

## **New species of *Buxus* (Buxaceae) from northeastern Cuba based on morphological and molecular characters, including some comments on molecular diagnosis**

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## New species of *Buxus* (*Buxaceae*) from northeastern Cuba based on morphological and molecular characters, including some comments on molecular diagnosis

### Abstract

González Gutiérrez P. A., Köhler E. & Borsch T.: New species of *Buxus* (*Buxaceae*) from northeastern Cuba based on morphological and molecular characters, including some comments on molecular diagnosis [Novitiae florum cubensis 40]. – Willdenowia 43: 125–137. June 2013. – Online ISSN 1868-6397; © 2013 BGBM Berlin-Dahlem. Stable URL: <http://dx.doi.org/10.3372/wi.43.43115>

Three new species of *Buxus* endemic to the serpentine areas of Sierra de Nipe and Sierra del Cristal, northeastern Cuba, are described. Morphological descriptions including pollen and leaf anatomy are provided as well as sequences of the plastid *trnK-matK* and *trnL-trnF* regions, serving as molecular descriptions. The substitutions were evaluated to find suitable characters for a molecular diagnosis that complements the morphological diagnosis. Using the newly described species of *Buxus* as an example, prospects and pitfalls of DNA characters to support species diagnosis are discussed. Furthermore, an assessment of the distribution, habitat, ecology, and conservation status of the three newly recognized endemic species is provided.

Additional key words: box, boxwood, serpentine, pollen, anatomy, endemism, conservation, DNA barcodes, Greater Antilles

### Introduction

The genus *Buxus* is the largest of the family *Buxaceae* and comprises c. 100 species distributed in all continents except Australia and Antarctica. About 40 % of these species occur on Cuba. The centres of morphological and ecological diversity of *Buxus* are the Caribbean (c. 50 spp.), East Asia (c. 40 spp.) and Africa and Madagascar (c. 15 spp.) (Köhler & Brückner 1990; Schatz & Lowry 2002).

In Alain's (1953) treatment of *Buxaceae* for the Flora de Cuba, 27 *Buxus* species were recognized. During the second half of the 20th Century, in the context of collabo-

ration between Cuban and East European botanical institutions, Borhidi & Muñiz (1973, 1977) published three further species. Starting in the seventies, Prof. Johannes Bisse and a young team of Cuban and East German botanists made collecting expeditions to all Cuban provinces in order to collect new plant material for the elaboration of a modern, critical Flora of Cuba. As a result of this, Köhler (1982, 1998, 2006) published nine new Cuban species of *Buxus*, raising their total number to c. 40. Recent field work, as well as morphological, palynological and anatomical studies and molecular phylogenetic analysis of the whole genus, led to the discovery of the three additional new species described below.

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*Buxus* represents one of the most important species radiations in the flora of Cuba and also shows an interesting distribution pattern in the Caribbean with a few species on various islands and also in México and Central America. The genus was therefore chosen as a model group to reconstruct its phylogeny at the species level and to test hypotheses concerning the origin and evolution of the Cuban flora (González & al., ongoing work). So far only few groups of the Cuban flora have been investigated using phylogenetic techniques, such as *Croton* (Ee & al. 2008), *Pachyanthus* (Bécquer-Granados & al. 2008), *Ginoria* (Graham 2010), *Spathelia* (Appelhans & al. 2011, 2012), *Leucocroton* (Jestrow & al. 2012) and *Brunfelsia* (Filipowicz & Renner 2012). However, sampling at the species level was often not complete and taxonomic questions including analyses of species limits were not part of these works, although this is of high importance in lineages that had previously just been treated with alpha-taxonomic approaches.

In the course of our analysis of the evolutionary diversification of *Buxus* on Cuba and in the Caribbean region, all previously described taxa as well as known morphologically deviant and geographically isolated populations are currently being included into molecular and morphological data sets. Molecular trees thereby facilitate the analysis of often more homoplastic phenotypic character states (e.g. flower morphology, pollen morphology, leaf anatomy) in a speciose clade. Phylogenetic analysis revealed well-resolved species-level trees (González & al. unpublished data) and indicated several of the deviating specimens to belong to distinct sub-lineages within the Cuban radiation of *Buxus*. The goal of this paper is to formally describe three new species of *Buxus* that can be unambiguously recognized by both phenotypic and sequence data. This study will thereby also serve as a basis for the preparation of the treatment of the genus in the Flora de Cuba (Köhler & González, ongoing work).

## Materials and methods

### Plant material

The mountains of Nipe-Sagua-Baracoa have been visited repeatedly during the last 15 years in order to collect *Buxus* material. Material from these expeditions also allowed the establishment of a living collection of *Buxus* at the National Botanical Garden of Cuba, University of La Habana (Rankin & al. 1999; Köhler 2001) with well-documented plants that could be studied at various stages of flowering and fruiting, and also served this study. The material included in this study is listed in both the information on types and the respective paragraphs on “additional specimens seen”. For those samples sequenced, the corresponding DNA sample codes (Bx026, Bx055, Bx117, Bx162, Bx163, Bx164 and Bx165) that are used for all *Buxus* samples in the ongoing evolutionary analysis of the genus are added.

### DNA isolation, sequencing, annotation and analysis

Sequences of the plastid *matK-trnK* and the *trnL-F* regions were generated for seven and five samples, respectively. Total DNA was isolated from silica-gel-dried leaf tissue or herbarium specimens using a triple CTAB extraction method (Borsch & al. 2003) or the Nucleo Spin Plant II extraction kit (Macherey Nagel, Düren, Germany). The amplification of each marker was performed in reaction volumes of 50 µL, containing 2 µL of extracted DNA (with a concentration of 10–20 ng/µL), 14.7 µL of H<sub>2</sub>O, 5 µL of 10× peqLab Taq.Buffer S containing MgCl<sub>2</sub>, 3 µL of MgCl<sub>2</sub> (25 mM), 10 µL of Betaine monohydrate (5 M), 1 µL of BSA (10 µg/µl), 2 µL of forward primer (20 pm/µl), 2 µL of reverse primer (20 pm/µl), 10 µL dNTPs (each 0.25 mM) and 0.3 µL Taq polymerase 5 units/µl (PeqLab, Erlangen Germany).

The *trnL-F* region was amplified using the primers trnTc and trnTf (Taberlet & al. 1991). The Polymerase Chain Reaction (PCR) program was: 30 cycles of denaturation (60 seconds at 96 °C), annealing (60 seconds at 50 °C), extension (120 seconds at 72 °C). Sequencing was carried out with the primer trnTf and the additional internal primer trnTd (Taberlet & al. 1991). The *trnK-matK* region was amplified and sequenced in two fragments using the primer pairs trnK-F (Wicke & Quandt 2009) and BxmatK-1270R (5'-ATTCCAATTATGATACTCG-3', designed for *Buxaceae* in this study), as well as BxmatK-467F (5'-TGTCAGATATACTAATACC-3', designed for *Buxaceae* in this study) and trnK-2R (Johnson & Soltis 1994). For the samples Bx164 and Bx165, isolated from older herbarium specimens, the use of further internal primers for amplification and sequencing was necessary that were either newly designed (BxtrnK-779R, 5'-TAAATATACTCCTGAAAGAG-3'; BxtrnK-1750R, 5'-AATTTTCTAGCATTTGACTC-3'), or taken from Müller & Borsch (2005, ACmatK-105F). The PCR program used was: 34 cycles of denaturation (60 seconds at 94 °C), annealing (60 seconds at 50 °C) and extension (120 seconds at 72 °C).

In all cases the amplification products were purified by electrophoresis in a 1.2 % NEEO agarose gel (Carl Roth, Germany) running during 3 hours at 100 Volts. The gel extraction was performed using the AveGene Gel/PCR DNA Fragments Extraction Kit (Avegene life science Corporation), following the protocol provided by the manufacturers. The concentration of the purified PCR products were measured with a NanoDrop spectrophotometer (ND-1000, PeqLab, Erlangen, Germany). Cycle sequencing, fragment purification and sequencing were performed by MacroGen Inc., South Korea (<http://www.macrogen.com>). The sequences were edited and manually aligned with a motif alignment approach (Löhne & Borsch 2005; Morrison 2009) using PhyDE v.0 995 (Müller & al. 2007). Boundaries of the genomic regions studied were annotated using a multiple sequence alignment in comparison with completely sequenced and annotated plastid genomes of *Nicotiana tabacum* (Z00044; Shi-

nozaki & al. 1986) and *Buxus microphylla* (NC009599; Hansen & al. 2007). The respective character positions were then determined with reference to each specific sequence because of length variability within the genomic regions.

### Micromorphology and anatomy

The palynological and anatomical methods applied in this study, as well as the technical terms employed in the discussion are those set out in Köhler (1981, 1982, 1998, 2006) and Köhler & Schirarend (1989).

## Results

*Buxus nipensis* Eg. Köhler & P. A. González, **sp. nov.** – Fig. 1–7.

Holotype: Cuba, province Holguín, Mayarí, Cabezas del río Piloto, en la zona de las cascadas, 20°27'44"N, 75°48'59"W, 500–700 m, 8 Mar 1998, J. Gutiérrez, E. Köhler, A. Leiva, R. Rankin & I. Silva HFC 75468 (HAJB; isotypes: B, BHU, JE) [= Bx165].

*Morphological diagnosis* — Leaves oblong to narrowly elliptic, apex retuse to emarginate and mucronulate. Male tepals broadly ovate to suborbicular, adaxially glabrous, margin narrowly membranous, apex apiculate. Ovary rounded-triangular, dorsal veins sunken at edges; nectaries well-developed, angular; styles obliquely erect. Capsule ellipsoid-globose, dorsal veins scarcely prominent; nectaries prominent, rounded; styles erect then recurved.

*Molecular diagnosis* — Nucleotide character state “A” in position 343 and “G” in position 448 of *matK* coding sequence.

*Morphological description* — *Shrub or tree* to 5 m tall; branchlets angular; internodal folds narrow with slightly prominent ribs, dorsally ± keeled or variably keeled on each side; internodes 2–5(–8) cm long, glabrous. *Leaves* dimorphic; *normal-sized leaves* with petiole 4–8 mm long, blade greenish yellow and slightly shiny adaxially, paler and dull abaxially, oblong to narrowly elliptic, 4–8 × 2–4 cm, coriaceous, glabrous, base broadly cuneate to shortly narrowed, apex obtuse, retuse to emarginate and mucronulate, midvein progressively sunken adaxially toward base, raised abaxially, secondary veins in 12–18 pairs, anastomosing in an adaxially prominent intramarginal vein 1–1.5 mm from revolute margin; *smaller decussate leaves* interspersed between normal ones, linear-lanceolate, 5–7 mm long, apex acute. *Inflorescences* sessile, 4–7 mm long, glabrous; bracts ovate-triangular to suborbicular, 1–1.8 mm long, margin scarcely ciliate, apex acute to apiculate with a bright membranous tip. *Male flowers* with pedicel 1.5–3 mm long; tepals broadly ovate to suborbicular, 1.2–2 mm long, adaxially glabrous, margin narrowly membranous, scarcely ciliate,

apex apiculate; stamens 2.5–4 mm long, filaments slightly flattened, anthers c. 1.2 mm long, with a prominent black tip; pistillode hemispherical, wrinkled. *Female flowers* with tepals apiculate with a bright membranous tip; ovary rounded-triangular, c. 2.5 × 2.5 mm, glabrous, dorsal veins sunken at edges, commissures narrowly protruding between collateral furrows, continued into angled nectaries; styles obliquely erect, apically curved, 2–3 mm long; stigmas narrowly folded, 2–3 mm long. *Capsule* brownish, ellipsoid-globose, c. 7 × 5–7 mm, glabrous, with scarcely prominent dorsal veins, crowned by c. 4 mm long erect then recurved styles; nectaries forming prominent rounded knobs. *Seeds* black, shining, rounded-triangular, c. 4 × 2.2 mm.

*Molecular description* — Sequences describe the type specimen (code Bx165) and are available in EMBL/GenBank/DBJ under accession numbers HG004439 (*matK-trnK*) and HG004432 *trnL-trnF*. Further sequences describe paratype specimens (codes Bx117, Bx162, Bx163) and are available in EMBL/GenBank/DBJ under accession numbers HG004436, HG004437 and HG004438 (*matK-trnK*) and HG004431 *trnL-trnF* (only Bx117).

*Pollen morphology* — Pollen 3- or 4(–6)-colporate, colpi 3- or 4-orate, reticulate, heterobrochate, higher murus segments broader than the lower ones, crenulated (conspicuously ribbed), bounding lumina of different size.

*Leaf anatomy* — *Buxus nipensis* is characterized by the absence of secretory cells. The adaxial epidermis consists of high, thin-walled cells, with anticlinal walls only slightly thickened in the apical part. The palisade parenchyma is composed of 2 or 3 layers of scarcely differentiated cells. The adaxial epidermis has a reticulate pattern of protruding anticlinal walls with sunken anticlinal borders (as in *B. retusa* Müll. Arg.) and slightly undulate periclinal walls. The stomata have a peristomal rim.

*Etymology* — The specific epithet alludes to the distribution area of this species, Sierra de Nipe, in the northeastern region of Cuba.

*Distribution* — *Buxus nipensis* is endemic to the Sierra de Nipe, municipality of Mayarí, current province of Holguín. In Sierra de Nipe it has been collected near to Woodfred, Brazo Dolores, La Casimba, La Plancha, loma de La Bandera, loma de La Estrella, río Piloto, Loma Mensura and near to Estación de Investigaciones de la Montaña. However during the last decade it has been refound only in the last three localities.

*Habitat and ecology* — *Buxus nipensis* grows on serpentine in subspiny xeromorphic thickets known in Cuba as “charrascal” or “charrascos”, in forest of *Pinus cubensis* Griseb., and in riverine rainforest along mountain brooks



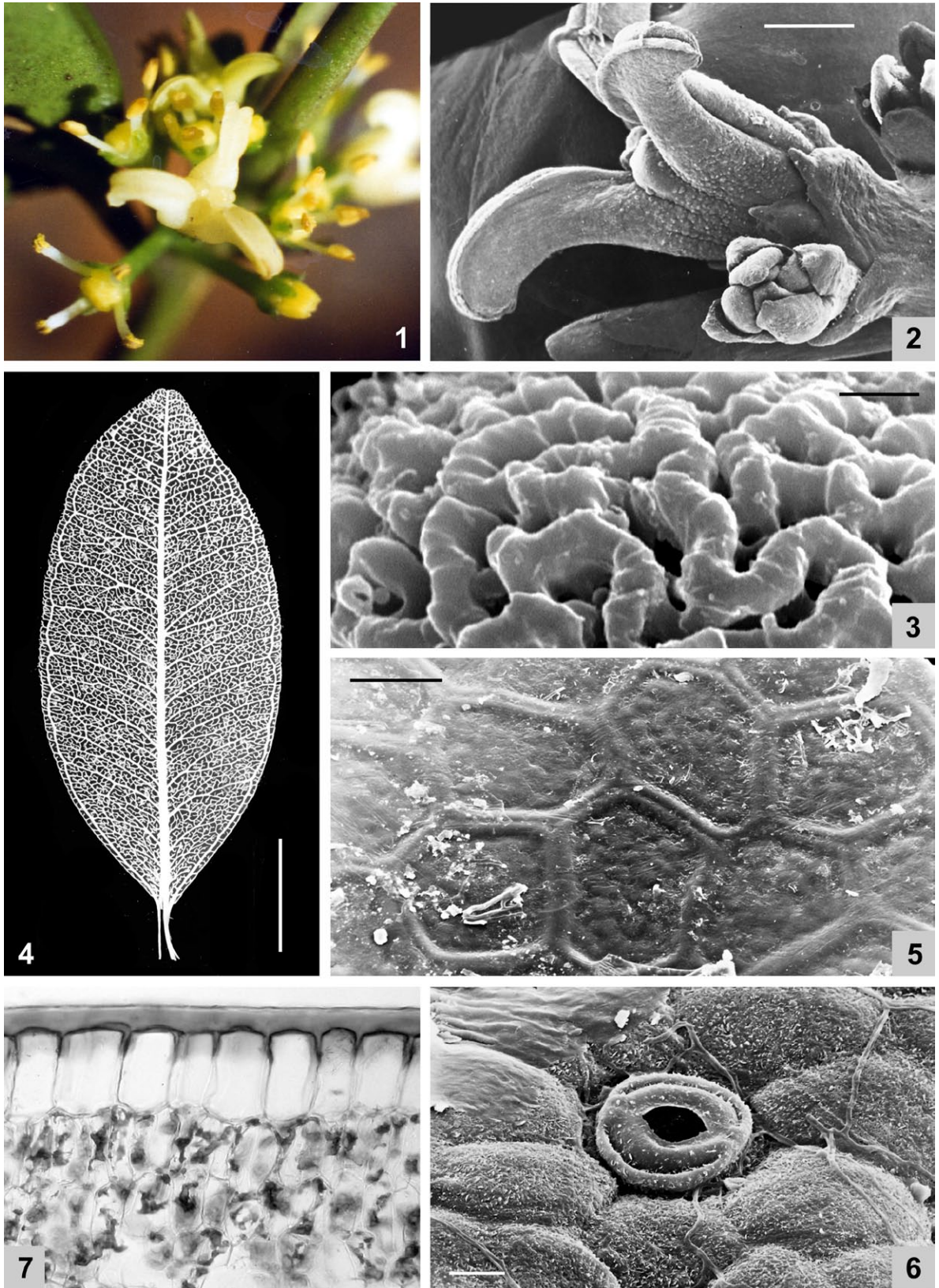


Fig. 1–7. *Buxus nipensis* – 1: inflorescence, female flower, recurved white stylodia, interstylary nectaries; 2: female flower, male flower before anthesis, scale bar = 1 mm; 3: exine detail, reticulate heterobrochate, with broad crenulate muri, 11 000 $\times$ ; 4: brochidodromous leaf venation pattern, scale bar = 1 cm; 5: adaxial leaf epidermis, reticulately raised anticlinal walls, sunken anticlinal borders, finely knobbed periclinal wall, 600 $\times$ ; 6: abaxial leaf epidermis, stoma with a peristomal rim, 1100 $\times$ ; 7: leaf cross-section, adaxial epidermis cells with light-line, absence of secretory cells. – 1, 2, 4, 5 from specimen *HFC 75431* (B); 3, 6 from *HFC 75468* (HAJB, holotype); 7 from specimen *HFC 80900* (B).

and rivers, at 500–700 m above the sea level. During field work carried out in February 2010 we saw bees (*Apis mellifera*) and an unidentified diptera visiting the flowers of *B. nipensis* in the locality of río Piloto.

*Phenology* — The species has been collected in flower from December to May and in fruit from April to August. When we visited the locality of río Piloto in February 2010 almost all adult plants were in flower.

*Conservation status* — *Buxus nipensis* has been recently confirmed in three of nine localities in which it had been collected earlier, according to the consulted herbarium specimens. Some historical localities of this species have been affected mostly by the nickel-mining industry and by the extraction of timber, and we suppose that some populations of *B. nipensis* could have been affected or may have disappeared. In the last ten years *B. nipensis* has been collected in río Piloto, Loma Mensura and in the ecologic path of Estación de Investigaciones de la Montaña. The populations of Loma Mensura and of the ecologic path of Estación de Investigaciones de la Montaña have six and ten mature individuals, respectively, which are close to one another; in the population of río Piloto we counted at least 50 individuals, both mature and immature, along c. 1000 m. The three populations are protected since Loma Mensura and río Piloto belong to the protected area “Mensura-Piloto” and the third population is protected as well for being included in an ecologic path managed by the Estación de Investigaciones de la Montaña. Based on a suspected population size reduction of  $\geq 50\%$ , a range of less than 500 km<sup>2</sup> and a decline in the number of locations, the species must be classified as Endangered (EN AB2b) according to IUCN criteria (IUCN 2012).

*Discussion of phenotypic characters* — Specimens of *Buxus nipensis* had been previously identified as *B. retusa*, but *B. nipensis* can easily be distinguished from that species by the well-developed angular interstylar nectaries and the obliquely upright styles. These develop into upright, apically recurved styles with prominent knob-like, rounded nectaries in the capsule. The internodal morphology of the new species and also the reticulate pattern of protruding anticlinal walls of the adaxial leaf epidermis with sunken anticlinal borders is reminiscent of *B. retusa* but also of *B. braimbridgeorum* Eg. Köhler and *B. triptera* Eg. Köhler. However, the leaf anatomy of *B. nipensis* differs from that of *B. retusa* by the complete absence of secretory cells and by the palisade parenchyma composed of 2 or 3 layers of little-differentiated cells, features that it shares with *B. triptera*. The exine sculpture of the pollen is heterobrochate with murus segments of different breadth, which are bounding smaller lumina. The muri are broadly crenulate (ribbed), reminiscent of *B. braimbridgeorum*.

*Discussion of molecular characters* — The first mentioned diagnostic character state for *Buxus nipensis* in the *matK* coding sequence (“A” in position 343) is a synapomorphy for the type and all so-far investigated paratypes with respect to the whole genus *Buxus*. The second diagnostic character state (“G” in position 448) is present in the type and two paratypes but paratype specimen Bx163 exhibits an “A” like all other species of *Buxus*. This case illustrates that there may be infraspecific variation at the molecular level that may affect some of the characters states considered as diagnostic. We argue that at least some diagnostic characters states are present in all so-far studied individuals of the newly described species, and also, most importantly, that the type specimen exhibits all character states defined as diagnostic. Therefore the species is well defined based on the type. Further research has to address how infraspecific variation can be explained, either through homoplasy in certain individuals as a result of on-site mutation after speciation, through ancient haplotypes still present in individuals of some populations, or even through recent introgression.

The *trnK-matK* region further has two microsatellite regions (poly A/Ts) starting in sequence position 371 of the *trnK* intron 5' part and in position 901 (each referring to the sequence of the type) of the *matK* coding sequence. Both microsatellites are highly variable, including infraspecific variability in *Buxus nipensis*. A sole individual (Bx163) shows a unique haplotype (13 Ts) in the second microsatellite. The patterns are in line with high mutational rates and high levels of homoplasy in most chloroplast microsatellites (e.g. Tesfaye & al. 2007; Weising & Gardner 1999). Therefore, these characters are unsuitable for use in diagnoses to describe species.

*Additional specimens seen (paratypes)* — CUBA: PROV. HOLGUÍN: Mayarí, Sierra de Nipe, near Woodfred, deciduous woods and thickets, 450–550 m, 20 Dec 1909, *J. Shafer 3219* (NY); near Woodfred, deciduous woods and thickets, 450–550 m, 1 Jan 1910, *J. Shafer 3408* (NY); in charrascales ad Brazo Dolores, c. 800 m, 20 Feb 1918, *E. Ekman 9124* (S); South of lumber camp, crest of Sierra de Nipe, 600–700 m, 16–17 Oct 1941, *C. V. Morton & J. Acuña 3066* (US); Fuente del Arroyo Naranjo, bosque húmedo, arbusto 1.5–2 m, 750 m, 20 Apr 1960, *Bro. Alain & J. Acuña 7833?* (HAC); arroyo cerca de La Casimba, 19 Apr 1960, *Bro. Alain & J. Acuña 7833* (HAC); Cayo de La Plancha, 7 Apr 1941, *Bro. Leon & al. 20032* (GH, HAC, NY); Sierra de Nipe, Oct 1966, *V. Samek 16193* (HAC); charrascales de la Loma de La Bandera, c. 400 m, Apr 1968, *J. Bisse & E. Köhler HFC 7336* (HAJB, JE); Pinares cerca de la Loma de La Estrella, 800 m, 12 Aug 1970, *J. Bisse & H. Lippold HFC 18102* (HAJB, JE); orillas del arroyo en el camino a Woodfred, 600 m, 2 Nov 1979, *A. Álvarez & al. HFC 36019* (B, HAJB, JE); orillas de las cabezadas del río Piloto, c. 800 m, 30 Oct 1977, *A. Álvarez & al. HFC 35736* (B, HAC, HAJB, JE); arroyo afluente del río Piloto, 10 Aug 1988, *R. Berazaín*



HFC 66166 (HAJB); orillas de arroyo del Medio cerca de Woodfred, c. 425 m, 7 Mar 1998, *J. Gutiérrez & al.* HFC 75431 (BHU, HAJB); arroyo Mensura (río Sabina) alrededores de la Estación de Investigaciones Integrales de la Montaña, 500–600 m, 9 Mar 1998, *J. Gutiérrez & al.* HFC 75472 (BHU, HAJB); cabezadas del río Piloto, 689 m, 7 Apr 2003, *J. Gutiérrez & al.* HFC 80900 (BHU, HAJB); río Piloto, cascadas altas, 690 m, 7 Apr 2003, *J. Gutiérrez & al.* HFC 80905 (BHU, HAJB); cabezadas del río Piloto, 710–724 m, 7 Apr 2004, *J. Gutiérrez & al.* HFC 81745 (HAJB); cabezadas del río Piloto, 20 Mar 2005, *J. Gutiérrez & al.* HFC 83242 (HAJB); detrás de la Estación de Investigaciones Integrales de la Montaña, c. 670 m, 20 Mar 2005, *J. Gutiérrez & al.* HFC 83269 (BHU, HAJB); río Piloto, *T. Borsch & al.* 4164 [= Bx117] (B, HAJB, ULV); Loma Mensura, en el margen de un arroyo con presencia de *Cyrilla* sp., *Tabebuia* sp., *Rondeletia* sp., *Arthrostylidium* sp. y *Leucocroton* sp., 700 m, 7 Sep 2011, *P. González & al.* HFC 87220 [= Bx162] (B, HAJB); sendero ecológico detrás de la Estación de Investigaciones de la Montaña, bosque pluvial con presencia de *Chionanthus domingensis*, *Bactris cubensis*, *Phyllanthus* sp., c. 700 m, 8 Sep 2011, *P. González & al.* HFC 87221 [= Bx163] (B, HAJB).

***Buxus cristalensis*** Eg. Köhler & P. A. González, **sp. nov.** — Fig. 8–15.

Holotype: Cuba, province Santiago de Cuba, Segundo Frente, Sierra del Cristal, Arroyo en el camino del Oro a Batista, 20°31'51"N, 75°26'14"W, 700 m, 8 Mar 1998, *J. Gutiérrez, E. Köhler, R. Rankin & I. Silva* HFC 75347 (HAJB; isotypes: B, BHU, JE) [= Bx164].

**Morphological diagnosis** — Leaves elliptic to oblong, apex obtuse, retuse to emarginate and mucronulate. Male tepals broadly ovate, adaxially finely pilose, apex apiculate. Ovary trilobate, dorsal veins deeply sunken; nectaries well-developed, angular, wrinkled; styles obliquely spreading, stout. Capsule ellipsoid, dorsal veins scarcely prominent proximally, deeply sunken distally; nectaries prominent, angulose, wrinkled; styles erect then recurved.

**Molecular diagnosis** — Nucleotide character state “A” in position 1507 of *matK* coding sequence.

**Morphological description** — *Shrub or tree* to 5 m tall; branchlets angular; internodal folds narrow with slightly prominent ribs, dorsally ± keeled, with 2 lateral keels or variably keeled on each side; internodes 2–5(–8) cm long. *Leaves* dimorphic; *normal-sized leaves* with petiole 5–8 mm long, blade green to yellowish green and slightly shiny adaxially, paler abaxially, elliptic to oblong, 5–7 × 1.5–4 cm, coriaceous, glabrous, base obtuse, apex obtuse, retuse and slightly mucronulate, midvein sunken adaxially, prominent abaxially, secondary veins in 12–15

pairs anastomosing in an intramarginal vein c. 2.5 mm from revolute margin, venation prominent on both surfaces; *smaller decussate leaves* interspersed between normal ones, narrowly lanceolate, c. 7 mm long, apex acute. *Inflorescences* ± sessile, c. 1 cm long, glabrous; bracts triangular, 1.5–2.5 mm long, margin scarcely ciliate, apex acute. *Male flowers* with pedicel 2–3.5 mm long; tepals broadly ovate, c. 2 mm long, adaxially finely pilose, margin narrowly membranous, scarcely ciliate, apex apiculate; stamens 3–4 mm long, filaments slightly flattened, anthers c. 1 mm long with a prominent rounded brownish tip; pistillode rounded-quadrangular, hemispherical, with lateral ellipsoid sinus, wrinkled. *Female flowers* with 5 tepals; tepals triangular with bright tip, 1–1.5 mm long, margin scarcely ciliate; ovary trilobate, 2–2.5 × c. 2.5 mm, dorsal veins deeply sunken, commissures with lateral furrows apically continued into nectaries; styles white, recurved, thick, glabrous; stigmas broad, deeply folded, 2–3.5 mm long; nectaries angular, wrinkled. *Capsule* green-brownish, ellipsoid, 6–8 × 5–7 mm, glabrous, crowned by 3–4 mm long erect then recurved styles, dorsal veins scarcely prominent proximally, sunken distally, commissures apically slightly protruding, with lateral furrows; nectaries prominent, angular, wrinkled. *Seeds* rounded-trigonous, c. 4 × 2 mm.

**Molecular description** — Sequences describe the type specimen (code Bx164) and are available in EMBL/GenBank/DDBJ under accession numbers HG004435 (*matK-trnK*) and HG004430 *trnL-trnF*. Further sequences describe a paratype specimen (code Bx026) and are available in EMBL/GenBank/DDBJ under accession numbers HG004434 (*matK-trnK*) and HG004429 *trnL-trnF*.

**Pollen morphology** — Pollen 3- or 4(or 5)-colporate, colpi 3–5-orate, reticulate, heterobrochate, higher murus segments broader than the lower ones, which are bounding smaller lumina, muri crenulate.

**Leaf anatomy** — *Buxus cristalensis* is characterized by the absence of secretory cells. The adaxial epidermis consists of high, thin-walled cells, with anticlinal walls only slightly thickened in the apical part, showing a light line. The palisade cells are scarcely differentiated. The periclinal walls of both epidermis are papilla-like and protruding, similar to the species of the *B. gonoclada* Müll. Arg. type (see Köhler & Schirarend 1989). The stomata have a peristomal rim.

**Etymology** — The specific epithet alludes to Sierra del Cristal, a mountainous region in the northeastern part of Cuba, where this species is endemic.

**Distribution** — *Buxus cristalensis* is endemic to Sierra del Cristal, municipality of Segundo Frente, in the province of Santiago de Cuba. In Sierra del Cristal the species

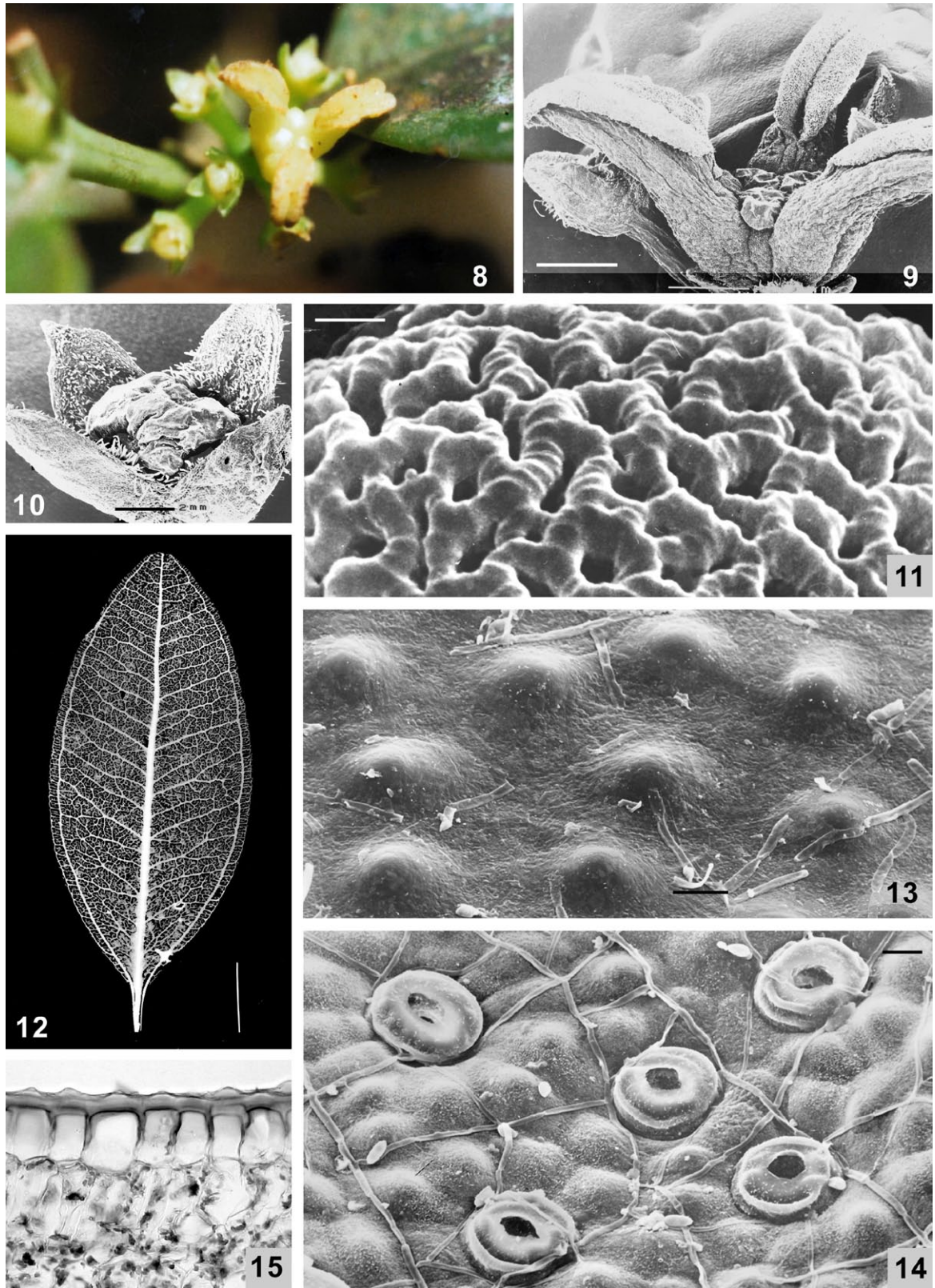


Fig. 8–15. *Buxus cristalisensis* – 8: inflorescence, female flower, spreading yellowish white thick stylodia, interstylary nectaries; 9: female flower, stout stylodia, nectaries, commissures with collateral furrows, scale bar = 1 mm; 10: male flower, tepals adaxially finely pilose, pistillode, scale bar = 2 mm; 11: reticulate exine, broad, crenulate muri, 10 000 $\times$ ; 12: brochidodromous leaf venation pattern, scale bar = 1 cm; 13: adaxial leaf epidermis, papilla-like (papilloid) protruding periclinal walls, 1000 $\times$ ; 14: abaxial leaf epidermis,  $\pm$  papilla-like protruding periclinal walls, stomata with peristomal rim 550 $\times$ ; 15: leaf cross-section, adaxial epidermis with protruding periclinal walls, little-differentiated palisade parenchyma, absence of secretory cells. – 8, 12–15 from *HFC 75347* (HAJB, holotype), 9, 10 from specimen *HFC 75349* (B), 12 from specimen *HFC 15938* (HAJB).



has been collected near to the rivers Miguel and Levisa (sometimes erroneously written as “Lebisa”), close to the top of Sierra del Cristal, between Los Moreiros and La Zanja, on the eastern slope of the hill El Gallego, along a brook on the way between El Oro and Batista.

*Habitat and ecology* — *Buxus cristalensis* grows on serpentine in subspiny xeromorphic thickets and riverine rainforest, at 600–1100 m above the sea level.

*Phenology* — The species has been collected in flower from December to May and in fruit from April to August.

*Conservation status* — Although the distribution of *Buxus cristalensis* is restricted to Sierra del Cristal and we have not visited all the recorded populations, it is known that all localities where this species occurs are included in the protected area National Park Pico Cristal managed by the Cuban Enterprise for the protection of the flora and fauna. However, an assessment of the populations in the field is needed before any more reliable conservation status according to IUCN criteria (IUCN 2012) can be determined.

*Discussion of phenotypic characters* — Herbarium specimens of *Buxus cristalensis* had also been identified as *B. retusa*, but *B. cristalensis* can easily be distinguished from that species by the ovary and capsule with well-developed angular interstyler nectaries. It differs from *B. nipensis* by the obliquely spreading styles rising stoutly out of the carpel, which have a deeply sunken dorsal vein, well pronounced in the upper part of the capsule in contrast to *B. nipensis* and *B. retusa*. In contrast to these species the commissures are narrowly protruding distally, with collateral furrows. The internode morphology of the new species is reminiscent of *B. braimbridgeorum*, *B. nipensis* and *B. retusa*, while the papilla-like protruding periclinal walls of both epidermis layers are different, pointing more to the *B. gonoclada* type (see Köhler & Schirarend 1989). The leaf anatomy of *B. cristalensis* differs from that of *B. retusa* by complete lack of secretory cells and by the palisade parenchyma composed of 1 or 2 layers of little-differentiated cells, features that it shares with *B. nipensis* and  $\pm$  with *B. triptera*. The anticlinal walls of the adaxial epidermis are only slightly thickened in the apical part, showing a light line, like in *B. nipensis*.

*Discussion of molecular characters* — The unique molecular diagnostic character states found in *matK* for *Buxus cristalensis* seems synapomorphic for this species. As in *B. nipensis* we did not find any distinctive character state in the *trnL-trnF* sequences of *B. cristalensis*.

*Additional specimens seen (paratypes)* — CUBA: PROV. SANTIAGO DE CUBA: Segundo Frente, Sierra del Cristal, prope río Lebisa in carrascales, 650–1000 m, 4 Mar 1916, *E. Ekman* 6792 (S); at the tributary of río Lebisa, in

carrascales, 600–1000 m, 15 Dec 1922, *E. Ekman* 15960 (S); charrascos y cumbres del Cristal, rocky places, c. 1000 m, 2–7 Apr 1956, *Bro. Alain & al.* 5655 (HAC, HAJB); charrascos y cumbres del Cristal, 2–7 Apr 1956, *Bro. Alain & al.* 5697 (HAC, HAJB); Sierra del Cristal, falta sur de la Sierra, cabezadas del río San Miguel, 600–800 m, April, 1968, *J. Bisse & E. Köhler* HFC 8174 (HAJB, JE); camino entre Los Moreiros y La Zanja, April 1970, *J. Bisse* HFC 15938 (HAJB, JE); Pinares y arroyos en la ladera este de la loma El Gallego, 2 May 1985, *A. Álvarez & al.* HFC 57280 (B, BHU, HAJB, JE); charrascos en la subida y firme del Pico Cristal, 800–1100 m, 4 Mar 1998, *J. Gutiérrez & al.* HFC 75298 (BHU, HAJB); Sierra del Cristal, arroyo en el camino del Oro a Batista, c. 700 m, 5 Mar 1998, *J. Gutiérrez & al.* HFC 75348 (BHU, HAJB); Sierra del Cristal, arroyo en el camino del Oro a Batista, c. 700 m, 5 Mar 1998, *J. Gutiérrez & al.* HFC 75349 (BHU, HAJB); Sierra del Cristal, arroyo en el camino entre El Halcón y Batista, 5 Mar 1998, *J. Gutiérrez & al.* HFC 75386 [= Bx026] (BHU, HAJB).

*Buxus koehleri* P. A. González & Borsch, **sp. nov.** – Fig. 16–23.

Holotype: Cuba, province Holguín, Mayarí, Sierra de Nipe, Sendero Salto del Guayabo, 20°35'N, 75°45'W, 24 Feb 2010, *T. Borsch, M. Ackermann, C. Panfet, K. Zoglauer, P. González, I. Castañeda & J. Gutiérrez* 4091 (HAJB; isotypes: B, ULV) [= Bx055].

*Morphological diagnosis* – Leaves oblong-lanceolate to narrowly elliptic, apex acute,  $\pm$  retuse, weakly mucronulate. Male tepals triangular to oblong. Filaments flattened. Ovary rounded, dorsal veins and commissures sunken; nectaries angulose. Capsule globose, dorsal veins little protruding; nectaries inconspicuous; styles erect then recurved, placed close to each other basally, connate with nectaries.

*Molecular diagnosis* — Nucleotide character state “A” in positions 612 and 915 of *matK* coding sequence. Nucleotide character state “A” in positions 359 and 392 of *trnL* group I intron and “G” in position 248 of *trnL-F* spacer.

*Morphological description* — Tree to 7 m tall; trunk 20–25 cm in diam.; bark furrowed; branchlets angular; internodal folds narrow with slightly prominent ribs, dorsally variably keeled; internodes 2–6 cm long. Leaves dimorphic; *normal-sized leaves* with petiole 4–7 mm long, blade green and shiny adaxially, paler abaxially, oblong-lanceolate to narrowly elliptic, 7–9(–10.5)  $\times$  2.5–3.5 cm, coriaceous, glabrous, base acute, apex acute to slightly acuminate,  $\pm$  retuse and weakly mucronulate, midvein sunken adaxially, prominent abaxially, secondary veins in 15–18 pairs, anastomosing in an intramarginal vein c. 1.5 mm from margin, venation conspicuous on both surfaces, slightly reticulate; *smaller decussate leaves*

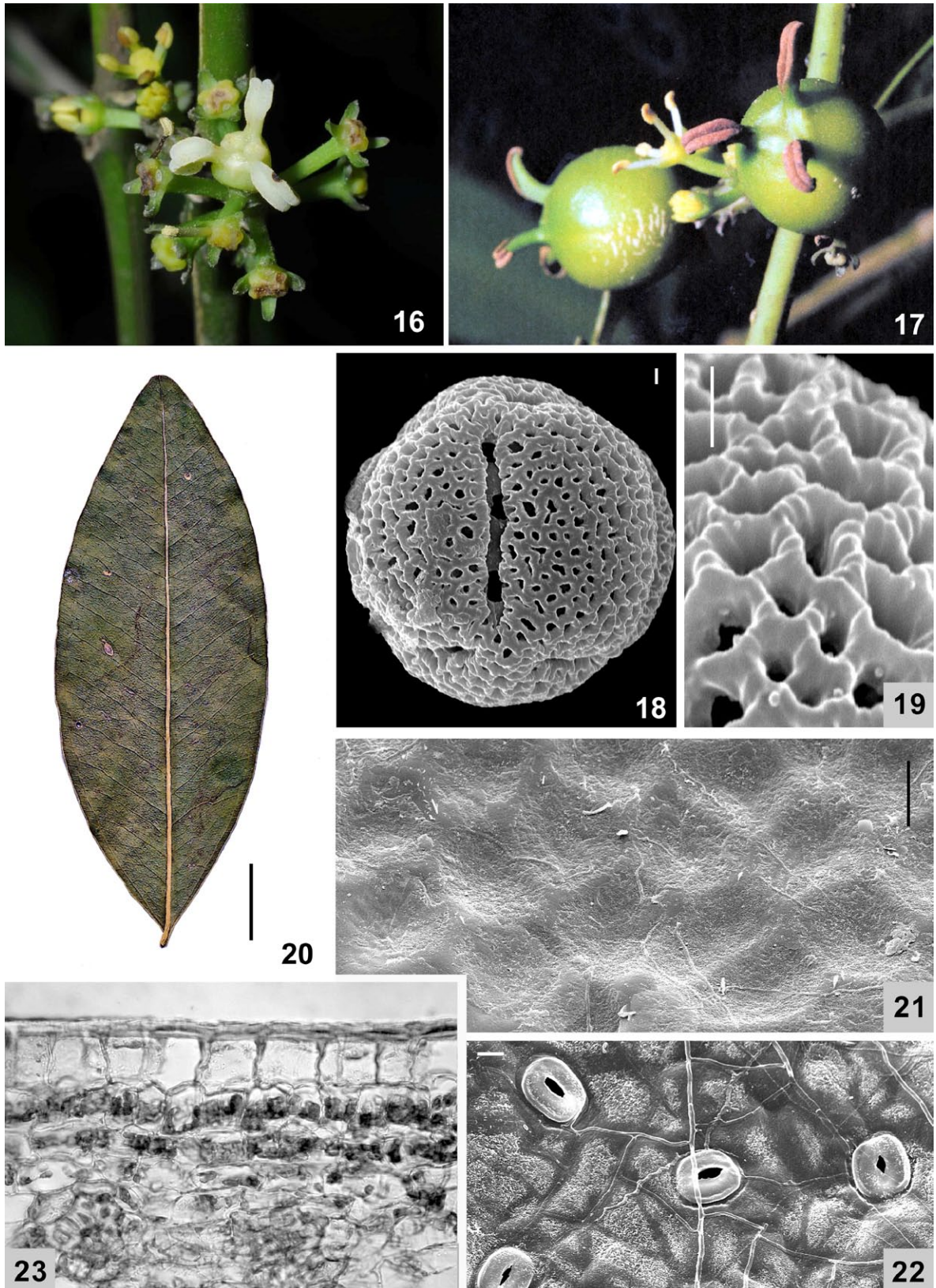


Fig. 16–23. *Buxus koehleri* – 16: inflorescence, female flower, male flowers; 17: immature capsule, inconspicuous nectaries; 18: pantocolporate pollen grain, colpus 3-orate, 3500 $\times$ ; 19: reticulate exine, pronounced crenulate muri, 10 000 $\times$ ; 20: brochidodromous leaf venation pattern, scale bar = 1 cm; 21: adaxial leaf epidermis, reticulate pattern of weakly protruding anticlinal walls, 500 $\times$ ; 22: abaxial leaf epidermis, pattern with sunken anticlinal walls, stomata with peristomal rim, 500 $\times$ ; 23: leaf cross-section, adaxial epidermis, little-differentiated palisade cells, absence of secretory cells, 500 $\times$ . – 16–23 from specimen *Borsch & al. 4091* (HAJB, holotype).



interspersed between normal ones, (4–)6–10 mm long. *Inflorescences* with axis 6–7 mm long; bracts triangular, 0.5–1 mm long, apex acute. *Male flowers* with pedicel 4–6 mm long; tepals triangular to oblong, 1–1.5 mm long; stamens 2–4 mm long, filaments white, flattened, anthers c. 1 mm long with a prominent brownish tip; pistillode rounded-quadrangular, with lateral ellipsoid sinus, wrinkled. *Female flowers* with 5 tepals; tepals triangular, c. 1 mm long, with scattered hairs along margin; ovary white to yellowish, rounded, c. 2.5 × 2.5 mm, glabrous, with sunken dorsal veins and commissures; styles erect, recurved, white, thick, c. 3 mm long; stigmas broad, plicate; nectaries prominent, angular. *Capsule* brownish green, globose, 7–10 × 7–8 mm, glabrous, crowned by c. 3.5 mm long erect then recurved styles approaching each other basally, dorsal veins slightly protruding, commissures slightly sunken; nectaries inconspicuous, connate to style bases. *Seeds* rounded-triangular, c. 6 × 2 mm.

*Molecular description* — Sequences describe the type specimen (code Bx055) and are available in EMBL/GenBank under accession numbers HG004433 (*matK-trnK*) and HG004428 *trnL-trnF*.

*Pollen morphology* — Pollen (3–)6–9-pantocolporate, colpi 1–3-orate, reticulate, muri thick, crenulate with protruding ribs.

*Leaf anatomy* — *Buxus koehleri* is characterized by the absence of secretory cells. The epidermis is formed by isodiametric cells with apically but slightly thickened anticlinal walls. The palisade parenchyma has 2 or 3 layers of little-differentiated cells. The adaxial epidermis has a weakly defined reticulate pattern of protruding anticlinal walls and slightly sunken periclinal walls. The abaxial epidermis has scarcely sunken anticlinal walls. The stomata have a peristomal rim.

*Etymology* — The name honours Professor Egon Köhler for his significant contributions to the knowledge of *Buxus*.

*Distribution* — *Buxus koehleri* is a local endemic of Sierra de Nipe, Mayarí, province of Holguín. In Sierra de Nipe it has been collected in Sendero Salto del río Guayabo and in arroyo Woodfred.

*Habitat and ecology* — In Sendero del Salto del Guayabo *Buxus koehleri* inhabits the understory of rainforest in association with other species such as *Bactris cubensis* Burret, *Calophyllum* sp. and *Dendropanax arboreus* (L.) Decne. & Planch., growing on black and alluvial soils mixed with serpentine, at c. 400 meters above the sea level.

*Phenology* — *Buxus koehleri* has been collected in flower in February and in fruit in February and September.

We have visited the population in Sendero del Salto del Guayabo five times, and saw only a few plants (and always the same plants) with flowers or fruits, which is perhaps due to the low availability of sunlight for most of plants in the understory of the rainforest.

*Conservation status* — *Buxus koehleri* has been collected in two localities of Sierra de Nipe. We have visited only the population in Sendero del Salto del Guayabo, where we have estimated the population to consist of 150–200 plants. Most are small trees of 3–7 m in height, but we also found seedlings and juvenile plants. This population is protected, being located in one area administrated by the Cuban Enterprise for the protection of the flora and fauna. Following IUCN criteria (IUCN 2012) the species must be classified as Endangered (EN B2a), mainly because of the small area of occupancy with less than five localities; the population size appears to be fewer than 250 individuals.

*Discussion of phenotypic characters* — The most relevant characters in the morphology of *Buxus koehleri* are its habit and the shape of the leaf blade. *B. koehleri* is among the tallest species of Cuban *Buxus* and the tallest growing in Sierra de Nipe. Its apiculate leaf blade also differentiates it from other *Buxus* species that occur in Sierra de Nipe. The leaf form and size are similar to *B. muelleriana* Urb., which possesses, however, broader internodal folds, larger, more petaloid tepals and broader white filaments. The internode morphology and the leaf dimorphism of *B. koehleri* may indicate a relationship to the species of the *B. gonoclada* group that do not have a sharp dorsal keel but are ± variably keeled, like *B. cristalensis*, *B. excisa* Urb., *B. nipensis*, *B. retusa* and *B. triptera*. *Buxus koehleri* is well-distinguished from these species by its capsule with long erect and only terminally recurved styles that are placed very close to each other and possess only inconspicuous nectaries. In leaf anatomy, the presence of a peristomal rim is indicative of the *B. gonoclada* group, while the absence of secretory cells and the weakly differentiated palisade tissue, which is shared with *B. cristalensis*, *B. nipensis* and *B. triptera*, differentiates it from *B. retusa*. The comparatively coarse reticulum of the pollen exine with thick crenulate muri is reminiscent of *B. triptera* and *B. braimbridgeorum*, the latter of which deviates by the well-developed secretory cells in leaf anatomy.

*Discussion of molecular characters* — In *Buxus koehleri* the two substitutions in the *trnL* intron and the substitution in the *trnL-trnF* spacer are unique among all taxa in the genus *Buxus* and therefore represent apomorphies. The same applies to the two substitutions in the *matK* coding region. But the “A” in position 612 is only an apomorphy for this species amongst the members of the Caribbean clade. The distant lineage of Eurasian *Buxus* (*B. colchica* Pojark., *B. sempervirens* L., etc.) exhibit the



same mutation (González & al. unpubl. data), which must be a convergence. Considering that the *matK* sequences in *B. koehleri* are typical Caribbean clade sequences with a considerable distance to the Eurasian clade sequences, the Caribbean clade can be unambiguously defined as a reference group for this substitution to be diagnostic. Ongoing sequencing of population samples further indicates that the so-far studied individuals do not show any variation in the diagnostic character states presented here. *B. koehleri* appears to be the most distinct from all three species newly recognized here when considering plastid genome sequence data.

*Additional specimens seen (paratypes)* — CUBA: PROV. HOLGUÍN: Mayarí, Sierra de Nipe, Sendero del Salto del Guayabo, al final del sendero, bosque pluvial con presencia de *Callophyllum* sp., *Dendropanax arborea*, *Bactris cubensis*, *Philodendron lacerum*, *Pharus* sp., 7 Sep 2011, P. González & al. HFC 87164 (B, HAJB, herb. Greuter, JE, ULV); arroyo Woodfred, 2 Apr 1999, H. Stenzel 742 (BHU).

### Molecular characters supporting the recognition and formal description of new species

The phylogenetic analysis of homologous DNA sequences has not only revolutionized our picture of organismic evolution but sequence data are also increasingly appreciated for identifying species (“DNA barcoding”; e.g. CBOL Plant working group 2009). On the other hand, the taxonomic work process has been traditionally based on morphological characters and the formal description of species relies on characters and their states described in the protologues. Fully integrating the wealth of information that can be obtained from sequence characters into the taxonomic work process, means to also include such data into diagnoses and descriptions of species. Conceptually, a sequence of a genomic region that is obtained from a type specimen describes this particular genomic region. Unlike morphological characters, a species description in a paper will not include the actual sequence in text format but rather the corresponding reference number of a data base such as EMBL or GenBank. Those sequence characters or their states that are found to be diagnostic, should, however, be included in the diagnosis of the taxon to be described. In the case of phenotypically complex species groups this will provide further data that can be unambiguously attached to the type specimen, and thus allow for a precise positioning of the type specimen amongst other specimens of the study group. The analysis of evolutionarily complex species groups will include the assessment of patterns such as reticulation and incomplete lineage sorting that typically require information from the genome. We therefore argue that diagnoses and descriptions of new species should be complemented by sequence data whenever possible. In our study we

have attempted to consider DNA characters in the formal description of three new species of *Buxus* for exactly this reason.

Several issues appear relevant when comparing the use of morphological versus molecular data in the taxonomic workflow. Morphological data are contained in the protologues for all previously described taxa, certainly with varying levels of precision. Along with further data obtained from additional specimens of the study group, and often from re-studying the type specimens, morphological characters can then be comprehensively evaluated during the research process by the specialist researcher who recognizes a taxon as new. Thereby, cladistic or phenetic methods can be applied. The important thing is that all other accepted species in a study group can be considered. Using molecular data this process is more complicated, simply because protologues do not contain such information and because generating new sequence data from historical type specimens is often limited. Using molecular data in the taxonomic work process, therefore, often requires retroactive generation of the sequence data from the previously described species for comparison. What is needed is a comprehensive comparative sequence database, comprising genomic regions that allow the distinction of the respective species. In this context, a phylogenetic tree including putatively new species will help to focus the study of characters supporting the delimitation of a new species on the respective closest relatives. Overview trees that include as many species of a study group (e.g. a genus) as possible with the best possible resolution are needed. This has recently been shown to be feasible by using, e.g. plastid intron sequences, for which large multiple sequence alignments can be constructed (Mansion & al. 2012). In contrast, the typical phylogenetic analysis still contains only between 20 % and 40 % of the species of a study group.

Another challenge in plants is to find molecular markers that provide sufficient information to distinguish closely related species in a taxonomic context. There seems to be increasing awareness that a few standard loci such as partial *matK* and *rbcl* (CBOL Plant Working Group 2009) will not allow to achieve this goal. Recent studies on angiosperm groups such as *Crocus* L. (Seberg & Petersen 2009) or *Rhipsalideae* of *Cactaceae* (Korotkova & al. 2011) indicate that a combination of various introns and spacer sequences may in fact allow recognition of species-specific character states for most species but this may require combination of some five loci (>3000 nt in total) with also lineage-specific differences as to what are the respective informative genomic regions. In our case, the plastid *trnK* intron including the complete *matK* gene provided diagnostic characters for all three new species of *Buxus*. However, the widely applied *matK* barcoding fragment (CBOL Plant Working Group 2009) contains the two diagnostic characters of *Buxus koehleri* only, while the diagnostic sites for the other two species are located either up- or downstream. In this study we have used

comprehensive molecular data sets that are currently being generated and include nearly all species of New World *Buxus* (González & al., in prep.) to find the diagnostic characters. Further genomic regions should definitely be sequenced for the types and other specimens during the further analysis of the evolution of *Buxus* in the Caribbean, including the nuclear genome.

Supporting the formal description of a flowering plant species, Filipowicz & al. (2012) recently recognized a deviant species of *Brunfelsia* L. (*Solanaceae*) from the Andes solely based on a molecular diagnosis. In this case, morphological characters were not apparent to support a morphology-based diagnosis while the newly recognized species and its close morphological allies could be shown to belong to distant subclades of the genus. This indicated that reproductive isolation exists and thus the species circumscription withstands further evolutionary study even in the absence of currently known deviating morphological characters. In other cases both morphology and sequence characters from the *matK* gene diagnosed *Pedersenia volubilis* Borsch & al. as a new species (*Amaranthaceae*, Borsch & al. 2011). However, only one of two diagnostic characters was in the range of the c. 850 nt long barcoding fragment used from *matK*. The available results on molecular diagnostic characters therefore strongly indicate that additional genomic regions should be sequenced rather than focusing on the markers recommended for barcoding.

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