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A phylogenetic road map to antimalarial *Artemisia* species

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ABSTRACT

Ethnopharmacological relevance: The discovery of the antimalarial agent artemisinin is considered one of the most significant success stories of ethnopharmacological research in recent times. The isolation of artemisinin was inspired by the use of *Artemisia annua* in traditional Chinese medicine (TCM) and was awarded a Nobel Prize in 2015. Antimalarial activity has since been demonstrated for a range of other *Artemisia* species, suggesting that the genus could provide alternative sources of antimalarial treatments. Given the stunning diversity of the genus (c. 500 species), a prioritisation of taxa to be investigated for their likely antimalarial properties is required.
Materials and methods: Here we use a phylogenetic approach to explore the potential for identifying species more likely to possess antimalarial properties. Ethnobotanical data from literature reports is recorded for 117 species. Subsequent phylogenetically informed analysis was used to identify lineages in which there is an over-representation of species used to treat malarial symptoms, and which could therefore be high priority for further investigation of antimalarial activity.
Results: We show that these lineages indeed include several species with documented antimalarial activity. To further inform our approach, we use LC-MS/MS analysis to explore artemisinin content in fifteen species from both highlighted and not highlighted lineages. We detected artemisinin in nine species, in eight of them for the first time, doubling the number of *Artemisia* taxa known to content this molecule.
Conclusions: Our findings indicate that artemisinin may be widespread across the genus, providing an accessible local resource outside the distribution area of *Artemisia annua*.

1. Introduction

Malaria is one of the most prevalent life-threatening diseases of humanity, with serious social and economic impacts (Sachs and Malaney, 2002). According to the World Health Organization (WHO), almost half of the world's population is at risk of contracting malaria (World Health Organization, 2015). In 2015 alone, 214 million new cases were reported, resulting in 438,000 fatalities, with Sub-Saharan Africa bearing 89% of global cases and 91% of deaths (World Health Organization, 2015). Currently, the main antimalarial treatment is based on the potent sesquiterpene lactone artemisinin.

The discovery of artemisinin was inspired by the use of *Artemisia annua* L. (Asteraceae), known as *qinghaosu* in traditional Chinese medicine (TCM) (Klayman, 1985). It is certainly considered one of the most significant success stories of ethnopharmacological research in recent times, and was awarded a Nobel Prize in Physiology or Medicine in 2015 (Normile, 2015). Artemisinin gained importance as an alternative to quinine-based treatments for malaria in the 1970's, at a time when the malaria parasite (e.g. *Plasmodium falciparum* Welch) was starting to develop resistance to existing treatments (Tu, 2011, 2016, 2017). Nonetheless, parasite resistance to artemisinin has been detected in Southeast Asia in recent years (World Health Organization, 2015), raising concerns about the future efficiency of artemisinin as an antimalarial agent. Drug resistance is a persistent problem in the treatment

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and prevention of malaria, and continuous discovery and development of alternative antimalarial agents are needed to ensure the availability of reliable treatment options in the future.

Antimalarial activity has recently been demonstrated for several other *Artemisia* species (Mojarrab et al., 2014, 2015), suggesting that this genus of circa 500 species could provide much-needed alternative sources of antimalarial treatments. *Artemisia* is widely distributed in the northern hemisphere, reaching South America and North Africa, across a diverse range of habitats, and presenting different life forms (Mucciarelli and Maffei, 2002). Given the stunning diversity in the genus, a prioritisation of taxa to be investigated for their likely antimalarial properties is required. Several species of *Artemisia* are used as food flavourings (e.g. tarragon: *A. dracunculus* L.), drink additives (e.g. absinth: *A. absinthium* L.; genipi: *A. genipi* Stechm.), and in traditional medicine across the distribution range of the genus (Abad et al., 2012). Approximately 300 *Artemisia* species have been investigated in variable level of detail for their chemistry and bioactivity, and some have demonstrated antimalarial bioactivity (Tan et al., 1998; Wilcox, 2009; Vallès et al., 2011, and references therein). Besides artemisinin, a remarkable diversity of bioactive compounds has been recorded to exist in species with demonstrated antimalarial bioactivity, such as in *A. abrotanum* L. (Cubukcu et al., 1990). In this species artemisinin is not present, but a range of several coumarins, sesquiterpenes and flavonoids has been reported (Cubukcu et al., 1990; Tan et al., 1998). This suggests that *Artemisia* species can both provide alternative sources of artemisinin as well as novel artemisinin analogues and other antimalarial compounds, potentially with different modes of action. Furthermore, different compound types may act synergistically to delay the development of drug resistance in the malaria parasite, suggesting that combination therapies or herbal extracts rather than single compounds may also result in more efficient approaches in the future (Rasoanaivo et al., 2011).

Recent research avenues have proposed that phylogenies can provide a rationale for the prioritisation of plant species for investigation. By mapping traditional medicinal uses (Ernst et al., 2016; Forest et al., 2007; Grace et al., 2015; Saslis-Lagoudakis et al., 2011, 2012), as well as chemical and bioactivity data (Larsen et al., 2010; Rønsted et al., 2012; Zhu et al., 2011) on phylogenetic trees, several studies have reported that both traditional uses and demonstrable medicinal properties are concentrated around certain lineages, and largely absent from others.

This phylogenetic pattern can help us mine traditional knowledge to identify lineages with desired medicinal properties. Further, lineages rich in traditional uses are also largely coincident with the ones overrepresented in species with pharmacological activity (Saslis-Lagoudakis et al., 2012). Therefore, by identifying “hot nodes” (Saslis-Lagoudakis et al., 2011) that are overrepresented in species with traditional uses, it might be possible to detect lineages that are most likely to demonstrate desired bioactivity. Hence, the use of phylogenies and traditional knowledge could increase hit rates in bioprospecting by narrowing down the number of potentially relevant species to be investigated for bioactivity. When combined with chemical data, it is possible to further focus investigations on the most promising species for alternative sources of artemisinin for local use, as well as species more likely to possess new leads with other modes of action.

The main objective of this study was to investigate how traditional medicinal knowledge combined with phylogenies could be used to identify *Artemisia* species with potential antimalarial bioactivity for further exploration. We collated a comprehensive database of traditional medicinal uses for *Artemisia*, which we used to highlight lineages with an overrepresentation of medicinally-used species on a comprehensive phylogeny of the genus. To validate our approach and explore if presence of artemisinin may explain the reported use, we then used high-performance liquid chromatography coupled to a mass/mass detection (LC-MS/MS) to analyse a subset of fifteen randomly-selected species for artemisinin content representing the phylogenetic diversity as well as species from both inside and outside hot nodes.

Table 1
Ethnobotanical use categories, analysis codes, and number of *Artemisia* species reported in the literature for each category. The plant taxa are considered as quoted in the original publications.

Use category	Analysis code	Focused malaria analysis	Number of species
MEDICINAL (when no specific category)	Med_0		19
ANTIHELMINTHIC	Med_1		23
ANTI-INFLAMMATORY	Med_2		21
ANTIMALARIAL	Med_3	Y	23
ANTITOXIC	Med_4		10
CANCER	Med_5		9
CIRCULATORY SYSTEM	Med_6		19
DERMATOLOGICAL	Med_7		15
DIGESTIVE SYSTEM	Med_8		34
FEVER	Med_9	Y	23
GENITOURINARY SYSTEM	Med_10		26
HALLUCINOGEN	Med_11		2
IMMUNITARY DISORDERS	Med_12		5
INFECTIONS	Med_13	Y	52
METABOLIC DISORDERS	Med_14		19
MUSCULOSKELETAL SYSTEM	Med_15		13
NERVOUS SYSTEM	Med_16		13
NUTRITIONAL DISORDERS	Med_17		20
PAIN	Med_18		12
PREGNANCY/BIRTH/ PUERPERIUM	Med_19		4
INSECT REPELLENTS	Med_20	Y	2
RESPIRATORY SYSTEM	Med_21		14
SENSORIAL DISORDERS	Med_22		5

2. Materials and methods

2.1. Medicinal uses

Information on the ethnobotanical uses of the genus *Artemisia* was collected based on a comprehensive literature review of published sources obtained from searching in online databases using the keyword "Artemisia" including reviews of the uses of the genus, primary ethnobotanical studies, and reports from local floras. A total of 124 published sources provided 1215 use records (Supplementary materials, Table S1), which were classified into 23 use categories (Table 1) following the Economic Botany Data Collection Standard (Cook, 1995) as also used in other studies (Cámara-Leret et al., 2017; Ernst et al., 2016; Grace et al., 2015). Additionally, we extracted records of uses for the prophylaxis or treatment of symptoms related to malaria, namely antimalarial (med_3), fever (med_9), infections (med_13) and insect repellents (med_20) (Table 1, Table S1).

2.2. Phylogenetic reconstruction

Nuclear ribosomal DNA sequences (ITS1–5.8S-ITS2 and 5′ETS) were downloaded from GenBank (Table S2) for a total of 244 *Artemisia* species representing all subgenera described in the genus, primarily from previous studies by Vallès et al. (2003), Tkach et al. (2008), Pellicer et al. (2010a), (2010b), Hobbs and Baldwin (2013), Pellicer et al. (2011), and Malik et al. (2017). Additionally, *Lepidolopsis turkestanica* (Regel and Schmalh.) Poljakov and *Tanacetum parthenium* (L.) Schultz Bip. were designated as outgroup species based on their phylogenetic proximity to *Artemisia* (Table S2; e.g. Malik et al., 2017). Sequences were edited for each DNA marker using BioEdit v.7.0.9 (Hall, 1999) and aligned using CLUSTAL X (Thompson et al., 1997). Base gaps and any inaccuracies found were manually adjusted. Preliminary Bayesian inference (BI) was conducted to assess incongruence among nuclear markers, and subsequent concatenated analysis was conducted

after confirming no conflicts on the phylogenetic signals. For this analysis, a partitioned BI of the combined dataset was conducted with MrBayes v.3.2.2 (Ronquist and Huelsenbeck, 2003) on the CIPRES server (Miller et al., 2010). The most appropriate models for nucleotide substitution were chosen with jModelTest v.0.1 (Posada, 2008), which were GTR + G + I for ITS and GTR + G for ETS. Two independent runs of four Markov Chain Monte Carlo (MCMC) analyses were run for 10^7 generations, sampling every 10^3 generations. The MCMC sampling was evaluated with Tracer v.1.6 (Rambaut et al., 2014), confirming that the effective sample size (ESS) was > 200 in each run. The first 2500 trees were discarded as the 'burn-in' period, and the remaining trees were used to produce a 50% majority-rule consensus tree, with the posterior probabilities (PP) of nodes calculated from the pooled samples.

2.3. Phylogenetic distribution of medicinal species

We explored the phylogenetic distribution of *Artemisia* species with described medicinal uses with two main approaches as recommended by Ernst et al. (2016) and described below. All analyses were performed in the R environment v.3.0.3 (Core Team, 2015), based on scripts developed by Ernst et al. (2016).

With the first approach, we aimed to estimate the overall degree of phylogenetic clustering of the different uses reported for *Artemisia* species on the *Artemisia* phylogeny. We used the D metric (Fritz and Purvis, 2010) implemented by the function "phylo.d" in the R package caper (Orme, 2013) with the default parameter of 1000 permutations. Thus, if a species trait - in our case species reported used in a use category - shows phylogenetic signal, it may be assumed that species in this category are not randomly distributed over the phylogeny, but are more phylogenetically clustered than expected by chance. Values of $D = 1$ indicate a phylogenetically random distribution, whereas $D < 1$ indicates phylogenetic clustering and $D = 0$ indicates that the trait is clustered as if it had evolved by Brownian motion of evolution (Fritz and Purvis, 2010). Two p-values are furthermore calculated for the D metric, $p(D < 1)$ indicating whether the D metric is significantly smaller than 1, meaning that the trait (reported use) is not randomly distributed over the phylogeny. The second p-value, $p(D > 0)$ indicates whether the D metric is significantly greater than 0, meaning that the trait has a significantly different distribution on the phylogeny from the Brownian model of evolution. To account for phylogenetic uncertainty, the D metric was calculated on 1000 randomly selected trees from within the 95% credibility set of trees from our Bayesian analyses. This was done for all medicinal species, as well as the different categories of use presented above for which there were more than ten species with described uses.

With the second approach, we wanted to identify lineages on the *Artemisia* phylogeny that are overrepresented in species used medicinally. To do that, we searched for "hot nodes" (nodes that are significantly overrepresented by species in a given category) (Saslis-Lagoudakis et al., 2011), using the "nodesigl" command in PHYLOCOM v4.2 (Webb et al., 2008). This option uses the same community sampling (the group of species used for a certain category of use) as described above and tests each node of the phylogeny for overabundance of terminal taxa distal to it. Observed patterns for each sample are compared to those from random samples to provide significance for the observed overabundance. For a node that is identified through this approach, the descendants of this node are significantly more likely to belong to the "community" under consideration than expected by chance alone. Accordingly, this technique identifies the exact position of phylogenetic clumping on the phylogeny, namely the "hot" nodes for a category of use (Saslis-Lagoudakis et al., 2011).

2.4. Artemisinin content determination

Plant material was obtained either directly in the field or after sowing achenes from wild populations and cultivating the plantlets in the Institut Botànic de Barcelona until adult age. The plant material was collected in all cases, irrespective of its provenance (germinated achenes or vegetative material), either before flowering or just when flowering period was starting. Vouchers are deposited in the herbaria BCN (Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona), LE (Botanical Institute, Russian Academy of Sciences, Saint Petersburg) or SSLP (Rocky Mountain Research Station, USDA Forest Service, Provo, Utah). Plant samples, consisting of leaves from the terminal third part of five plants per accession, were dried on absorbent paper at room temperature in absence of light and humidity for fifteen days. Dried samples were pulverised in an electrical grinder; particle size varied among species, with textures from granulate to spongy. Following Misra et al. (2014) microwave-assisted extraction was carried out for all samples, mixing 0.2 g of pulverised plant material in 20 ml of hexane:acetone 1:1 in a recipient of a microwave extractor and under the following conditions: Initial temperature ramp (from 100 °C to 115 °C in 10 min), extraction temperature and time: 115 °C during 15 min. Initial and final pressure: 50 psi and 155 psi, respectively. Finally, samples were left to cool down to room temperature before the extracted samples were filtered with polytetrafluorethylene (*Teflon®*) meshes of 0.2 µm of pore diameter. The extracted samples were brought to dryness and resuspended in acetonitrile, and were then analysed with high-performance liquid chromatography coupled to a mass/mass detection (LC-MS/MS) carried out on an API3000 (ABsciex) triple quadrupole mass spectrometer integrated on an Acquity (Waters) UPLC system. Two replicates of 10 µl were analysed for each accession. A Luna 2.5 µm C18 column (20 HST 50 × 2.00 mm, producer) was used as stationary phase. The column temperature was set at 40 °C. The mobile phase consisted of a mixture of (A) MilliQ Water with 0.1% formic acid and (B) acetonitrile. The gradient elution profile consisted of: Initial conditions: A: 2%, B: 98%; 1 min A: 2%, B: 98%; 3 min A: 100%, B: 0%; 4 min: A: 100%, B: 0%; 4.1 min A: 2%, B: 98%; 6 min A: 2%, B: 98%. Flow was set at 0.7 ml/min. Analytes were detected in positive electrospray ionisation (ESI) mode. The ion spray voltage was set at 4500 V and source temperature at 350 °C. Data was acquired in MRM (multiple reaction monitoring) mode. Three replicates of the extraction were used per accession, and two analyses were performed per replicate. Transition selected was 283.3/209.1 for artemisinin. Artemisinin content in weight/weight percentage of dried plant material is reported in Table 2. Limit of detection is 1.2 ppb (ng/ml) and limit of quantification is 6 ppb (ng/ml) determined as 3 times and 10 times the minimum value that could be detected respectively.

3. Results

3.1. Medicinal use of Artemisia species

We found reports of medicinal use for 117 scientific names (including 8 subspecies and/or varieties) corresponding to 109 currently accepted species (21% of the genus) of *Artemisia* in 23 medicinal use categories. The primary categories included digestive system disorders (34 species), infections (52 species), and genitourinary disorders (26 species) (Fig. 1, Table 1). Of these, 66 species (13% of the genus) were specifically used as prophylaxis or treatment of symptoms related to malaria, in the following categories: antimalarial (23 species), fever (23 species), infections (52 species) and insect repellents (2 species) (Table 1, Table S1). Note that of the 117 species with reported ethnobotanical uses, 77 were included in the phylogeny. Likewise, of the 66 species with malaria-related symptoms, 46 were included in the phylogeny.

3.2. Phylogenetic relationships in Artemisia

Our preliminary analyses (data not shown) did not reveal robust incongruence on the overall tree topologies concerning supported nodes, so the resulting 50% majority rule consensus phylogram

Table 2
Artemisinin content in fifteen investigated *Artemisia* species, and one *Tanacetum* species used as outgroup. Artemisinin reports in bold are considered confirmed (above limit of detection).

Taxon	Reported antimalarial use	Present in hot nodes	Voucher (collectors, herbarium acronyms and numbers)	Provenance	Mean artemisinin content in weight/weight percentage of dried plant material (standard deviation)
Artemisia absinthium L.	Antimalarial, fever, infections, insect repellents	Yes	M. Mumbrú (BCN 142757)	Spain, Catalonia: Campelles	**0.000390 (0.000057)**
Artemisia anethifolia Weber ex Stechm.	None	No	A.A. Korobkov (LE-K-06 to 22; MV1)	Russia, Selenge: Selenduma-Shanan	**0.000497 (0.000053)**
Artemisia anethoides Mattf.	None	No	S. Dariimaa, S. Tsooj, J. Vallès (BCN 23790; MV12)	Mongolia, Selenge: Shaamar	0.000064[a] (0.000006)
Artemisia annua L.	Antimalarial, fever, infections	Yes	J. Vallès, S.W. Zhao (BCN 130815; MV8)	People's Republic of China, Inner Mongolia: Helan Shan	**0.069100 (0.005657)**
Artemisia annua L.	Antimalarial, fever, infections	Yes	M. Mumbrú, J. Vallès (BCN 142758)	Spain, Catalonia: Barcelona	**0.142000 (0.045255)**
Artemisia arborescens L.	Fever, infections	Yes	T. Garnatje GR − 128, J. Luque (BCN 28644; MV14)	Greece, Crete: Kalyves	0.000010[2] (0.000014)
Artemisia barrelieri Besser	None	No	T. Garnatje, J. Vallès (BCN 129030)	Spain: Benferri	0
Artemisia frigida Willd.	Infections	No	A.A. Korobkov (LE-K-03–00; MV2)	Russia, Tuva: Kyzyl	**0.000073[3] (0.000030)**
Artemisia gmelinii Weber ex. Stechm.	None	No	A.A. Korobkov (LE-K − 00 to 04: MV27)	Russia, Sakhalin island: Shebunino	**0.000385 (0.000016)**
Artemisia herba-alba Asso	Fever, infections	No	T. Garnatje, J. Vallès (BCN 129022)	Spain: Aranjuez	0
Artemisia integrifolia Richards.	None	No	A.A. Korobkov (BCN 13934; MV19)	Russia, Primorie: cape Gamova	**0.000361 (0.000025)**
Artemisia macrocephala Jacquem. ex Besser	None	Yes	S. Dariimaa, S. Tsooj, J. Vallès (BCN 23801; MV11)	Mongolia, Uvur Khangi: Arvay Kheer	**0.000115[3] (0.000021)**
Artemisia messerschmidtiana Besser	None	No	A.A. Korobkov (LE-K-95-00; MV28)	Russia, Dauria: upper course of river Gazymua	**0.000324 (0.000016)**
Artemisia sieversiana Ehrh.	Fever, infections	Yes	A. Ivaschenko, A. Susanna S-2114, J. Vallès (BCN 11692, 142756; MV13)	Kazakhstan: Jamatalap	0
Artemisia thuscula Cav. (*A. canariensis* Less.)	None	Yes	A. Santos, J. Vallès (BCN 97854: MV-10)	Spain, Tenerife island: Tejina de Isora	**0.000449 (0.000026)**
Artemisia tridentata Nutt. subsp. *vaseyana* (Rydb.) Beetle	Fever, infections	Yes	Collector MV18 (SSLP-EDM-2874; MV18)	United States of America, Utah: Salt Creek Canyon	0.000005[2] (0.000007)
Tanacetum vulgare L.	None	n.a.	I. Canals (BCN 131678)	Spain, Catalonia: Manresa	0

[a] Under quantification limit and practically equal to the detection limit. [2]Under detection limit. [3]Under quantification limit.

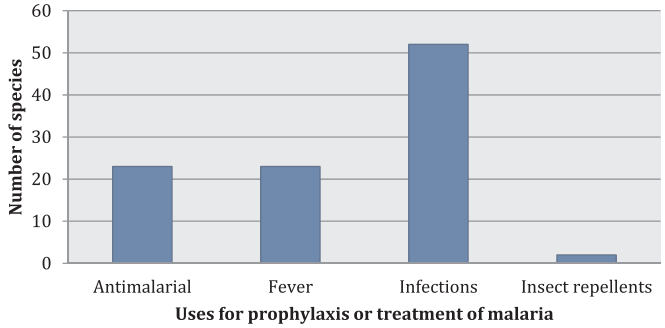

Fig. 1. Number of *Artemisia* species cited in the literature for the prophylaxis or treatment of malaria. In total 66 species are reported to be used for at least one of the four categories. 46 of these are present in the phylogeny.

obtained from the concatenated ETS and ITS datasets is depicted in Fig. S3. Note that the 5.8 S gene was excluded from the phylogenetic analysis since it was missing in several species (Table S2). The tree topology we obtained largely corresponds to our current understanding of the evolutionary relationships in *Artemisia* [see Malik et al. (2017) for the most recent phylogenetic study of the genus]. The monophyly of the genus is strongly supported (PP = 95%). Overall, the backbone of the tree is largely resolved (PP > 90%), with the exception of some lineages (see Fig. S3), as already found in previous attempts to untangle the evolutionary relationships in the genus (e.g., Hobbs and Baldwin, 2013; Malik et al., 2017; Riggins and Seigler, 2012). Likewise, there are several cases in which the traditional classification of the species does not fully correspond with the lineage segregation resulting from the analysis of DNA sequence (Fig. S3).

3.3. Phylogenetic distribution of species with medicinal use

We mapped reported medicinal use of *Artemisia* species on the most comprehensive phylogeny of the genus to date (Fig. 2) to investigate the degree of phylogenetic clustering of species with medicinal uses using two independent approaches, the D-metric, which explores overall phylogenetic signal of a trait, and the hot nodes approach, which explores overabundance of a trait on individual nodes across the phylogeny (Table 3), focussing primarily on uses treating symptoms related to malaria.

The D-statistic for clustering of medicinal uses on the tree was smaller than 1 (D-median = 0.808; p(D < 1): ns; Table 3), corresponding to phylogenetic clustering, although this was not significant. Likewise, species used against malaria-related symptoms were clustered based on D < 1, but again this was not significant (D = 0.746; p (D < 1): ns; Table 3).

Using the "nodesig" command to identify hot nodes of species used against malaria-related symptoms on the phylogeny (Fig. 2), we were able to highlight four hot nodes including a total of 15 species that are most likely to possess antimalarial properties based solely on the phylogenetic exploration of traditional medicinal uses (Table 4). These lineages comprise species with documented antimalarial activity (Table 4, Table S1), including *Artemisia annua*, *A. absinthium*, and *A. gorgonum,* as well as *A. arborescens, A. sieversiana* and *A. tridentata* with use reports for fever or infections, corroborating the validity of our approach. Eight of the fifteen species in the hot nodes are not associated with any use reports in the literature, and therefore represent entirely new lines of enquiry. It is likely that additional species could belong to the identified hot nodes because the phylogenetic analysis included only about half of the ca. 500 *Artemisia* species.

3.4. Artemisinin content

Artemisinin was detected in nine of the 15 investigated species selected from both within and outside the hot nodes (Fig. 2, Table 2). The two included accessions of *Artemisia annua* contained 140–286 times higher levels of artemisinin (0.069100% and 0.142000%) than *A. anethifolia*, (0.000497%) which was the species with the second highest content. This confirms the value of *A. annua* as the hitherto best source of artemisinin. Naturally, artemisinin is produced in small quantities, which leads to a shortage of global supply. Artemisinin is difficult to chemically synthesize due to its complex structure, and *A. annua* remains the main source of the drug, although current advances in genetic and metabolic engineering may improve *in planta* production of artemisinin in the future (Ikram and Simonsen, 2017). The detection of artemisinin in four of seven species (*A. absinthium*, *A. annua*, *A. macrocephala*, and *A. thuscula*) from hot nodes and five of eight (*A. anethifolia*, *A. frigida, A. gmelinii, A. integrifolia,* and *A. messerschmidtiana*) from outside the hot nodes suggests that the occurrence of artemisinin is common throughout the genus. A further four species from the hot nodes for which artemisinin content was tested but not confirmed in the present study (Table 2, Table S4), namely *A. arborescens, A. herba-alba*, *A. sieversiana* and *A. tridentata,* have reported uses against fever and infections.

4. Discussion

4.1. Phylogenetic prediction of antimalarial activity in Artemisia

We conducted a bibliographic review searching for *Artemisia* species with reported traditional uses against malaria-related symptoms (66 species). In addition to the nine species found here to contain detectable levels of artemisinin (Table 2), several other *Artemisia* species have previously been reported to contain artemisinin (Table S4): *A. abrotanum* (Suresh et al., 2011) *A. apiacea* (Liersch et al., 1986), *A. cina* (Aryanti et al., 2001), *A. dubia* (Kiani et al., 2012), *A. hedinii* (Ling, 2007), *A. lancea* (Tan et al., 1998), *A. pallens* (Suresh et al., 2011), *A. scoparia* (Singh and Sarin, 2010), and *A. sieberi* (Arab et al., 2006) (18 species, Table 2, Table S4). Artemisinin has been detected for the first time in eight species (Table 2), almost doubling the number of species in the genus known to contain this compound. It is notable that of the several species found here to contain artemisinin, only *A. annua* was previously known as a main source of the compound. Certainly, the occurrence of artemisinin in so far 18 species across the phylogeny (Table S4), suggests that the artemisinin biosynthetic pathway might be an ancestral trait and the biosynthetic machinery needed to produce artemisinin and related compounds is likely to be common for many *Artemisia* species. The widespread distribution of the genus therefore enables potentially a local use of alternative species for the treatment of malaria.

Our phylogenetic predictions are only informed by traditional uses, which are difficult to classify (Staub et al., 2015), and not biochemistry or bioactivity data. Using the D-statistic, we did not find significant phylogenetic signal of neither medicinal use nor antimalarial activity, but using the hot node approach, we were able to identify several lineages within *Artemisia* with overrepresentation of species used for antimalarial bioactivity and seven of the 15 species in the hot nodes (including *A. annua*) have reported antimalarial use (Table 2).

Weak phylogenetic signal using the D-statistic was also reported by Grace et al. (2015) for medicinal use in the genus *Aloe* and by Ernst et al. (2016) for several use categories in the genus *Euphorbia*. Grace et al. (2015) did not explore the hot nodes approach in their study, but Ernst et al. (2016) was able to identify nodes that were significantly overrepresented by species showing inflammatory response although the D-statistic of this category was not showing significant clustering (p (D < 1) ns).

Whereas the hot nodes approach has been able to identify nodes overrepresented by species with a specific trait both in the present study and in the study by Ernst et al. (2016), the D-statistic approach appears to be more conservative and potentially less useful for

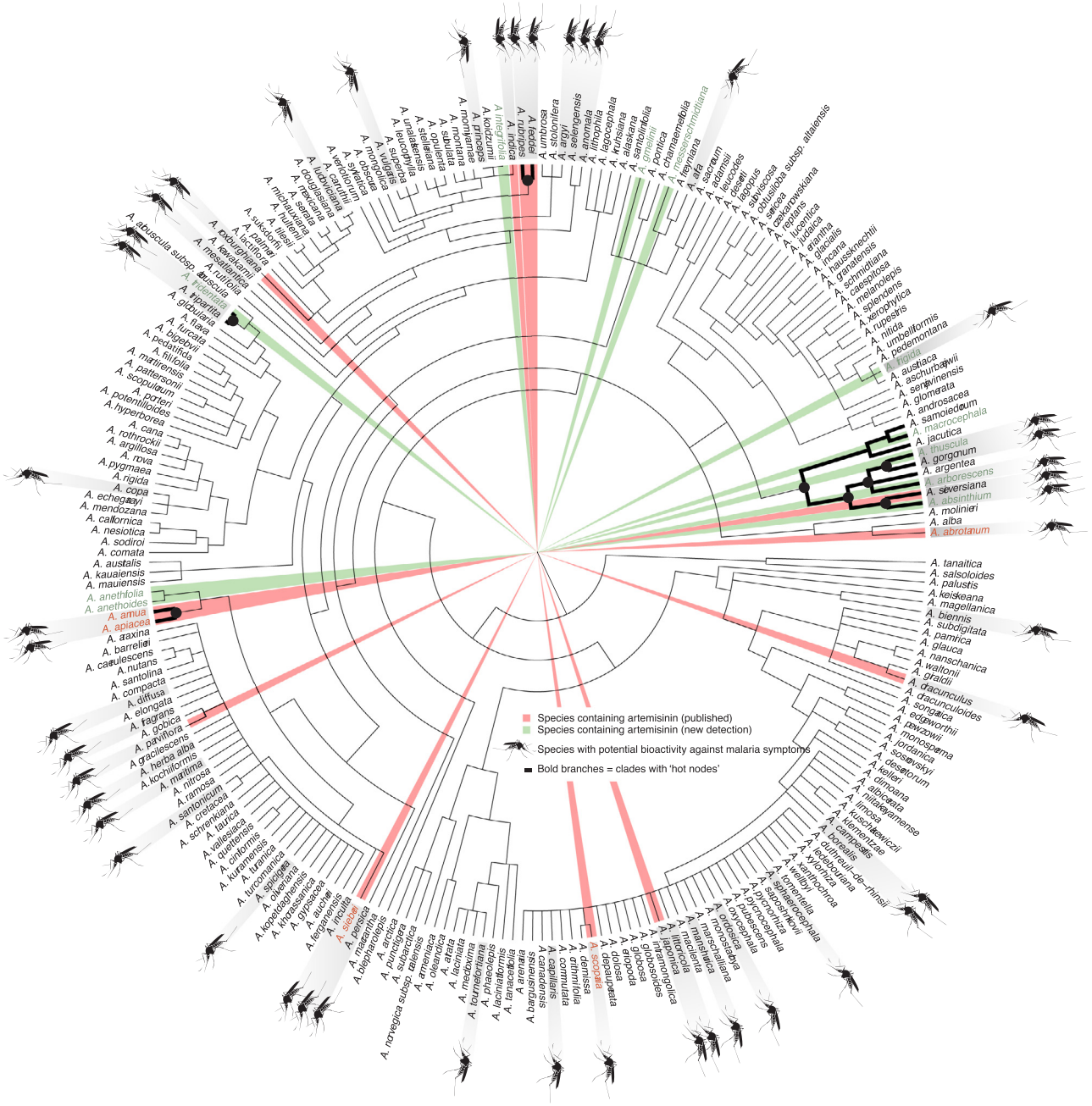

Fig. 2. Hot nodes of *Artemisia* species used against malaria-related symptoms plotted on the 50% majority-rule consensus phylogram from Bayesian inference. Published (red) and novel (green) records of artemisinin presence in *Artemisia* are also indicated.

Table 3
Phylogenetic signal (D-statistic) on 1000 phylogenetic trees of *Artemisia* per Economic Botany Data Collection Standard categories all medicinal (med_0-med_22) and for prophylaxis or treatment of malaria related symptoms (antimalarial (med_3), fever (med_9), infections (med_13) and insect repellents (med_20). The prevalence expresses the number of species within a category versus the total number of species (244).

Category	N	Prevalence	D-statistic		Phylogenetic signal	
			Median	Range	p(D < 1)[a]	Strength[b]
All medicinal	77	0.32	0.81	0.65–0.94	ns	Weak
Malaria related	46	0.19	0.75	0.46–0.95	ns	Weak

N: number of species. [a*] $p < 0.05$ in 95% of trees, ** $p < 0.05$ in 95% of trees; ***$p < 0.005$ in all trees. [b]weak: < 90% $p(D > 0) > 0.05$; moderate: $p(D > 0) > 0.05$ in 90% of trees; strong: $p(D > 0) > 0.05$ in 95% of trees; very strong: $p(D > 0) > 0.05$ in all trees.

Table 4
Antimalarial use and artemisinin presence reports in the 15 *Artemisia* species included in the hot nodes of potential antimalarial interest. The confirmed artemisinin reports are marked in bold.

Species	Known antimalarial use	Previous reports of artemisinin	Artemisinin content analysed in this study	Artemisinin found in this study
A. absinthium L.	**Antimalarial, fever, infections, insect repellents**	**No**	**Yes**	**Yes**
A. annua L.	**Antimalarial, fever, infections**	**Yes**	**Yes**	**Yes**
A. apiacea Hance	**Antimalarial, infections**	**Yes**	**No**	–
A. arborescens L.	Fever, infections	No	Yes	No
A. argentea L'Her.	None	No	No	–
A. feddei H.Lév. & Vaniot	None	No	No	–
A. gorgonum Webb.	Antimalarial	No	No	–
A. jacutica Drobow	None	No	No	–
A. macrocephala Jacquem. ex Besser	**None**	**No**	**Yes**	**Yes**
A. rubripes Nakai	None	No	No	–
A. samoiedorum Pamp.	None	No	No	–
A. sieversiana Ehrh. ex Willd.	Fever, infections	No	Yes	No
A. thuscula Cav.	**None**	**No**	**Yes**	**Yes**
A. tridentata Nutt.	Fever, infections	No	Yes	No
A. tripartita Rydb.	None	No	No	No

identifying phylogenetic clustering. Future modelling studies may provide a better understanding of whether the D-statistic approach is underestimating phylogenetic signal under certain conditions and we would also urge for the exploration of additional approaches to be used for phylogenetic prediction in order to continue to develop this emerging field.

It should also be emphasized that phylogenetic prediction relies on community sampling of a trait of interest and the approach is therefore limited by availability of relevant trait data. Furthermore, while phylogenetic exploration offers a new approach to exploration of medicinal potential, many other approaches to enable drug discovery exists ranging from ethnopharmacological surveys to combinatorial chemistry approaches, each with different advantages and limitations, which may all be relevant tools depending on the objectives.

Despite the hypothesis that biosynthesis of artemisinin may be an ancestral trait for the genus, previous studies (Tan et al., 1998) have found that not all bioactive species appear to contain artemisinin. For example, Rustaiyan et al. (2009) and Rustaiyan and Masoudi (2011) reported in *A. diffusa* a new sesquiterpene lactone (tehranolide), which they consider could be responsible of the antimalarial activity of this species, given the relevance of the peroxide bridge in this medicinal property (Shandilya et al., 2013), though such activity yet awaits to be tested. However, variation of composition and quantity of specialised metabolites within species is well known and can depend on collection site, seasonality, or induction by for example herbivory (Wink, 2003; Moore et al., 2014; Maldonado et al., 2017). It is therefore likely that further investigation of multiple specimens per species may confirm the ability to produce artemisinin also in these species. It is also possible, that artemisinin analogues or other compound types (e.g. coumarins, polymethoxyflavones or flavonoids) could be responsible for the activity or possibly act in synergy with artemisinin. More detailed analysis of compounds and their potential antimalarial activity would be required to explore this further.

Having comprehensive understanding of the evolutionary relationships among species can help to systematically identify candidate taxa for further exploration. In *Artemisia*, this is even more important since traditional and molecular classifications sometimes reveal intricate and conflicting evolutionary relationships.

Species lacking morphological affinities can be closely related based on DNA sequence data, and more interestingly, such relationships have been further supported when the biochemical pathways have been taken into account (Greger, 1988). As an example, while there is lack of morphological affinity between *A. keiskena* and *A. palustris* (subg. *Artemisia*) with representatives of subg. *Dracunculus*, their close phylogenetic placement (Fig. S3) is also supported by the fact that they share specific dehydrofalcarinone derivatives and aromatic acetylenes, which are absent in other lineages (Greger, 1988). Therefore, future drug discovery efforts should focus on exploring the full metabolome across the phylogenetic diversity of the genus to account for other potentially useful compounds. A main conclusion of the present study is that artemisinin is much more widespread in the genus than previously documented.

4.2. Traditional knowledge and ethics

Naturally, extreme caution regarding conservation of genetic resources and intellectual property of indigenous peoples needs to be taken while carrying out bioprospecting efforts stemming from traditional knowledge. The Convention on Biological Diversity (CBD) (www.cbd.int) has been an important milestone to assigning ownership of traditional knowledge and genetic resources to local communities. However, our study reveals a gap in the existing framework of intellectual rights. The phylogenetic approach to bioprospecting presented here combines traditional knowledge and phylogeny to identify lineages with high bioactivity potential. As we demonstrate, these lineages may include species for which no use reports exist in traditional knowledge. Although we argue that this feature is one of the main strengths of our approach, it also unearths a grey area in the existing framework. If novel biological resources are identified, to whom may intellectual rights be assigned? This is a complex ethical issue when knowledge from several cultures is advised, like in the present study. Although creating interdisciplinary bioprospecting efforts can safeguard the ethical aspects of bioprospecting (Saslis-Lagoudakis and Clarke, 2013), we call for the CBD and policy makers to formally discuss this issue, in order to be able to fully acknowledge the power of traditional knowledge from local communities.

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Author contributions

J.P., C.H.S.L., J.V. and T.G. jointly conceived and designed the project. J.V., T.G., E.C. A.G. and O.M.G. collated the ethnobotanical dataset. J.P., T.G. and D.V. generated the phylogeny. T.G., M.M. and J.V. collected plants. M.M., J.V. and T.G. conducted the LC-MS/MS analysis. M.E. contributed bioinformatics pipelines. J.P. and C.H.S.L. conducted the phylogenetic analyses. J.P. and C.H.S.L. drafted the manuscript with N.R., and all authors commented on the manuscript and approved the final version.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2018.06.030.

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