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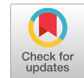
Sustainable use of mangroves as sources of valuable medicinal compounds: Species identification, propagation and secondary metabolite composition



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ABSTRACT

Mangroves are able to withstand a number of stress factors, such as high salt concentrations, tidal flooding, strong wind, solar radiation and heat. Their ability to grow under these circumstances is based on morphological and physiological adaptations, among them the high abundance of plant secondary metabolites. We are interested to investigate and exploit their medicinal and biotechnological potential for new bioactive compounds, without collecting material in the countries of origin and in a sustainable way. Therefore, a simple identification system based on molecular marker analysis, and a sustainable greenhouse propagation protocol for the continuous supply of fresh plant material, were established. DNA barcoding of the internal transcribed spacer (ITS) including ITS1, the 5.8S rRNA region and ITS2 as a molecular marker was applied for several mangrove species. The obtained data and GenBank sequences were used for species identification. Three mangrove species are cultivated in our greenhouse and propagated in different ways: *Avicennia* species produced many propagules in the greenhouse, however, further propagation by cuttings was not successful. *Laguncularia racemosa* was propagated by cuttings in a fog house whereas *Bruguiera cylindrica* was difficult to cultivate and propagation was not successful. Finally, the concentration of secondary phenolic compounds, including flavonoids, and the content of major elements were compared among naturally and greenhouse-grown mangroves indicating comparable amounts and composition.

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1. Introduction

Mangroves are trees or shrubs that are able to grow in saline water along tropical and sub-tropical coasts around the world (Kathiresan and Bingham, 2001). These plants are able to withstand a number of environmental stress factors: high salt concentrations, tidal flooding, strong wind, solar radiation and heat (Spalding et al., 2010). Their ability to grow under these circumstances is linked to various morphological, physiological and biochemical adaptations, such as stilt and air roots, salt excretion systems and secondary metabolites.

A mangrove forest provides protection against erosion and high waves for coastal regions (Alongi, 2008). Mangroves also offer an important habitat for many species. For example, many fish species use the sheltered root systems to breed, which is important for local fisheries (Phillips et al., 1993). However, the area of mangrove forests worldwide is decreasing at a rate of 1%–2% every year (FAO, 2003). In Asia, almost half of the area used for aquacultures (42%) was previously covered by mangroves (ADB/NACA, 1998). In a counter movement, there are several reforestation programs, e.g. in India, Vietnam and Bangladesh (Benthem et al., 1999; MFF Vietnam, 2015; Chow, 2018). Studies of the economic value of replanted mangrove forests show that the benefits outweigh the input costs (Tuan and Tinh, 2013).

In general, woody plant species can be propagated in different ways. An easy, low cost method is the use of seeds. In some woody plant species, seed development and germination are time-consuming processes, which limits the availability. In that case, preparation of cuttings or in vitro culture can be applied. Cuttings are branches of a tree, which can have a variable length and can be directly put in soil or growth media to generate roots at the cutting site. The plant hormone auxin can be used to improve root growth, and a humid atmosphere supports the rooting of vegetative cuttings (Milbocker, 1983; Dirr, 1992). In vitro propagation requires more equipment and is more cost-intensive, but is independent of seasons.

Species determination and taxonomy of mangroves is not fully resolved (Ragavan et al., 2014). Even the taxonomy on the family level is not conclusively determined. For example, the taxonomic placement

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of the genus *Avicennia* is controversial. In some classifications, it has been placed in the family Verbenaceae (Moldenke and Moldenke, 1980; Li et al., 2016) but recent phylogenetic studies have suggested that *Avicennia* is derived from within the Acanthaceae (The Angiosperm Phylogeny Group, 2016). In addition, the designation of species remains difficult due to the great variability and morphological plasticity, such as within *Avicennia marina*. Therefore, the application of an identification system that is based on molecular markers could support the correct identification of species and even subspecies, as was previously shown for other taxa (Lucas et al., 2012; Nguyen et al., 2015). Recently, the efficacy evaluation of a multilocus marker system for delineating mangrove species from the West Coast of India could successfully demonstrate the mangrove species resolution based on several genes (Saddhe et al., 2016, 2017). However, for most applications such as a fast species identification, a simpler DNA barcode system is preferable.

Mangroves have a long tradition of medicinal use and are rich in secondary metabolites (Bandaranayake, 1998, 2002). Some of these secondary compounds from mangrove species have been reported to have antimicrobial, antioxidant and other effects, which is mainly based on ethnobotanical reports (Patra and Thatoi, 2011). For researchers in the temperate zones, it is only possible to have access to fresh plant material when mangroves are cultivated successfully in the greenhouse. In addition, the use of greenhouse plants makes it unnecessary to take plant material out of the wild. Prerequisites for the successful use of greenhouse-grown plants are high contents of secondary metabolites and a similar composition as in naturally grown plants.

For our study, several mangrove species were selected from three main mangrove families, which is based on ethnobotanical studies. The species *Avicennia marina* (Forssk.) Vierh. and *A. germinans* (L.) L. (Acanthaceae) use different strategies to survive in high salinities such as salt rejection, elimination, and a slight concentration. *Bruguiera cylindrica* (L.) Blume (Rhizophoraceae) uses rejection, concentration, and ultrafiltration. The species develops salt glands and accumulation of salt in old leaves has been observed. For *Laguncularia racemosa* (L.) C.F. Gaertn. (Combretaceae), several bioactive effects have been reported, such as protein kinase inhibition and insecticidal activity (Shi et al., 2010). The high salt tolerance is based on salt excretion through glands close to the petiole and conservative water use at high salinities (Sobrado, 2005).

The aims of this study were to clearly identify mangrove species for further propagation and as a resource of valuable secondary compounds. ITS including ITS1, the 5.8S rRNA region and ITS2 were tested as a phylogenetic marker for fast and simple species determination for mangroves. Methods for propagation in the greenhouse in temperate zones were determined. Secondary metabolites were characterized in mangroves grown in the greenhouse and outdoors, to reveal if greenhouse-grown mangroves can be considered for further exploitation of secondary compounds.

2. Material and methods

2.1. Plant material

The mother plants used for propagation in the greenhouse have different origins. A small tree of the species *Avicennia marina* (Forrsk.) Vierh., Acanthaceae, was donated by Prof. Dr. H. Lieth, University of Osnabrück, in 1999, but is of unknown origin. In the course of this study, the identification as *A. marina* was found to be incorrect. Five ca. 15-year-old plants of *Avicennia germinans* (L.) L. (Acanthaceae), and a ca. 15-year-old plant of the species *Laguncularia racemosa* (L.) C.F. Gaertn. (Combretaceae), originally collected in South America, were donated by Prof. R. and Dr. M. L. Schnetter, University Gießen, Germany. *Bruguiera cylindrica* (L.) Blume (Rhizophoraceae) plants were purchased as propagules originally collected in Indonesia (Marek Mangroven, Wien, Austria). The plants were cultivated in the greenhouse of the Institute of Botany, Leibniz University Hannover, Hannover, Germany. Further plant material used for molecular analysis was collected in Bangladesh, Cuba, Guatemala, Egypt, India and Vietnam at various collection sites (Fig. 1, Table 1).

2.2. Internal transcribed spacer (ITS) analysis

2.2.1. DNA extraction and PCR

DNA was extracted from leaf material dried at ambient temperature using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany). The procedure followed the manufacturer's instruction modified according to Lucas et al. (2012). The success of DNA extraction was determined by visualizing bands on 1% agarose gels stained with Midori green (Biozym-Diagnostik GmbH, Hess. Oldendorf, Germany). The concentration of DNA was measured on micro-volume plates with a microplate reader (Synergy Mx Multi-Mode, BioTek, Bad Friedrichshall, Germany).

The nuclear ITS region including ITS1, the 5.8S rRNA region and ITS2, with a size of 700 to 720 bp, was amplified by PCR. The primers P674 5′-CCTTATCATTTAGAGGAAGGAG-3′ (ITS5a) (Stanford et al., 2000) and P675 5′-TCCTCCGCTTATTGATATGC-3′ (ITS4) (White et al., 1990) were used. A PTC 200 thermocycler (Biozym-Diagnostik GmbH) with lid heating was used for the PCR reactions, with following: 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 μl Dream *Taq* polymerase (Thermo Fisher Scientific, Waltham, USA), 1 × Dream *Taq* Green buffer, 10–30 ng template DNA, 1 pmol of each primer in a total volume of 25 μl.

An initial denaturation step at 95 °C for 4 min was followed by 30 cycles with the following steps: denaturation at 95 °C for 25 s, primer annealing at 52 °C for 30 s and primer extension at 72 °C for 35 s. The reaction was terminated with a final hold at 10 °C. To avoid possible errors in the final consensus sequence (due to the *Taq* polymerase) each PCR reaction for every specimen was repeated two to four times independently. Sequencing of the PCR products was done by GATC Biotech (Konstanz, Germany) using the primers P674 5′-CCTTATCATTTAGAGG AAGGAG-3′ and P675 5′-TCCTCCGCTTATTGATATGC-3′.

2.2.2. Data analysis

In this study, 27 sequences of Acanthaceae, 3 sequences of Rhizophoraceae and 5 sequences of Combretaceae were retrieved from the mangrove species collected in Bangladesh, Cuba, Egypt, Guatemala, India, Vietnam and the greenhouse (Table 1). For comparison, known ITS sequences of Acanthaceae, Combretaceae and Rhizophoraceae species retrieved from GenBank were added to the dataset. These sequences were aligned by CLUSTAL X (Thompson et al., 1997) in MEGA 7 (Kumar et al., 2016) and the alignment controlled visually. Gaps between nucleotides in the alignment were considered as missing data. Identical sequences within each species were excluded from the alignment. Additional in-group sequences were obtained from GenBank (Table 1), and included in the alignment. The total length of the alignment was 604 bp. jModelTest version 2.1.6 (Darriba et al., 2012) and the corrected Akaike Information Criterion was used to find the best model for the analysis. Phylogenetic analyses were performed using Maximum Likelihood (ML) in RAxML version 8.1 (Stamatakis, 2014) with the model General Time Reversible (GTR) (Lanave et al., 1984). Maximum Parsimony (MP) (Felsenstein, 1978) and Neighbor Joining (NJ) (Saitou and Nei, 1987) with the GTR model were estimated using MEGA 7 (Kumar et al., 2016). Bayesian inference, using the Metropolis coupled Markov-chain Monte-Carlo method, was performed in MrBayes v.3.2.2 (Ronquist et al., 2012). Two parallel runs with four chains each (three heated and one cold) were performed for 3 million generations, sampling a tree every 1000 generations. The 530,000 burn-in period was identified graphically using Tracer 1.7.1 software (Rambaut et al., 2018) by tracking likelihoods at each generation to determine whether the likelihood values had reached a plateau, and the average deviation of split frequencies fell below 0.01. The 7952 trees sampled at stationary were used to infer Bayesian posterior. The

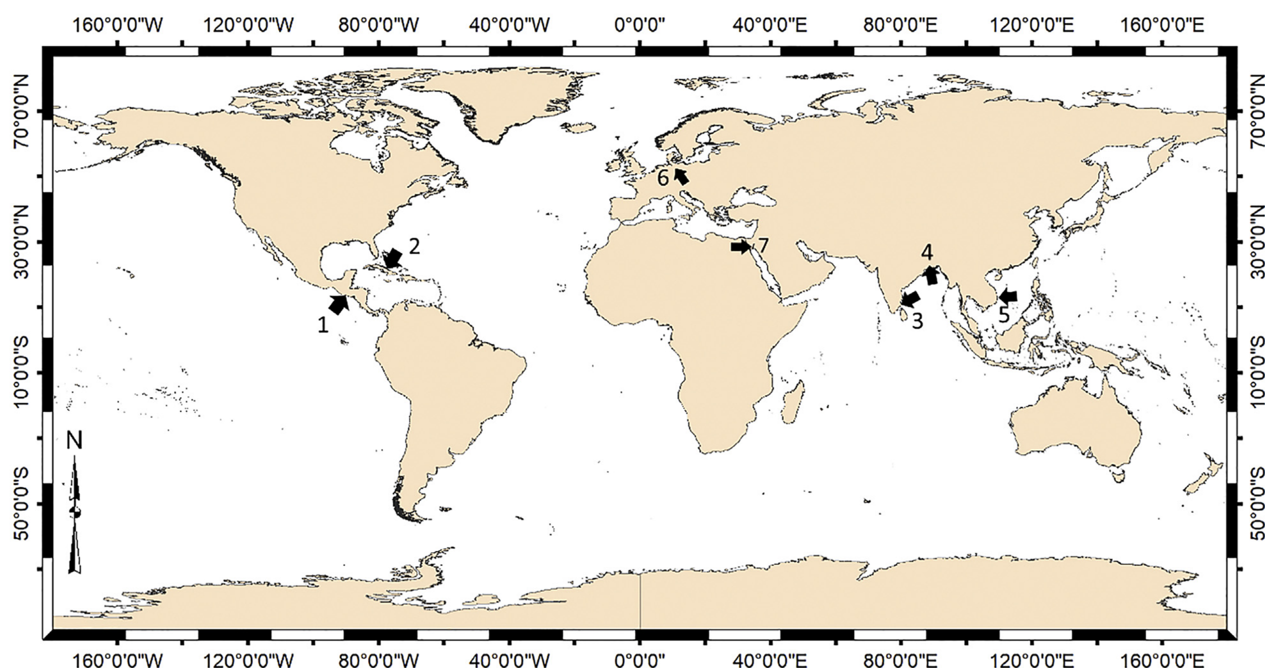

Fig. 1. The world map (Source: The National Oceanic and Atmospheric Administration (NOAA), USA) shows the sampling sites (solid arrows). Thirty five samples were collected at 1 (Guatemala, six samples), 2 (Cuba, one sample), 3 (India, three samples), 4 (Bangladesh, thirteen samples), 5 (Viet Nam, six samples), 6 (at greenhouse, LUH, Germany, five samples) and 7 (Egypt, one sample). The map was processed by MapInfo Pro™, version 12.5.5 (Pitney Bowes Software Inc., NY, USA).

consensus tree based on four different trees (achieved from the four methods) was constructed by Dendro Scope software, version 3.2.10 (Huson and Scornavacca, 2012).

2.3. Greenhouse experiments

2.3.1. Greenhouse conditions

The illumination by sunlight was supported by sodium vapor lamps (SON-T Agro 400, Philips, Amsterdam, Netherlands) to raise the quantum fluence rate to approximately 350 μmol m^{-2} s^{-1} over the day period of 12 h. The average temperature was 25 °C. Water used for irrigation was tap water, which was occasionally enriched with artificial sea salt (Seequasal GmbH, Münster, Germany). Plants were grown either in soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany), sand (0–2 mm grain size, Hornbach, Hannover, Germany) or a mixture of both. Macro- and micronutrients were added within the irrigation solution with 0.8% (winter) or 1% (summer) liquid fertilizer (Wuxal Top N, Manna, Düsseldorf, Germany) once per week. The fertilizer is composed of the following nutrients: 12.0% N, 4.0% P_2O_5, 6.0% K_2O, 0.01% B, 0.004% Cu, 0.02% Fe, 0.012% Mn, 0.001% Mo, 0.004% Zn.

2.3.2. Propagation and growth conditions

For each species, cuttings for propagation were taken from mother plants of 5–15 years old and approximately 2 m high. Propagation was tested in the greenhouse, in a fog house with a high humidity of 90%, and subsequently covered with a hood for eight weeks to acclimatize in the greenhouse. In addition, root growth promoting methods were applied to increase rooting, such as covering with humid sphagnum moss and the application of different concentrations of the plant hormone auxin.

Plants of *Avicennia germinans* were grown in soil and watered with tap water, which was enriched every 4 weeks with 10 Practical Salinity Units (PSU) with sea salt. During a biofilter experiment, 1.5-year-old *A. germinans* plants were grown in sand and the salt concentrations were increased to 15 and 30 PSU for 4 weeks. In another biofilter experiment using about 9-month-old *Laguncularia racemosa* plants the influence of salt at 15, 30 and 45 PSU was followed for 6 weeks.

2.3.3. Analysis of secondary compounds

The content of secondary compounds in plants from natural populations and greenhouse-grown plants of *Avicennia germinans* were compared using liquid chromatography coupled to a mass spectrometer (LC–MS). From six plants grown in the greenhouse, leaves growing at the top, middle and bottom of each plant were harvested and dried at 30 °C for five days. Already dried samples from Guatemala and the dried greenhouse samples were milled with a bead mill (Retsch, Haan, Germany). For extraction of secondary metabolites, 20 mg of milled material was weighed into a reaction tube and 800 μl of 80% methanol (MeOH) was added. After 10 min incubation with regular vortexing, the tubes were centrifuged for 5 min at 7800*g*. The supernatant was transferred into a new reaction tube with a pipette. These steps were repeated three times with 400 μl MeOH 80% each. The extracts were stored at −20 °C over night and centrifuged again the next day. In a glass vial, 500 μl of the supernatant was diluted with 500 μl MeOH 80% for LC–MS analysis. Standards for quantification (rutin and naringenin) were dissolved in ethanol and MeOH, respectively, and thereafter diluted in 80% MeOH. Concentrations of 0.001, 0.01, 0.1, 0.5, 1 and 10 μmol L^{-1} for naringenin and 0.5, 1, 10 and 100 μmol L^{-1} for rutin were measured. The LC–MS consisted of a HPLC (Shimadzu, Darmstadt, Germany) with a controller, two pumps, an auto sampler, column oven and photo diode array detector (PDA) and a time-of-flight mass spectrometer (AB Sciex TripleTOF 4600, Canby, USA). A Knauer Vertex Plus column (250 × 4 mm, 5 μm particle size, packing material ProntoSIL 120–5 C18-H) with pre-column (Knauer, Berlin, Germany) was used for sample separation, with 10 μl injections. Water and MeOH supplemented with 2 mM ammonium acetate and 0.01% acetic acid were used as solvents. The flow rate was 0.8 ml min^{-1} with a linear gradient from 10% to 90% MeOH over 35 min, 2 min of 90% MeOH, switch to 10% MeOH in 1 min and subsequent equilibration at 10% MeOH for 2 min. In the photodioide array, UV–vis spectra between 190 and 800 nm were recorded. Mass spectrometry was conducted in negative ionization mode at a nebulizer temperature of 600 °C and an ion spray voltage floating of −4500 V. Masses from 100 to 800 Da

Table 1
List of taxa, locations, and GenBank number used for the analysis. *: First recorded as *Avicennia marina*. **: First recorded as *A. marina*. # First recorded as *Rhizophora x annamalayana*. ##: First recorded as *Rhizophora apiculata*. LUH: Leibniz University Hannover. —/—: as above. na: not available.

No	Taxa	Family	Country	Location	GenBank accession number	Source
1	*Avicennia alba* Blume	Acanthaceae	India	na	KF848261	Direct submission
2	*Avicennia alba* Blume	—/—	Thailand	na	KX641594	Li et al. (2016)
3	*Avicennia alba* Blume	—/—	Indonesia	na	EF540977	Nettel et al. (2008)
4	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880028	This study
5	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880029	This study
6	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880030	This study
7	*Avicennia alba* Blume	—/—	Bangladesh	Patakhali	MG880031	This study
8	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880032	This study
9	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880033	This study
10	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880034	This study
11	*Avicennia alba* Blume*	—/—	Bangladesh	Patakhali	MG880035	This study
12	*Avicennia alba* Blume	—/—	Viet Nam	Cam Hai Dong	MG880036	This study
13	*Avicennia germinans* (L.) L.	—/—	Dominica	na	EF136923	Nettel and Dodd (2007)
14	*Avicennia germinans* (L.) L.	—/—	—/—	na	DQ469854	Nettel and Dodd (2007)
15	*Avicennia germinans* (L.) L.	—/—	Guadeloupe	na	EF136925	Nettel and Dodd (2007)
16	*Avicennia germinans* (L.) L.	—/—	Costa Rica	na	EF540979	Nettel et al. (2008)
17	*Avicennia germinans* (L.) L.	—/—	Angola	Soyo	DQ469860	Nettel and Dodd (2007)
18	*Avicennia germinans* (L.) L.	—/—	Germany	Greenhouse Hannover	MG880037	This study
19	*Avicennia germinans* (L.) L.**	—/—	Germany	Greenhouse Hannover	MG880038	This study
20	*Avicennia germinans* (L.) L.	—/—	Germany	Greenhouse Hannover	MG880039	This study
21	*Avicennia germinans* (L.) L.	—/—	Germany	Greenhouse Hannover	MG880040	This study
22	*Avicennia germinans* (L.) L.	—/—	Cuba	Cayo Coco	MG880041	This study
23	*Avicennia germinans* (L.) L.	—/—	Guatemala	Iztapa	MG880042	This study
24	*Avicennia germinans* (L.) L.	—/—	Guatemala	Iztapa	MG880043	This study
25	*Avicennia germinans* (L.) L.	—/—	Guatemala	Manchón-Guamuchal	MG880044	This study
26	*Avicennia germinans* (L.) L.	—/—	Guatemala	Manchón-Guamuchal	MG880045	This study
27	*Avicennia germinans* (L.) L.	—/—	Guatemala	San Andrés Villa Seca	MG880046	This study
28	*Avicennia germinans* (L.) L.	—/—	Guatemala	Tulate	MG880047	This study
29	*Avicennia integra* N.C. Duke		Australia	na	KX641598	Li et al. (2016)
30	*Avicennia marina* (Forssk.) Vierh.	—/—	China	na	AF477771	Shi et al. (2003)
31	—/—	—/—	—/—	na	AF477770	—/—
32	*Avicennia marina* (Forssk.) Vierh.	—/—	Egypt	Nabq Nature Reserve	MG880048	This study
33	*Avicennia marina* (Forssk.) Vierh.	—/—	Viet Nam	Cam Hai Dong	MG880049	This study
34	*Avicennia marina subsp. australasica* (Walp.) J. Everett	—/—	Australia	na	AF365978	Schwarzbach and McDade (2002)
35	*Avicennia marina* (Forssk) Vierh. subsp. *marina*	—/—	China	na	KX641593	Li et al. (2016)
36	*Avicennia marina subsp. eucalyptifolia* (Valeton) J. Everett		Australia	na	KX641592	Li et al. (2016)
37	*Avicennia officinalis* L.	—/—	Thailand	na	KX641597	Li et al. (2016)
38	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880050	This study
39	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880051	This study
40	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880052	This study
41	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880053	This study
42	*Avicennia officinalis* L.	—/—	Bangladesh	Patakhali	MG880054	This study
43	*Avicennia rumphiana* Hallier f.	—/—	Malaysia	na	KX641595	Li et al. (2016)
44	*Avicennia schaueriana* Stapf & Leechm. ex Moldenke	—/—	Brasil	na	AB861236	Mori et al. (2015)
45	*Avicennia schaueriana* Stapf & Leechm. ex Moldenke	—/—	Guadeloupe	na	EF540986	Nettel et al. (2008)
46	*Laguncularia racemosa* (L.) C.F. Gaertn.	Combretaceae	China	na	AF425685	Tan et al. (2002)
47	*Laguncularia racemosa* (L.) C.F. Gaertn.	—/—	Germany	Greenhouse Hannover	MG880055	This study
48	*Lumnitzera littorea* (Jack) Voigt	—/—	China	na	AF160468	Tan et al. (2002)
49	*Lumnitzera littorea* (Jack) Voigt	—/—	Vietnam	Thuan An	MG880056	This study
50	*Lumnitzera littorea* (Jack) Voigt	—/—	Vietnam	Cam Hai Dong	MG880057	This study
51	*Lumnitzera x rosea* C. Presl	—/—	Vietnam	Thuan An	MG880058	This study
52	*Lumnitzera racemosa* Willd.	—/—	China	na	AF160467	Tan et al. (2002)
53	*Lumnitzera racemosa* Willd.	—/—	Vietnam	Ninh Ich	MG880059	This study
54	*Bruguiera cylindrica* (L.) Blume	Rhizophoraceae	Australia	na	HM366078	Sun and Lo (2011)
55	*Bruguiera cylindrica* (L.) Blume	—/—	Australia	na	HM366079	—/—
56	*Bruguiera gymnorrhiza* (L.) Savigny	—/—	Indonesia	na	HM366082	Sun and Lo (2011)
57	*Bruguiera gymnorrhiza* (L.) Savigny	—/—	China	na	HM366083	Sun and Lo (2011)
58	*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griff.	—/—	Australia	na	HM366110	Sun and Lo (2011)
59	*Bruguiera parviflora* (Roxb.) Wight & Arn. ex Griff.	—/—	Australia	na	HM366111	Sun and Lo (2011)
60	*Bruguiera sexangula* (Lour.) Poir.	—/—	China	na	HM366122	Sun and Lo (2011)
61	*Bruguiera sexangula* (Lour.) Poir.	—/—	Indonesia	na	HM366134	Sun and Lo (2011)
62	*Ceriops tagal* (Perr.) C.B. Rob.	—/—	na	na	AF130329	Schwarzbach and Ricklefs (2000)
63	*Ceriops tagal* (Perr.) C.B. Rob.	—/—	na	na	EF119031	Direct submission
64	*Kandelia candel* (L.) Druce	—/—	na	na	AF130327	Schwarzbach and Ricklefs (2000)
65	*Kandelia candel* (L.) Druce	—/—	na	na	EF119071	Direct submission
66	*Rhizophora apiculata* Blume	—/—	Malaysia	na	HQ337923	Lo et al. (2014)
67	*Rhizophora apiculata* Blume	—/—	Micronesia	na	HQ337918	Lo et al. (2014)
68	*Rhizophora x annamalayana* Kathiresan	—/—	India	na	KF848256	Direct submission
69	*Rhizophora mangle* L.	—/—	Mexico	na	HQ337958	Lo et al. (2014)

Table 1 (*continued*)

No	Taxa	Family	Country	Location	GenBank accession number	Source
70	*Rhizophora mangle* L.	—/—	na	na	AF130332	Schwarzbach and Ricklefs (2000)
71	*Rhizophora mucronata* Poir.	—/—	Kenya	Gazi Bay	HQ337949	Lo et al. (2014)
72	*Rhizophora mucronata* Poir.	—/—	Kenya	Mida Creek	HQ337948	Lo et al. (2014)
73	*Rhizophora mucronata* Poir.	—/—	India	Parangipettai, Vellar Estuary	MG880060	This study
74	*Rhizophora mucronata* Poir.#	—/—	India	Parangipettai, Vellar Estuary	MG880061	This study
75	*Rhizophora mucronata* Poir.##	—/—	India	Parangipettai, Vellar Estuary	MG880062	This study
76	*Rhizophora stylosa* Griff.	—/—	Taiwan	na	HQ337934	Lo et al. (2014)
77	*Rhizophora stylosa* Griff.	—/—	Malaysia	na	HQ337935	Lo et al. (2014)

were measured in TOF mode. In addition, MS/MS spectra from 50 to 800 Da at a collision energy of −30 eV were recorded. The resulting peaks of extracted secondary compounds were evaluated using PeakView and MultiQuant (AB Sciex, Canby, USA). Masses and MS/MS spectra were compared to database entries from MassBank (Horai et al., 2010) and ReSpect (Sawada et al., 2012). Calibration curves and quantifications were calculated with MultiQuant.

2.3.4. Determination of the elemental composition of mangrove leaves

Dry plant material was ground to a fine powder (MM 400 grinder, Retsch GmbH, Haan, Germany) and 38 mg was incinerated for 8 h in a muffle furnace at 480 °C (M104, Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA). After cooling the samples to about 22 °C, 1.5 ml of 66% nitric acid was added and, after 10 min, 13.5 ml of ultra-pure water. The solutions were filtered (0.45 μm pore size, Carl Roth, Karlsruhe, Germany) and stored in vials at 4 °C before final analysis. The samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific).

3. Results

3.1. Phylogenetic analysis

Results of the four phylogenetic analyses (maximum likelihood, neighbor joining, maximum parsimony and Bayesian inference) showed that all sequences were distributed into three main clades consisting of Acanthaceae, Rhizophoraceae and Combretaceae (Fig. 2). For the Acanthaceae family, four samples labeled as *Avicennia marina* collected in Bangladesh clustered with the *A. alba* branch instead of the *A. marina* group. This result indicated the misidentification of *A. marina* samples from Bangladesh. Samples of *A. officinalis* collected in Bangladesh clustered with known *A. officinalis* samples. Interestingly, *A. germinans* collected in Cuba clustered with *A. germinans* “Caribbean sea clone” and the materials collected in Guatemala grouped together with the “Pacific clone” with very high posterior probability and bootstrap values of 1.0, 99%, 93% and 72%, respectively. As illustrated in Fig. 2, it became obvious that *A. germinans* cultivated in the greenhouse of the Institute of Botany, Leibniz University Hannover, Hannover, Germany, was grouped within the *A. germinans* “Caribbean sea clone”. Since the origin of the *A. germinans* mother plant was hitherto unknown, it can now be concluded that only areas of the Central and South American North Atlantic coast are possible origins. The putative *A. marina* growing in the greenhouse of the Institute of Botany clustered with *A. germinans*. Based on the ITS analysis results, two more misidentifications were detected. Two samples identified as *Rhizophora x annamalayana* and *R. apiculata* (Rhizophoraceae) were found to have closer affinities to *R. mucronata* instead.

3.2. Different ways of propagation

A trial of 200 *Avicennia germinans* cuttings were prepared and grown under the same conditions as the mother plant. In addition, longer cuttings (30 cm) were taken and grown in the same way. To improve the survival rate of the cuttings, the fresh cut branches were grown in a fog house for 13 weeks with a humidity higher than 90%. Afterwards, the plants were transferred back to the greenhouse and covered with a hood for eight weeks to acclimatize. Rooting experiments with cuttings of different lengths were performed at high humidity, based on rooting results obtained with other woody species in a fog house experimenting with different length of cuttings (Mateja et al., 2007). Cuttings showed a low survival rate of only 10 rooted cuttings among the 200 cuttings tested (Table 2). Also longer cuttings of 30 cm and keeping the fresh cuttings in a fog house with high humidity did not lead to better results. The treatment with different concentrations of the growth hormone auxin did not induce root formation, neither the application of the sphagnum moss method (data not shown). Plants of *A. germinans* are flowering in the greenhouse and produce viable seeds. These seeds were laid on wet soil without any further treatment and had a high germination rate. In comparison to propagation by cuttings, for *A. germinans*, the propagation by seeds was the easier and more effective strategy.

Propagules from *Bruguiera cylindrica*, which come from Indonesia, grew slowly and 9 of 23 plants were put in the fog house to regenerate. The propagation of *B. cylindrica* by cuttings was also not very productive (2 of 10 cuttings rooted) (Table 2). For *Lumnitzera racemosa*, 66 cuttings were prepared and placed in the fog house for 13 weeks, and 18 cuttings were grown in the green house without further treatment. Cuttings of *L. racemosa* from the fog house showed a much higher rooting rate (95%) than cuttings grown in the normal greenhouse (50%) (Table 2).

3.3. Identification of suitable culturing conditions and influence of salinity

For *Avicennia germinans* and *Laguncularia racemosa* sand, soil or a mixture of both, provided good growing media. Watering the plants without letting the soil dry worked well for *A. germinans* and *L. racemosa*. *Bruguiera cylindrica* plants grew best in a sand/soil mixture of 1:2 when watered only 1–2 times per week, depending on the ambient temperature. Nonetheless, *B. cylindrica* plants grown from propagules showed slow growth and yellow leaves, even though the plants were supplied with nutrients through regular fertilization.

All mangrove plants were regularly watered with saline water (10 PSU) every 4 weeks, which lead to typical morphological adaptations to salinity. On *Avicennia* leaves, small salt crystals were visible on the top of the leaves, whereas *L. racemosa* excretes salt-enriched sap through salt glands close to the petiole. To find out more about the influence of higher salinity on the plant growth, different salt concentrations

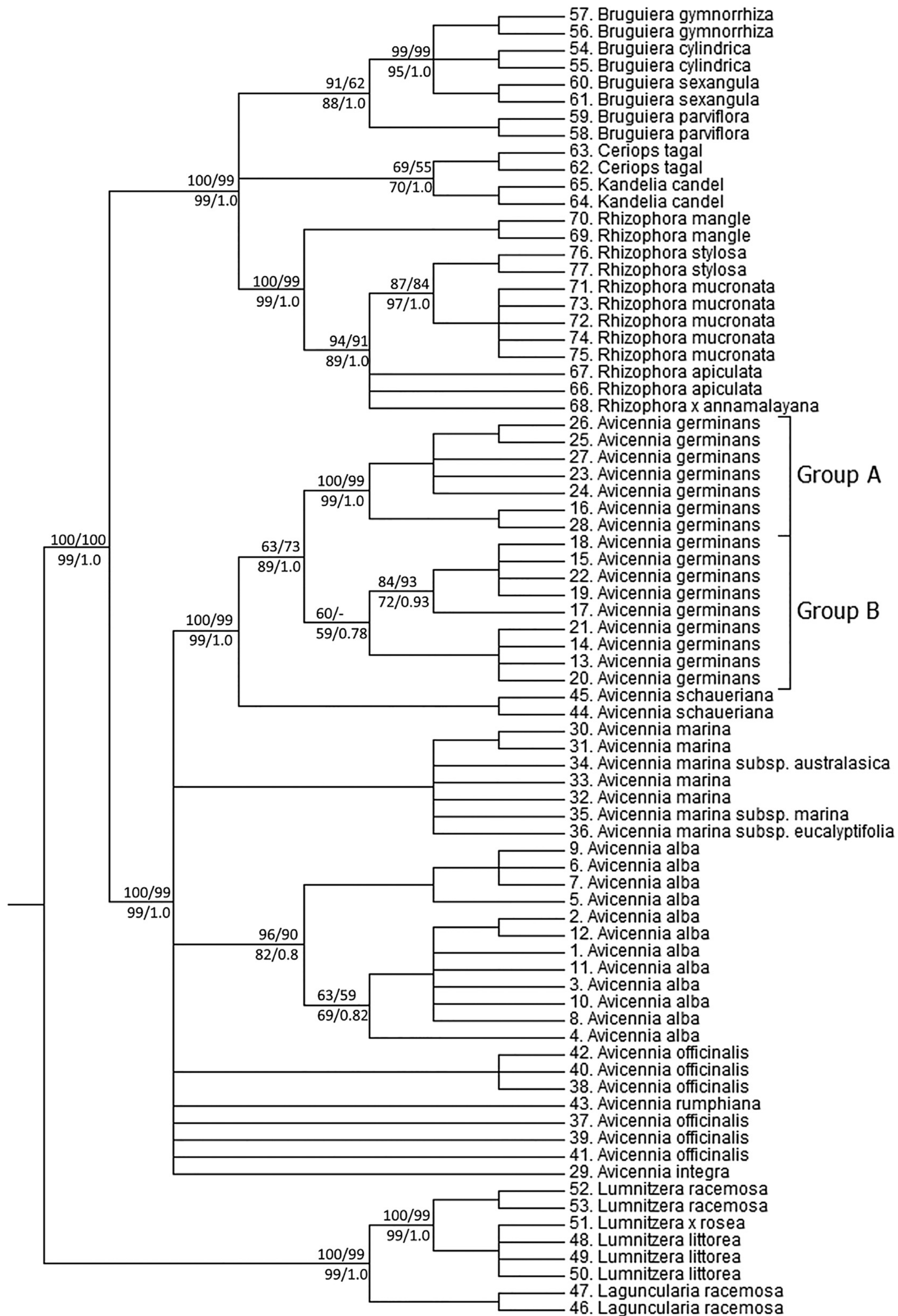

Fig. 2. Phylogeny of members of mangroves inferred from Bayesian Iinference, maximum likelihood, neighbor joining and maximum parsimony. The data set based on 600 bp (including gaps) of nrDNA sequences comprising ITS-1, 5.8SrDNA and ITS-2. The posterior probability and bootstrap values of each method are shown in each node: above nodes, left: Bayesian Inference, right: Maximum Likelihood; below nodes, left: Maximum Parsimony, right: Neighbor Joining. Species names printed in bold were this study. Group A: Pacific clone; Group B: North Atlantic clone. See Table 1 for the number in front of each taxon.

were tested on *A. germinans* (15, 30 PSU) and *L. racemosa* (15, 30, 45 PSU). Leaves and shoots of *A. germinans* plants showed a significantly lower growth rate at 30 PSU compared to 15 PSU (Fig. 3A). Complete plant biomass of *L. racemosa* showed no difference after two weeks of growth. After four weeks, plants grown at 15 PSU had a significantly higher biomass weight compared to 30 and 45 PSU. At the last measurement after 6 weeks, plants treated with 15 and 30 PSU both had a higher weight than when grown at 45 PSU (Fig. 3B).

Table 2
Growth conditions and rooting rates of cuttings from *A. germinans*, *L. racemosa* and *B. cylindrica*.

Species	Cuttings	Fog House [weeks]	Hood [weeks]	Rooted	% rooting
A. germinans	200	–	–	10	5
A. germinans	52	13	8	6	12
A. germinans	9 (long)	–	–	3	33
A. germinans	9 (long)	13	8	5	56
L. racemosa	18	–	–	9	50
L. racemosa	66	13	8	63	95
B. cylindrica	10	8	8	2	20

3.4. Comparison of secondary compounds from naturally and greenhouse-grown mangrove plants

Leaf extracts from *Avicennia germinans* plants grown in the greenhouse were compared to those from naturally grown plants from Guatemala. The extracted secondary metabolites were analyzed by LC–MS and the resulting peaks identified with database comparison (Figs. 4 and 5, Table 3). A total of 32 compounds were detected. For the compounds producing peaks 2, 20 (kaempferol-3-glucoside), 21 (kaempferol-3-glucoside-3-rhamnoside) and 29 (kaempferide) the content in plants from Guatemala was significantly higher than in greenhouse-grown plants. The content of the compounds producing peaks 6, 9 and 23 was higher in greenhouse plants than in the samples from Guatemala. The compound concentration in the six individual samples of *A. germinans* grown outdoors or in the greenhouse was variable, resulting in high standard deviations like for peak 22.

Eight compounds were found only in extracts from greenhouse plants. Peaks were considered as present if more than four samples presented the peak at a height at least 3 × above the background value. Two were identified as sugars, namely galactinol and modified palatinose monohydrate. The flavonoids flavanomarein, prunin and a modified form of marein were found. Two peaks were identified as modified forms of eriodyctiol-7-O-glucoside. One compound was identified as a gentisic acid derivate. Leaf extracts from Guatemalan samples comprised three unknown compounds, which did not occur in greenhouse samples that could not be further identified with the help of the databases.

The quantification of the two flavonoids naringenin and rutin is shown in Fig. 6. Calibration curves for naringenin and rutin were linear through zero with $y = 8.714e^5$ x, $r = 0.999$ and $y = 1.241e^5$ x, $r = 0.999$, respectively. Naringenin showed very low concentrations in samples from Guatemala, with values between 0.001 and 0.006 µmol g^{-1} DW. In greenhouse plants two and three, the values were comparably low. Greenhouse plants one, four, five and six contained concentrations between 0.191 and 1.234 µmol g^{-1} DW. Rutin was present in low concentrations in half of the samples from Guatemala (0.018–0.025 µmol g^{-1} DW) and in higher concentrations in the other half (0.553–1.231 µmol g^{-1} DW). In the greenhouse plants, all samples had higher rutin concentrations between 0.169 and 0.835 µmol g^{-1} DW.

3.5. Elemental composition

Results of the elemental analysis using ICP-OES are shown for twelve essential elements (Fig. 7 A, B). In leaf samples collected in Guatemala, iron, sodium, sulfur and magnesium were detected in higher amounts compared to the greenhouse samples, but only the latter being statistically significant (2.54/0.89 mg g^{-1} DM). The leaf samples from the greenhouse were significantly richer in calcium (16.93/6.38 mg g^{-1} DM), manganese (0.15/0.07 mg g^{-1} DM), phosphorus (7.21/3.97 mg g^{-1} DM), and zinc (0.04/0.01 mg g^{-1} DM).

4. Discussion

The use of a DNA marker is an advantageous method to identify the phylogenetic affiliation of an organism. In this work, phylogenetic analysis using just one molecular marker was applied for characterizing different mangrove species collected at various sites. The aim was to apply a fast and cheap identification method of plant species already identified based on morphological characters. The different mangrove species analyzed were chosen in a way that the broad distribution of *Avicennia* species is covered, including the Atlantic Caribbean East-Pacific (ACEP) and the Indo West-Pacific (IWP) region. Samples from the Acanthaceae family were misidentified in some cases based solely on the analysis of morphological traits. *A. marina* collected in Bangladesh was found to be *A. alba* instead. One plant from the greenhouse in the Institute of Botany, which was designated as *A. marina*, was also misidentified and belongs to *A. germinans* "Caribbean sea clone". *A. marina* and the closely related species *A. alba*, *A. officinalis* and *A. germinans* have a very similar leaf and flower morphology, which makes them not easy to distinguish. Similarly, two *Rhizophora* subspecies from India were identified as *R. mucronata* instead of *R. x annamalayana* and *R. apiculata.* However, the use of a multilocus marker system (Saddhe et al., 2017) or a different marker system, such as amplified fragment length polymorphisms (AFLP) (Garcia et al., 2004), might result in a better resolution but is also more time consuming and expensive. The ITS analysis clearly

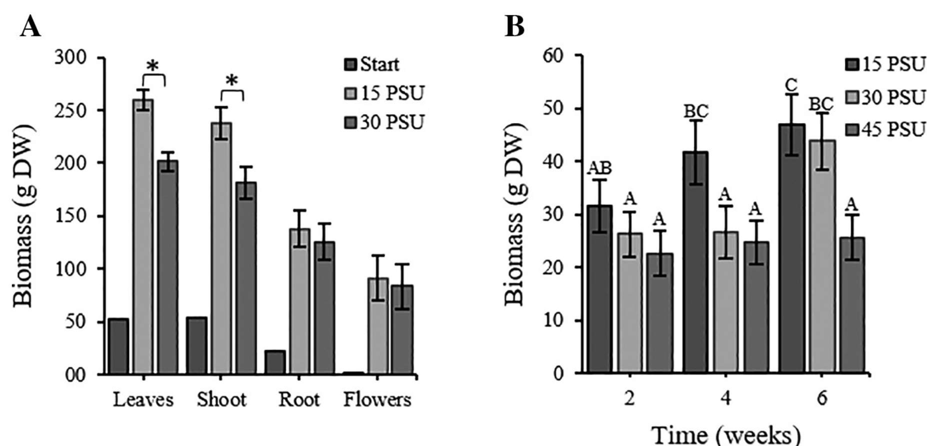

Fig. 3. Growth of *A. germinans* plants growing in different salt concentrations (A). One plant was harvested in the beginning of the experiment and the dry weight of leaves, shoot, root and flowers was measured (Start). For each treatment, three plants were grown in containers at 15 and 30 PSU. After 20 weeks, the dry weight was also measured. A star above the bar indicates a significant difference ($p < .05$) tested by an ANOVA analysis. Biomass of *L. racemosa* grown at different salt concentrations (B). The plants have been grown at 15, 30 and 45 PSU and three plants of each treatment were harvested after 2, 4 and 6 weeks. The plant material was dried for 4 d at 85 °C and then the dry weight was measured. Means with a common letter are not significantly different ($p > .05$) according to ANOVA results with Duncan alpha test.

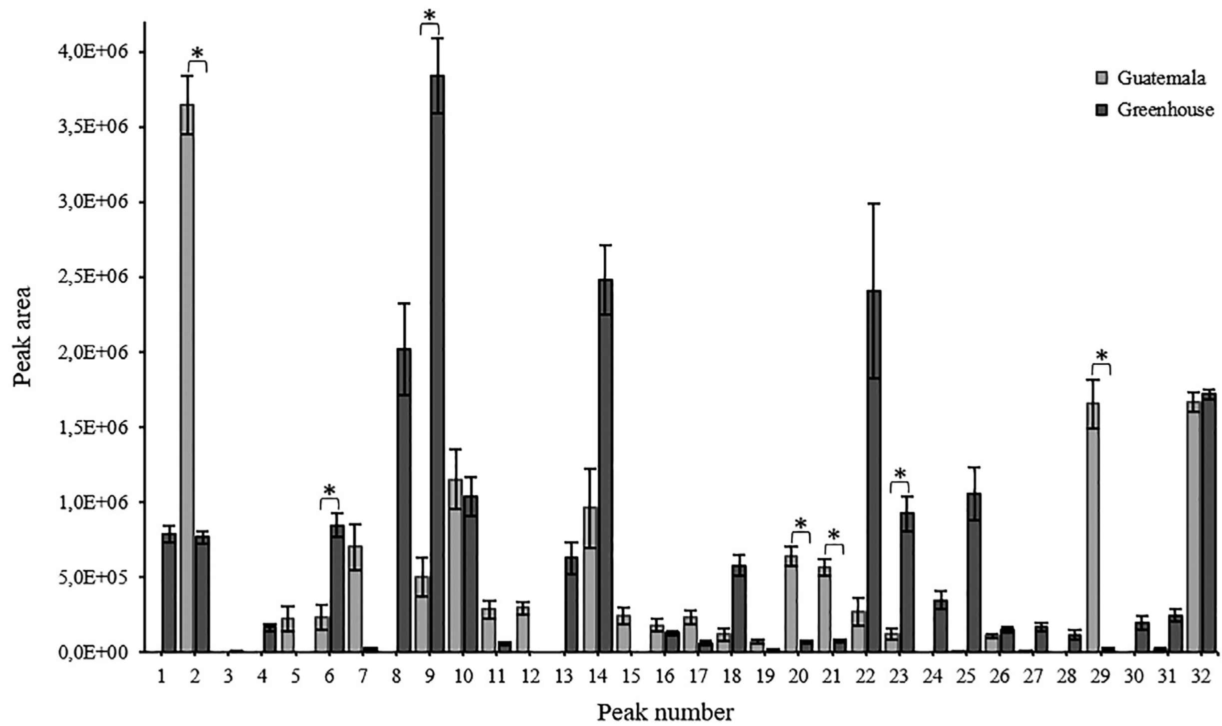

Fig. 4. Relative content of metabolites in *A. germinans* leaf samples from Guatemala and the greenhouse. Six samples each were extracted with MeOH 80% and analyzed by LC–MS. The mean peak area and standard error are shown. Statistically significant difference with $p < .05$ tested by t-test is indicated with a star above the bar.

shows also the division of *A. germinans* into two groups: the Pacific and the Caribbean sea clone. These results provide valuable information about the origin of mangroves grown in the greenhouse and from different collection sites, as it was found that on-site species identification could be incorrect.

The natural habitat of mangroves is characterized by complex environmental factors. Tide, water currents, strong winds and high solar radiation cannot be imitated in a greenhouse. Nevertheless, it was shown that cultivation of mangroves in the greenhouse in temperate zones is possible: *A. germinans* and *L. racemosa* were successfully grown and propagated in our greenhouse. Plants grown from propagules of *Bruguiera cylindrica* can also be grown in a greenhouse in the temperate zone, but further optimization is necessary.

Many findings in the search for optimized growth conditions for mangroves in the greenhouse were found by chance and observation of plant growth over the last 10 years. For all three species examined

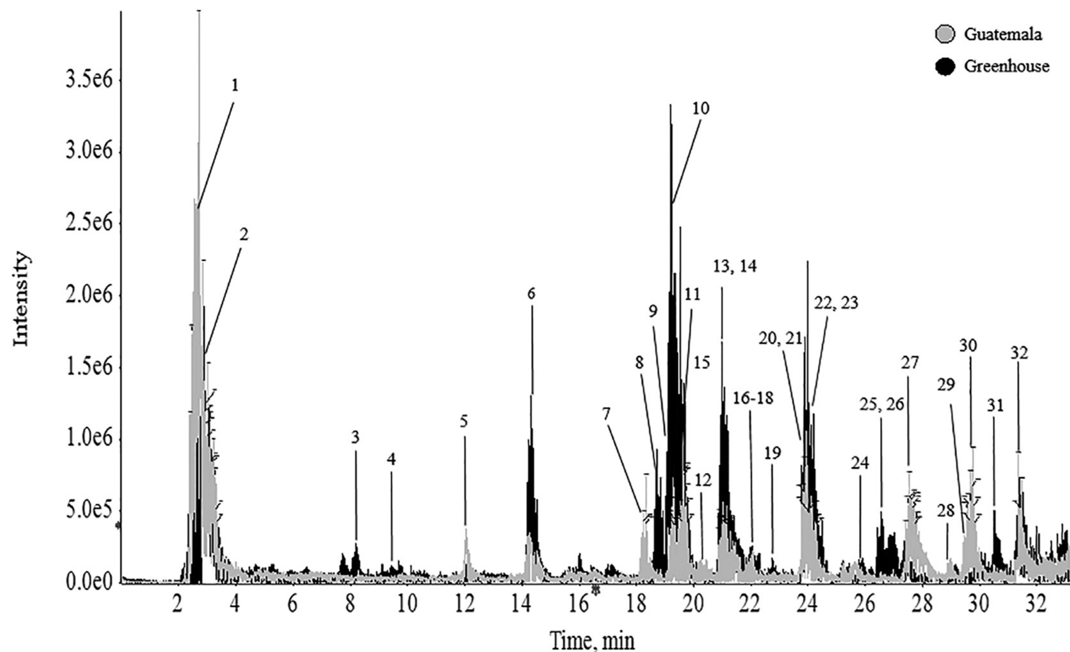

Fig. 5. Total ion current (TIC) chromatogram from LC–MS in negative electrospray ionization. Individual chromatograms for six samples from Guatemala and the greenhouse were summed up. Numbers indicate peaks described in Table 3.

Table 3
Compounds identified in *A. germinans* leaf extracts by database comparison of MS/MS fragment masses. No = number of peak, RT = retention time, Mass = mass of precursor ion in Da, MS/MS = fragment masses obtained at −30 eV collision energy in Da, n. i. = not identified, mod. = modified.

No	RT	Mass	MS/MS	Name	Accession	Database
Guatemala and greenhouse						
2	3.1	201.02	157.02, 59.01	n. i.	–	–
6	14.2	703.16	623.21, 541.13, 461.17	n. i.	–	–
7	18.2	461.07	285.04, 175.03, 133.02	Luteolin mod.	PT204040	ReSpect
9	19.2	623.21	461.17, 161.02,	n. i.	–	–
10	19.6	521.17	357.12, 213.07, 163.04, 145.02, 119.04	n. i.	–	–
11	20.2	475.09	299.04, 284.02, 175.01, 113.02	Kaempferide mod.	PS040309	ReSpect
14	21.2	623.21	461.17, 161.02	n. i.	–	–
16	21.8	609.15	300.01	Rutin	PS045410	ReSpect
17	22.0	463.09	300.01, 271.01, 255.01	Hyperoside	PT204320	ReSpect
18	22.1	535.15	329.08, 179.02, 135.04	Caffeic acid derivative	PS044608	ReSpect
19	23.0	489.10	285.02, 255.01, 227.02	Kaempferol-3-glucoside mod.	PT209270	ReSpect
20	23.8	447.09	285.02, 255.01, 227.02	Kaempferol-3-glucoside	PT209270	ReSpect
21	23.8	593.15	285.02, 255.02	Kaempferol-3-glucoside-3-rhamnoside	PT209200	ReSpect
22	24.0	519.16	313.11, 193.05, 163.04, 149.06	Sinapic acid derivative	PT210880	ReSpect
23	24.2	549.17	343.12, 325.11, 193.05, 175.04, 149.06	Sinapic acid derivative	PT210880	ReSpect
25	26.5	271.06	177.01, 151.00, 119.04, 107.01	Naringenin	PS040709	ReSpect
26	26.5	301.03	285.02, 257.03	Hesperetin	PS078009	ReSpect
27	27.5	541.24	379.18, 355.10, 335.18, 193.05, 185.11, 175.04, 149.06	Sinapic acid derivative	PT210880	ReSpect
29	29.6	299.06	284.02, 256.02	Kaempferide	PS040309	ReSpect
31	30.5	531.19	357.10, 195.05, 173.05, 151.07	n. i.	–	–
32	31.6	293.18	236.10, 221.15, 192.11	n. i.	–	–
Only greenhouse						
1	2.8	341.11	179.05, 119.03, 89.02, 71.01	Galactinol	PT211910	ReSpect
3	8.2	373.11	211.06, 167.07, 149.06, 123.04	Gentisic acid derivative	PS055907	ReSpect
4	9.4	353.14	221.1, 179, 161.04, 101.02	Palatinose monohydrate mod.	PT212460	ReSpect
8	18.7	449.11	287.05, 151.00, 135.04	Flavanomarein	PS084609	ReSpect
13	21.1	433.12	271.06, 151.00, 119.05	Prunin (naringenin-7-O-glucoside)	PR040149	MassBank
24	25.8	597.17	287.05, 151.00, 135.04	Marein mod.	PR100806	MassBank
28	29.1	615.22	449.11, 287.05, 151.00	Eriodyctiol-7-O-glucoside mod.	PR040090	MassBank
30	29.9	617.23	287.06, 151.00	Eriodyctiol-7-O-glucoside mod.	PR040090	MassBank
Only Guatemala						
5	12.0	637.08	351.04, 285.03	n. i.	–	–
12	20.5	291.13	96.96	n. i.	–	–
15	21.3	289.11	96.96	n. i.	–	–

for this work, it was found that both sand and soil or a mixture of both can be used as a base. Soil offers more stability for the plants, while sand is useful for experiments in which the roots are weighed, as the sand can be washed off more easily. Websites for commercially available mangroves also recommend sand mixtures rather than soil (mangrove.at).

Adding low amounts of salt did not affect plant growth, only concentrations higher than 15 PSU lead to a reduced growth rate for *A. germinans* and *L. racemosa* plants. In a study on *R. mangle*, it was shown that salinity of 15 ppt had no significant influence on plant growth compared to fresh water (Doyle, 2003). In another study, *A. germinans* plants had a lower photosynthesis rate when grown at increasing salinities (López-Hoffman et al., 2007). Stress is known to induce the biosynthesis of plant secondary metabolites and increases the overall yield of secondary compounds (Boestfleisch and Papenbrock, 2017).

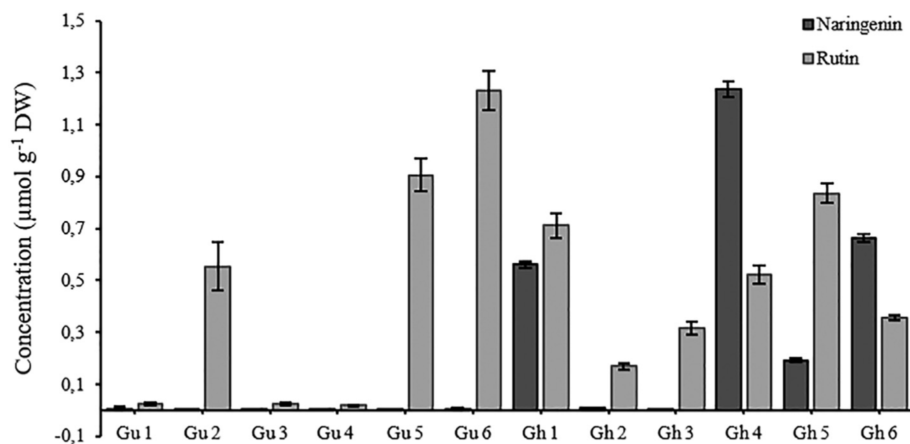

Fig. 6. Quantification of rutin and naringenin in *A. germinans* leaf extracts from plants collected in Guatemala (Gu) and the greenhouse (Gh) given in µmol g^{-1} dry weight (DW). Means and standard deviation are shown from three individual measurements in negative electrospray ionization mode. Concentrations of rutin and naringenin were calculated according to calibration curves.

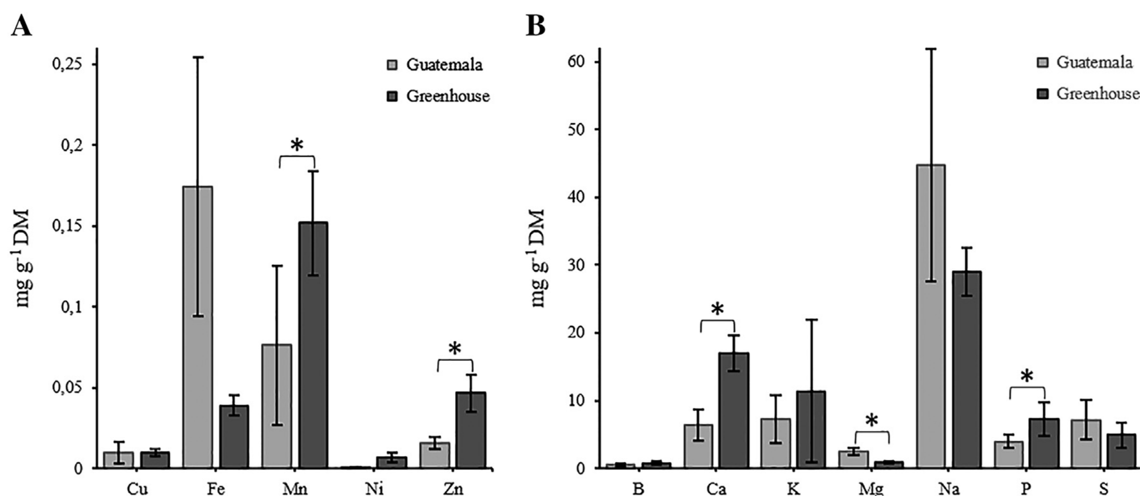

Fig. 7. Elemental composition of leaf material from samples collected in Guatemala and in the greenhouse measured by ICP-OES in mg g^{-1} dry mass. Mean values of six individual *A. germinans* plant samples from Guatemala and the greenhouse in Hannover, Germany, are shown for elements with low concentrations (A) and high concentrations (B). A star above the bar indicates statistically significant difference with $p < .05$ analyzed by *t*-test.

Reforestation of mangrove forests is achieved by growing plants in nurseries from seeds or by cuttings from mature trees (Eganathan et al., 2000). The costs of this investment are outweighed by the benefits of re-planted mangrove forests (Tuan and Tinh, 2013). For the propagation of greenhouse-grown mangroves, rooting rates of up to 95% can be obtained (in this study, for *L. racemosa* cuttings grown in a fog house for 13 weeks before transfer into the greenhouse). The rooting rate of *A. germinans* was also improved by growing cuttings in the fog house (12/56%). In contrast, *B. cylindrica* cuttings showed a low survival rate of 20%, despite also being grown in the fog house. The fog house helped to provide the high humidity, which is found in the natural habitat of mangroves. The use of fog to improve the rooting and survival of cuttings has been described in the literature and is a recommended procedure for woody species (Milbocker, 1983). The sensitive cuttings can build roots more easily and can later be adapted to the greenhouse conditions with less humidity.

Vegetative propagation or in vitro multiplication are likely to be more reliable than seed propagation of mangroves. Reproduction of *A. germinans* was also achieved by seeds, the plants are able to cross-pollinate via insects in the greenhouse (flies and bees) or possibly self-pollinate (Kathiresan and Bingham, 2001). *Laguncularia racemosa* has a variable pollination system: plants can be either male or hermaphroditic, so that self-pollination can occur (Landry et al., 2009). The species flowered in the greenhouse but did not set seed, suggesting that the plants have male flowers or the physiological conditions are suboptimal. *Bruguiera cylindrica* plants did not flower in the greenhouse during this study. The overall low growth rate and yellowing of leaves could be caused by a suboptimal nutrient composition, even though essential macro- and micronutrients were supplied by fertilization or the absence of essential endophytic or mycorrhizal fungi, which could be essential for nutrient uptake and availability (D'Souza and Rodrigues, 2013). In vitro propagation remains a possibility to reproduce mangroves, if cuttings have a low survival rate and there are no seeds available. Cousins and Saenger (2002) have investigated in vitro propagation of *A. marina* on different media, but only with minor success, and further tests on a variety of species are needed.

Mangrove species are valuable sources of secondary metabolites with potential for new drug development (Wu et al., 2008). Diseases like rheumatism and throat pains are treated with *A. germinans* bark and leaves in folk medicine (Bandaranayake, 2002; Table A.1). The active compounds, which are responsible for the effectiveness, are in many cases still unknown (Spalding et al., 2010). LC–MS analysis of methanolic extracts from *A. germinans* grown in the greenhouse and from a natural habitat in Guatemala revealed the presence of mainly phenolic secondary metabolites. One caffeic acid derivative and three sinapic acid derivatives could not be identified. Several polyphenolic compounds were detected, including eight flavonols (kaempferide modified, kaempferol-3-glucoside modified, kaempferol-3-glucoside-3-rhamnoside, kaempferide, rutin, hyperoside), two flavanones (naringenin and hesperetin) and one flavone (luteolin modified). Five peaks of the chromatograms could not be identified in this way.

Two of the substances identified in both wild and greenhouse-grown sample groups of *A. germinans*, namely naringenin and rutin, show a high discrepancy in their content in individual plant samples. In the class of flavonoids, naringenin belongs to the subgroup of flavanones and rutin to the flavonols as a glycosidic form of quercetin (Zhang et al., 2013). These compound classes play an important role for plants, e.g. in UV protection and plant defense against herbivores (Simmonds, 2003). The concentration of quercetin and rutin as well as their biosynthesis activity has been found to be increased by water, salt and UV-B stress (Lucci and Mazzafera, 2009; Kreft et al., 2002). In this context, differing concentrations can be explained by the stress factors affecting each individual plant or the collected leaves. In the greenhouse, those factors are more consistent, which is revealed by a more comparable content of the two metabolites. Here, naringenin was present in four out of six samples and rutin in all, whereas in samples from Guatemala rutin was only present in three out of six samples. Naringenin is a precursor in the biosynthesis of flavones and flavonols such as apigenin and kaempferol (Zhang et al., 2013). The low concentration of naringenin in samples from Guatemala matches to higher concentrations of kaempferol derivatives represented by peaks 19, 20, 21 and 29.

Eight compounds were detected only in the samples of *A. germinans* plants grown in the greenhouse. Two of these were identified as sugars (galactinol and palatinose monohydrate modified), whereas the other six belong to the group of flavanones (flavanomarein, prunin, eriodyctiol-7-O-glucoside modified) and chalcones (marein). This indicates a higher diversity of flavanones in the greenhouse plants compared to naturally growing plants, which comprise only of two flavanones. In the plant samples from Guatemala, three unidentified peaks might belong to a group of phenolic compounds as well, and are possibly not yet characterized in the literature. In total, the majority of compounds was found in the greenhouse samples as well as in samples collected in Guatemala, which speaks for a comparable composition even though they grow in different environments. An advantage of plant material from the greenhouse is the stable production of secondary metabolites under controlled conditions, as shown for the medicinal plant perilla (Lu et al., 2017).

The elemental composition of *A. germinans* leaf samples from Guatemala and the greenhouse was measured by ICP-OES for 25 elements. Of these, 13 were detected only in trace amounts and are not discussed. From the remaining 12, 5 showed significant differences between the two sample groups. Magnesium was more abundant in the samples collected in Guatemala. In saline soils, magnesium belongs to the main cations, together with sodium and calcium (Szabolcs, 1989). This can explain the higher concentration of magnesium, even though the calcium content is lower compared to greenhouse plants. The nutrient availability can also vary strongly in individual mangrove forests, depending on, e.g. soil texture, microbial activity and plant species (Boto and Wellington, 1984; Reef et al., 2010).

In greenhouse leaf material, the four elements manganese, zinc, calcium and phosphorus were found in significantly higher concentrations compared to the samples from Guatemala. Mangrove soils in general have a low nutrient content (Alongi et al., 1992). In the greenhouse experiments, all elements are provided in high amounts in regular fertilization. In addition, sodium was found in higher concentrations in outside grown mangroves (44.7 compared to 29.0 mg g^{-1} DM) but with no statistical significance between the two sample groups. This can be explained by the growth in saline water, as *A. germinans* takes up salt and excretes it through salt glands on the upside of the leaves. The stable supply of nutrients is an advantage for the propagation of mangroves in the greenhouse. Together with the gained knowledge about optimal growth parameters, mangrove plant material can be harvested throughout the whole year for further studies on mangrove physiology and secondary metabolites.

Contributions

YG cultivated the mangroves and performed the secondary compound analysis. YG, IK and XVN did the phylogenetic analysis. YG, IK, XVN and JP evaluated the data. YG, XVN and JP wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sajb.2018.11.020.

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