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Molecular confirmation of two medicinal species of *Jatropha* L. (Euphorbiaceae) used by the *Agusan Manobo* in the Philippines

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The *Agusan Manobo* in the Philippines is known to use complex plant vernacular names. One of their interesting medicinal plants having local name similarity is called "tubatuba" in Bisayan and Minanubu dialects. The local name refers these plants to two different specimens; one is "tubatuba puti," and the other is "tubatuba tapol," which are classified by the tribe based on their distinct color shades of white and red, respectively. This study verifies the true identity of the two "tubatuba"-named specimens using molecular data and morphology. Molecular confirmation using BLAST*n* nucleotide database query revealed that the two confused samples are members of Euphorbiaceae and its verified generic affinity is *Jatropha* L. Phylogeny of 24 *Jatropha* specimens using ITS sequences revealed "tubatuba puti" as *Jatropha curcas* L. and "tubatuba tapol" as *Jatropha gossypiifolia* L. with strong Bootstrap support (BS = 100%). Morphological descriptions and field photographs of the two *Jatropha* species, as well as their conservation status based on the international and national red listings, are provided here.

Keywords: *Agusan Manobo*, BLAST*n*, Euphorbiaceae, ITS, *Jatropha* species, molecular confirmation

Introduction

Jatropha L. of the family Euphorbiaceae is composed of 175 species diversified across the subtropical and tropical regions of the world [1, 2]. Classified into two subgenera: *Jatropha* is found in South America, Antillean Islands, Africa, and India, while *Curcas* inhabits the lands of North America and Mexico [2, 3]. Currently, there are five *Jatropha* species (*J. curcas* L., *J. gossypiifolia* L., *J. integerrima* Jacq., *J. multifida* L., and *J. podagrica* Hook.) known to be present in the Philippines [4].

Based on the folk medicine of *Jatropha* species in the Philippines, two species, namely *J. curcas* and *J. gossypiifolia*, are widely used as medicine. Dapar *et al.* [5] documented the ethnopharmacological use of these two

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medicinal plants among the *Agusan Manobo* as a treatment for both internal and external conditions. Drinking decoctions of leaves and roots can alleviate colds, malaria, typhoid fever, pulmonary tuberculosis, diarrhea, arthritis, rheumatism, dysmenorrhea, irregular menstruation, backache, body ache, fever, weakness, fatigue, cramp, spasm, relapse, gas pain, and flatulence [5]. Also, applying decocted roots and pounded scraped barks are effective against scabies, ringworm, ear infection, discharging ear, toothache, mouth sore, cuts and wounds, fracture, dislocation, and bites (animal and insects). This plant can also be used as a bath or wash using decocted leaves in treatment for ringworm, boils, carbuncles, dermatitis, swollen muscles, swellings, muscle pain, backache, body ache, fever, cuts, and wounds [5]. Decocted leaves of these two species were recently reported by Dapar *et al.* [6] among *Agusan Manobo* to wash on burns, injuries, cuts, and wounds. Other ethnobotanical accounts mentioned that both *J. curcas* and *J. gossypiifolia* are used in treatments to cure arthritis, body pain, fatigue, tuberculosis, diarrhea, menstrual problems, and related spasms [7, 8].

Folklorically, both *J. curcas* and *J. gossypiifolia* are referred to as the local name "tubatuba". Based on the ethnoclassification of *Agusan Manobo*, each plant is distinguished based on its coloration. Accordingly, the one having shades of white is known as "tubatuba puti," while for red is known as "tubatuba tapol," with the latter being more effective than the former. However, the ethnoclassification of *Agusan Manobo* poses the complexity of the correct species identity based on color differences. Dapar *et al*. [9] deduced the importance of integrating molecular data and morphology of plants to evaluate traditional limits of identification. Although the main distinction is in the color of the vegetative parts, molecular data are needed to examine further the true familial and generic affinities as well as the phylogenetic placement of the species. Thus, this study aims to (1) confirm the familial and generic affiliations, and (2) resolve phylogenetic placements of the two "tubatuba"-named specimens used by the *Agusan Manobo* of Agusan del Sur, Philippines.

Materials and methods

All necessary ethics approval, permit, certification, and fee prior informed consents were secured before fieldwork and plant collection. The collection of the plant samples was then followed through guided field walks with tribal healers who are knowledgeable of their "tubatuba"-named medicinal plants. A plant sample classified as "tubatuba puti" and one "tubatuba tapol" (Fig. 1) was collected in Bayugan City, Agusan del Sur. Voucher specimens were deposited in the University of Santo Tomas Herbarium (USTH) with accession numbers, as presented in Table 1.

The total genomic DNA was extracted from silica gel-dried leaf tissues of "tubatuba puti" and "tubatuba tapol" samples following the protocols of DNeasy Plant Minikit (Qiagen, Germany). The internal transcribed spacer (ITS) of nuclear DNA (nrDNA) marker was amplified and sequenced using the primer pairs, P17F and 26S-82R. PCR reactions were administered on a Biometra T- Gradient with a total volume of 25 L following the PCR parameters and mixture of Alejandro *et al.* [10]. Amplified DNA was purified using QiaQuick PCR Purification Kit (Qiagen, Germany) before sending it to MACROGEN Inc., Seoul Korea, for bidirectional sequencing.

The sequences of studied samples were assembled and edited using Codon Code Aligner v8.0.2, trimmed, and checked for ambiguous nucleotides. A BLASTn search was performed to check for species identity using the BLAST*n* algorithm available in the GenBank (www.ncbi.nim.nih.gov), as shown in Table 1. Available sequences of all reported Philippine

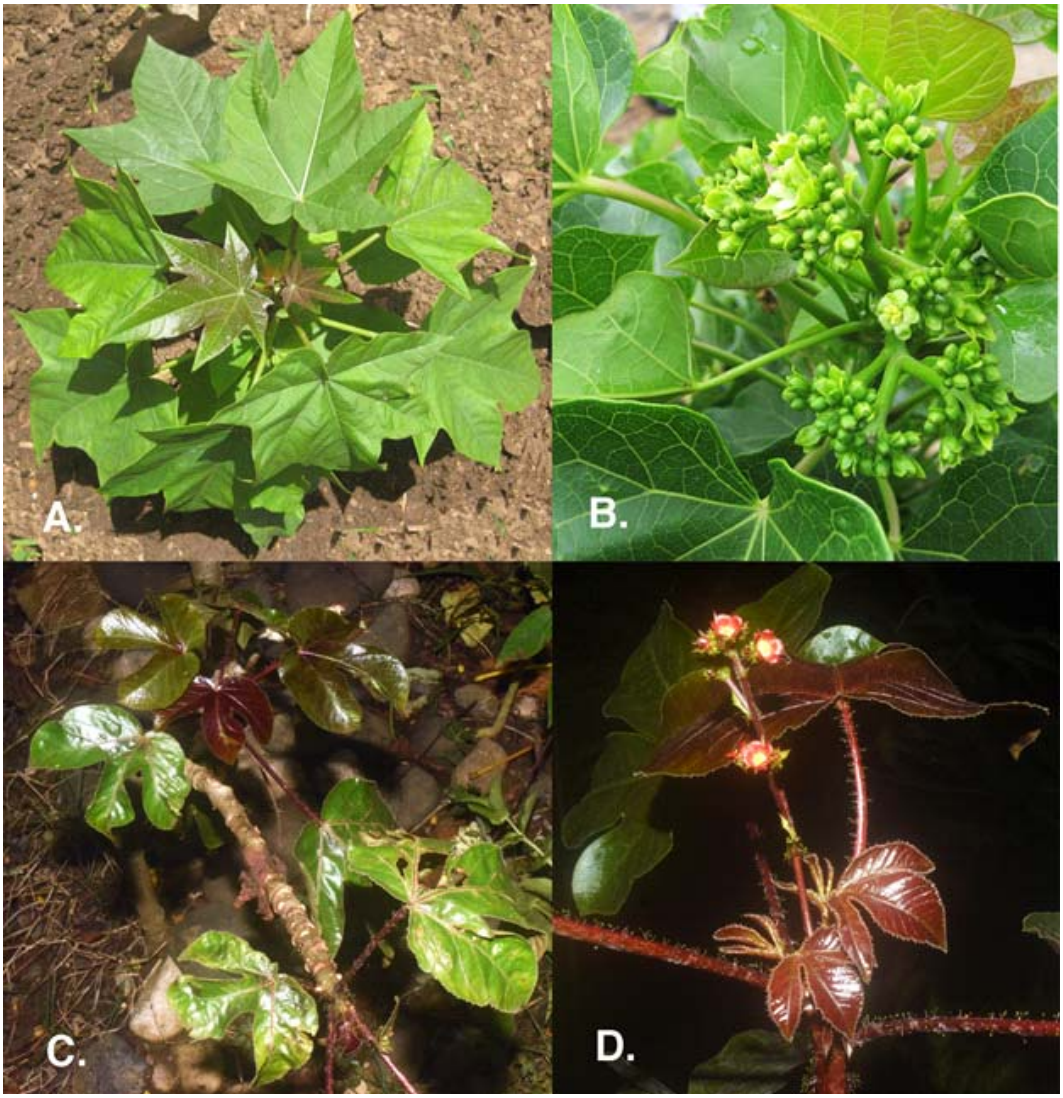

Figure 1. Field photographs of "tubatuba"-named specimens: (A) "tubatuba puti" (*Jatropha curcas* L.) leaves, (B) inflorescence, (C) "tubatuba tapol" (*Jatropha gossypiifolia* L.) leaves, and (D) inflorescence. Photos taken by M.LG. Dapar.

Table 1. BLAST*n* results of newly sequenced samples based on the ITS marker.

Local name	Sample code / Voucher	(nrDNA) ITS		
		BLAST ID	*e-value*	*% identity*
"tubatuba puti"	19MD-019/ USTH015595	*Jatropha curcas*	0.0	100%
"tubatuba tapol"	19MD-129/ USTH015621	*Jatropha gossypiifolia*	0.0	100%

Table 2. Nucleotide sequence database accession numbers of taxa used in the ITS phylogenetic analysis.

Species	Genbank accession code	(nrDNA) ITS	
		e-value	*% identity*
Outgroup			
Croton draco Schltdl.	EU478006.1	0	100%
Ingroup			
Jatropha curcas L.	KP190942.1	0	100%
	KP190943.1	0	100%
	KP190956.1	0	100%
	KP190957.1	0	100%
	KP190959.1	0	100%
	KP190961.1	0	100%
	KP191042.1	0	100%
	KP190971.1	0	100%
	KP190976.1	0	100%
	KP190982.1	0	100%
	KP190989.1	0	100%
	KP190997.1	0	100%
	KP190998.1	0	100%
	KP191024.1	0	100%
	KP191029.1	0	100%
	KP191031.1	0	100%
	KP191033.1	0	100%
	KP191034.1	0	100%
	KP191035.1	0	100%
	KP191036.1	0	100%
Jatropha gossypiifolia L.	KP191041.1	0	99.83%
	EU340792.2	0	100%
	EU340793.2	0	80.35%
	KF500510.1	0	100%
Jatropha integerrima Jacq.	EU340795.2	0	99.83%
	MN688731.1	0	92.02%
Jatropha multifida L.	EU340789.2	0	97.95%
	EF599630.2	0	98.12%
Jatropha podagrica Hook.	KP868740.1	0	99.12%
	KF500509.1	0	99.83%

Jatropha species [4], including from the study of Guo *et al.* [11] of key *Jatropha* species, were included in the analysis. A total of 30 accessions of *Jatropha* species and an outgroup (*Croton draco* Schltdl.) were included in a parsimony analysis of the ITS marker (Table 2).

Before the final visual inspection, several alignments of sequences were conducted in MEGA v.7.0.18 [12] using the MUSCLE alignment [13]. Bootstrap replicates of 1000 were utilized to run and perform parsimony analysis using the TBR search method with 100 random incorporations of initial trees. Homoplasy was approximated by quantifying the consistency index (CI) [14] and retention index (RI) [15].

Results and discussion

Nucleotide analysis. The BLAST*n* results of successfully sequenced ITS of the two "tubatuba"-named specimens identified these

as members of the genus *Jatropha* (Euphorbiaceae) with e-values = 0 and identities of 100% (Table 1).

Molecular confirmation. In verifying the species identity of medicinal plants with similar local names or uncertain species identity, the use of molecular sequences has become a powerful tool [11]. The two "tubatuba" voucher specimens were verified as *J. curcas* for "tubatuba puti" and *J. gossypiifolia* for "tubatuba tapol" based on the ITS marker. As a universal marker, ITS renders high interspecific divergence and greater discriminatory power over plastid regions [16, 17]. In general, it is utilized for species-level phylogenetic studies between plants and fungi that are associated with the recently diverged taxa [18]. Furthermore, due to its small size, it can be easily amplified with a universal marker designed by White *et*

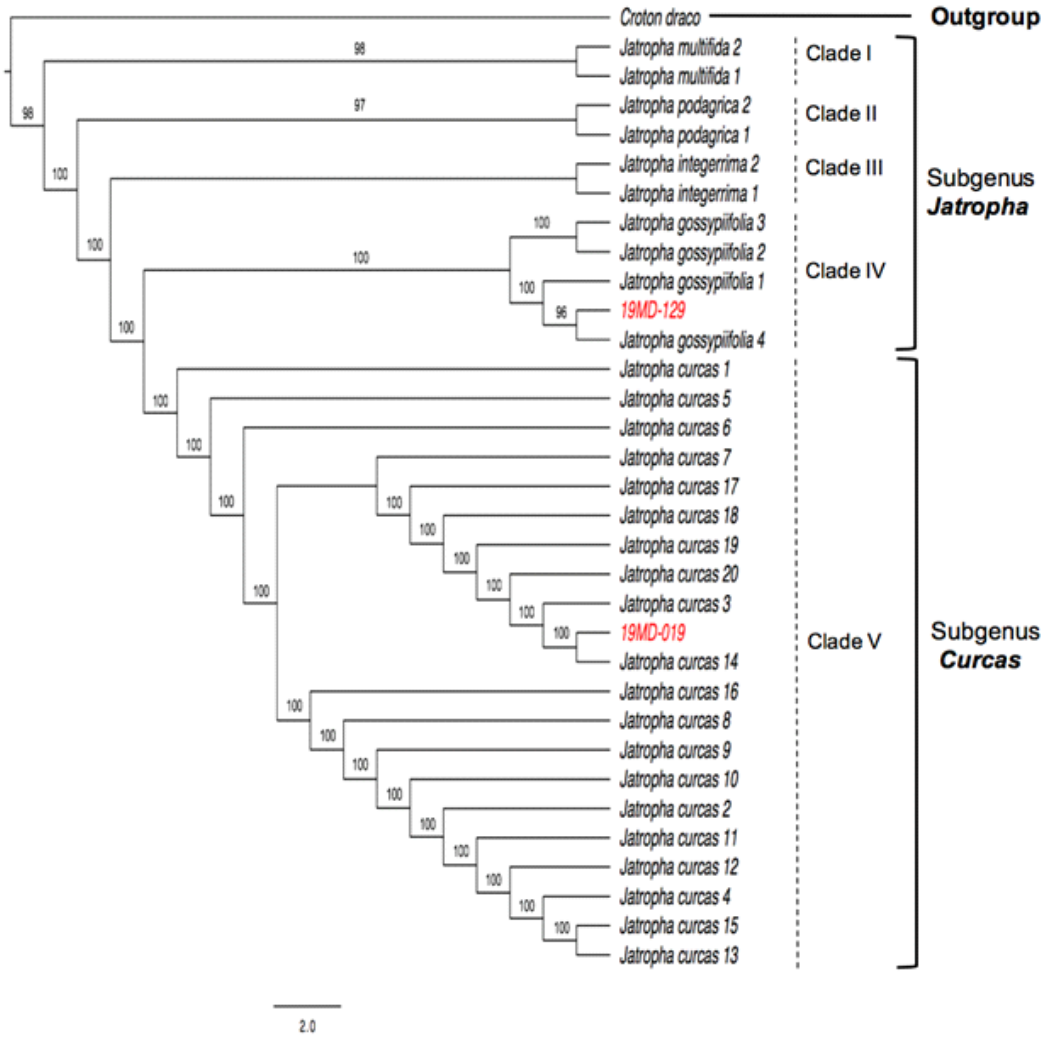

Figure 2. Strict consensus tree derived from eight equally parsimonious trees based on the phylogenetic analysis of ITS sequence data. Length = 438 steps; Consistency index = 0.83; Retention index = 0.82. Branch lengths are drawn to scale. Percentage of 1000 bootstrap replicates is given when higher than 50%. Taxa in red emphasize where the newly included samples (19MD-129 and 19MD-019) are nested.

al. [19] and is taken into account as a supplementary locus for plant barcode [17].

Specimens that were amplified using the ITS marker resulted in a 100% success rate. They were identified down to their species level by simple BLAST-search query. The molecular confirmation verified the morphological identities examined.

Phylogenetic analysis. The obtained maximum parsimonious tree (Fig. 2) is governed by a bisection between two subgenera classifications, namely *Jatropha* and *Curcas.* The first section of the phylogenetic tree shows the representative members of the subgenus *Jatropha,* composed of *J. multifida, J. podagrica, J. integerrima,* and *J. gossypiifolia*. This subgenus, however, is subdivided into different lineages (I, II, III, and IV) per species. Although all species of subgenus *Jatropha* are known to be naturalized in the Philippines, the observed interspecific relation between them implies that these obtained specimens belong to different countries of origin [4].

As for the subgenus *Curcas*, all *J. curcas* specimens were grouped in Clade V, wherein the majority, including the collected sample, was branched from one same lineage. The other three *J. curcas* (labeled as 1, 5, and 6) separated from the majority of *J. curcas*, which suggests that these were the probable cause of varied geographical and environmental conditions [11]. As opposed to the obtained accessions from Myanmar, other parts of China, India, Mali, Burkina Faso, and Philippine tropics, these three divergent *J. curcas* (labeled 1,5 and 6) share their origins from the arid-upland climate of Southwestern China [20].

Both *J. gossypiifolia* and *J. curcas* voucher specimens are nested together with their corresponding accession counterparts from India, in clades I and V, respectively. These intraspecific relationships are mainly because of the similar tropical climate conditions they were propagated from [3].

Comparative morphology of J. curcas and J. gossypiifolia. This section contains the general vegetative and reproductive morphology along with the distribution, habitat, and conservation status of *J. curcas* and *J. gossypiifolia*. A comparative summary of the two *Jatropha* species is presented in Table 3.

Jatropha curcas is an upright shrub or small tree that grows ca. 2–4 m tall. Stem woody brown, 1.5–2 cm thick, exudes watery sap when broken. Pedicel 3–7 cm long with racemose inflorescence. Leaves glabrous, divided into 3–5 palmately lobed. Leaf blades round to ovate, 10–19 cm × 5–15 cm, acute at apex, cordate at base, glabrous on the upper surface, 7–10 lateral nerve pairs. Flowers small, yellow to green. Fruits oval, 3–4 cm, crowned by calyx lobes. Calyx ovate, 0.4 cm long; lobes 2–4, 3.2–5 cm long. Corolla green, ovate, 3–5 mm in length; anther unequal. Style 2–5.6 mm long, connate at 2/3 of its length [21].

Jatropha gossypiifolia is an upright shrub or small tree that usually grows ca. 1–3 m tall. Stem woody green, 1–3 cm thick, and exude a soapy sap when broken. Pedicel 2–10 cm long, inflorescence racemose. Leaves are alternately arranged, with 3 or 5 pointed lobes, and dark reddish-purple when young. Leaf blades 4.5–10 cm × 5–13 cm, palmately lobed to partite with lobed segments and palmate venation, acute to acuminate at apex, shallow at base, glabrous on the upper surface, 7–11 lateral nerves pairs. Flowers small, glabrous, deep red with yellow centers, obovate, 3–5 mm long. Flowers are borne in clusters in the upper leaf forks. Calyx ovate to lanceolate, 0.7 cm long; lobes 3–5 lobes, 3 cm long. Capsules 3-lobbed seeds, fleshy, usually bright, glossy green, sometimes sparsely hairy. Fruits crowned by calyx lobes, oblong, 1.2–1.3 cm long. Style 1 mm, anther unequal length [22].

Table 3. Comparative morphology of *J. curcas* and *J. gossypiifolia.*

	Jatropha curcas	*Jatropha gossypiifolia*
Habit	Shrub	Shrub
Height	2–4 m	1–3m
Stem		
Thickness	1.5–2 cm	1–3 cm
Structure & Color	Woody covered in brown	Woody covered in green
Petiole		
Length	6–18 cm	7–12cm
Leaf Blade		
Shape	Round to ovate	Palmately lobed to partite with lobbed segments
Venation	Pinnate/palmate	Palmate
Length	10–19 cm	4.5–10 cm
Width	5–15 cm	5–13 cm
Apex	Acute	Acute to acuminate
Base	Cordate	Shallowly
Surface	Glabrous on upper surface	Glabrous on upper surface
Lateral nerve pairs	7–10 pairs	7–11 pairs
Inflorescence		
Type	Racemose	Racemose
Pedicel		
Length	3–7 cm	2–10 cm
Calyx		
Shape	Ovate	Ovate to lanceolate
Length	0.4 cm	0.7 cm
Lobes number	2–4	3–5
Lobes length	3.2–5 cm	3 cm
Corolla		
Color	Green	Deep red
Shape	Ovate	Obovate
Length	3–5 mm	3–5 mm
Surface	Glabrous adaxially	Glabrous adaxially
Anther		
Size	Unequal length	Unequal length
Style		
Length	2–5.6 mm, connate to about two-thirds of its length	1 mm
Fruits		
Calyx lobes	Persistent	Persistent
Shape	Long-ovoid	Oblong
Length	3–4 cm	1.2–1.3 cm

Distribution and habitat. *Jatropha curcas* was first introduced in the early colonial times from Mexico, then distributed to the tropical regions of Thailand, Sumatra, Peninsular Malaysia, Java, Borneo, Philippines, Sulawesi, Lesser Sunda Islands, Isles of Moluccas, and New Guinea. This plant is commonly found in hedges throughout the Philippines. On the other hand, *J. gossypiifolia* is endemic to Mexico to South America and the Caribbean and naturalized throughout the Malesia region, including the Philippines [4].

Conservation status. As per IUCN [23], *J. curcas* is categorized as endangered, while *J. gossypiifolia* as least concern. However, based on CDFP [4], both *Jatropha* species in the Philippines have not been evaluated.

Conclusion

Molecular sequences inferred from the nuclear ITS with comparative morphology confirmed the identity of "tubatuba puti" as *J. curcas* while "tubatuba tapol" as *J. gossypiifolia*. This study reinforced the ethnoclassification of *Jatropha* species as different species and supported the application of molecular data for confirmation of uncertain plant species identity.

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