| *V. inodora* | 292.02 | 151.90 | Reyes-López *et al.,* 2014 |
| *V. pompona* | 286.20 | 183.47 | Reyes-López *et al.,* 2014 |
| *V. insignis* | 323.51 | 267.88 | Reyes-López *et al.,* 2014 |
| *V. planifolia** | 286.08 | 232.17 | Reyes-López *et al.,* 2014 |
| *V. planifolia* | 250 | 220 | Bouriquet, 1947 |
| *V. trigonocarpa* | 600 | - | Soto-Arenas & Dressler, 2010 |
| *V. planifolia* | 500 | - | Barthlott *et al.*, 2014 |

*Average for 10 accessions

midsection of the fruit of at least two fruits per species were extracted and washed following Knudson protocol (Knudson 1950). Seeds were deposited in 5 mL Eppendorf tubes and dehydrated in 3 mL of absolute ethanol and shacked with ultrasound twice for five minutes. For optic microscopy, seeds were mounted over slides with gelatine-glycerine and sealed with paraffin. Slides were observed in an optical microscope (NIKON ECLIPSE NI-U90) at the Image laboratory at Universidad del Valle, Cali, Colombia. Seed length and width measurements, were obtained using a microscope imaging software (NIS-Elements Br) to 40 seeds per species. Additionally, a dissection microscopy (SEM; NIKON SMZ 800) was used for describing the seed color. For detailed observation of seed coat traits, cleaned seeds were dried at room temperature for 24 hours, then seeds were fixed on carbon tape and covered with gold-palladium for 80 seconds. Samples were observed using a SEM (HITACHI S4800) at the Image laboratory at Universidad de Valencia, Valencia, Spain. Statistical analysis (Descriptive statistics, ANOVA and Tukey test) of length, width and length/width ratio were performed using Statistica 7.0 and R 3.1.1 software. Comparison among means were done considering statistically significant differences if p<0.05.

**Results.** There were significant differences in seed length ($F_{3,\ 156}$ = 410.36, p<0.005), width ($F_{3,156}$ = 297.84, p<0.005) and length/width ratio ($F_{3,\ 156}$ = 48.35, p<0.005) among species. For seed length, the tukey test (Table 2) indicated that all species were different. *Vanilla oroana* had the largest seeds (431 ±17 µm x 312±27 µm), while *V. odorata* had the smaller ones (310±15 µm x 222±18 µm). For seed width, there were two groups significantly different from each other; the first form by *V. oroana* (312.12 ± 27.30) and *V. calyculata* (320.82 ± 14.78) and the second included *V. rivasii* (228.21 ± 14.26) and *V. odorata* (222.10 ± 17.92). The length/width ratio of *V. calyculata* (1.23±0.06) was close to one as a consequence of the circular shape pattern, while in *V. rivasii* that had ellipsoid seeds, its ratio was higher than one (1.49±0.10)-On the other hand, *V. oroana* (1.39±0.12) y *V. odorata* (1.41±0.12), which had an intermediate shape between spherical and ellipsoid, don't showed differences (Table 2).

Microscopic images of *V. calyculata* (Fig. 1), *V. oroana* (Fig. 2), *V. odorata* (Fig. 3) and *V. rivasii* (Fig. 4) are presented. *Vanilla calyculata*, *V. odorata* and *V. rivasii* had an oval-planar corpus (Figs. 1, 3 y 4, respect.), while *V. oroana*, had an oval-globular corpus with protuberances that gave an irregular appearance

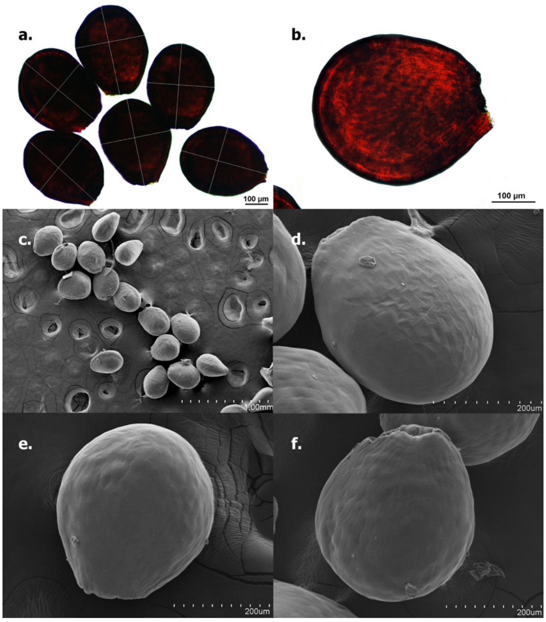


Figure 1. Seeds of *Vanilla calyculata*. a–b. Optic microscopy images. c–f. Scanning electron microscopy (SEM) images.

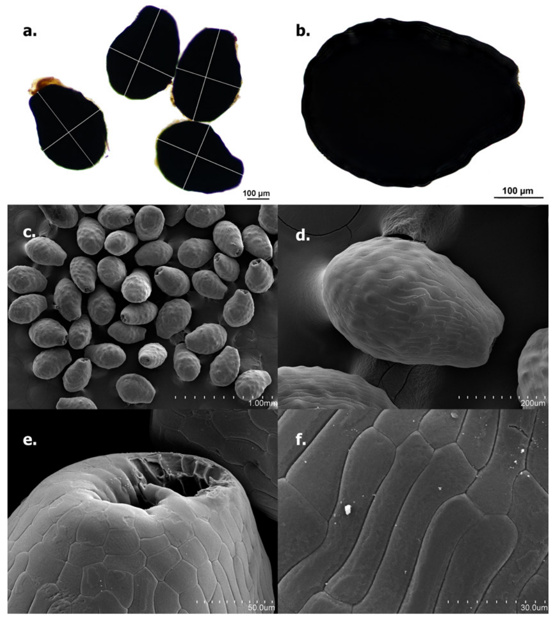


Figure 2. Seeds of *Vanilla oroana.* a–b. Optic microscopy images. c–f. Scanning electron microscopy (SEM) images.

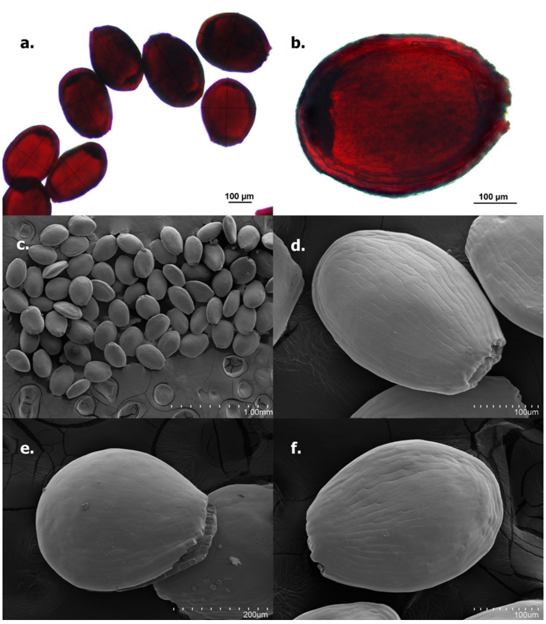


Figure 3. Seeds of *Vanilla odorata.* a–b. Optic microscopy images. c–f. Scanning electron microscopy (SEM) images.

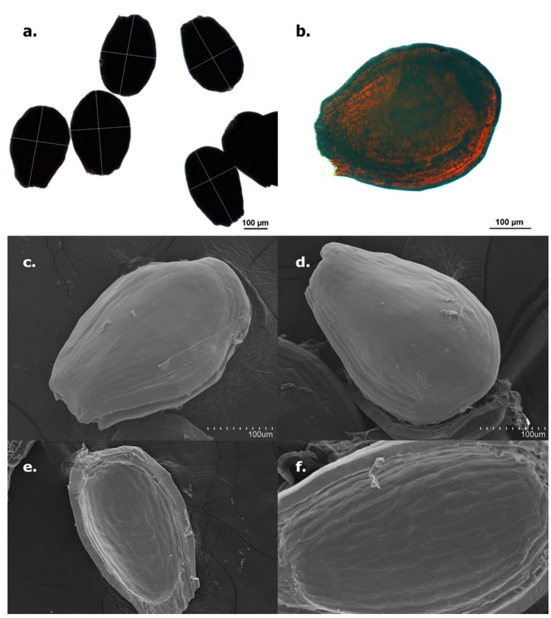


Figure 4. Seeds of *Vanilla rivasii*. a–b. Optic microscopy images. c –f. Scanning electron microscopy (SEM) images.

(Fig. 2c-d). The cells of the seed coats of the four studied species were smooth, with a longitudinal aperture and without reticulation patterns. In all studied species, the cells around the micropile are square or polygonal, contrasting with the rectangular shape of the cells in the central part of the seed (Figs. 1d, 2d, 3d, 4d). Relative to the seed color, all species had brown seeds; however, a tonality variation was observed. The darkest seeds were those of *V. oroana* and the lightest ones were from *V. odorata* (Figs. 1a-b, 2a-b, 3a-b, 4a-b).

**Discussion.** We found a seed morphological variation among *Vanilla* studied species that is consistent with other comparative studies (Table 3). The seeds of the Colombian *Vanilla* species were within the known seed size range. The smallest reported *Vanilla* seeds are those from *V. planifolia* (250 ± 80 µm x 220 ± 60 µm; Bouriquet 1947). However, Barthlott *et al.* (2014) reported seed length of 500 µm for *V. planifolia.* Thus, it is necessary to conduct rigorous studies to evaluate intraspecific variation. Other studies with mexican vanillas have reported that *Vanilla* species with the biggest seeds was *V. insignis* Ames (323.51 µm x 267.99 µm), while *V. inodora* Schiede had the smallest seeds 292.02 µm x 151.90 µm (Reyes-López *et al.* 2014; Table 3). Taking into account of color of the seeds, *Vanilla* species have been reported as dark-brown (Knudson 1950; Hernández-Hernández 2011). In our study we found that there is variation in the tonality from light to dark -brown, close to black among species. Given the kwon variation in *Vanilla* seed size, this parameters show the potential for *Vanilla* taxonomy. Nevertheless, it is necessary more studies on *Vanilla* seeds to cover the nearly 110 described species.

*Vanilla* seed morphology could be useful in dispersal mechanisms studies. The *Vanilla* dispersion mechanisms are not well known; however, some studies have suggested a possible dispersion through endozoochory, mainly by birds (Nakamura & Hamada 1978, Barthlott *et al.* 2014). In addition, it has been suggested that euglossinae bees disperse *Vanilla* seeds due to the collection of vanillin by the male of this species for female attraction (Soto-Arenas & Dressler 2010). Identification of *Vanilla* seeds from animal feces may shed light on this old and interesting question.

After dispersion, seeds require developing into the seedling stage, and morphological trades recognized in seeds may be related with the germination process. The thickness of the seed coat could be involved in dormancy process and development of seed banks (Baskin & Baskin 1998). Alternatively, it could help the seeds to survive passing through an animal's digestive system during seed dispersion as in other seeds with lignified seed coat (Traveset *et al.* 2007, Suetsugu *et al*. 2015). As with other orchids, *Vanilla* seeds require mycorrhizal fungi for germination under field conditions (Porras-Alfaro & Bayman 2007). In Colombia, *Ceratobasidium* and *Tulasnella* fungi were reported as mycorrhizal in *Vanilla* spp. (Mosquera-Espinosa *et al.* 2010, Alomía 2014).

Regarding the techniques used in this study, combining the optical microscopy with the use of permanent slides allowed to observe the coat color and take easily morphometric measurements, all at low cost. On the other hand, images in SEM at high resolution allowed the observation of the external structures of seeds, highlighting the smooth ornamentation in all studied species. Therefore, both approaches are complementary for a complete characterization of the seeds. Given the economic importance of *Vanilla*, this study reports new data about morphometric characteristics that may be useful for future taxonomic and ecological studies. Additionally, the techniques here reported could be applied for determining the species of *Vanilla* used on commercial vanilla products.

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