Vepris amaniensis: A morphological, biochemical, and molecular investigation of a species complex.

Mary Ciambrone[1], Moses K. Langat[2], Martin Cheek[3], Félix Forest[2], Eduard Mas-Claret[2]

[1]SEEB, Queen Mary University of London, London, United Kingdom
[2]Trait Diversity and Function, Royal Botanic Gardens Kew, London, United Kingdom
[3]Accelerated Taxonomy, Royal Botanic Gardens Kew, London, United Kingdom

Corresponding Author:
Mary Ciambrone[1]
Email address: msciambrone@gmail.com

Abstract

Vepris Comm. ex A. Juss. is a genus of 96 species extending from Africa to India that are~~is~~ distinct in their unarmed stems and their digitately (1-)3(-5) foliolate leaflets, and whose many secondary compounds earn them uses in traditional medicine. Mziray (1992) subsumed six related genera into *Vepris*, with *Vepris amaniensis* becoming somewhat of a dustpan for ambiguous specimens (Cheek & Luke, 2023). This study, using material from the Kew herbarium, sought to pull out novel species from those previously incorrectly filed as *Vepris amaniensis*, and here describes the new species *Vepris usambarensis*. This species is morphologically distinct from *Vepris amaniensis* with its canaliculate to winged petioles, 0.5-2.3cm long inflorescences, 1-3 foliolate leaflets, and hairs on inflorescences and stem apices. Phytochemical analysis attributed seven compounds to *Vepris usambarensis*~~;~~: tecleanthine (**1**), evoxanthine (**2**), 6-methoxytecleanthine (**3**), tecleanone (**4**), 1-(3,4-methylenedioxyphenyl)-1,2,3-propanetriol (**5**), lupeol (**6**), and arborinine (**7**). This is a~~A~~ unique mixture of compounds for a species of *Vepris*, though all are known to occur in the genus, with the exception of 1-(3,4-methylenedioxyphenyl)-1,2,3-propanetriol (**5**), which was characterized from a species in the Asteraceae. An attempt at constructing a phylogeny for *Vepris* using the ITS and *trnL-F* regions was made, but these two regions could not be used to differentiate ~~at~~ species ~~level~~ and it is suggested that 353 sequencing is used for further research. Originally more than one new species were~~as~~ hypothesized to be within the study group~~;~~, however, separating an additional species was unsupported by the data produced. Further phylogenetic analysis is recommended to fully elucidate species relationships and identify any cryptic species that may be present within *Vepris usambarensis*.

Introduction

Vepris Comm. ex A. Juss. is a genus consisting of 96 species ("Plants of the World Online", accessed 18 July 2023) distributed widely in Africa and Madagascar, with one species

on the Arabian peninsula and one in India. Generally evergreen trees and shrubs, they are distinct from other African genera in the Rutaceae due to their digitately (1-)3(5-) foliate leaflets and their unarmed stems. Species can be found in tropical lowland to submontane forest, with a few found in drier habitats. *Vepris* species are also used as indicators of healthy, relatively undisturbed forests as they are not known to be pioneers (Cheek et al., 2019).

Like other members of~~in~~ Rutaceae, *Vepris* species are characterized by gland dots ~~on leaves~~ that are filled with aromatic compounds on the leaves. Many species are also known to have important secondary metabolites in root and stem tissue (Ombito, Chi & Wansi, 2021). The secondary metabolites in these tissues are utilized all over Africa in traditional medicine (Ombito, Chi & Wansi, 2021). The compounds produced are used in various forms to treat a large number of ailments, from everyday problems such as wounds and sores, to more long lasting issues such as rheumatic pains, infertility, and malaria (Ombito, Chi & Wansi, 2021). A recent review reports that 213 compounds have been isolated from various *Vepris* species, including alkaloids, quinolones, terpenoids, triterpenoids, flavonoids, and coumarins (Ombito, Chi & Wansi, 2021). Some of these compounds~~which~~ have been tested for bioactivity and have displayed antimicrobial, cytotoxic, anti-protozoal, and insecticidal properties (Mwangi et al., 2010; Langat, 2011; Atangana et al., 2017; Ombito, Chi & Wansi, 2021; Ojuka et al., 2023). These properties make the genus a promising one for pharmaceutical research.

The genus underwent a major taxonomic rearrangement in the 1990~~'~~s. Mziray (1992) collapsed the genera *Araliopsis* Engl., *Diphasia* Pierre, *Diphasiopsis* Mendonça, *Oricia* Pierre, *Teclea* Delile, and *Toddaliopsis* Engl. into *Vepris* based on morphological analysis. This reorganization was later confirmed with molecular work done by Morton (2017). However, in Morton's analysis, species were not well delimited and a better supported and more complete tree would be desirable. In subsuming six genera into *Vepris,* Mziray transferred the names of~~renamed~~ 31 species, most of which were from the former genus *Teclea.* One such species was *V. amaniensis* (Engl.) Mziray; described (as *Teclea amaniensis* Engl.) in the Flora of Tropical East Africa as a glabrous shrub with ~~elliptic~~ unifoliolate ~~leaflets~~ or~~with~~ occasionally 2-3 foliolate leaves, elliptic leaflets~~,~~ with a short broad acumen, numerous gland dots on lower leaflet surfaces, terete or occasionally winged petioles, and glabrous or pubescr~~ul~~ent inflorescences (Kokwaro, 1982). However, in a taxonomic review of unifoliolate African *Vepris,* it was found that many of the specimens ascribed to *V. amaniensis* were disparate from the few that agreed with the protologue of *Teclea amaniensis* (Cheek & Luke, 2023).Very recently, the description of *V. amaniensis* has been amended to better match the protologue and so is defined as being completely glabrous, having terete to canaliculate petioles, and always being unifoliolate (Cheek & Luke, 2023). This new delimitation has been utilized here to study the c. 30 specimens, collected from Kenya and Tanzania, that were found to disagree with the *Teclea~~V.~~ amaniensis* protologue.

Commented [RG1]: The "protologue" can only refer to the original publication of the basionym *Teclea amaniensis*, not to any description of the later combinaton *Vepris amaniensis*.

This study aimed to determine how many distinct taxa reside within this group of specimens. Morphological, biochemical, and molecular methodologies were used to address this question.

Materials and Methods

Morphology

We studied twenty-nine herbarium specimens from the herbarium at the Royal Botanic Gardens, Kew (K) that were previously filed as *Vepris amaniensis* but were reconsidered here as possibly distinct following an inventory of the available specimens at K by Cheek. All the specimens were collected in the East Usambara, West Usambara, Nguru, and Uluguru Mountains~~Morogoro and eastern Usambara mountain regions~~ of Tanzania, or from the south eastern coast of Kenya. Measurements of vegetative and floral traits were taken with a ruler or a Leica S6E microscope using a graticule eyepiece measuring to 0.025mm at maximum magnification. Where appropriate fruit or floral material was available, dissections were performed after rehydration and were photographed under a Leica M165 C dissecting microscope. Measurements of floral parts were taken from these photos using ImageJ (Schneider, Rasband & Eliceiri, 2012). The specimens were sorted into groups based on two distinctive vegetative character states:~~,~~ the absence or presence of winged petioles, and the number of leaflets per leaf~~petiole~~. These traits resulted in four groups; winged petiole with unifoliolate leaflets (WU), winged petioles with 1-3 foliate leaflets (WT), canaliculate petioles with unifoliolate leaflets (GU), and canaliculate petioles with 1-3 foliate leaflets (GT). The GU group was then split into two subsections, one with proportionally narrower leaflets (GU lance) and one with proportionally broader leaflets (GU broad), to account for two specimens with distinctively narrower leaflets. Specimens studied, their morphological groupings, biochemical sampling, and GenBank accessions can be found in Table 1.

Mapping of specimens was done with coordinates directly as recorded, or with a combination of locality data and the Index of Collecting Localities for the Flora of Tropical East Africa (Polhill, 1988). Mapping was done with ArcPro (v3.1).

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants (ICN [Shenzhen Code]; Turland et al., 2018), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. In addition, new names contained in this work that~~which~~ have been issued with identifiers by IPNI will eventually be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix "http://ipni.org/". The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central SCIE, and CLOCKSS

Commented [RG2]: Cite in Bibliography.

Chemistry

Samples were collected from well preserved and representative herbarium specimens within the morphological groupings, as well as one dried sample sent directly from Kenya (*Luke WRQ* 18906), which was included due to the large amount of its mass that could be sacrificed for extraction. Material was ground to a powder in a spice grinder and/or a pestle and mortar. Extractions were then done in methylene chloride (CH_2Cl_2, abbreviated DCM) and then methanol (MeOH). Purification, analysis, and characterization of compounds were done with liquid

chromatography, thin plate chromatography (TLC), nuclear magnetic resonance spectrometry (NMR), and high-resolution mass spectrometry (HR-MS).

Separation of compounds was done using a 2 cm diameter column packed with silica (40-63 micron Davisil®). Solvent systems and the corresponding compounds eluted are outlined in Table 2. Extracts yielded 7 compounds; compound **1** was determined to be tecleanthine (Atangana et al., 2017), compound **2** to be evoxanthine (Ombito, Chi & Wansi, 2021), **3** to be 6-methoxytecleanthine (Atangana et al., 2017), **4** to be tecleanone (Casey & Malhotra, 1975), **5** to be 1-(3,4-methylenedioxyphenyl)-1,2,3-propanetriol (Rahman and Moon 2007), **6** to be lupeol (Ombito, Chi & Wansi, 2021), and **7** to be arborinine (Langat, Kami & Cheek, 2022).

Fractions were tested for purity using aluminum-backed TLC plates (silica gel 60 F254, Sigma-Aldrich). Visualization of the plates was done with UV radiation at 245nm, an anisaldehyde spray reagent (1% *p*-anisaldehyde: 2% H_2SO_4: 97% cold MeOH), and heat. 1D and 2D NMR data was recorded from a Bruker 400MHz Advance NMR instrument at room temperature in either $CDCl_3$ or CD_3OD. Chemical shifts (δ) are expressed in ppm with reference solvent peaks in H^1 and ^{13}C NMR spectra placed at δ_H 7.26 ppm and δ_C 77.23 ppm for $CDCL_3$ and δ_H 3.31 ppm and δ_C 49 ppm for CD_3OD. Where samples were small and crude extracts were near identical within groups, samples were combined to produce a stronger signal for easier chemical characterization (table 1). The two WU herbarium samples were not pooled despite their small mass due to their distant geographical origins.

Compounds **1, 2,** and **3,** isolated from *Luke WRQ* 18906 were dissolved in MeOH and confirmed by mass on a Thermo Scientific Orbitrap fusion mass spectrometer. Compound **5,** also isolated from *Luke WRQ* 18906, could not be confirmed with MS, and the remaining compounds could not be isolated in sufficient quantity or quality to be analyzed.

DNA

The sampling and methods used here are informed by those used in Morton (2017). All samples studied for morphology were sampled for DNA, as well as several other *Vepris* species for comparison (Table 1). Extractions were carried out with 20-30 mg of leaf material ground in a Mixer Mill until powdered. Samples were incubated for 30 min in a 65°C isolation buffer solution of CTAB (747µL) and 2-mercaptoethanol (3 µL). SEVAG solution (750 µL, $CHCl_3$: isoamyl alcohol = 24:1) was added and samples were shaken in an orbital shaker at 250 rev/min for 30 min and then centrifuged at 13000 rpm for 15 min. The supernatant of each sample was transferred into new tubes with 500 µL isopropanol and stored at -20°C for three days. The samples were then centrifuged for another 15 min, after which the aqueous phase was decanted, and the pellet washed with 70% ethanol twice, with 15 min of centrifuging between washes. The pellets were allowed to dry overnight at room temperature and then resuspended in 100 µL of water. A 1% agarose gel with ethidium bromide was then loaded for quality check.

Two markers were sequenced, the nuclear transcribed spacer (ITS) region and the plastid trnL-F intron and spacer. For ITS primers 101, 102, 2, and 3 were used. PCR Methodology followed Sun et al. (1994). PCR of the whole region (primers 101-102) was attempted, as well as from the combinations 101-2 and 3-102 to maximize likelihood of successful sequencing. The

PCR solution was 25 µL consisting of 1µL sample DNA, water (5 µL), TBT (5 µL), Dream Taq (12.5 µL, Thermo Sci, 4 mM $MgCl_2$), DMSO (2%, 0.5 µL) and 0.5 µL of each primer (0.2 µM). PCR conditions were an initial denaturation and activation for 2 minutes at 94°C, then 28 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C, and one minute of extension at 72°C. A final 7-minute extension at 72°C completed the process.

For trnL-F ~~the~~ we used the primers c and f following the method of Taberlet et al. (1991). Once again PCR of the whole region was attempted, with c-d and e-f also performed to maximize likelihood of success. The PCR solution was 25 µL, consisting of 1µL sample DNA, water (5.5 µL), TBT (5µL), Dream Taq (12.5 µL, Thermo Sci, 4 mM $MgCl_2$), and 0.5 µL of each primer (0.2 µM). PCR conditions were an initial denaturation and activation for 2 minutes at 94°C, then 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 50°C, and two minutes of extension at 72°C. A final 4-minute extension at 72°C finished the process.

PCR products were cleaned by using a binding buffer (125µL, Buffer PB Qiagen) and a PCR clean-up column (NucleoSpin), centrifuging (13,000 rpm, 1 min) the solution through and then washing the column twice with 600µL of AW1 wash buffer (Qiagen). The columns were transferred to clean tubes and 30µL of 65°C EB elution buffer (Qiagen) was added. After 10 minutes samples were centrifuged at 13,000 rpm for 1 min to draw the DNA through the column.

Cycle sequencing was performed for each successful PCR product. The mixture for ITS primers was 1µL sequencing buffer, 0.15 µL water, 0.1 µL (2%) DMSO, 0.25 µL BigDye Premix 3.1(Thermo Sci), and 0.5 µL of 1pmol/ µL of corresponding primer to make 2 µL of solution. Depending on DNA concentration, 1-3 µL of PCR product was added, with water added for a final reaction volume of 5 µL. The solution for *trnL-F* primers is the same as above, except for the exclusion of DMSO. Samples were then all subject to 26 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C. Products were then cleaned with NaAC and 100% EtOH, resuspended in water and Sanger sequenced on an Applied Biosystems 3730xl DNA analyzer.

Phylogenetic analysis

Geneious Prime (Geneious Prime, 2023.1.2) was utilized to combine complementary strands and check base-calling, produce alignments of the obtained sequences using the MUSCLE algorithm, and concatenating the resulting alignments into one matrix. Available ITS and *trnL-F* sequences for *Vepris* in GenBank were included in the alignment (Table 3), as well as two species of *Zanthoxyl~~e~~um* and *Fagaropsis angolensis* (Engl.) Dale to act as outgroup taxa in subsequent analysis (Morton, 2017). Seven specimens; *Ruffo and Mmari* 1785*, Greenway* 4895*, Mgaya* 157*, Luke and Robertson* 1900*, Paulo* 168*, Luke and Robertson* 1716*, and Drummond and Hemsley* 3456 failed to be amplified at the PCR stage or produced sequences too noisy to be used and so were not included in the analysis. All other specimens had at least partial sequences of either the ITS or the trnL-F markers and were included.

A Bayesian inference analysis was performed with MrBayes (Huelsenbeck & Ronquist, 2001, v3.2.7). The ITS and *trnL-F* regions were treated as independent partitions in the analysis. The General Time Reversible (GTR) model with a proportion of invariable site and a gamma

shape to account for rate heterogeneity among sites (GTR+I+G) was assigned to both partitions. *Zanthoxylum chevalieri* P.G Waterman was selected as the outgroup taxon. Posterior probability distribution was estimated using Markov chain Monte Carlo sampling (MCMC) over 7 million generations, sampled every 1000th generation and saving branch length.

Tracer (Rambaut et al., 2018, v1.7.2) was used to determine if all the parameters of the analysis reached a stationary phase. A final consensus tree was compiled in MrBayes (v3.2.7) with a burnin phase of 10% (1000 trees) and using the option contype=allcompat. The resulting tree was visualized using FigTree v1.4.4 (Rambaut, 2010).

Results and Discussion

Morphology

Vepris amaniensis is delimited~~fined~~ as being glabrous, unifoliolate, having terete petioles, and inflorescences 0.9-4(-5)cm long (Cheek & Luke, 2023). The samples measured for this study are morphologically distinct, and are separated from *V. amaniensis* by their canaliculate to winged petioles, 0.5-2.3cm long inflorescences, 1-3 foliolate leaflets, and hairs on both inflorescences and stem apices. As such, they are here described as the new species *Vepris usambarensis*.

It was originally hypothesized that there might be more than one new taxon within the specimens studied. However, this study could not generate enough support to clearly separate additional taxa other than the proposed new species *V. usambarensis.* Samples were originally separated into four groups; those with winged petioles and unifoliolate leaflets (WU), winged and 1-3 foliolate leaflets (WT), canaliculate petioles and unifoliolate leaflets (GU), and canaliculate and 1-3 foliolate leaflets (GT). These two characters, petiole morphology and leaflet number, were the only distinctive characters by which the samples could be divided. All other characters, both vegetative and reproductive, appeared to have too little variation across specimens to be credibly utilized in differentiating groups. The availability of mature flowering structures in this study group was low and so with more complete resources, distinct reproductive characters may become apparent.

The use of leaflet number as a delimiting~~fining~~ character proved fairly weak across specimens; many nearly exclusively expressing unifoliolate leaflets only to have one or two multi-foliolate leaflets, or vice versa. Others had a relatively even mix of 1, 2, and 3 foliolate leaflets. The plasticity in leaflet number displayed on a single specimen suggests that even on specimens that only included unifoliolate material, it was not possible to rule out a higher number of leaflets elsewhere on the plant from which these specimens were taken. In *Vepris*, petiole morphology and leaflet number are generally important characters used in delimiting species (Cheek & Luke, 2023), and species are generally able to be defined by a single leaflet and petiole state. That this species has so much variation makes it an interesting one for further taxonomic and phylogenetic study. More reproductive specimens and further DNA sequencing may also prove the presence of more taxa.

Commented [RG3]: Why?

Commented [RG4]: Almost entirely within the range given aove for *V. amaniensis*.

This ~~flip flopping~~variability of leaflet number poses a question concerning the evolution and ecological relevance of having a single leaflet versus multiple leaflets. That these plants have and express the genes for both could suggest a maximizing of efficiency by using two states with differing benefits, or it could simply be an evolutionary artifact going from one state to another.

With support for leaflet number as a taxon identifier low in this case, demarcating additional taxa solely on petiole morphology could not be justified, and so here the study specimens are all classified together as a new, unusually morphologically variable species separate from *V. amaniensis*.

Vepris usambarensis *Ciam. & Cheek*, sp. nov. Holotype: Tanzania, Lushoto district, Mazumbai, West Usambaras, University Forest reserve, 1480m, fr, fl, March 1984, *Jon Lovett* 263 (estimated ~~38.5 S 4.8 E~~4.8°S, 38.5°E, herbarium specimen, K!).

Dioecious evergreen shrub 0.5-2.5(-5)m tall, alternate branching, grey bark, internodes 0.45-5.3cm long, stem diameter at lowest leafy node (1-)1.5-5mm, puberulent at stem apex, rapidly becoming glabrous within 4 nodes of the stem apex, lenticels sub-elliptic, raised, c. 0.3-0.5(-0.8) by 0.2-0.3mm, coverage up to 40% of surface area after 7 nodes from stem apex, leaflets 1-3 foliolate (where a plant is predominantly 1 or 3 foliolate there will generally be at least one leaflet displaying a higher or lower leaflet number).

Leaflets elliptic, 5.8-17.7(-22.7) cm long, c. (2.2-)2.8-5(-7) cm wide, margin simple, entire, where 2-3 foliolate, lateral leaflets are overall 40-60% more reduced than the median; leaflets acuminate, acumen 0.3-1.9cm long, 2.5-7mm wide; secondary veins 13-22 on each side of midrib, brochidodromous, gland dots conspicuous on abaxial side, clear in transmitted light, pale green in reflected light, c. 0.1mm diameter, density 5-7(-11) dots/mm^2.

Formatted: Superscript

Petiole (0.4-)0.9-3.6(-5.1) cm long, articulated at top and bottom, base puberulent when it falls within 4 nodes of stem apex, apex and abaxial surface with occasional hairs, hairs simple, thickened pulvinus at apical articulation sometimes present, canaliculate to winged, wings up to c. 2.4(-4) mm wide at apex.

Petiolule 0.1-0.3(-0.5) cm long, inconspicuous, terete, glabrous.

Inflorescences axillary, paniculate, 0.5-2.3(-3.8) cm long, all parts densely hairy when young, becoming less dense with age, hairs simple, patent, 0.02-0.07mm long, yellow upon drying.

Peduncle 0.1-0.3(-0.7) cm long, sparsely hairy to puberulent, hairs simple.

Rachis 0.5-1.1(-1.5) cm long, sparsely hairy to puberulent, hairs simple, internodes 0.1-0.5cm long, alternate, node bearing 1-3 flowers.

Bracts four lobed, lobes triangular, c. 0.25 x0.5mm, cupuliform, resembling sepals, surrounding peduncle, puberulent when young, becoming sparsely hairy with age, hairs simple, not seen intact on mature flowers.

Bracteoles subtending pedicels, as for bracts.

Pedicel 1.3-1.8mm long, sparsely puberulent, hairs simple, yellow.

Flowers dioecious, male and female both 2.3-3.3mm long.

Sepals four, triangular, c. 0.6mm long x 0.8mm wide, united from base to c. one third of length, ciliate, occasional gland dots.

Petals four, elliptic, c. 2-2.4mm long, 0.9-1.39mm wide, drying golden yellow, thickened tip, petal becomes fully reflexed with age, occasional persistent hairs on outer surface, gland dots present on bud, clustered near apex, drying yellow.

Stamens in male flowers 4(-5), filaments 2-2.7mm long, dorsiventrally flattened, tapering at top, anthers ovoid to discoid, diameter 0.46-0.59mm, medifixed. Staminodes ~~U~~unobserved in available female flowers.

Ovary in male flowers vestigial, cone shaped, 0.83-1mm long, c. 0.34-0.38mm wide at base, densely covered in semi-appressed simple yellow hairs~~,~~ c. 0.5-0.6mm long, unilocular, yellow-orange, slightly lobed~~ing~~ at bottom around stamens (suspected vestigial disk). In mature female flowers ovary is sub-ovoid, c. 2mm long and 1.4mm wide, unilocular, vesicular, scabrid near base, drying brown, ~~vesicular,~~ 4-5 orange lobes near base around where staminodes~~ens~~ emerge, stigma discoid, c. 1.4mm diameter, convex, style minute, c. 0.1mm long.

Fruit a single seeded~~,~~ berry, ellipsoid to apex slightly beaked, 9-13mm long x 3-8mm wide, thinly fleshy, c. 0.4mm thick, ripening green, brown purple on drying, 4-5 orange lobes generally persistent on bottom, conspicuous gland dots, yellow to brown, slightly raised, 0.1-0.26mm diameter, occasional persistent hairs, pedicel accrescent, 2-6mm long.

Seed tan, ellipsoid, dimensions slightly smaller than in fruit, single longitudinal groove.

Representative specimens examined: (All specimens were seen and housed at K) Tanzania, ~~Lushoto~~Muheza dist, Amani-Kwamkoro road 2miles SE of Amani, fr, July 15, 1953, *R.B. Drummond and J.H. Hemsley* 3456 (estimated 5° 7' 16.8594"S 38° 37' 18.084"E, K003470026), Tanzania, Muheza dist, Kwamkoro forest reserve, Monga, fr, July 18, 1986, *Ruffo and Mmari* 2354 (est 5º 06~~10~~'S 38º 3~~7~~6'E, K003470022), Tanzania, Muheza dist, east Usambara mt, Kwamkoro forest trail 3, fl, March 1998, *Luke & al.* 5242 (est 5º 10'S 38º 36'E, K003470027), Tanzania, Lushoto district, Usambara mts, Mahezangulu forest reserve, fl , Jan 24, 1985, *Mziray* 85240 (est 4° 56' 43.6488"S 38° 30' 51.7746"E, K003470028), Tanzania, Lushoto distr, Mazumbai, west Usambara, university forest reserve, 4º 48'S 38 º 30'E, fr, fl, Mar 1984, *Jon Lovett* 263(K003470013), Tanzania, Lushoto dist, near Mazumbai HQ, M~~u~~azu~~a~~mbai forest reserve, west Usambara Mts, 4º 48'S 38º 30'E, fl, July 3, 2008, *Andrew R. Marshall* 1423 (K003470011), Tanzania, Korogwe dist, Ambangulu tea estate, fr, July 17, 1983, *R.M. Polhill, J.C. & J.M. Lovett* 5007 (est 5º 2' S 38º 23'E, K003470024), Tanzania, Muhez~~r~~a dist, fl, fr, June 28, 1987, *Ruffo and Mmari* 2170 (est 5 º 10'S 38º 48' E, K003470023), Tanzania Korogwe dist, Lutindi forest reserve, fr, Aug 1989, *Ruffo and Mmari* 2306 (est c.4º 53'S 38º 38'E,

Commented [RG5]: But female flowers described below as having 4-5 lobes "near base around where stamens emerge" – which is true?

Commented [RG6]: Isn't this a constant generic character?

Commented [RG7]: What is c. 04mm thick? The exocarp? Certainly not the whole fruit!

Commented [RG8]: Post facto geo-referencing of lat/long coordinates to small fraction of seconds makes no sense given that one second is approximately 30 metres long. Nearest-minute estimates are as close as can be reliably calculated for these old collections.

Commented [RG9]: Coordinates of Monga in Polhill (1988)

Commented [RG10]: This collection number almost certainly belongs to A.L. Borhidi with Mziray as one of the co-collectors; recheck specimen label.

Commented [RG11]: Round to nearest minute – see above.

Commented [RG12]: No definite locality?

K003470021), Tanzania, ~~Korogwe~~Muheza dist, Kilanga forest reserve, fr, Aug 24, 1986, *Ruffo and Mmari* 1785 (est 5º 18.5'S 38º 38' E, K003470020), Tanzania, Korogwe dist, ~~l~~Lutindi forest reserve, fr, Aug 1986, *Ruffo and Mmari* 2304 (est c.4º 53'S 38º 38'E, K003470032), Tanzania, Muh~~n~~eza dist, Kwamkoro forest reserve, fr, July 1, 1985, *Ruffo and Mmari* 2243 (est 5º 10'S 38º 36'E, K003470030), Tanzania, Muheza dist, Mgue Sangerawe, fl, Oct 2, 1937, *P.J. Greenway* 4895 (est 5º 8'S 38º 37'E, K003470029), Tanzania, Korogwe dist, West Usambaras, Ambangulu~~is~~ estate, st (leaves), Oct 1940, *E.B. Wallace* 939 (est 5º 5'S 38º 26' E, K003470031), Kenya, Kwale dist, Buda Mafisini forest reserve, fr, Feb 24, 1989, *Luke and Robertson* 1716 (est 4º 27'S 39º 24' E, K003470014), Kenya, Kilifi dist, Kombeni reserve valley edge of Kaya ~~f~~Fimboni, fl, Aug 21, 1989, *Luke and Robertson* 5848 (est 3º 54' S 39º 36'E, K003470012), Kenya, Kwale dist, Buda Mafisini forest reserve, fr, Nov 3, 1959, *D. Napper* 1380 (est c.4º 27'S 39º 24'E, K003470010), Kenya, Kwale dist, Muhka forest, fr, Feb 19, 1987, *Robertson and Luke* 4538 (est 4º 20'S 39º 31'E, K003470019), Kenya, Kwale dist, Buda Mafisini forest reserve, fl, Aug 16, 1953, *R.B. Drummond and J.H. Hemsley* 3802 (est 4º 27~~8~~'S 39º 24~~2~~'E, K003470009), Tanzania, Morogoro Rural dist, Mkungwe forest reserve, fr, fl, Aug 13, 2000, *B. M~~o~~horo UMBCP* 329 (est 6º 53'S 3~~.7~~7º 55'E, K003470017), Tanzania, Muheza dist, Mtai forest reserve, fl, Sep 13, 1996, *Kisena* 1631 (est c.4º 50'S 38º 46'E, K003470035), Tanzania, Muheza dist, Kiwanda~~buwa~~ to Bulwa foot path, st (leaves), Nov 10, 1981, *S.P. Kibuwa* 5459 (est 5° 2' 55.6074"S 38° 41' 7.3314"E, K003470015), Tanzania, Korogwe~~got~~ dist, Lutindi forest reserve, fl , Aug 24, 1986, *Ruffo and Mmari* 1724 (est c. ~~5~~4º 53'S 38º 38' E, K003470018), Tanzania, Korogw~~i~~e dist, fr, Jan 22, 1987, *Ruffo and Mmari* 2307 (est 5º 9'S 38º 28' E, K003470016), Tanzania, Mvomero dist, Turiani ~~Morogoro dist~~, fr, Nov 1953, *S. Paulo* 168 (est 6º 9'S 37º 35'E, K003470038), Tanzania, Mvomero~~orogoro~~ district, Mtibwa forest reserve, fr, Nov 1953, *S.R. Semsei* 1508 (est c. 6º 7'S 37º 39'E, K003470037), Tanzania, Mvomero~~orogoro~~ district, Manyangu forest reserve, fl, July 1957, *Mgaya* 157 (est c.6º ~~5~~07'S 37º 34'E, K003470033), Kenya, Mwele Mdogo forest, ~~s~~Shimba ~~h~~Hills 12 miles SW of Kwale, fr, Feb 4, 1953, *Drummond and Hemsley* 1101 (est 4º 18'S 39º 21'E, K003470034), Kenya, Kilifi dist, Pangani Rocks, fl, Aug 16, 1989, *Luke and Robertson* 1900 (est 3º 51'S 39º 40'E, K003470036).

Distribution: Coastal south~~-~~eastern Kenya, ~~the~~East and West Usambara mountains, Nguru mountains, and ~~the Morogoro region~~Uluguru Mountains of Tanzania. (Figure 3.)

Habitat: Restricted to relatively undisturbed habitat, in Tanzania relegating it to submontane to montane evergreen tropical forest. Kenyan specimens are also found in protected areas, though at much lower elevations.

Etymology: Named after the Usambara mountains, where the majority of the specimens were collected.

Phenology: Flowers March to September, and fruits August to February.

Recognition:

Vepris usambarensis can be distinguished from *Vepris amaniensis* Engl. by its canaliculate to winged petioles, the presence of an indumentum at stem apices and on inflorescences, generally shorter panicles (0.5-2.3(-3.8) cm long), and 1-3 foliolate leaflets.

Commented [RG13]: Is this a post facto estimate, or taken directly from the specimen label?

Commented [RG14]: Or from label?

Commented [RG15]: Or from label?

Commented [RG16]: Coordinates of Buda Forest in Polhill (1988)

Commented [RG17]: Round to nearest minute; see above.

Commented [RG18]: No definite locality?

Commented [RG19]: Or from label?

Vepris amaniensis Engl. has terete to canaliculate petioles, glabrous stems and inflorescences, 0.9-4(-5) cm long panicles~~etioles~~, and unifoliolate leaflets (Table 4).

Chemistry

Of the specimens that were sampled, 7 compounds were found in high enough concentration to be described; tecleanthine (**1**), evoxanthine (**2**), 6-methoxytecleanthine (**3**), tecleanone (**4**), 1-(3,4-methylenedioxyphenyl)-1,2,3-propanetriol (**5**) (synonym 3',4'-methylene ether), lupeol (**6**), and arborinine (**7**) (Figure 4). All compounds described were found in CH_2Cl_2 extracts, methanol extracts yielded tecleanthine (**1**) and evoxanthine (**2**) as well, but any other compounds were too dilute to be discerned.

As with the attempt to create morphological groupings within the studied specimens, the NMR profiles of the samples were too similar to each other to lend credible support to more than one taxon being present (Figures 5-6). All samples had tecleanthine (**1**) as the dominant compound, in some samples such as GT(1) and WU(K) it was nearly the only one present. Evoxanthine (**2**) was the second most dominant compound, and the rest were notably less dominant~~more minor~~ (Table 5). The only differentiation between samples were the varying concentrations of compounds, which can be attributed to life stage, time of collection, and local climate, or the absence of very minor ones. Absences could be due to the small sizes of some samples. Minor compounds like lupeol (**6**) and arborinine (**7**) were able to be isolated from *Luke WRQ* 18906 and not others likely due to it being over six times the mass of many of the other samples.

The exact combination of chemicals found in this study are not reported in any other *Vepris* species studied for biochemistry, though all but one of the compounds identified is known to occur in the genus. *Vepris grandifolia* (Engl.) Mziray and *Vepris trichocarpa* (Engl.) Mziray, both species that occur in the same geographical range as *Vepris usambarensis*, are reported with similar mixtures including tecleanthine, tecleanone, evoxanthine, lupeol, arborinine, and 6-methoxytecleanthine (Ombito, Chi & Wansi, 2021). The only compound not described before from a *Vepris* species is the lignan 1-(3,4-methylenedioxyphenyl)-1,2,3-propanetriol (**5**). It was first described from extracts taken from the roots of *Dendranthema zawadskii* var. *latilobum* (Maxim) Kitam. (synonymized with *Chrysanthemum naktongense* Nakai), a temperate member of the Asteraceae native to north-east Asia and used medicinally in Korea (Rahman & Moon, 2007). It is not known from any other species. That these two species from disparate clades, continents, and climates produce the same chemical seems unlikely, and while its presence was confirmed with NMR analysis, it could not be confirmed with mass spectrometry analysis. Further investigation of the presence of this lignan in this species is recommended.

Lignans and alkaloids have been recognized for their potent pharmacological potential, and are known to contain compounds important to medicine (Saleem et al., 2005; Barker, 2019; Cui et al., 2020). Tecleanthine (**1**), evoxanthine (**2**), 6-methoxytecleanthine (**3**), lupeol (**6**), arborinine (**7**), and 1-(3,4-methylenedioxyphenyl)-1,2,3-propanetriol (**5**) are known to have bioactive properties including antioxidant, antiprotozoal, cytotoxic, antimicrobial,

Commented [RG20]: No, *V. grandifolia* is only in far western Tanzania and Kenya, so the ranges do not overlap. Maybe better as "both species also occurring in Tropical East Africa"?

Formatted: Font: Not Italic

antiplasmodial, and antifeedant properties (Popp & Chakraborty, 1964; Lwande et al., 1983; Muriithi et al., 2002; Rahman & Moon, 2007; Mwangi et al., 2010; Dongfack et al., 2012; Nouga et al., 2016; Atangana et al., 2017). Tecleanone (**4**) has not been studied for any bioactivity, but it is considered an intermediary in the chemical pathway to make both tecleanthine (**1**) and evoxanthine (**2**) (Dagne et al., 1988; Singh & Bharate, 2006).

Phylogeny

The use of the ITS and *trnL-F* regions here was modeled on the approach of Morton (2017), as this study is the most complete molecular work done at species level on *Vepris* to date. In Morton's study, it is stated that discerning species with these regions is difficult, a conclusion supported here. Utilizing these two regions, isolated from each specimen studied, as well as those included from other *Vepris* species on GenBank, our consensus tree did not have any node with posterior probability over 0.26 (Figure 7). There is a loose grouping of the specimens that were collected from Kenya, as well as a loose separation of the winged specimens from the canaliculate specimens. However, with the very small support for these relationships, these conclusions cannot be supported.

It appears that these two regions are sufficiently invariable across *Vepris* and so should not be relied upon alone in future phylogenetic analyses. Future studies in this group would require approaches that provide a greater amount of information, such as RADseq and targeted enrichment (e.g. Angiosperm353; Johnson et al., 2019) to produce a better supported phylogeny and reveal any cryptic species that *Vepris* may contain.

Conclusion

The new species *Vepris usambarensis* is here described as being distinct from *V. amaniensis*, the name under which its specimens had previously been filed. This species can be confirmed to produce tecleanthine (**1**), evoxanthine (**2**), 6-methoxytecleanthine (**3**), tecleanone (**4**), 1-(3,4-methylenedioxyphenyl)-1,2,3-propanetriol (**5**), lupeol (**6**), and arborinine (**7**). ~~Which~~These are all chemicals known to be found in *Vepris*, save for the lignan which is known from a *Chrysanthemum* native to east Asia. The known bioactivity of both the lignan and the alkaloids present, like in many *Vepris*, give it pharmacological potential.

It was originally hypothesized that there would be more than one taxon within the study group due to the high levels of morphological variation observed in characters usually of value in differentiating species in *Vepris*. However, morphologically and biochemically any further delineations could not be supported. Molecular work was undertaken to try to~~and~~ add additional support~~;~~, however, the chosen regions~~,~~ ITS and *trnL-F*, failed to produce a tree at the species level with enough support. These regions were chosen following Morton (2017), and we conclude that in *Vepris*, they have little to no value for differentiating species.

Given the variation of morphology observed, it is recommended that further phylogenetic research be conducted with other sequencing approaches. A more complete tree of *Vepris* than

the one produced by Morton (2017) with more support is desired and further investigation of the phylogenetic diversification of species versus morphology in the genus would shine light on the evolution and ecology of the genus as a whole.

Acknowledgements

Thank you to Yanisa Olaranont for help in the lab and instrument training. Thank you to Lazlo Csiba for training and assistance with molecular work. Thank you to Quentin Luke for providing the substantial specimen from Kenya.

References

Appelhans MS, Wen J. 2020. Phylogenetic placement of *Ivodea* and biogeographic affinities of Malagasy Rutaceae. *Plant Systematics and Evolution* 306:7. DOI: 10.1007/s00606-020-01633-3.

ArcGIS Pro. 2021.

Atangana AF, Toze FAA, Langat MK, Happi EN, Mbaze LLM, Mulholland DA, Waffo AFK, Sewald N, Wansi JD. 2017. Acridone alkaloids from Vepris verdoorniana (Excell & Mendonça) Mziray (Rutaceae). *Phytochemistry Letters* 19:191–195. DOI: 10.1016/j.phytol.2017.01.001.

Barker D. 2019. Lignans. *Molecules* 24:1424. DOI: 10.3390/molecules24071424.

Casey AC, Malhotra A. 1975. Tecleanone: A new alkaloid from teclea grandifolia (Engl.). *Tetrahedron Letters* 16:401–404. DOI: 10.1016/S0040-4039(00)71877-6.

Cheek M, Luke WRQ. 2023. A taxonomic synopsis of unifoliolate continental African Vepris (Rutaceae). Kew Bull. 78: 469-497. DOI: 10.1007/s12225-023-10120-0.

Cheek M, Onana J-M, Yasuda S, Lawrence P, Ameka G, Buinovskaja G. 2019. Addressing the Vepris verdoorniana complex (Rutaceae) in West Africa, with two new species. *Kew Bulletin* 74. DOI: 10.1007/s12225-019-9837-y.

Cui Q, Du R, Liu M, Rong L. 2020. Lignans and Their Derivatives from Plants as Antivirals. *Molecules* 25:183. DOI: 10.3390/molecules25010183.

Dagne E, Yenesew A, Waterman PG, Gray AI. 1988. The chemical systematics of the Rutaceae, subfamily Toddalioideae, in Africa. *Biochemical Systematics and Ecology* 16:179–188. DOI: 10.1016/0305-1978(88)90093-2.

462 Dongfack MDJ, Lallemand M-C, Kuete V, Mbazoa CD, Wansi J-D, Trinh-van-Dufat H, Michel
463 S, Wandji J. 2012. A new sphingolipid and furanocoumarins with antimicrobial activity
464 from Ficus exasperata. *Chemical and Pharmaceutical Bulletin* 60:1072–1075.
465 Geneious Prime. 2023.
466 Groppo M, Pirani JR, Salatino MLF, Blanco SR, Kallunki JA. 2008. Phylogeny of Rutaceae
467 based on two noncoding regions from cpDNA. *American Journal of Botany* 95:985–
468 1005. DOI: 10.3732/ajb.2007313.
469 Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees.
470 *Bioinformatics (Oxford, England)* 17:754–755. DOI: 10.1093/bioinformatics/17.8.754.
471 Johnson MG, Pokorny L, Dodsworth S, Botigué LR, Cowan RS, Devault A, Eiserhardt WL,
472 Epitawalage N, Forest F, Kim JT, Leebens-Mack JH, Leitch IJ, Maurin O, Soltis DE,
473 Soltis PS, Wong GK, Baker WJ, Wickett NJ. 2019. A Universal Probe Set for Targeted
474 Sequencing of 353 Nuclear Genes from Any Flowering Plant Designed Using k-Medoids
475 Clustering. *Systematic Biology* 68:594–606. DOI: 10.1093/sysbio/syy086.
476 Kokwaro JO. 1982. Rutaceae. In: Polhill, RM (ed.), Flora of Tropical East Africa. ~~In: Polhill RM~~
477 ~~ed.~~ A.A.Balkema/Rotterdam,.
478 Langat M. 2011. Flindersiamine, a Furoquinoline Alkaloid from Vepris Uguenensis (Rutaceae)
479 as a Synergist to Pyrethrins for the Control of the Housefly, Musca Domestica L.
480 (Diptera: Muscidae). *Journal of the Kenya Chemical Society* 6:9–15.
481 Langat MK, Kami T, Cheek M. 2022. Chemistry, taxonomy and ecology of the potentially
482 chimpanzee-dispersed Vepris teva sp.nov. (Rutaceae) endangered in coastal thicket in the
483 Congo Republic. *PeerJ* 10:e13926. DOI: 10.7717/peerj.13926.

Lwande W, Gebreyesus T, Chapya A, Macfoy C, Hassanali A, Okech M. 1983. 9-Acridone insect antifeedant alkaloids from Teclea trichocarpa bark. *International Journal of Tropical Insect Science* 4:393–395. DOI: 10.1017/S1742758400002459.

Morton CM. 2017. Phylogenetic relationships of Vepris (Rutaceae) inferred from chloroplast, nuclear, and morphological data. *PLOS ONE* 12:e0172708. DOI: 10.1371/journal.pone.0172708.

Muriithi MW, Abraham W-R, Addae-Kyereme J, Scowen I, Croft SL, Gitu PM, Kendrick H, Njagi ENM, Wright CW. 2002. Isolation and in Vitro Antiplasmodial Activities of Alkaloids from Teclea trichocarpa: In Vivo Antimalarial Activity and X-ray Crystal Structure of Normelicopicine. *Journal of Natural Products* 65:956–959. DOI: 10.1021/np0106182.

Mwangi E, Keriko J, Machocho A, Wanyonyi A, Malebo H, Chhabra S, Tarus P. 2010. Antiprotozoal activity and cytotoxicity of metabolites from leaves of Teclea trichocarpa. *J Med Plants Res* 4:726–31.

Mwaura A. 2020. Vepris simplicifolia voucher NMK:EA 13545 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

Mziray W. 1992. Taxonomic studies in Toddalieae Hook. f. (Rutaceae) in Africa. Acta Univ. Upsal., Symb. Bot. Upsal. 30:1–95.

Nouga AB, Ndom JC, Mpondo EM, Nyobe JCN, Njoya A, Meva'a LM, Cranwell PB, Howell JAS, Harwood LM, Wansi JD. 2016. New furoquinoline alkaloid and flavanone glycoside derivatives from the leaves of Oricia suaveolens and Oricia renieri (Rutaceae). *Natural Product Research* 30:305–310. DOI: 10.1080/14786419.2015.1057727.

Ojuka P, Kimani NM, Apollo S, Nyariki J, Ramos RS, Santos CBR. 2023. Phytochemistry of the Vepris genus plants: A review and in silico analysis of their ADMET properties. *South African Journal of Botany* 157:106–114. DOI: 10.1016/j.sajb.2023.03.057.

Ombito JO, Chi GF, Wansi JD. 2021. Ethnomedicinal uses, phytochemistry, and pharmacology of the genus Vepris (Rutaceae): A review. *Journal of Ethnopharmacology* 267:113622. DOI: 10.1016/j.jep.2020.113622.

Plants of the World Online. *Available at https://powo.science.kew.org/results?f=species_f%2Caccepted_names&q=vepris* (accessed July 18, 2023).

Polhill D. 1988. *Index of collecting localities*. Kew: Royal Botanic Gardens.

Popp FD, Chakraborty DP. 1964. Alkaloids of the Bark of Teclea grandifolia. *Journal of Pharmaceutical Sciences* 53:968. DOI: 10.1002/jps.2600530830.

Rahman MdAA, Moon S-S. 2007. Antimicrobial phenolic derivatives from Dendranthema zawadskii var. latilobum ~~k~~Kitamura (Asteraceae). *Archives of Pharmacal Research* 30:1374–1379. DOI: 10.1007/BF02977359.

Rambaut A. 2010. Figtree.

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. *Systematic Biology* 67:901–904. DOI: 10.1093/sysbio/syy032.

Saleem M, Kim HJ, Ali MS, Lee YS. 2005. An update on bioactive plant lignans. *Natural Product Reports* 22:696–716. DOI: 10.1039/B514045P.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9:671–675. DOI: 10.1038/nmeth.2089.

530 Singh IP, Bharate SB. 2006. Phloroglucinol compounds of natural origin. *Natural Product*
531 *Reports* 23:558–591. DOI: 10.1039/B600518G.
532 Sun Y, Skinner DZ, Liang GH, Hulbert SH. 1994. Phylogenetic analysis of Sorghum and related
533 taxa using internal transcribed spacers of nuclear ribosomal DNA. *Theoretical and*
534 *Applied Genetics* 89:26–32. DOI: 10.1007/BF00226978.
535 Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-
536 coding regions of chloroplast DNA. *Plant Molecular Biology* 17:1105–1109. DOI:
537 10.1007/BF00037152.
538 Veldman S, Ju Y, Otieno JN, Abihudi S, Posthouwer C, Gravendeel B, van Andel TR, de Boer
539 HJ. 2020. DNA barcoding augments conventional methods for identification of medicinal
540 plant species traded at Tanzanian markets. *Journal of Ethnopharmacology* 250:112495.
541 DOI: 10.1016/j.jep.2019.112495.