Supplementary material

Kratom (*Mitragyna speciosa*) validation: quantitative analysis of indole and oxindole alkaloids reveals chemotypes of plants and products

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Table 1S List of publications covering the analytical analysis of kratom plants and products.

Biological matrix	LOD (ng/mL)	LOQ (ng/mL)	Calibration range	Compounds detected	Concentration	Concentration (%)	Internal standard(s)	Analytical technique	Column	Solvent system	Run time	Reference
kratom cocktail' (4 x 100)	1.000 mg/L	3.000 mg/L	5.000–100.000 mg/L	MG	90.021 mg/L			HPLC-DAD	Eclipse XDB-C8	methanol:water (80:20, v/v)	10 min	[1]
commercial products, live plant (US)		5 μg/mL	5–100 μg/mL	MG	2.76–20.05 mg/g		mitragynine-d_3	DART-HRMS		methanol		[2]
kratom products, *M. speciosa* and *M. hirsuta* dried leaves (Thailand)			1.0–10.0 μg/mL 10–1000 ng/mL	MG 7-HMG SPG SPC PAY	0.8 ± 0.2–62.6 ± 1.6 mg/g 0.031 ± 0.004–0.393 ± 0.026 mg/g	0.08–6.3 % 0.003–0.04 %	betamethasone valerate	HPLC-DAD, LC-ESI-MS	Atlantis dC18	A: water (10 mM ammonium formate, pH 3.5), B: methanol	66 min	[3]
commercial products, police sample, fresh and dried leaves			0.5 mg/mL	MG				HPLC-UV-Vis, TLC	Luna C18	MeCN/0.01% ammonia (70:30, v/v), pH 10.3	20 min	[4]
leaf extracts, drinks, dried plants	0.25 μg/mL	0.50 μg/mL	0.5–10.0 μg/mL	MG	0.80 ± 0.11–24.72 ± 0.67 mg/g, 30.95 ± 1.61–444.23 ± 3.55 μg/mL			HPLC-DAD	Inertsil C8	MeCN:0.5% formic acid, pH 5 (50:50, v/v)	6 min	[5]
leaf extracts, commercial products		1 ng/mL	1–200 ng/mL	MG 7-HMG SPG SPC PAY COR COX COX-B		0.7–38.7 % w/w 0.01–0.04 % w/w 0.1–5.3 % w/w 0.4–12.3 % w/w 0.3–12.8 % w/w 0.02–1.16 % w/w 0.03–2.75 % w/w 0.03–1.44 % w/w	phenacetin	UHPLC-ESI-MS/MS	BEH C18	A: water (10 mM ammonium acetate, pH 3.5), B: MeCN	22.5 min	[6]

	ICOR	0.02–0.13					
	MTP	< LLOQ					
leaf extract, alkaloid extract	COX-B		UHPLC-API-MS-DAD, SFC-DAD, GC-MS	Zorbax RRHD SB-C8, Rx-SIL, DB-5MS	A: water (10 mM ammonium acetate, pH 7.7), B: MeCN; CO2 with MeOH (10 mM ammonium acetate)	35 min, 10 min	[7]
	COX						
	PAY						
	3-IPAY						
	MG						
	SPG						
	SPC						
	7-HMG						
commercial leaves and products	7-HMG		UHPLC-ESI-MS/MS	Zorbax RRHD SB-C8	A: water (0.1% formic acid), B: MeCN (0.1% formic acid)	40 min	[8]
	ISPF						
	ISPFE						
	IROF						
	COX-B						
	COX						
	7*ß*-hydroxy-7*H*-MTC						
	PAY						
	MG						
	SPG						
	3-IPAY						
	SPC						
commercial product, seized product "Krypton"	MG		CE-HRMS, CE-MS/MS	capillary (50 µm i.d., 67.5 cm length)	MeCN (58 mmol/L ammonium formate and 1.01 mol/L acetic acid)		[9]
	SPG						
	SPC						
	MTC						
	O-DMT						
	7-HMG						

leaves and local drink extracts				MG			GC-MS, GC-FID	DB-5, HP-5			[10]
commercial products		1 ng/mL	10–0.05 µg/mL	MG	9.7–19.0 µg/mg, 190.7–396.4 ng/µL	ondansetron	UHPLC-ESI-MS/MS	BEH C18			
				7-HMG	93.0–593.2 ng/mg, 1.96–2.51 ng/µL						[11]
commercial products	0.2 µg/mL	0.6 µg/mL	1.0–500 µg/mL	MG	0.379–68.52 mg/g		HPLC-DAD	Kinetex EVO C18	A: water (5.0 mM ammonium bicarbonate, pH 9.5), B: MeCN	18 min	[12]
	0.4 µg/mL	1.1 µg/mL		7-HMG	0.59–2.59 mg/g						
Malaysian *M. speciosa* leaves		1 ng/mL	1–200 ng/mL	COX	0.03–0.46 mg/g	phenacetin	UHPLC-ESI-MS/MS	BEH C18	A: water (10 mM ammonium acetate, pH 3.5), B: MeCN	22.5 min	[13]
				COX-B	0.05–0.15 mg/g						
				ISPF	0.03 mg/g						
				MG oxindole B	0.05–0.21 mg/g						
				COR	0.03–0.42 mg/g						
				MG	9.38–18.85 mg/g						
				SPG	0.97–1.99 mg/g						
				PAY	0.01–1.90 mg/g						
				SPC	0.43–14.76 mg/g						
				SPC *N*(4)-oxide	0.02–0.06 mg/g						
				7-HMG	0.03–0.15 mg/g						
				ICOR	0.03–0.17 mg/g						
				AJM							
				MTP							
M. speciosa leaves	32.47 µg/mL		32.92–250 µg/mL, 1.56–25 µg/mL	MG	8.96–56.26 mg/g, 8.45–55.52 mg/g		ELISA, HPLC-DAD	Vertisep UPS C18	water (5 mM phosphate buffer, pH 6.0):MeCN (35:65)		[14]
commercial products			0–750 µg/mL	MG	0.640–187 mg/g	thymol	GC-MS	Varian VF-5MS			[15]
				7-HMG							
				PAY							
				SPC							
				SPG							
				COX A							

				COX-B								
commercial products			0.01–2 mg/g	MG	*ca.* 2–19 mg/g		yohimbine	TLC, GC-MS	Agilent DB-5MS UI			[16]
commercial products	0.5 ng, 2 ng/mL	6 ng/mL	0.1–5 ng/µL, 10–100 ng/mL	MG	0.03–862 µg/mL			IMS, HPLC-ESI-MS	Xbridge C18	A: water (0.1% formic acid), B: MeCN (0.1% formic acid)	20 min	[17]
commercial products			10 or 20 µg/mL, 10–100 ng/mL, 1–10 µg/mL	MG	189–308 µg/mL	0.536–3.100 % w/w, 0.540–3.540 % w/w		GC-MS, HPLC-ESI-MS/MS, UHPLC-PDA	Agilent DB-5 MS, XBridge BEH C18, Acquity BEH C18	A: water (0.1% formic acid), B: MeCN	20 min, 7 min	[18]
commercial products, *M. speciosa* leaves	1.1 ng/mL		9.77–5000 ng/mL	IMTP	0.943–1.47 mg/g			UHPLC-ESI-HRMS	Kinetex F5	A: water (0.1% formic acid), B: MeCN (0.1% formic acid)	20 min	Present method
	0.67			7-HMG	0.1240–1.10 mg/g							
	0.79			ISPFE	0.382–1.675 mg/g							
	0.66			SPF	0.1222–5.90 mg/g							
	0.69			COX	0.2322–11.40 mg/g							
	0.77			COXE	0.290–1.217 mg/g							
	1.0			RYP	0.0480–0.239 mg/g							
	1.0			AJM	0.0262–0.883 mg/g							
	1.5			PAY	5.79–70.4 mg/g							
	1.4			MG	0.53–270 mg/g							
	1.3			SPG	3.18–33.4 mg/g							
	0.94			IPAY	0.512–3.80 mg/g							
	0.59			SPC	3.68–41.7 mg/g							
	0.70			MTC	0.647–4.75 mg/g							

Abbreviations: MG, mitragynine; 7-HMG, 7-hydroxymitragynine; SPG, speciogynine; SPC, speciociliatine; MTC, mitraciliatine; PAY, paynantheine; IPAY, isopaynantheine; COR, corynantheidine; ICOR, isocorynantheidine; COX, corynoxine; COX-B, corynoxine B; COXE, corynoxeine; MTP, mitraphylline; IMTP, isomitraphylline; AJM; ajmalicine; SPF, speciofoline; ISPF, isospeciofoline; ISPFE, isospeciofoleine; IROF, isorotundifoline; RYP, rhynchophylline; O-DMT, *O*-desmethyltramadol; HPLC, high performance liquid chromatography; UHPLC, ultra-high performance liquid chromatography; TLC, thin-layer chromatography; CE, capillary electrophoresis; GC, gas chromatography; SFC, supercritical fluid chromatography; DART, direct analysis in real time; DAD, diode array detector; PDA, photodiode array detector; UV-Vis, ultraviolet-visible light detector; FID, flame ionization detector; MeCN, acetonitrile; CO2, carbon dioxide; MS, mass spectrometry; MS/MS, tandem mass spectrometry; IMS, ion mobility spectrometry; HR, high resolution; API, atmospheric pressure ionization; ESI, electrospray ionization; ELISA, enzyme-linked immunosorbent assay.

Refer to Kerrigan et al. [19] for analytical methods that identify and quantify *Mitragyna* alkaloids in biological matrices.

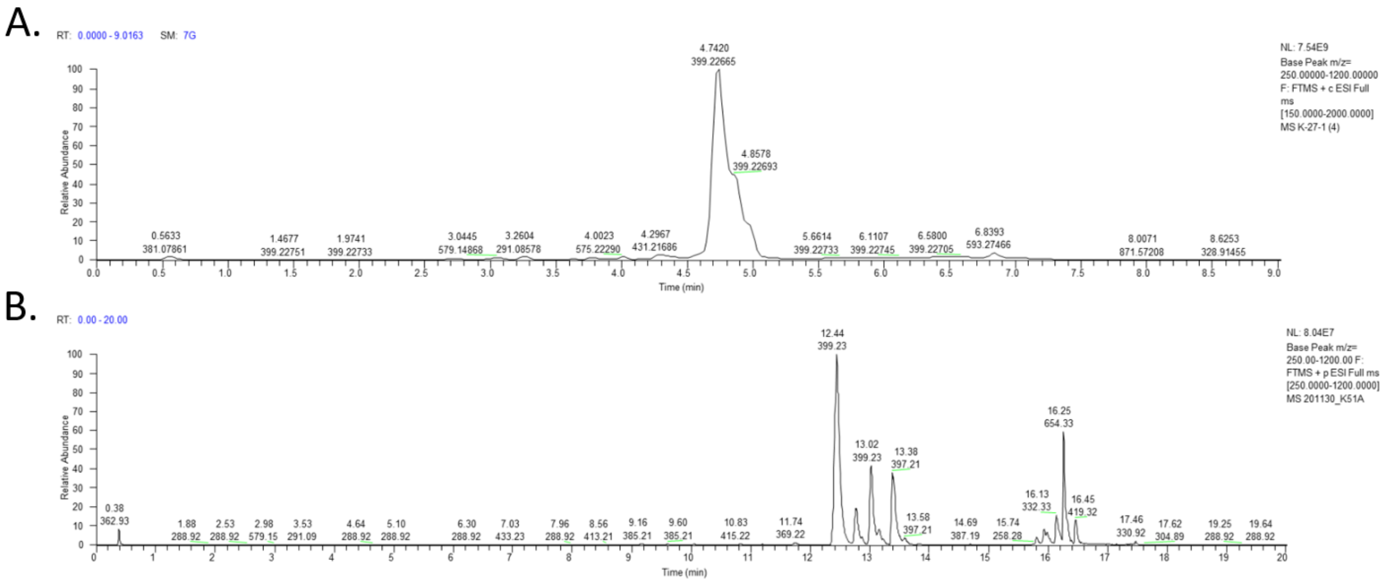


Fig. 1S Comparison of a kratom extract analyzed using a generic screening method with a BEH C18 column (A) and the developed method using a Kinetex F5 column (B).

Table 2S Intraday and interday precision (as measured by RSD) and accuracy (as measured by RE) of the method for kratom alkaloid quantification.

Analyte	Concentration of standard solution (ng/mL)	Intraday		Interday	
		RSD[a] (%)	RE[b] (%)	RSD[a] (%)	RE[b] (%)
isomitraphylline (**1**)	5000	1.8	1.6	6.8	4.4
	2500	0.90	7.6	5.8	5.0
	1250	0.30	7.4	5.8	2.7
	625	1.7	1.8	4.9	0.88
	313	1.3	2.6	7.3	1.0
	156	2.2	5.4	10	5.5
	78.1	1.1	3.2	4.4	0.59
	39.1	2.2	10	4.9	6.4
	19.5	1.0	0.70	4.3	3.6
	9.77	2.9	3.6	3.1	3.7
7-hydroxymitragynine (**2**)	2500	2.7	4.5	5.8	1.4
	1250	5.2	2.7	4.5	3.3
	625	6.9	6.4	6.8	3.4
	313	5.2	4.9	6.2	6.6
	156	11	11	9.9	2.9
	78.1	3.8	1.9	6.3	1.3
	39.1	4.7	6.9	6.3	7.4
	19.5	1.0	1.4	4.0	4.4
	9.77	1.8	3.1	3.3	4.1
isospeciofoleine (**3**)	2500	7.3	0.043	7.4	0.54
	1250	3.6	3.3	4.4	4.3
	625	5.1	7.7	6.5	5.0
	313	3.6	5.2	5.6	6.5
	156	12	8.9	9.8	3.8
	78.1	2.1	1.4	7.2	3.0
	39.1	2.4	7.1	5.9	8.5
	19.5	0.39	2.0	3.4	4.9
	9.77	2.1	3.2	3.0	4.9
speciofoline (**6**)	5000	1.6	7.4	7.0	2.7
	2500	0.24	0.92	4.6	1.6
	1250	0.41	5.8	3.6	4.1

	625	1.0	7.4	5.9	3.3
	313	0.60	4.0	6.7	4.6
	156	1.1	0.36	11	4.0
	78.1	0.36	2.7	5.6	0.36
	39.1	4.3	9.8	5.9	6.5
	19.5	1.0	0.8	4.5	3.6
	9.77	1.7	3.0	3.8	3.5
corynoxine A (**7**)	2500	0.90	5.4	5.8	3.4
	1250	1.3	2.6	3.9	1.6
	625	1.0	6.7	6.4	2.9
	313	1.1	5.3	6.8	6.9
	156	0.95	2.0	11	1.5
	78.1	0.84	3.0	6.8	0.44
	39.1	3.8	9.9	6.0	6.0
	19.5	0.84	1.5	4.9	4.3
	9.77	1.7	3.2	3.7	3.4
corynoxeine (**8**)	5000	1.2	0.4	6.1	3.8
	2500	1.1	6.6	4.9	4.9
	1250	1.1	6.7	3.7	4.4
	625	0.28	3.8	4.9	0.7
	313	1.0	0.64	6.2	2.3
	156	1.4	2.3	11	5.8
	78.1	1.2	5.6	6.2	2.7
	39.1	5.1	12	6.3	7.5
	19.5	0.81	2.6	4.7	5.1
	9.77	1.8	4.9	3.0	5.0
rhynchophylline (**9**)	2500	1.8	7.3	8.0	1.4
	1250	0.12	0.74	5.8	1.3
	625	0.68	8.1	7.8	1.7
	313	0.63	6.0	7.3	7.0
	156	0.17	1.9	13	3.8
	78.1	0.54	1.3	8.9	2.2
	39.1	5.3	10	7.2	6.1
	19.5	0.20	0.34	7.2	4.3
	9.77	2.6	2.5	5.0	3.4
ajmalicine	5000	6.1	3.1	7.7	4.5

(**10**)	2500	0.76	5.1	4.9	1.3
	1250	2.4	5.1	3.4	4.9
	625	5.9	2.6	5.8	2.6
	313	3.7	4.8	8.3	6.4
	156	14	14	12	2.8
	78.1	2.6	1.9	7.1	0.11
	39.1	6.0	6.8	7.6	6.8
	19.5	2.0	0.68	7.6	4.9
	9.77	2.7	2.9	5.1	4.1
paynantheine (**11**)	2500	1.2	7.4	5.8	4.1
	1250	1.3	0.66	5.4	0.17
	625	0.66	2.5	6.0	0.0064
	313	0.19	3.7	7.4	5.5
	156	0.57	3.2	12	1.7
	78.1	1.1	3.1	7.3	4.1
	39.1	5.7	6.8	7.3	2.7
	19.5	0.73	2.7	7.7	2.6
	9.77	4.3	0.37	4.6	1.4
mitragynine (**12**)	5000	0.24	11	8.5	3.4
	2500	1.2	1.3	5.5	0.11
	1250	0.81	4.1	5.7	2.7
	625	1.2	4.2	5.7	0.64
	313	1.4	4.3	8.4	4.9
	156	1.3	1.6	14	3.2
	78.1	0.95	2.6	8.3	3.0
	39.1	4.6	7.3	7.5	3.7
	19.5	0.59	2.1	8.4	3.3
	9.77	4.1	0.11	4.7	2.2
speciogynine (**13**)	2500	2.7	2.8	7.4	1.5
	1250	1.0	6.9	5.4	8.3
	625	1.6	10	6.9	3.1
	313	0.30	2.1	11	3.6
	156	0.52	3.6	12	8.0
	78.1	1.3	4.4	8.0	1.5
	39.1	6.2	12	8.3	7.0
	19.5	1.3	0.043	8.1	4.4
	9.77	3.2	3.5	5.0	4.4

isopaynantheine (**14**)	2500	4.4	2.6	7.8	5.8
	1250	5.5	2.2	5.8	0.27
	625	6.1	3.1	7.9	0.24
	313	3.4	3.4	8.7	5.4
	156	17	12	14	0.0020
	78.1	1.2	2.5	9.8	6.1
	39.1	7.3	3.2	8.2	3.0
	19.5	2.8	0.13	7.5	4.9
	9.77	2.7	1.0	4.0	2.3
speciociliatine (**15**)	2500	2.2	1.2	9.8	2.5
	1250	2.9	7.8	6.2	4.1
	625	1.0	5.5	7.4	1.9
	313	1.3	1.8	11	6.6
	156	1.0	2.7	14	3.6
	78.1	1.3	4.8	11	1.3
	39.1	6.4	11	12	6.0
	19.5	1.2	1.9	8.7	6.2
	9.77	1.5	4.3	3.7	4.4
mitraciliatine (**16**)	2500	5.1	3.0	8.3	1.6
	1250	7.0	4.4	7.9	2.5
	625	1.1	7.0	5.9	2.8
	313	2.6	3.8	7.8	6.7
	156	2.5	0.20	12	2.9
	78.1	0.88	2.8	9.6	1.0
	39.1	6.9	11	9.0	7.3
	19.5	1.3	1.7	6.6	5.6
	9.77	1.8	3.8	2.8	4.4

[a] $relative\ standard\ deviation\ (RSD) = 100\% \times \left(\frac{s_i}{\bar{x}}\right)$

$standard\ deviation\ (s_i) = \sqrt{\frac{\sum(x_i-\bar{x})^2}{(n-1)}}$

[b] $relative\ error\ (RE) = \left|\frac{\overline{x_i}-x_t}{x_t}\right| \times 100\%$

$\overline{x_i} = mean\ of\ the\ actual\ values\ observed$

$x_t = true\ (or\ nominal)\ value$

Table 3S Concentration (µg/mL) and Percent recovery (%) of internal standard mitragynine-d_3, which was added to all solutions at a concentration of 125 µg/mL.

Sample	Conc. (µg/mL)	% Recovery
K49	109	88
K51	120	96
K52	110	88
K55	115	92
K59	114	91
K76	111	89
K77	114	91

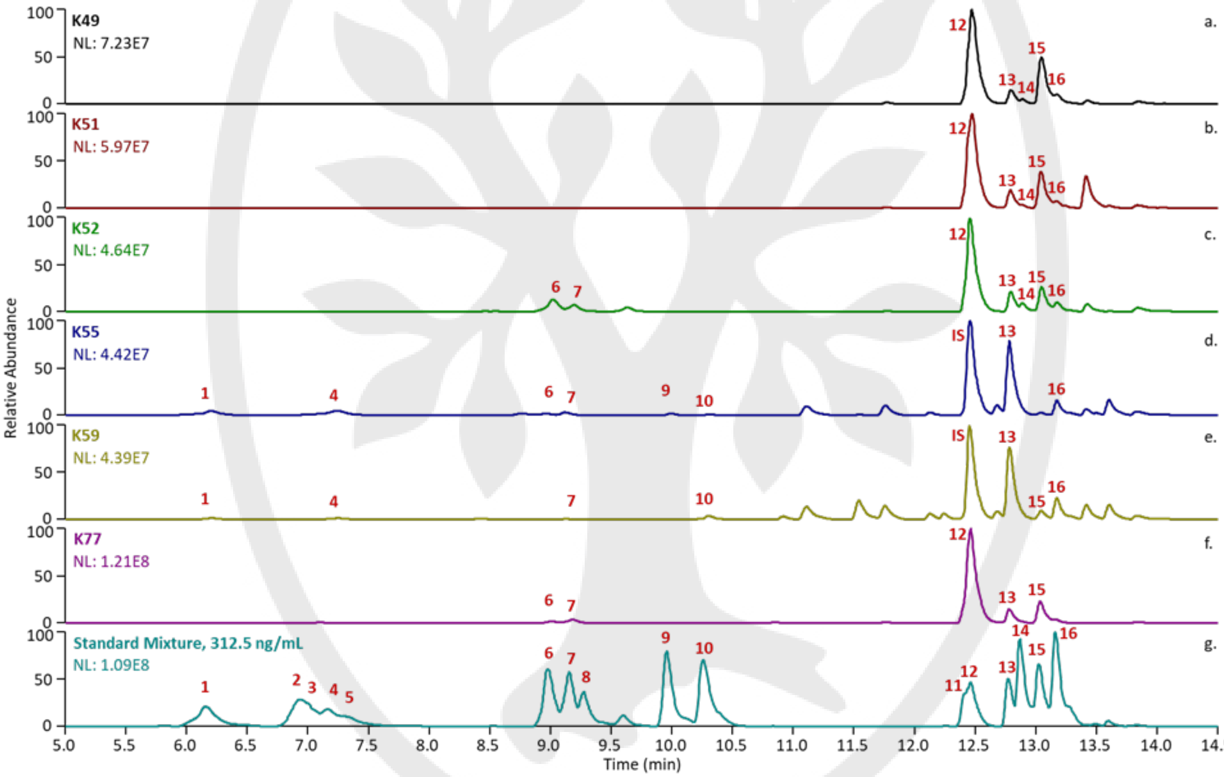


Figure 2S Base peak MS chromatograms representing the methanolic extracts of kratom material quantified in Table 2. Specifically, from top to bottom, (panel a) a commercial kratom powder (K49), (panel b) a commercial kratom powder (K51), (panel c) a high-speciofoline containing commercial kratom powder (K52), (panel d) a UNCG grown kratom cutting “Rifat” (K55), (panel e) an Ohio grown kratom cutting “Rifat” (K59), (panel f) a commercial kratom pill, and (panel g) an equimolar mixture of kratom alkaloids at 313 ng/mL. Structures corresponding to each peak (Figs. 1 and 2) were assigned by matching retention time, fragmentation pattern, and accurate mass with authentic standards. Compounds are numbered in order of elution. NL is the normalization level, i.e., the base peak intensity.

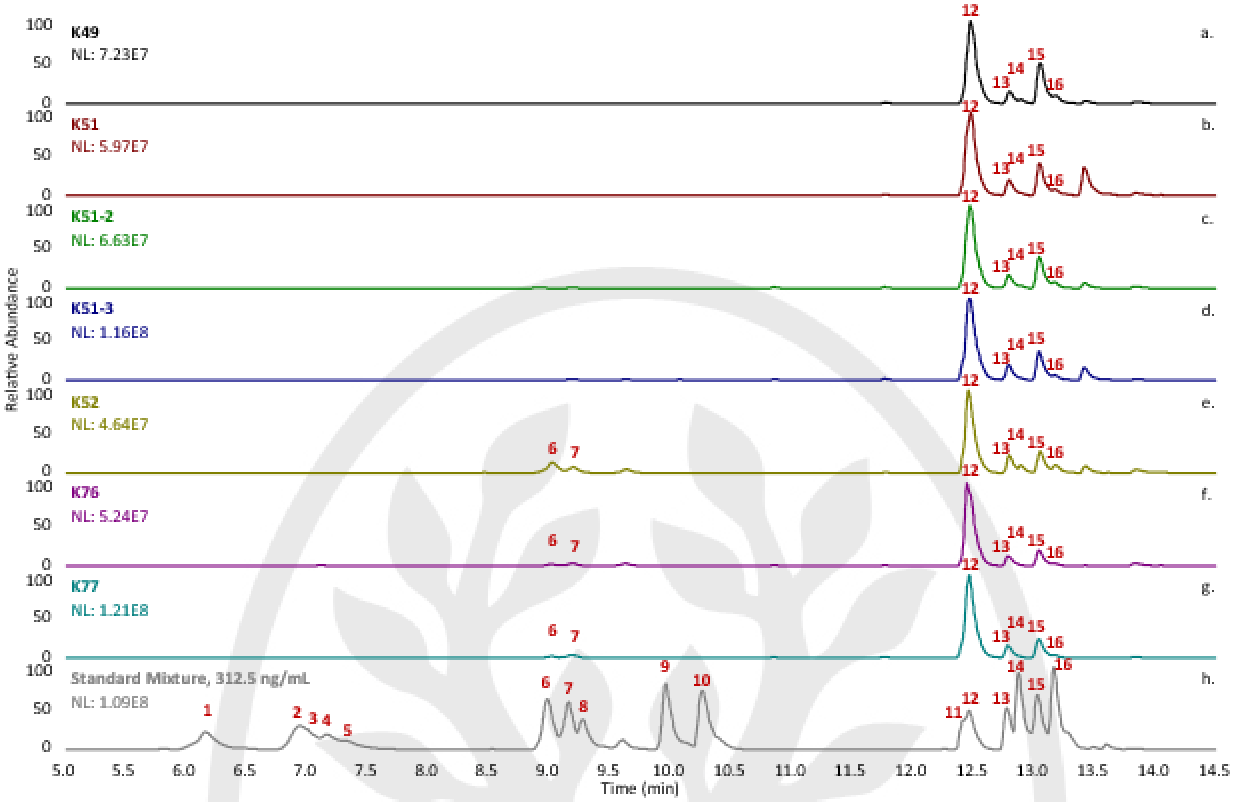


Figure 3S Base peak MS chromatograms representing the extracts of kratom material quantified in Table 3. Specifically, from top to bottom, (panel a) a methanolic extract of a commercial kratom powder (K49), (panel b) a methanolic extract of a commercial kratom powder (K51), (panel c) the alkaloidal partition from a $CHCl_3$–MeOH extract of K51 (K51-2), (panel d) a hot water (tea) extract of K51 (K51-3), (panel e) a high-speciofoline containing methanolic extract of commercial kratom powder (K52), (panel f) a liquid kratom product (K76), (panel g) a methanolic extract of a commercial kratom pill, and (panel h) an equimolar mixture of kratom alkaloids at 313 ng/mL. Structures corresponding to each peak (Figs. 1 and 2) were assigned by matching retention time, fragmentation pattern, and accurate mass with authentic standards. Compounds are numbered in order of elution. NL is the normalization level, i.e., the base peak intensity.

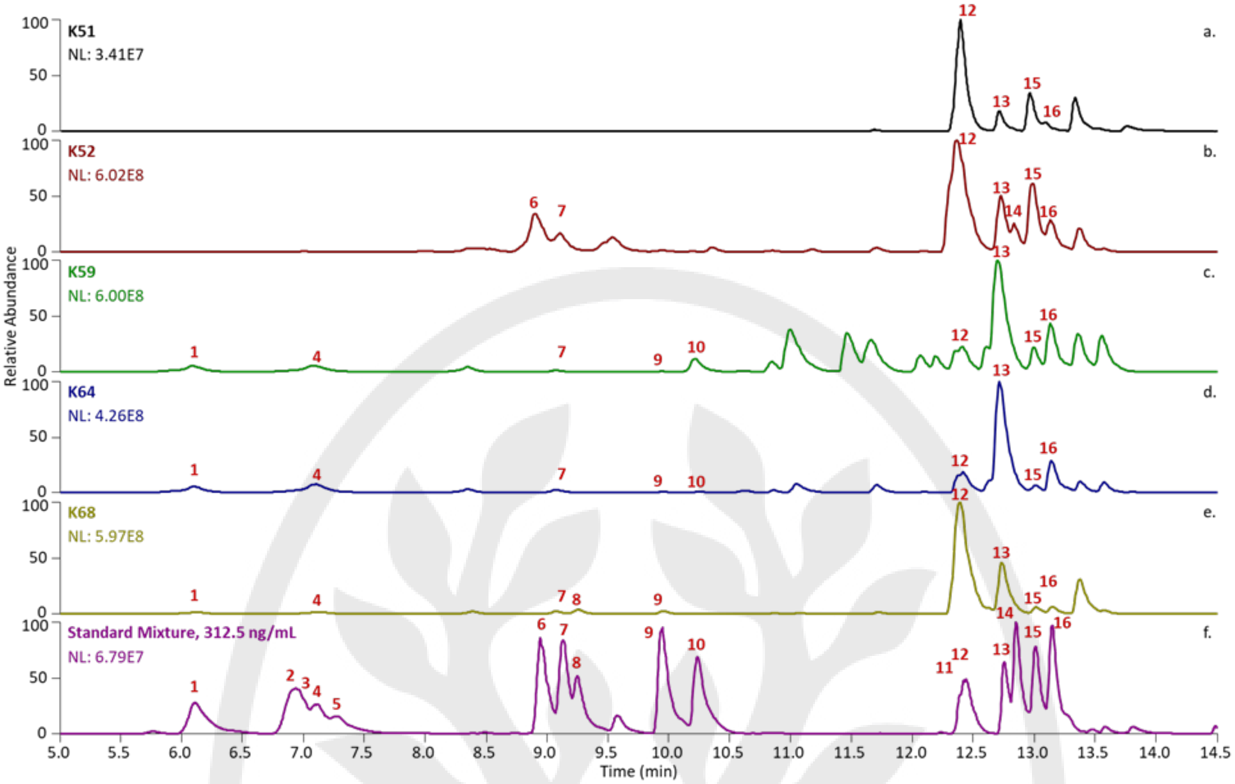


Figure 4S Base peak MS chromatograms representing the methanolic extracts of kratom material quantified in Table 4. Specifically, from top to bottom, (panel a) a commercial kratom powder (K51), (panel b) a high-speciofoline containing commercial kratom powder (K52), (panel c) an Ohio grown kratom cutting "Rifat" (K59), (panel d) a UNCG grown kratom cutting "Rifat" (K64), (panel e) a UNCG grown kratom seedling (K68), and (panel f) an equimolar mixture of kratom alkaloids at 313 ng/mL. Structures corresponding to each peak (Figs. 1 and 2) were assigned by matching retention time, fragmentation pattern, and accurate mass with authentic standards. Compounds are numbered in order of elution. NL is the normalization level, i.e., the base peak intensity.

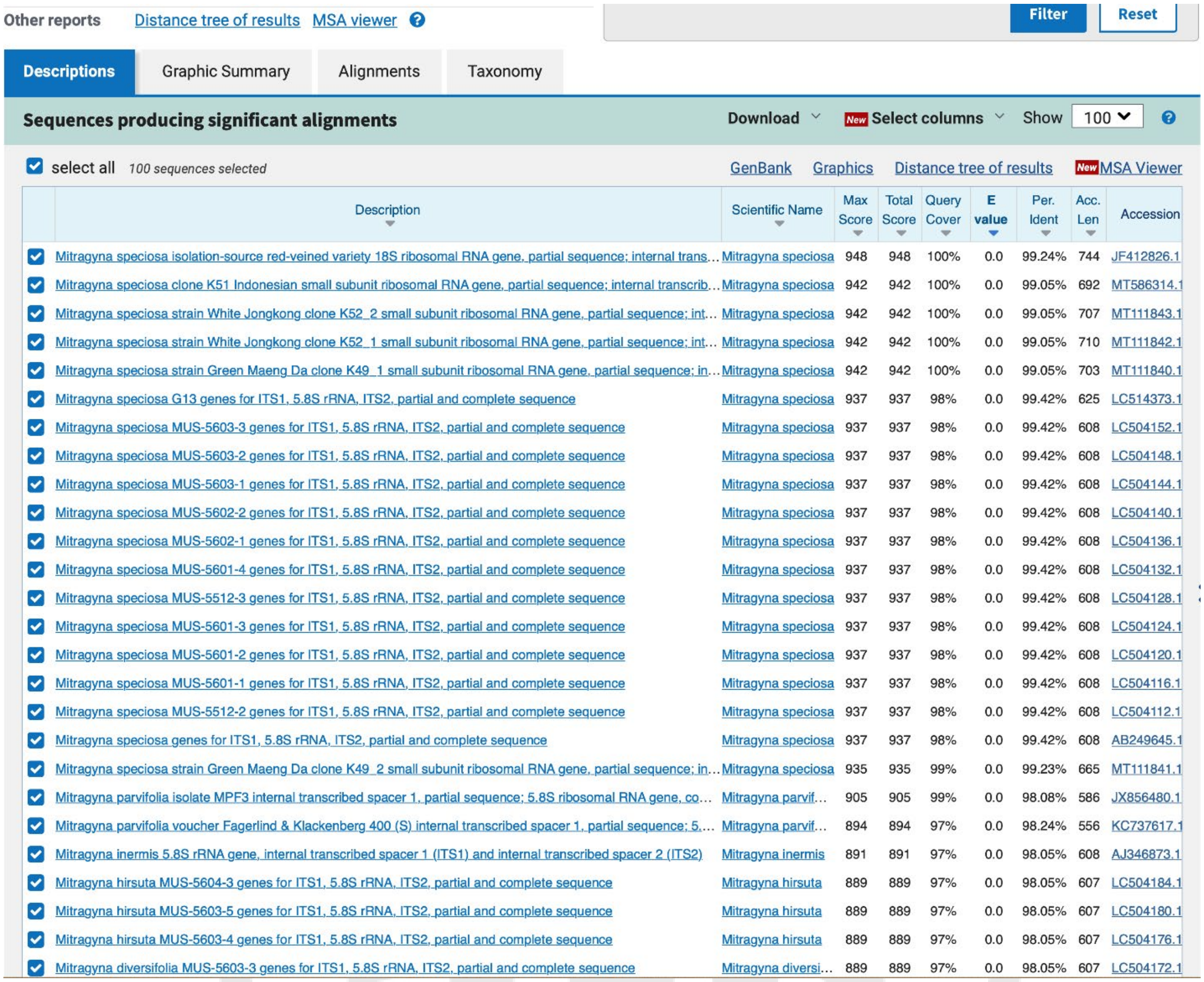
Table 4S Literature references for purchased indole and oxindole alkaloid standards.

Compound	Reference(s)
7-hydroxymitragynine (**2**)	[20]
ajmalicine (**10**)	[21, 22]
isomitraphylline (**1**)	[23, 24]
speciophylline (**5**)	[24]
mitraphylline (**4**)	[23, 24]
rhynchophylline (**9**)	[25]

Table 5S Commercial kratom products and *M. speciosa* specimens analyzed in this study.

Code	Product Name	Purported Origin	Description	Voucher	GenBank # for ITS
K49	Green Maeng Da	Thailand	Cut leaf; low speciofoline group	-	MT111840, MT111841
K51	Yellow Indonesian	Indonesia	Powder; low speciofoline group	-	MT586314
K52	White Jongkong	Jongkong, West Kalimantan, Indonesia	Powder; high speciofoline group	-	MT111842, MT111843
K76	Kratom Extract	Unknown	Liquid; leaf extract 50:1	-	-
K77	Botanical Extract	Unknown	Capsule; powdered extract 50:1	-	-
K55	Living UNCG plant	Cutting from Ohio grown mother tree	Rifat, *ca.* 9-month-old	NCU00433756	MT586315
K59	Living Ohio plant	Leaves from Ohio grown mother tree	Rifat, *ca.* 7-year-old	-	-
K64	Living UNCG plant	Cutting from US supplier grown “Rifat”	Rifat, *ca.* 8-month-old	NCU00675498	OM181964
K68	Living UNCG plant	Mempawah, West Kalimantan, Indonesia seedling from US supplier	Borneo seedling, *ca.* 14 months	NCU00675499	OM181965

Products K49, K51, K52, and K55 were identified previously as *M. speciosa* [26, 27].

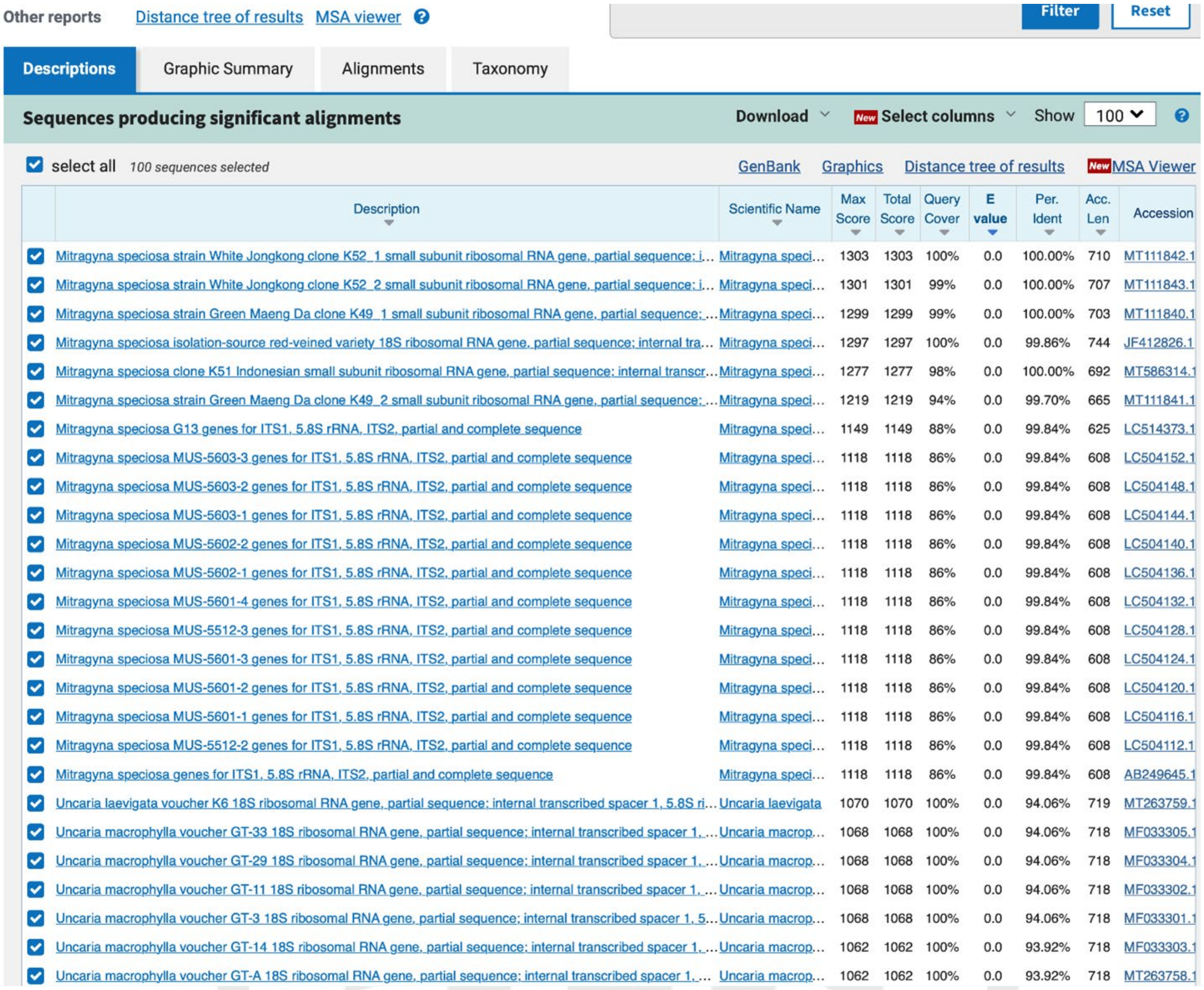


Sequences producing significant alignments

☑ select all *100 sequences selected*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Mitragyna speciosa isolation-source red-veined variety 18S ribosomal RNA gene, partial sequence; internal trans…	Mitragyna speciosa	948	948	100%	0.0	99.24%	744	JF412826.1
☑	Mitragyna speciosa clone K51 Indonesian small subunit ribosomal RNA gene, partial sequence; internal transcrib…	Mitragyna speciosa	942	942	100%	0.0	99.05%	692	MT586314.1
☑	Mitragyna speciosa strain White Jongkong clone K52_2 small subunit ribosomal RNA gene, partial sequence; int…	Mitragyna speciosa	942	942	100%	0.0	99.05%	707	MT111843.1
☑	Mitragyna speciosa strain White Jongkong clone K52_1 small subunit ribosomal RNA gene, partial sequence; int…	Mitragyna speciosa	942	942	100%	0.0	99.05%	710	MT111842.1
☑	Mitragyna speciosa strain Green Maeng Da clone K49_1 small subunit ribosomal RNA gene, partial sequence; in…	Mitragyna speciosa	942	942	100%	0.0	99.05%	703	MT111840.1
☑	Mitragyna speciosa G13 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	625	LC514373.1
☑	Mitragyna speciosa MUS-5603-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504152.1
☑	Mitragyna speciosa MUS-5603-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504148.1
☑	Mitragyna speciosa MUS-5603-1 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504144.1
☑	Mitragyna speciosa MUS-5602-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504140.1
☑	Mitragyna speciosa MUS-5602-1 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504136.1
☑	Mitragyna speciosa MUS-5601-4 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504132.1
☑	Mitragyna speciosa MUS-5512-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504128.1
☑	Mitragyna speciosa MUS-5601-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504124.1
☑	Mitragyna speciosa MUS-5601-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504120.1
☑	Mitragyna speciosa MUS-5601-1 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504116.1
☑	Mitragyna speciosa MUS-5512-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	LC504112.1
☑	Mitragyna speciosa genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speciosa	937	937	98%	0.0	99.42%	608	AB249645.1
☑	Mitragyna speciosa strain Green Maeng Da clone K49_2 small subunit ribosomal RNA gene, partial sequence; in…	Mitragyna speciosa	935	935	99%	0.0	99.23%	665	MT111841.1
☑	Mitragyna parvifolia isolate MPF3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, co…	Mitragyna parvif…	905	905	99%	0.0	98.08%	586	JX856480.1
☑	Mitragyna parvifolia voucher Fagerlind & Klackenberg 400 (S) internal transcribed spacer 1, partial sequence; 5.…	Mitragyna parvif…	894	894	97%	0.0	98.24%	556	KC737617.1
☑	Mitragyna inermis 5.8S rRNA gene, internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2)	Mitragyna inermis	891	891	97%	0.0	98.05%	608	AJ346873.1
☑	Mitragyna hirsuta MUS-5604-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna hirsuta	889	889	97%	0.0	98.05%	607	LC504184.1
☑	Mitragyna hirsuta MUS-5603-5 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna hirsuta	889	889	97%	0.0	98.05%	607	LC504180.1
☑	Mitragyna hirsuta MUS-5603-4 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna hirsuta	889	889	97%	0.0	98.05%	607	LC504176.1
☑	Mitragyna diversifolia MUS-5603-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna diversi…	889	889	97%	0.0	98.05%	607	LC504172.1

Fig. 5S Graphical overview of the BLAST results (January 2022) in NCBI GenBank database using Internal Transcribed Spacer (ITS) region. Sample K64 shows ≥ 99% similarity with *Mitragyna speciosa.* The ITS sequence of K64 shows high homology with *M. speciosa* samples identified previously (K49, K51 and K52) [26, 27]. One sample, K55 showed ≥98% similarity with ITS data, but did not group with other *M. speciosa* samples in the ITS phylogeny, and showed phylogenetic affiliation with *M. speciosa* samples based on combined *matK* and *trnH-psbA* regions [27].



Sequences producing significant alignments

☑ select all 100 sequences selected

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Mitragyna speciosa strain White Jongkong clone K52_1 small subunit ribosomal RNA gene, partial sequence; i...	Mitragyna speci...	1303	1303	100%	0.0	100.00%	710	MT111842.1
☑	Mitragyna speciosa strain White Jongkong clone K52_2 small subunit ribosomal RNA gene, partial sequence; i...	Mitragyna speci...	1301	1301	99%	0.0	100.00%	707	MT111843.1
☑	Mitragyna speciosa strain Green Maeng Da clone K49_1 small subunit ribosomal RNA gene, partial sequence;...	Mitragyna speci...	1299	1299	99%	0.0	100.00%	703	MT111840.1
☑	Mitragyna speciosa isolation-source red-veined variety 18S ribosomal RNA gene, partial sequence; internal tra...	Mitragyna speci...	1297	1297	100%	0.0	99.86%	744	JF412826.1
☑	Mitragyna speciosa clone K51 Indonesian small subunit ribosomal RNA gene, partial sequence; internal transcr...	Mitragyna speci...	1277	1277	98%	0.0	100.00%	692	MT586314.1
☑	Mitragyna speciosa strain Green Maeng Da clone K49_2 small subunit ribosomal RNA gene, partial sequence;...	Mitragyna speci...	1219	1219	94%	0.0	99.70%	665	MT111841.1
☑	Mitragyna speciosa G13 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1149	1149	88%	0.0	99.84%	625	LC514373.1
☑	Mitragyna speciosa MUS-5603-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504152.1
☑	Mitragyna speciosa MUS-5603-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504148.1
☑	Mitragyna speciosa MUS-5603-1 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504144.1
☑	Mitragyna speciosa MUS-5602-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504140.1
☑	Mitragyna speciosa MUS-5602-1 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504136.1
☑	Mitragyna speciosa MUS-5601-4 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504132.1
☑	Mitragyna speciosa MUS-5512-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504128.1
☑	Mitragyna speciosa MUS-5601-3 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504124.1
☑	Mitragyna speciosa MUS-5601-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504120.1
☑	Mitragyna speciosa MUS-5601-1 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504116.1
☑	Mitragyna speciosa MUS-5512-2 genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	LC504112.1
☑	Mitragyna speciosa genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence	Mitragyna speci...	1118	1118	86%	0.0	99.84%	608	AB249645.1
☑	Uncaria laevigata voucher K6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ri...	Uncaria laevigata	1070	1070	100%	0.0	94.06%	719	MT263759.1
☑	Uncaria macrophylla voucher GT-33 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Uncaria macrop...	1068	1068	100%	0.0	94.06%	718	MF033305.1
☑	Uncaria macrophylla voucher GT-29 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Uncaria macrop...	1068	1068	100%	0.0	94.06%	718	MF033304.1
☑	Uncaria macrophylla voucher GT-11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Uncaria macrop...	1068	1068	100%	0.0	94.06%	718	MF033302.1
☑	Uncaria macrophylla voucher GT-3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5...	Uncaria macrop...	1068	1068	100%	0.0	94.06%	718	MF033301.1
☑	Uncaria macrophylla voucher GT-14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Uncaria macrop...	1062	1062	100%	0.0	93.92%	718	MF033303.1
☑	Uncaria macrophylla voucher GT-A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Uncaria macrop...	1062	1062	100%	0.0	93.92%	718	MT263758.1

Fig. 6S Graphical overview of the BLAST results (January 2022) in NCBI GenBank database using Internal Transcribed Spacer (ITS) region. Sample K68 shows ≥99-100% similarity with *Mitragyna speciosa.* The ITS sequence of K68 shows high homology with *M. speciosa* samples identified previously (K49, K51 and K52) [26, 27].

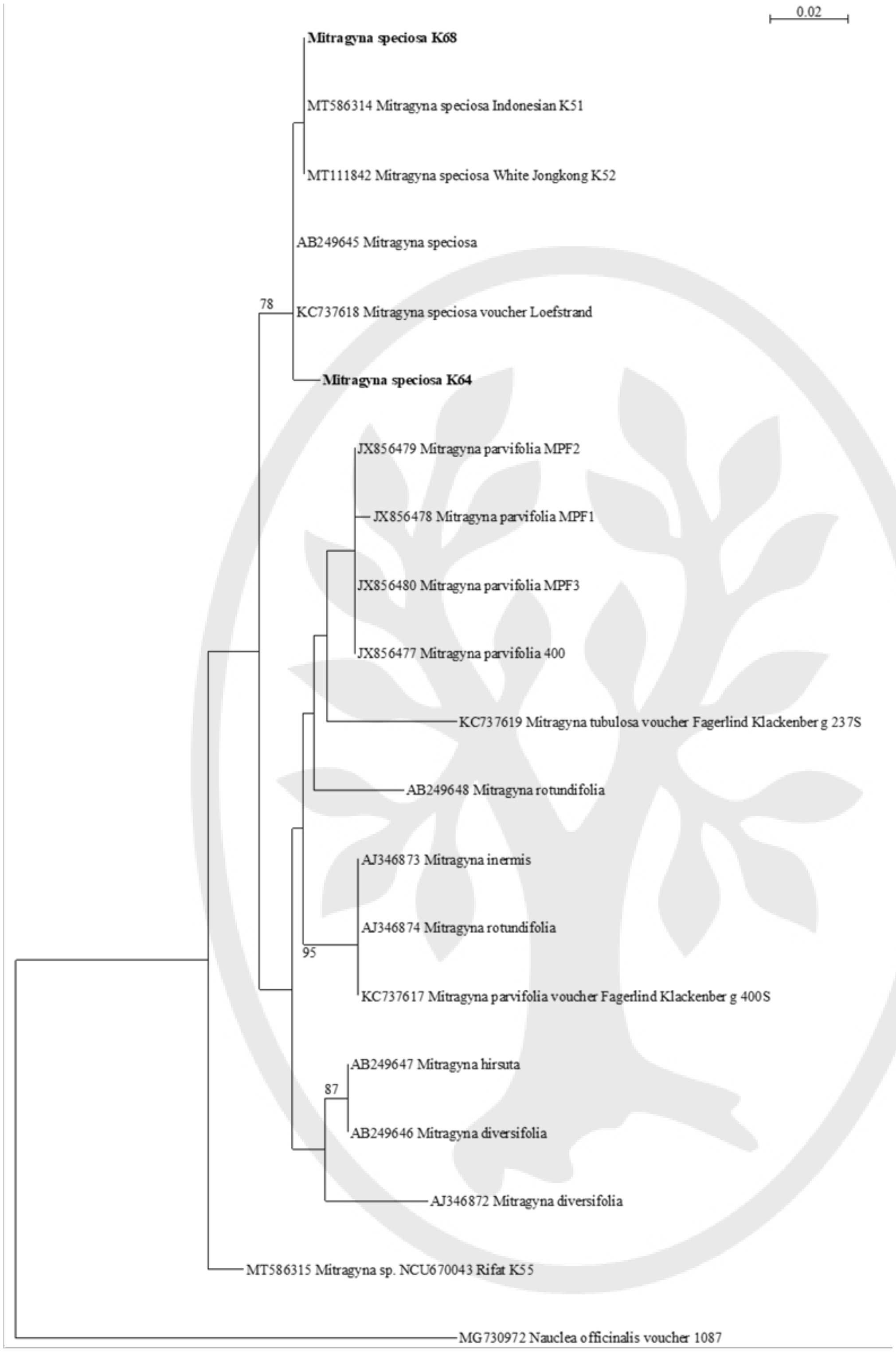


Fig. 7S Phylogram of the most likely tree (-lnL = 1320.66) from a RAxML analysis of 20 taxa based on ITS region (608 bp). Numbers refer to PhyML bootstrap support values ≥ 70% based on 1000 replicates. K64 and K68 group with *Mitragyna speciosa* with ≥78% PhyML bootstrap support. The tree is rooted with *Nauclea officinalis.* Bar indicates nucleotide substitutions per site. Phylogenetic tree was constructed using methodology described previously [26, 27].

Table 6S Precursor ion inclusion list for MS^2 analysis.

Analyte	Precursor ion (m/z) $[M+H]^+$	Molecular Formula
ajmalicine (**10**)	353.18597	$C_{21}H_{24}N_2O_3$
isomitraphylline (**1**), speciophylline (**5**), mitraphylline (**4**)	369.18088	$C_{21}H_{24}N_2O_4$
corynoxeine (**8**)	383.19653	$C_{22}H_{26}N_2O_4$
corynoxine A (**7**), rhynchophylline (**9**)	385.21218	$C_{22}H_{28}N_2O_4$
paynantheine (**11**), isopaynantheine (**14**)	397.21218	$C_{23}H_{28}N_2O_4$
isospeciofoleine (**3**), mitragynine (**12**), speciogynine (**13**), speciociliatine (**15**), mitraciliatine (**16**)	399.19145	$C_{22}H_{26}N_2O_5$
speciofoline (**6**)	401.20710	$C_{22}H_{28}N_2O_5$
7-hydroxymitragynine (**2**)	415.22275	$C_{23}H_{30}N_2O_5$

Table 7S Constrain peak width settings used for peak integration.

	Constrain peak width	
	Peak height (%)	Tailing factor
isomitraphylline (**1**)	30	2
7-hydroxymitragynine (**2**)	20	2
isospeciofoleine (**3**)	40	2
speciofoline (**6**)	30	2
corynoxine A (**7**)	30	2
corynoxeine (**8**)	30	2
rhynchophylline (**9**)	10	2
ajmalicine (**10**)	2	3
paynantheine (**11**)		
D3-mitragynine	5	2
mitragynine (**12**)	2	3
speciogynine (**13**)	20	2
isopaynantheine (**14**)	5	2
speciociliatine (**15**)	5	2
mitraciliatine (**16**)	15	2

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